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Diagnostic Virology Protocols

Second Edition

Edited by
John R. Stephenson
Alan Warnes

Humana Press

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Preface

Recent outbreaks of swine influenza and avian influenza, along with the remaining and in some cases expanding threats from HIV, dengue virus, and the viruses causing hepatitis, have reinforced the need for rapid, accurate, and cost-effective diagnosis of viral disease.

Since the first edition of this book, there have been several major changes in the virus diagnostic laboratory. The first edition contained protocols for a number of techniques, including electron microscopy, ELISA, virus neutralisation, heamagglutinin inhibition, and electrophoresis, but nearly all the chapters in this current edition describe protocols involving nucleic acid detection, most often utilising some form of the polymerase chain reaction (PCR). We also included a chapter on the then emerging technology of robotics, and now every well-founded diagnostic microbiology laboratory will contain several robotic analyzers, releasing the staff to give more informed scientific support to clinicians. Moreover, reliable and sophisticated robots which can carry out in vivo analysis, including mammalian cell culture, are readily available, and bespoke systems can be designed and purchased from several manufacturers.

This second edition contains two chapters which also aim to look to the future. Over-the-counter analytical systems for pregnancy testing and blood sugar analysis are now commonplace. Several companies are developing microfluidics systems which could be applied to similar devices to detect viral infection in blood spots, urine, and saliva. Many modern diagnostic protocols can provide detailed information on small samples within a few minutes, and the much longer turn-around times experienced by most clinicians are nearly always due to transportation times and the need for careful identification of samples. Consequently point-of-care (POC), or point-of-collection diagnostic devices, especially if they are hand-held, are very attractive, and we have asked Drs. Christopher C. Blyth, Robert Booy, and Dominic E. Dwyer to discuss these issues in Chap. 22. The objection that many have raised to POC devices is the validation of the readouts. The rapid sophistication of mobile phone technology may provide the answer, enabling diagnostic cassettes to be plugged into a mobile phone-like device and the data and its interpretation being sent to and from a central laboratory or data analysis centre within minutes. This is not new technology, we all use it every time we use a credit card and a mobile phone. If these technologies become widely available and popular, professional national microbiology laboratories may see their roles change or even expand in the future. In the clinical setting, they would be providing confirmatory tests and data validation, with nearly all routine analyses being carried out at the bedside. Moreover, these laboratories could also find themselves providing an entirely new kind of service, data validation and clinical advice for members of the general public who have purchased over-the-counter tests and submitted data through a mobile phone network.

Over the past decade or so, the use of mathematical modelling has become increasingly important in assessing the severity of disease outbreaks, either at the local level or internationally. These models have become increasingly sophisticated as computing power has risen exponentially. Modelling can also be used to identify critical bottlenecks in the

delivery of prophylactic and therapeutic measures and help both local hospitals and governments plan in advance. But of course, any model is only as good as the data used to populate it and closer links between the diagnostic laboratory and the mathematicians will be an important factor in controlling infectious diseases in the future. Therefore, we have asked Steve Leach to discuss the power and limitations of mathematical models in Chap. 23 of this edition.

The past few years have seen dramatic changes in the virus diagnostic laboratory, and as the threats to public health from infectious diseases remain ever present, the next few years are likely to bring more significant change. New viral diseases will almost certainly appear, but we can also expect to see the appearance of technologies that can only be imagined today.

London, UK — *John R. Stephenson*
Middlesex, UK — *Alan Warnes*

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Chapter 1

Detection of Adenoviruses

Kanti Pabbaraju, Sallene Wong, and Julie D. Fox

Abstract

The human adenovirus (hAdV) group is represented by 52 serotypes that have been reported to cause a broad range of clinical manifestations including respiratory tract infections, acute conjunctivitis, cystitis, gastroenteritis, and systemic infections. Conventional methods for detection of hAdVs include electron microscopy, antigen detection, and virus isolation in cell culture. Implementation of real-time PCR assays has increased the sensitivity and speed of detection, and allowed for rapid quantification and serotyping. This chapter describes the design and validation of a multiplex real-time PCR assay for the detection of a broad range of hAdV serotypes in respiratory samples, blood, or urine. This assay targets the conserved region of the hAdV hexon gene and utilizes hydrolysis probes for the detection of amplified products. The assay can be adapted to provide quantitative results to evaluate the change in viral load, and products can be sequenced for serotype designation. PCR-based methods for hAdV detection are sensitive, specific, allow for rapid diagnosis, and facilitate epidemiological studies.

Key words: Adenovirus, DNA, Nucleic acid, Multiplex PCR, Serotype, Sequencing

1. Introduction

1.1. Human Adenoviruses

Human adenoviruses (hAdVs) are nonenveloped, icosahedral viruses containing a single linear, double-stranded DNA genome. They belong to the *Adenoviridae* family and *Mastadenovirus* genus. They are divided into seven species, from A to G based on immunologic, biologic, and biochemical characteristics (1). Fifty two different hAdV serotypes have been described based on neutralization of specific animal antisera (2, 3). Transmission of hAdVs can be from person to person via the fecal-oral and respiratory routes. They can also spread through water, fomites, and instruments. Human AdVs have been widely reported to cause gastroenteritis, haemorrhagic cystitis, respiratory and ocular infections in children and military recruits (4). Due to the different

John R. Stephenson and Alan Warnes (eds.), *Diagnostic Virology Protocols*, Methods in Molecular Biology, vol. 665,
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tissue tropisms (1) hAdVs can cause diverse clinical syndromes in immunocompetent individuals; they are also associated with endemic infections in the community or outbreaks, which are typically mild and self-limiting. Asymptomatic, persistent hAdV infections are thought to be relatively common, with intermittent viral shedding occurring during periods of immune suppression (1). The high incidence and clinical impact of adenoviral infections are recognized increasingly in the immunocompromised, sometimes leading to high morbidity and even mortality in these vulnerable individuals (5). In stem cell transplant patients, hAdV infections are associated generally with disseminated disease, while in solid organ transplants the primary site of infection tends to be the transplanted organ (6).

1.2. Diagnosis of Adenovirus Infections

1.2.1. Conventional Methods

Conventional methods for detection of hAdVs have been reviewed recently (1) and include electron microscopy, antigen detection, and virus isolation in cell culture. Electron microscopy has some utility for detection of gastroenteric hAdV serotypes in stool samples but may not be very sensitive. Commonly used direct antigen identification methods for detection of respiratory and gastrointestinal hAdVs include immunofluorescence, radioimmunoassay, or ELISA techniques. These have good specificity and provide a fast turn-around time. However, these tests are less sensitive than culture which is still the gold standard method for detection of hAdVs associated with respiratory and/or systemic infections, although gastroenteric type hAdVs generally grow poorly in vitro. Commonly used cell lines include human epithelial cell lines such as A549, Hep-2, and HeLa. The cytopathic effect of hAdVs is characterized by enlarged, rounded, refractile cells, which eventually aggregate into clusters or develop intranuclear inclusions with flattened cells and a web-like monolayer degeneration (7). Although virus isolation and propagation is relatively sensitive for many serotypes, it is slow and can be inhibited by neutralizing antibodies and other interfering substances, including the presence of fungal and bacterial contamination. Histological examination of tissues for presence of hAdV inclusions and immunohistochemical staining can also be performed. Diagnosis of adenoviruses by serological methods has limited sensitivity, requires paired sera and may not be reliable because of inadequate antibody production, especially in immunocompromised individuals. Serological testing would also only provide a retrospective diagnosis, although it may still be useful for epidemiological investigations and to confirm the association between virus detection and an unusual clinical outcome.

1.2.2. Nucleic Acid Amplification Tests

Detection and analysis of hAdVs is being increasingly performed by nucleic acid amplification tests (NATs). Methods based on PCR amplification have become the front-line diagnostic procedures

for many laboratories. As PCR involves amplification of viral DNA directly from patient specimens, it provides a fast turn-around time with good sensitivity. Generally, specificity would also be good for PCR assays with careful design of primers and probes.

Several in-house PCR assays using either gel-based or real-time detection of amplified products have been reported for the detection and analysis of hAdVs from a variety of specimen types. These assays may be designed to detect either the whole virus family or they may be species-specific or designed and used to infer serogroup. Commonly used targets for amplification are the hexon and fiber genes (8–10). In addition, conserved regions like the polymerase gene may be used for detection of a broad range of sequenced hAdVs (11).

The high sequence heterogeneity within the virus family makes detection of all serotypes in a single PCR assay challenging, particularly for a quantitative assay demanding equal sensitivity for the different serotypes. The hexon gene contains highly conserved regions allowing the design of specific primers for detection of all sequenced hAdVs; amplification can be undertaken across the hexon hypervariable regions that code for serotype-specific epitopes making more detailed phylogenetic analysis possible (12). In addition to the hexon gene, the fiber gene displays antigenic determinants on the viral surface, and assays targeting the fiber gene have also been used for detection and analysis of adenovirus serotypes (8, 13).

Several commercial assays based on NATs are available for detection of hAdVs in respiratory specimens. Conventional PCR approaches (based on end-point detection) provide, in general, only qualitative results and are slower than real-time assays. Kits using end-point detection include the Seeplex® range of assays (Seegene, Soeul, Korea) for detection of viruses (including hAdVs) in respiratory specimens (14). The adenovirus consensus assay from Argene (Varilhes, France) is an end-point PCR detection method using a biotinylated probe. This kit is CE marked for the detection of 51 serotypes and typing of seven hAdV species from nasopharyngeal, stool, biopsy, and plasma samples. The VaRNA gene kit from Argene can be used for the detection and typing of seven hAdV species.

Real-time PCR assays can be designed to be qualitative or quantitative and provide excellent sensitivity and fast turn-around time. The Adenovirus r-gene kit (Argene) is a 5′ nuclease real-time amplification and detection assay for six hAdV subtypes. A real-time PCR kit, proAdenoplus™ is also available from Prodesse Ltd (Wisconsin, USA) for use on a variety of real-time amplification platforms. The Adenovirus real-time PCR kit from Z-J Biotech Co., Ltd. (Shanghai, China) can also be used on a variety of real-time platforms.

Kits using nucleic acid amplification with suspension microarray detection of a broad range of respiratory viruses (including some hAdVs) have been developed and evaluated. Such assays include the xTAG™ Respiratory Viral Panel (RVP) from Luminex Molecular Diagnostics (Austin, Texas, USA), which is FDA-approved (USA) and CE-marked in Europe (15, 16). Other similar format assays, which are at various stages of regulatory approval, include the Respiratory MultiCode-PLx assay (EraGen Biosciences Ltd, Wisconsin, USA) (17–19) and ResPlex assays (Qiagen Molecular Diagnostics, California, USA) (20, 21).

1.3. Adenovirus Typing

Analysis of hAdVs to provide species and serotype-specific information may be valuable to predict outcome and severity of infection (4) as well as possible susceptibility to anti-viral therapy (22). Traditionally, hAdV typing was performed by neutralizing or hemagglutination inhibition assays on virus isolates, but these can be slow, and suitable antibody preparations are not available commercially for all serotypes. Serotyping can be incorporated into antigen detection assays using fluorescent labeled serotype-specific antibodies (e.g., assays from Argene Ltd). However, the sensitivity of this approach for direct application to clinical material (without culture) is not very high.

Molecular methods can help to overcome problems with traditional antibody-based methods for hAdV typing as they do not require pre-culture and specific antibodies. Also, methods based on analysis of nucleic acid provide additional information as changes in genotype are not always associated with serotype changes (23). The reported molecular methods used to infer serotype include a combination of PCR and restriction fragment length polymorphism, multiplex PCR, and amplification of hypervariable regions of the hexon gene followed by sequencing (24).

1.4. Interpretation of NATs for Detection and Analysis of Adenoviruses

Sensitive and specific detection with added sequence based typing of hAdVs using NATs can provide valuable information for effective antiviral therapy, outbreak investigations, and institution of infection control measures. However, latent hAdV infections with persistence of the viral genome have been reported (25); thus, care must be taken in interpreting results based on sensitive PCR amplification procedures. Infections in immunocompetent individuals may be self-limiting, and positive results for hAdV sequences must be interpreted in the clinical context. It is also important to differentiate localized disease from invasive disseminated disease as the latter may be associated with high mortality rates in vulnerable individuals (8). It has been reported that detection of hAdVs in blood precedes the onset of life-threatening virus disease and requires preemptive treatment (26), hence, due consideration should be given to the specimen type from which hAdV positive results were obtained.

It is possible to monitor viral kinetics in terms of viral load using quantitative PCR assays; this may help to distinguish between acute and low-level persistent infections in immunocompromised and other "at risk" individuals. This can also help to assess the success of antiviral therapy. Viral load determination in a patient at a single time point is not useful because severity of disease or outcome cannot be predicted from a single test. To gain maximum utility from quantitative PCR assays for hAdVs, it is better to monitor viral kinetics in sequential samples and evaluate the change in viral load over time.

1.5. Characteristics of the Reported PCR Assay

A multiplex real-time PCR method for the detection of a broad range of hAdV serotypes from a variety of specimens is described. This assay targets the hexon gene of hAdVs and uses hydrolysis probes for detection of amplified products. The assay can be adapted for use as a quantitative assay in combination with a standard curve. Details of the assay performance have been published (27), and the salient features are included below.

The AdV-2 primer and probe set detects serotypes 1, 2, 5, 6, 7, 8, 10, 19, 40, and 41 but not serotypes 3, 4, and 31. The AdV-4 primer and probe set detects serotypes 1, 3, 4, 5, 6, 7, and 40, but not serotypes 2, 8, 10, 19, 31, and 41. All hAdV serotypes are detected using the multiplex assay except serotype 31. Serotype 31 can be detected most efficiently upon reduction of the annealing temperature from 60 to 57°C, but samples with high serotype 31 viral loads may still be detected. The assay is specific for the detection of hAdVs in respiratory samples and does not cross-react with high copies of other respiratory pathogens. The limit of detection for the assay is 0.62, 1.01, 0.84, and 3,600 $TCID_{50}$/ml for serotypes 2, 4, 10, and 40 titrated viral stocks, respectively. Based on probit analysis, the limit of detection for the assay is 33 and 22 copies of hAdV2 and hAdV4 DNA, respectively, as shown in Fig. 1.

The linear range of the assay was between 9.1×10^0 to 9.1×10^7 and 3.8×10^0 to 3.8×10^7 copies of plasmid DNA/reaction for hAdV2 and hAdV4, respectively. Figure 2 shows amplification curves for tenfold serial dilutions of hAdV4 plasmid DNA used as a template with the standard curve as an inset. The standard curves generated using hAdV2 and hAdV4 plasmid DNA can be used in a quantitative assay to provide a copy number for estimating the viral load. The intra-assay coefficient of variation ranged from 0.22 to 1.93% for the hAdV2 plasmid and 0.18 to 1.81% for the hAdV4 plasmid. The inter-assay variability ranged from 0.57 to 2.22% and 0.72 to 1.27% for the hAdV2 and hAdV4 plasmid dilutions, respectively. The intra- and inter-assay coefficient of variation for four clinical samples tested in triplicate on three independent runs was ≤2.04% and ≤1.69%, respectively.

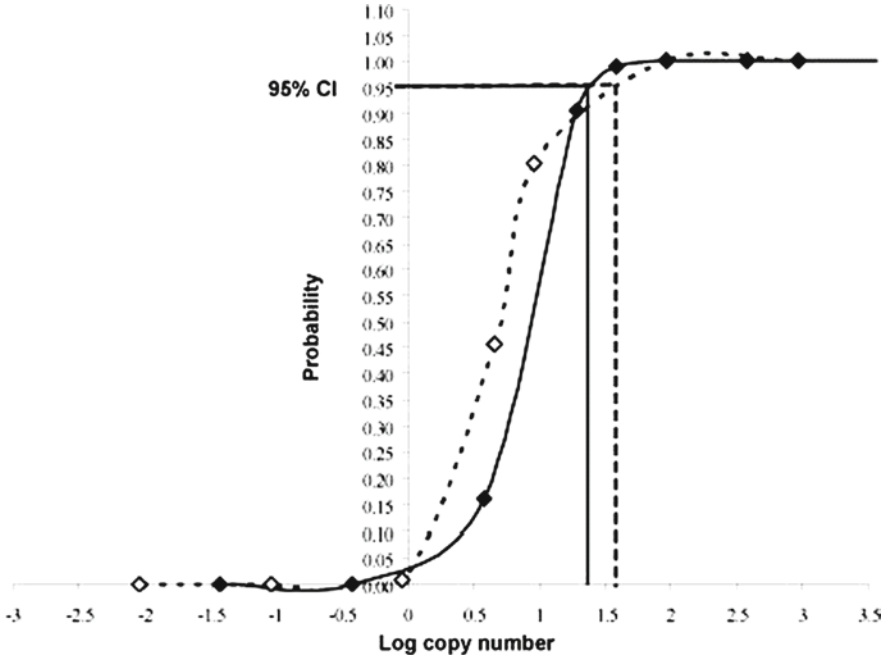

Fig. 1. Adenovirus PCR sensitivity by probit analysis. Results are based on testing 24 replicates at each dilution. Probit analysis shows that 33 and 22 copies of AdV2 and AdV4 plasmid, respectively, could be detected with 95% CI.

2. Materials

The source of equipment and specific reagents are indicated where it is critical to the success of the method. Specifics for equipment or reagents that are generally available and not critical to the method are not provided.

2.1. Extraction of DNA from Clinical Specimens

All easyMAG™ and NucliSens® equipment and reagents were obtained from bioMérieux Inc. (bioMérieux, Durham, NC, USA).

1. Powder-free gloves.
2. Transfer pipettes (single use plastic).
3. Micropipettes and aerosol-resistant tips (20–1,000 µl).
4. PCR-grade 1.5 ml screw-capped microfuge tubes.
5. PCR-grade water (550 µl aliquots).
6. easyMag extractor.
7. easyMag disposable plastics.
8. NucliSens lysis buffer (2 ml tube).
9. NucliSens easyMAG extraction buffers #1, #2, and #3.
10. NucliSens easyMAG magnetic silica.

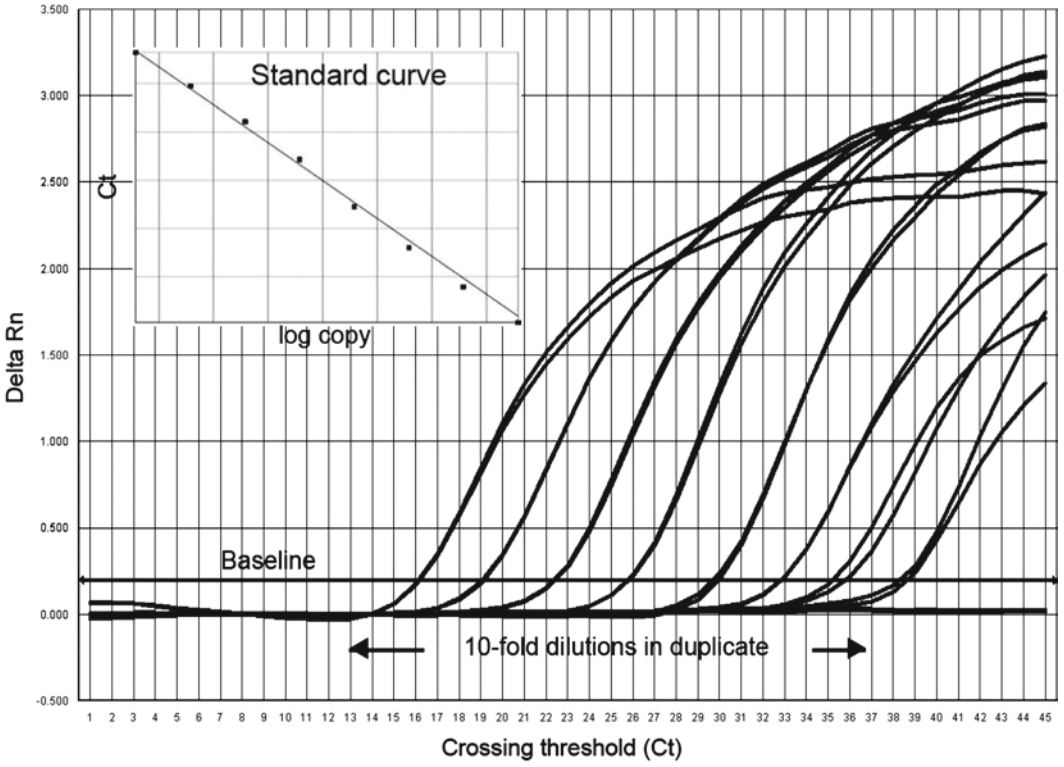

Fig. 2. Dynamic range and standard curve for adenovirus PCR. Amplification curves for tenfold serial dilutions of AdV4 plasmid DNA template in duplicate with concentration ranging from 3.8×10^0 to 3.8×10^7 copies/reaction. PCR was performed on the ABI 7500 SDS as described in the methods. Characteristics of the standard curve include slope: –3.14; *Y*-intercept: 40.52; R^2: 0.994. A similar standard curve was generated using tenfold serial dilutions of AdV2 plasmid DNA template with concentration ranging from 9.1×10^0 to 9.1×10^7 copies/reaction. Characteristics of this standard curve were; slope: –3.3; *Y*-intercept: 40.37; R^2: 0.998.

11. Protease diluted to 1 AU/ml (Qiagen, catalog no. 19157).
12. Vortex mixer.
13. Microfuge.
14. Thermomixer with temperature control.

See Note 1.

2.2. Real-Time Amplification and Detection of Adenoviruses

All MicroAmp®, TaqMan®, and Prism® equipment and reagents were obtained from Applied Biosystems Inc. (ABI, Foster city, California, USA).

1. Powder-free gloves.
2. Micropipettes and aerosol-resistant tips (20–1,000 µl).
3. Picofuge with standard and strip rotors.
4. 96-well plate centrifuge.
5. Cabinet with UV lamp in clean room for PCR set-up.

6. PCR-grade 1.5 ml screw-capped microfuge tubes.
7. PCR-grade water.
8. ABI Prism 7000 or ABI 7500 Real-Time Sequence Detection System (SDS).
9. ABI Prism reaction tube strips with support base and optical caps or MicroAmp optical 96-well reaction plates with support base, optical adhesive covers, and sealing device.
10. TaqMan Universal PCR Master Mix (2×) (ABI, catalog no. 4304437).
11. HAdV primers (gel purified): can be purchased from core DNA synthesis services or commercial sources.
12. HAdV probes (suitable for a hydrolysis probe real-time assay) can be purchased from ABI or other commercial sources.

See Note 2.

3. Methods

3.1. Extraction of Nucleic Acid from Clinical Specimens

3.1.1. Clinical Specimen Types

The main respiratory specimen types validated using the assay described were nasopharyngeal swabs, nasopharyngeal aspirates, throat swabs, endotracheal aspirates, and bronchoalveolar lavage. Other specimen types tested were plasma, cerebrospinal fluid, urine, eye swabs, tissue biopsies, stool, and pericardial fluid.

3.1.2. Isolation of DNA

Viral DNA was extracted from all respiratory specimen types using the easyMAG automated extraction system (bioMérieux) following the manufacturer's instructions. Table 1 shows the pretreatment, input and elution volumes for extraction of nucleic acid from the different specimen types used in the assay evaluation.

3.1.2.1. Procedure (See Note 3)

1. Combine 200 µl specimen with 25 µl 1 AU/ml protease in a screw-capped tube and vortex briefly (use 50 µl of protease if the sample is very viscous).
2. Incubate at 56°C with shaking for 15 min in a thermomixer. Viscous lower respiratory tract specimens should be incubated further to liquify the sample.
3. Spin the sample at 1000 rpm for 10 min and add it to the 2-ml tube of lysis buffer provided by the manufacturer and vortex briefly.
4. Using a transfer pipette, transfer the samples in lysis buffer to the sample cartridge, being careful to avoid cross-contamination between samples and creation of bubbles.
5. Add 550 µl of PCR-grade water to the 600 µl aliquot of magnetic silica provided in the kit and vortex.

Table 1
Methodology for extraction of different specimen types using the easyMAG

Specimen	Pretreatment	Input volume (µl)	Output volume (µl)
Respiratory[a]	Protease	200	110
Stool filtrate	Suspended in 500 µl PCR water	200	110
Urine	None	200	110
Eye swabs	None	200	110
Pericardial fluid	None	200	110
CSF	None	200	55
Plasma	None	1,000	55

Pretreatment, input and elution volumes for extraction of nucleic acid from different specimen types are listed
[a]Respiratory tract specimens evaluated included nasopharyngeal or throat swabs (collected into 3 ml of universal transport medium), nasopharyngeal aspirates, tracheal aspirates, and bronchoalveolar lavage

6. Add 100 µl of magnetic silica and water mixture to each sample cartridge and mix thoroughly.
7. Load the sample cartridge onto the easyMAG.
8. Run the program on the easyMAG using the appropriate input and elution volumes based on the specimen type as listed in Table 1.
9. Upon completion of extraction, transfer the extract to appropriately labeled tubes for testing.

See Note 4.

3.2. Amplification and Detection of Adenovirus DNA

All primer and probe manipulation as well as preparation of the master mix should be undertaken in a cabinet with a UV lamp in a clean room/dedicated space, where specimens and extracts are not handled.

3.2.1. Primers and Probes

Two sets of primers and probes (AdV-2 and AdV-4) were designed and utilized to facilitate detection of genetically diverse hAdV serotypes. Primers purified by polyacrylamide gel electrophoresis were used for the assay (available from any commercial or local core synthesis facility). The hydrolysis probes were labeled with 6-carboxyfluorescein (FAM) at the 5′ end and carboxytetramethyl-rhodamine (TAMRA) as a quencher at the 3′ end; these were purchased from ABI. Concentrated primer and probe stock solutions (200 µM and 100 µM, respectively) were stored at −40°C, and the working solutions (20 µM and 10 µM, respectively) were stored at −20°C.

The primer and probe sequences are listed below:

AdV-2 set	
Ad2-F	CCA GGA CGC CTC GGA GTA
Ad2-R	AAA CTT GTT ATT CAG GCT GAA GTA CGT
Ad2-probe	FAM-AGT TTG CCC GCG CCA CCG-TAMRA
AdV-4 set	
Ad4-F	GGA CAG GAC GCT TCG GAG TA
Ad4-R	CTT GTT CCC CAG ACT GAA GTA GGT
Ad4-probe	FAM-CAG TTC GCC CGY GCM ACA G-TAMRA

3.2.2. Preparation of Master Mix for DNA Amplification

1. Determine the total number of specimens and controls to be tested.
2. Use the table below to calculate the volume of reagents needed to make the master mix. Calculate the volume of each reagent required for (n+1) number of specimens (n = number of patient specimens + controls).

See Note 5.

Enzymes/primers/probes	Working stock concentration	Final concentration	Volume per test	Volume for master mix
Taqman Universal PCR Master Mix	2×	1×	12.5 µl	
Ad2-F	20 µM	0.4 µM	0.5 µl	
Ad2-R	20 µM	0.4 µM	0.5 µl	
Ad4-F	20 µM	0.4 µM	0.5 µl	
Ad4-R	20 µM	0.4 µM	0.5 µl	
Ad2-probe	10 µM	0.2 µM	0.5 µl	
Ad4-probe	10 µM	0.2 µM	0.5 µl	
PCR grade water	–	–	4.5 µl	
Total volume	–	–	20 µl	

3. Record the lot numbers and preparation dates of reagents, primers, and probes.
4. Label a 1.5-ml microfuge tube for master mix preparation and another microfuge tube for TaqMan Universal PCR Master Mix only (this will be used to monitor the baseline ROX signal). The ROX signal varies in different instruments

and must be monitored for laboratory quality control purposes.

5. Refer to the table above for preparing PCR Master Mix and, using aerosol-resistant pipette tips and pipettes, pipette the calculated amount of enzyme, primers, probes, and PCR water into the appropriately labeled 1.5-ml microfuge tube.
6. Pipette 28 µl of TaqMan Universal PCR Master Mix into a labeled tube.
7. Place the microfuge tubes containing master mix in an appropriate transport box to transfer to the extraction room/area.
8. Place the master mix in a cold block on the designated clean reaction set-up bench.

3.2.3. Setup of PCR

This is the stage where the clean master mix and reagents are combined with the extract for amplification.

If using strip tubes:

1. Place the appropriate number of ABI Prism optical reaction tube strips in a support base tray and label the strips.
2. Pipette 20 µl of master mix in to appropriate wells.
3. Pipette 5 µl of extracted sample or control to corresponding wells of strip tubes.
4. Pipette 25 µl of TaqMan Universal PCR Master Mix into one tube for monitoring the ROX signal.
5. Place ABI Prism optical caps on the reaction tube strips and close tightly.
6. Tap the capped strips gently to mix and then spin quickly in the picofuge to remove liquid from the caps and release bubbles.

If using reaction plates:

1. Place a 96-well reaction plate in the MicroAmp support base.
2. Pipette 20 µl of master mix to the appropriate wells.
3. Pipette 5 µl of extracted sample or control to corresponding wells.
4. Mix by pipetting up and down three times.
5. Press and smooth on an adhesive cover with the plastic sealing device to ensure a good seal.
6. Spin at 250 *g* for 2 min in the 96-well plate centrifuge to remove liquid from the cover and to release bubbles.

3.2.4. Taqman Assay

1. Use the following cycling conditions on the 7000 or 7500 SDS.

Step	Time (minutes)	Temperature
UNG incubation	2 min	50°C
Enzyme inactivation	10 min	95°C
PCR (45 cycles)		
Denaturation	15 s	95°C
Annealing/extension/acquisition	1 min	60°C

2. Place the tubes in the SDS and run the assay using the above cycling conditions.
3. Upon completion of assay analyze the run and print results.

3.3. Future Enhancements to NATs for Detection and Analysis of hAdVs

1. As mentioned above, many of the NATs described for detection of hAdVs can be adapted for evaluation of viral load, which may prove useful for assessing clinical relevance and for monitoring a patients' response to therapy. The increasing use of PCR-based procedures to amplify and detect hAdVs means that isolates are not readily available for assessment of serotype using antibodies. As sequence can be used to infer serotype (and provide additional genotype-specific information), longer regions of the hexon gene can be amplified for this purpose. This can be achieved using the same nucleic acid extract prepared for diagnostic screening as described above. This larger PCR product can then be subjected to capillary-based or other high-throughput sequencing to provide detailed epidemiological information.
2. Figure 3 shows an example of a phylogenetic tree based on sequences from different hAdV serotypes obtained from GenBank. Sequences from patients' samples were obtained using previously published primers (28); based on clustering with known serotypes, their relationship can be evaluated. Degenerate consensus primers have also been used for hAdV serotyping (29). Such a combination of PCR amplification and sequencing will prove useful in enhancing our understanding of hAdV distribution and clinical impact.

4. Notes

1. Extraction of DNA from clinical specimens: the easyMAG extractor gives total nucleic acid. Although the authors' experience is in using this extraction method for respiratory, plasma, and stool samples, other kit based procedures giving high quality DNA may be utilized.

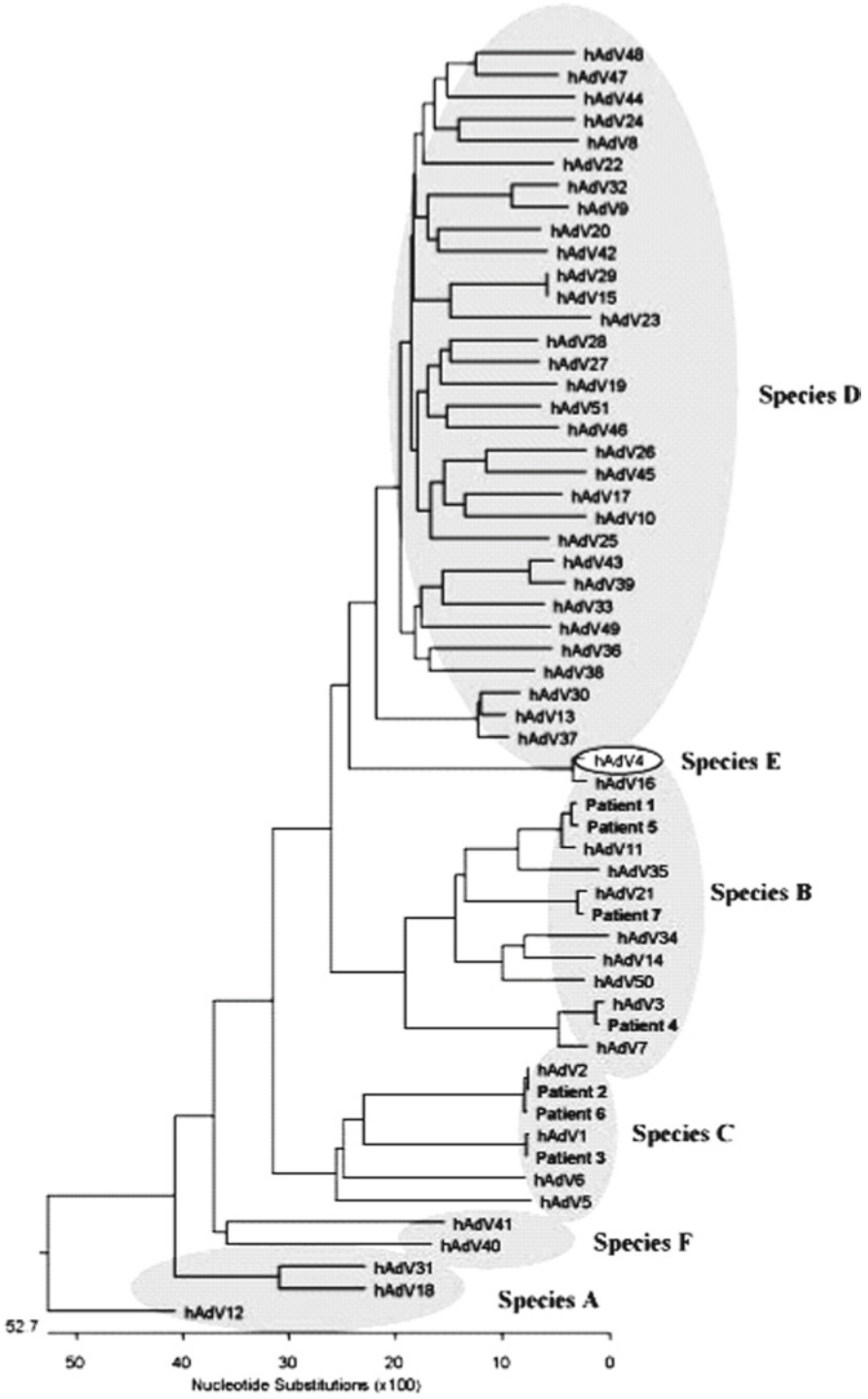

Fig. 3. Phylogenetic tree showing clustering of adenovirus serotypes. Tree was prepared based on partial hexon gene sequence using previously published primers (28). The hAdV species (A–F) is indicated. Hexon gene sequence from representative hAdV serotypes were obtained from GenBank. Also included are sequences amplified directly from patient specimens using previously published primers (28). The phylogenetic tree was constructed in the megalign module provided in Lasergene v6.

2. Real-time amplification and detection of adenoviruses: This assay was validated using the ABI Prism 7000 and 7500 SDS, but the methodology should be broadly applicable to a variety of real-time PCR amplification platforms.
3. Isolation of DNA: handling should be performed in a biological safety cabinet away from the clean PCR set-up area.
4. Isolation of DNA: if extracts are to be stored prior to setting up the amplification reaction, store at 4°C for <24 h or freeze at –80°C for >24 h.
5. Preparation of master mix for DNA amplification: add one extra sample for every ten samples (*n*) to allow for loss of volume during pipetting.

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Chapter 2

Alphaviruses

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Abstract

Alphaviruses remain important emerging mosquito-borne, zoonotic pathogens that cause both localized human outbreaks and epizootics (e.g., Venezuelan equine encephalitis) and large human epidemics (e.g., Chikungunya). Alphaviruses are globally dispersed, and each continent has humans at risk from one or more of these arthropod-borne viruses (arboviruses). Symptoms of human alphaviral disease range from frank, severe encephalitis (e.g., eastern and western equine encephalitis) to polyarthritis (e.g., Ross River). Diagnostic techniques to identify human alphaviral infections have changed dramatically with the development and implementation of standardized nucleic acid amplification tests (NAAT). The NAAT is rapidly replacing virus isolation and typing using indirect fluorescent antibody (IFA) assay with monoclonal antibodies (MAbs) as the preferred method of virus identification. The older techniques still have value, however, since alphaviral growth in cell culture is rapid, and IFA with MAbs is inexpensive. This chapter provides detailed, standardized protocols for the identification of alphaviruses from clinical specimens and the serological characterization of human infection-immune sera. Both laboratory approaches are needed to identify and confirm human infections with these agents.

Key words: Alphavirus, Nucleic acid amplification test, ELISA, Monoclonal antibodies

1. Introduction

Alphaviruses are enveloped, positive-stranded RNA viruses that are the etiologic agents of severe encephalitis and polyarthritis. These viruses can be divided into seven serocomplexes (1, 2). Four of these serocomplexes – represented by eastern equine encephalitis (EEE), western equine encephalitis (WEE), Venezuelan equine encephalitis (VEE), and Semliki Forest viruses – contain most of the medically important alphaviruses. The VEE serocomplex can be further divided into at least six subtypes (1–6), with subtype 1 having at least five different variations (1AB, 1C, 1D, 1E, and 1F). The importance of VEE virus subtyping is that varieties 1AB and 1C viruses cause epidemic/epizootic VEE infection, while disease

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caused by other VEE viruses is endemic/enzootic. Ross River, Chikungunya, Mayaro, and Getah viruses are members of the Semliki Forest serocomplex. Sindbis and Ockelbo viruses are members of the WEE virus serocomplex.

Laboratory diagnosis of human alphavirus infections has changed greatly over the last few years. In the past, identification of alphavirus relied on four tests involving the detection of antibodies: hemagglutination inhibition, complement fixation, plaque reduction neutralization test, and the indirect fluorescent antibody (IFA) test. Positive identification using these immunoglobulin M (IgM)- and IgG-based assays required a fourfold increase in titer between acute and convalescent serum samples. Good procedural reviews contain the specifics of these older assays (3).

With the advent of solid-phase antibody-binding assays, such as enzyme-linked immunosorbent assays (ELISA), the diagnostic algorithm for identification of viral activity has changed. Rapid serologic assays such as IgM-capture ELISA (MAC-ELISA) are now employed early in infection (4). In many cases, a positive MAC-ELISA with an acute serum sample precludes the need for testing of a convalescent serum sample. Early in infection, IgM antibody is more serocomplex specific, while later in the infection, IgG antibody is more serocomplex cross-reactive. Inclusion of monoclonal antibodies (MAbs) with defined virus specificities in these solid-phase assays has allowed for a level of standardization that was not previously possible. All tests described in this chapter are equally applicable to all alphaviruses.

Virus isolation and identification have also been useful in defining viral agents in serum, cerebrospinal fluid (CSF), or mosquito vectors. While virus isolation still depends upon growth of an unknown virus in cell culture or neonatal mice, virus identification has also been greatly facilitated by the availability of virus-specific MAbs for use in IFA assays. Similarly, MAbs with avidities sufficiently high to allow for specific binding to virus antigens in a complex protein mixture (e.g., mosquito pool suspensions) have enhanced our ability to rapidly identify virus agents in situ. Standardized polymerase chain reaction (PCR) nucleic acid amplification tests (NAATs) have now been developed to identify a number of alphaviruses (5, 6). These NAATs are now routinely used for rapid and routine identification of alphaviruses in the clinical or ecological settings.

2. Materials

2.1. General ELISA Materials List

The following materials list is employed in all subsequent ELISA procedures:

1. 96-well Immulon 2 microtiter plates (Dynatech Industries, Inc., Chantilly, VA).

2. Carbonate–bicarbonate (pH 9.6) coating buffer: 1.59 g of Na_2CO_3, 2.93 g $NaHCO_3$ in 1 L of distilled water (4).
3. Phosphate-buffered saline (PBS): BBL FTA-buffered saline (9.23 g/L, Becton Dickinson, Cockeysville, MD).
4. Blocking buffer: 5% skim milk, 0.5% Tween 20 in PBS.
5. Rinse buffer: 0.05% Tween 20 in PBS.
6. ELISA plate reader.
7. Refrigerator.
8. Humid incubator, 37°C.

2.2. Antigen Detection ELISA in Virus-Infected Mosquitoes

1. Grinding apparatus (Ten Broeck homogenizers or mortars and pestles).
2. Microcentrifuge with accompanying 1.5-ml microcentrifuge tubes.
3. Probe sonicator.
4. BA-1 diluent: 1× cell culture medium M199, 0.05 M Tris-HCl, 1% bovine serum albumin, 0.35 g/L $NaHCO_3$, final pH 7.6. Filter sterilize.
5. Lysis buffer: 5% Tween 20 in PBS.
6. Substrate: 3-3′,5-5′-tetra-methyl benzidine (TMB). Commercial source: TMB-ELISA reagent (GIBCO BRL, Gaithersburg, MD).
7. Stopping reagent: 1 N H_2SO_4.
8. Positive control antigen (suckling mouse brain [SMB] antigen of either EEE virus strain NJ-60, or WEE virus strain Fleming). Procedures for preparation of SMB antigens have been previously described (3).
9. Capture antibody: murine MAb, 1A4B-6, for EEE virus or 2A3D-5, for WEE virus (7, 8).
10. Detector antibody: murine MAb, 1B5C-3, conjugated to horseradish peroxidase (HRP) for EEE virus, or 2B1C-6, conjugated to HRP for WEE virus (7, 8).
11. Polyclonal control antibodies: procedures for producing murine polyclonal antivirus antibodies for use in the inhibition assay have been previously described (3).

2.3. MAC-ELISA

1. Previously titered goat antihuman IgM-capture antibody (Cappel Labs, Organon Teknika, Durham, NC).
2. Previously titered virus and control SMB antigens (3).
3. Previously titered HRP-conjugated MAb detector, 2A2 C-3 (8).
4. Known-positive human serum or CSF samples reactive with test viruses to serve as positive controls.

5. Known-negative human serum or CSF samples to serve as negative controls.

2.4. IgG ELISA

1. Capture antibody: Murine MAb, EEE 1A4B-6 (7).
2. Previously titered virus and control SMB antigens (3).
3. Detecting antibody: Goat antihuman IgG (Fc-specific)-alkaline phosphatase (AP) conjugate (Jackson Immunochemicals, West Grove, PA).
4. Known-positive human serum samples reactive with test viruses to serve as positive controls.
5. Known-negative serum samples to serve as negative controls.
6. Substrate: 3 mg/ml Sigma 104 in 1 M Tris pH 8.0 (Sigma Chemical Co., St. Louis, MO).
7. 3 M NaOH (120 g in 1 L of water) to stop reaction.

2.5. IFA Assay

1. Unconjugated MAbs of various specificity (Table 1).
2. Fluoresceinated antimouse antibody (Jackson Immunochemicals, West Grove, PA).
3. Sodium azide (as preservative).
4. Penicillin–streptomycin.
5. PBS.
6. Counterstain: Trypan blue diluted 1:4,000 in PBS.
7. Mounting solution: Aqua-mount (Lerner Labs, Pittsburgh, PA).
8. High-quality fluorescence microscope with epifluorescence interference filters and a tungsten light source.
9. Twelve spot IFA slides (Erie Scientific Co., Portsmouth, NH).
10. Cover slips (Corning Co., Corning, NY).

2.6. Alphavirus Reverse Transcriptase Polymerase Chain Reaction NAAT

1. RNA extraction kit (Qiagen, Valencia, CA) or Trizol (Invitrogen, Carlsbad, CA).
2. Chloroform.
3. Eppendorf tubes.
4. Primers.
5. Nucleotide triphospate mix (dNTPs).
6. RNasin (Promega, Madison, WI; or other equivalent RNase-inhibitor).
7. Reverse transcriptase (RT).
8. Thermocycler.
9. Dithiothreitol (DTT).

Table 1
Monoclonal antibodies useful in alphaviruses serology

MAb	Virus	Specificity	Serologic reactivity	Reference
1A2B-10	VEE peptide	E2	All VEE except TC-83	(10)
5B4D-6	VEE TC-83	E2	TC-83 specific	(11)
3B4C-4	VEE TC-83	E2	VEE 1AB, 1C, 1D, 2	(11)
1A3A-9	VEE TC-83	E2	VEE 1AB, 1C, 1D, 1E, 1F	(12)
1A1B-9	VEE Mena 2	E2	VEE 1D, 1E, 1F, 3	(13)
1A3B-7	VEE TC-83	E2	VEE complex	(12)
2B1C-6	WEE McMillan	E1	WEE specific	(8)
2A6C-7	WEE McMillan	E1	WEE complex	(8)
2A3D-5	WEE McMillan	E1	WEE complex	(8)
2D4-1	HJ original	E2	HJ specific	(14)
49	Sindbis Ar339	E2	Sindbis specific	(15)
1B5C-3	EEE NJ-60	E1	North American EEE	(7)
1B1C-4	EEE BeAn5122	E1	EEE complex	(7)
UM5.1	Semliki Forest	E2	Semliki Forest specific	(16)
2A2C-3	WEE McMillan	E1	All alphaviruses	(8)
1A4B-6	EEE NJ60	E1	All alphaviruses	(7)

10. PCR amplification kit.
11. Taqman polymerase.
12. Molecular weight markers.
13. Gel analysis supplies.
14. RNase-free water.
15. Isopropanol.
16. Magnesium chloride ($MgCl_2$).

2.7. Alphavirus Real-Time PCR NAAT

1. Bio-Rad iQ4 real-time detection system or equivalent (Hercules, CA).
2. Quantitect probe reverse transcriptase polymerase chain reaction (RT-PCR) kit (Qiagen no. 204445) or equivalent.
3. Optical plates in a 96-well or 48-well format (Bio-Rad no. 2239441) with sealing tape (Bio-Rad no. 2239444).
4. SYBR green super mix kit for optimization step (Bio-Rad no. 170-8880).

5. Various consumable (tips, 15 ml tubes, water, etc).
6. Optimized sequence specific oligonucleotides (primers and probes) see Table 2.
7. Standard curve, if applicable.

3. Methods

3.1. Antigen Detection ELISA in Virus-Infected Mosquitoes

Monitoring levels of virus in vector mosquitoes allows for rapid assessment of disease threat. The antigen-capture ELISA does not require use of expensive isolation techniques such as cell culture or animal inoculation. The test is not as sensitive as virus isolation in cell culture; however, if mosquito pools are kept below 25 individuals, the test has appropriate sensitivity to detect virus at levels necessary for alphavirus transmission (≥5.0 $\log_{10}$ Pfu).

3.1.1. Mosquito Pool Preparation

1. Using sterile procedures, triturate pools of suspect mosquitoes (25 mosquitoes/pool or less) in 1.5 ml BA-l buffer by standard protocol. We have used two methods. Method one employs homogenization in Ten Broeck tissue homogenizers. Method two employs homogenization in mortars and pestles (8). The method of trituration seems to be less important than the pool size. Preliminary data indicate that as pool size increases from 25 to 50 or 100, the ELISA signal is diminished. The diminution of ELISA signal with larger pools is probably associated with the larger concentrations of irrelevant material in the larger pools.
2. Following trituration, centrifuge the suspension in a microfuge at 12–14,000 *g* for 2 min. At this point, a small amount of the sterile supernatant can be removed for subsequent virus isolation in plaque assay in Vero cells. Split the remaining volume into two aliquots. Reserve one aliquot for confirmation of testing. The second aliquot serves as antigen for the antigen-capture ELISA.
3. Also prepare at least six independent pools of normal, noninfected mosquitoes to serve as ELISA-negative antigens.
4. Resuspend one 0.25 ml aliquot (including the pellet). Sonicate each sample in a biosafety cabinet using a microprobe at 100 W for 10 s. Centrifuge in a microfuge at 12–14,000 rpm for 2 min. Transfer the supernatant to new vial. Immediately before ELISA testing, add 10 µl lysis buffer per 100 µl mosquito pool sample. Addition of the lysis buffer frees virus antigens from larger particles. Incubate for 15 min at room temperature. After incubation, centrifuge in a microfuge at 12–14,000 rpm

Table 2
Real-time PCR primers for alphaviruses

Chikungunya		**Tm**
Fwd primer:	Chik 243- 5′ GAY CCC GAC TCA ACC ATC CT	52.6
Rev primer:	Chik 330- 5′ CAT MGG GCA RAC GCA GTG GTA	55.8
Probe(FAM):	Chik 273- 5′ AGY GCG CCA GCA AGG AGG AKG ATG T	65.6
Chikungunya (alternate)		**Tm**
Fwd primer:	Chik 10051- 5′ TCA ACA GAC CGG GCT ACA G	50.8
Rev primer:	Chik 10091- 5′ CTG TAC CGC AGC ACT TCA CG	50.8
Probe(FAM):	Chik 10137- 5′ CGT GCG AGT ACA AAA CCG TCA TC	58.3
Chikungunya (alternate)		**Tm**
Fwd primer:	Chik 874- 5′ AAA GGG CAA GCT CAG CTT CAC	49.2
Rev primer:	Chik 961- 5′ GCC TGG GCT CAT CGT TAT TC	48.7
Probe(FAM):	Chik 899- 5′ CGC TGT GAT ACA GTG GTT TCG TGC G	55.9
O'nyong-nyong		**Tm**
Fwd primer:	Onn 10692- 5′ GCA GGG AGG CCA GGA CAG T	56.2
Rev primer:	Onn 10840- 5′ GCC CCT TTT TCY TTG AGC CAG TA	57.7
Probe(HEX):	Onn 10759- 5′ AAA GAC CAG CGG CAG GAG CAA TAC AC	64.2
Western equine encephalitis		**Tm**
Fwd primer:	WEEV 8028- 5′ ACA AAC CAC CGG GCT TCT ACA	55.9
Rev primer:	WEEV 8126- 5′ GTC GCC TTT TCC GCC CAC TCC T	55.0
Probe(FAM):	WEEV 8058- 5′ CAC CAT GGC GCA GTC CAG TAT GAG AAT	64.8
Semliki Forest		**Tm**
Fwd primer:	SFV 1243- 5′ GATTGTGGCCGTCGCATTTAGC	60.3
Rev primer:	SFV 1353- 5′ GCCCACAAGCAGCAGCAAGTAA	59.1
Probe(FAM):	SFV 1266- 5′ AGTGGGCGAGGGAATACAAGGCAGACC	67.4
Mayaro		**Tm**
Fwd primer:	MAYV 9666- 5′ CATGGCCTACCTGTGGGATAATA	54.6

(continued)

Table 2 (continued)

Rev primer:	MAYV 9797- 5′ GCACTCCCGACGCTCACTG	56.4
Probe(FAM):	MAYV 9734(–)- 5′ TCGGGCGCAACATGTAGTCAGGATAA	64.1
Highlands J		**Tm**
Fwd primer:	HJV 9741- 5′ ACTTGCCGCACTTATCATCCTGTT	57.9
Rev primer:	HJV 9866- 5′ CCACTAGCGCTTTATACGGGACTC	57.3
Probe(FAM):	HJV 9838- 5′ CATGCGACCACTGTGCCAAATGTTCC	66.7
Eastern equine encephalitis – North American Strains		**Tm**
Fwd primer:	EEEV 9391- 5′ ACACCGCACCCTGATTTTACA	60.0
Rev primer:	EEEV 9459- 5′ CTTCCAAGTGACCTGGTCGTC	62.0
Probe(FAM):	EEEV 9414- 5′ TGCACCCGGACCATCCGACCT	70.0
Venezuelan equine encephalitis – subtype IE		**Tm**
Fwd primer:	VEEV 2800- 5′ GACGCGGAAAAGTGTCTATGC	54.0
Rev primer:	VEEV 2901- 5′ TCTTCCGTGCGGGTCAACAA	58.6
Probe(FAM):	VEEV 2843- 5′ AACCCCTTGTACGCACCCACCTCAGA	66.1
Fort Morgan		**Tm**
Fwd primer:	FMV E1- 5′ GTGCTGCGGGATTCTGGAGT	56.4
Rev primer:	FMV E1- 5′ GGCCCCTCCCCACATAAAC	55.3
Probe(FAM):	FMV E1- 5′ CAACAGCGAGCCGACTATGCCTGTAGAGT	66.1

for 2 min. The supernatant from this centrifugation will be the mosquito pool antigen used in the antigen-capture ELISA (see Subheading 3.1.2).

3.1.2. Antigen-Capture ELISA

1. Dilute the capture antibody (1:20,000 of MAb 1A4B-6 for EEE virus or 1:5,000 of MAb 2A3D-5 for WEE virus (7, 8) in coating buffer. Coat wells of a 96-well Immulon 2 microtiter plate with 100 µl capture antibody per well. Incubate coated plates for 18 h at 4°C.
2. Rinse plates five times with ELISA rinse buffer.
3. Block plates with 300 µl per well blocking buffer for 1 h at 37°C. Repeat the rinse step.
4. Add 100 µl per well detergent-treated mosquito pool antigen. Test mosquito pools in triplicate. Incubate plates for

18 h at 37°C. Include space for six normal uninfected mosquito pool homogenates. These normal homogenates will be used to calculate the test background. Also include space for positive control antigen diluted 1:1,000 and treated with lysis buffer (step 4, Subheading 3.1.1). Use positive control antigen at 100 µl per well. Repeat the rinse step.

5. Add 100 µl per well detector antibody 1B5C-3-HRP-conjugate, diluted 1:1,000 for EEE virus detection or 2B1C-6-HRP conjugate diluted 1:5,000 in ELISA rinse buffer for WEE virus. Incubate for 1 h at 37°C.
6. Rinse ten times with ELISA rinse buffer.
7. Add 100 µl per well of substrate (TMB-ELISA). Incubate for 30 min at room temperature and stop the reaction with 50 µl per well 1 N H_2SO_4. Measure the absorbance at 450 nm (A 450 nm) in a microplate reader.

3.1.3. Inhibition Assay

All mosquito pools presumed to be positive for viral antigen should be tested in the inhibition assay.

1. Dilute EEE, or WEE, and St. Louis encephalitis virus polyclonal antibodies 1:20 in PBS (9).
2. Mix 100 µl mosquito supernatant with 20 µl of either EEE virus or WEE virus polyclonal antibody. Also mix 100 µl mosquito supernatant with 20 µl St. Louis encephalitis virus polyclonal antibody, incubate at 37°C for 1 h. If there is enough mosquito supernatant, perform the procedure in duplicate.
3. Add the mixture to an ELISA plate, see step 4 in the above procedure. Incubate overnight at 4°C. Perform the ELISA as described in Subheading 3.1.2, step 4.
4. If the mean absorbance value of the pool is reduced by 50% or more when it is preincubated with the polyclonal antialphavirus antibody, the sample is considered specific for alphavirus antibodies.

3.1.4. Data Analysis

1. Derive the mean A 450 nm for each duplicate or triplicate and also the mean of the six normal mosquito pool samples. The negative cutoff will be twice the A 450 nm of the mean of the six normal mosquito pools. Any experimental pool with a mean A 450 nm greater than twice the mean of the A 450 nm of the six negative control pools should be considered presumptive for the presence of EEE or WEE virus antigen. These pools should be verified by testing in the inhibition assay (see Note 1).
2. The experimental sensitivity of this assay is 3.5–4.0 $\log_{10}$ Pfu per 0.1 ml. Pools with titers lower than this cutoff will give negative or variable results.

3.2. MAC-ELISA

Assays that detect virus-specific IgM are advantageous because they detect antibodies produced within days of infection, obviating the need for convalescent-phase specimens in many cases. The MAC-ELISA is the optimum approach to detect IgM because capturing the antiviral IgM antibody negates the competitive effects seen with antiviral IgG in the more standard indirect ELISA format. The MAC-ELISA is simple, sensitive and applicable to serum and CSF samples. False-positive reactions owing to rheumatoid factor are also minimized.

3.2.1. ELISA

1. Coat 96-well Immulon 2 plates with 75 µl per well of goat antihuman IgM in coating buffer pH 9.6. Coat enough wells to test each sample against both positive and negative antigens in triplicate. Do not use the outer wells on the plate. Incubate for 18 h at 4°C. Wash the plates in a microplate washer five times with rinse buffer.
2. Block the plates with 300 µl per well blocking buffer. Incubate covered plates at room temperature for 30 min. Repeat the wash step.
3. Add 50 µl per well of the patient's serum diluted 1:400 in rinse buffer or add the patient's CSF undiluted to six wells. Incubate for 1 h at 37°C. Also test appropriately diluted positive control human serum and a normal human serum. Repeat the wash step.
4. Dilute virus-infected SMB antigen in rinse buffer according to the previous titration. Add 50 µl per well to three wells of each test sample. To the other three wells add 50 µl per well of normal SMB antigen diluted in the same manner. Incubate overnight at 4°C. Repeat the wash step.
5. Add 50 µl per well of HRP-conjugated MAb, 2A2C-3, diluted as per the previous titration in the blocking buffer. Incubate for 1 h at 37°C.
6. Repeat the wash step twice.
7. Add 75 µl per well of TMB substrate. Incubate at room temperature for 10 min.
8. Add 50 µl per well of 1 N H_2SO_4 to stop the reaction. Leave at room temperature for 1 min. Read the plates in a microtiter plate reader at 450 nm.

3.2.2. Data Analysis

Calculate the positive/noise (P/N) values as follows:

$$\frac{\text{Average 450 nm reading of patient's serum plus antigen } (P)}{\text{Average 450 nm reading of normal human serum plus antigen } (N)}$$

The P/N ratio must be at least 2. The positive human serum control P/N ratio should be at least 2 and the normal human

serum control *P/N* ratio should be less than 2. If any OD readings or control serum *P/N* values fall outside these threshold values, the test must be repeated. All patient *P/N* values greater than or equal to 2 should be reported as positive (see Note 2).

3.3. IgG ELISA

Rapid testing for IgG antibody in a solid-phase assay precludes the necessity for other IgG measuring tests such as hemagglutination inhibition, complement fixation, and plaque reduction neutralization tests. Serologic cross-reactivity in the IgG response to the alphaviruses makes the IgG ELISA less specific than the MAC-ELISA. The ELISA assay design of this IgG test allows for concurrent application with the MAC-ELISA. The use of a MAb capturing antibody allows for easy standardization of antigen quantities between laboratories.

3.3.1. ELISA

1. Dilute the MAb 1A4B-6 1:10,000 in coating buffer and coat wells of 96-well microtiter plates with 75 µl for 16 h at 4°C. Coat enough wells to test each sample against both positive and negative antigens in triplicate. Wash the plates in a microplate washer five times with rinse buffer.
2. Block the plates with 300 µl of blocking buffer per well for 30 min at room temperature. Repeat the wash step.
3. Add 50 µl per well of appropriate SMB virus or control antigen (see MAC-ELISA Subheading 3.2.1, step 3) diluted in rinse buffer and incubate overnight at 4°C. Rinse the plate five times with rinse buffer.
4. Add 50 µl per well of unknown sera diluted 1:400 in rinse buffer and incubate for 1 h at 37°C. Rinse the plates five times with rinse buffer.
5. Add 50 µl goat antihuman IgG (Fc-specific)-AP conjugate diluted 1:1,000 in rinse buffer per well and incubate for 1 h at 37°C.
6. Rinse the plates ten times with rinse buffer.
7. Add 75 µl per well of substrate (Sigma 104) and incubate for 30 min at room temperature. Stop the color development, if necessary, by adding 25 µl 3 M NaOH per well, and read absorbance at 405 nm.

3.3.2. Data Analysis

P/N ratios are determined as with the MAC-ELISA. Ratios greater than or equal to 2 are considered positive (see Note 3).

3.4. IFA Assay

Immunofluorescence tests provide a useful means of identifying viral antigen directly in clinical specimens and of providing specific immunologic identification of isolates in the laboratory (17–19). If the antigen is known, the presence of specific antibodies in a test serum may also be documented. After incubation of

antiserum and antigen, the presence of a reaction is detected by observation of fluorescence in a microscope that is equipped with a source of ultraviolet light. A sequence of filters is used to generate exciting light of optimal wavelength and to block light of harmful wavelengths before viewing.

3.4.1. Infecting Cells and Preparing Spot Slides

1. Select a cell culture type appropriate for the virus to be used. Inoculate a monolayer culture less than 1 week old with the virus seed stock.
2. Incubate at 37°C and observe daily for virus cytopathic effects. When it involves at least 25% of the cell sheet, harvest the cells, saving the media for virus seed (if necessary).
3. Dilute the harvested cells so that sufficient cells are added to each spot on the slide. Add about 10 µl of diluted cells to each well on the slide.
4. Allow slides to air dry for at least 2 h. Fix the slides in cold acetone for 15 min, dry and store at –70°C.

3.4.2. IFA Assay

1. Remove antigen slides from –70°C and allow to air dry or optionally, refix in cold acetone for 10–15 min.
2. Dilute all antibodies in PBS with 0.1% sodium azide and 2% penicillin/streptomycin. Dilute the MAb ascites to appropriate concentrations. Add 10–12 µl of diluted antibody to one well on the spot slide. Run all necessary control slides (see controls).
3. Incubate in a moist chamber for 1 h at 37°C.
4. Wash slides for 15 min in PBS. Allow to air dry.
5. Add 10–12 µl of pretitrated antimouse antibody (see Subheading 3.4.3, step 1) conjugated to fluorescein isothiocyanate made up in 1:4,000 trypan blue with 0.1% sodium azide to each well. Incubate for 1 h at 37°C in a moist chamber. Repeat the wash step.
6. Add the mounting solution and coverslip. Then examine the slides by fluorescence microscopy no later than 24 h after completing the procedure. Store the slides at 4°C. A positive reaction appears as apple-green fluorescence against a background of red counterstained cells.

3.4.3. Test Controls

3.4.3.1. Titrating MAbs and Conjugates

1. Each new lot of MAb and commercial conjugate must be titrated before use. This is best performed in a box titration. Choose several antiviral antibodies for which you have homologous antigen slides.
2. Serially dilute the MAb in PBS with 0.1% sodium azide and 2% penicillin–streptomycin, starting at 1:100 in twofold dilutions to 1:10,240.

3. Refix the antigen slides as described in Subheading 3.4.
4. Add 10–15 µl of each detecting antibody in the dilution series to one spot on the antigen slide. Incubate at 37°C for 1 h. Wash the slides as described in Subheading 3.4.2.
5. Prepare a dilution series for the fluorescein isothiocyanate conjugate in 1:4,000 trypan blue with 0.1% sodium azide.
6. Add 10–15 µl of each dilution in the series to one set of the dilutions of detecting antibody. Incubate at 37°C for 1 h. Wash the slides as described in Subheading 3.4.2.
7. Affix coverslips as described in Subheading 3.4.
8. Read the slides with a fluorescence microscope. There must be at least 100 cells per well to assess accurately the extent of the antibody–antigen reaction. Optimum conjugate dilution is that dilution which yields intense 4+ fluorescence at the highest antibody dilution (see Subheading 3.4.4). This dilution is used in all subsequent tests performed with this conjugate lot.

3.4.3.2. Normal Tissue Culture Cell Slide

A slide of uninfected cells prepared in the same manner as for infected cells must be run in the same manner as the unknowns in all tests. This slide indicates any nonspecific reaction between the antibody and normal tissue culture cells of the type used.

3.4.3.3. Serum and MAbs

1. Use normal sera from the species in which the antibody was produced to show the level of nonspecific fluorescence between the species and the tissue culture cell type used.
2. Use a homologous antigen slide and an unrelated antigen slide for each MAb used in the test. Use the homologous MAb, and another unrelated antibody on each of the above slides. This will demonstrate that the MAb is specific for the antigen and shows no cross-reactivity. Use a "normal" MAb which is an ascites produced from mice inoculated with parental Sp2/0-Ag14 myeloma cells. Alternatively, any MAb specific for an antigen other than alphaviruses (e.g., flaviviruses) can be used as a negative control. This preparation will show if the procedure for producing the MAb causes any nonspecific reactions between the antibody and the tissue culture cells used.

3.4.4. Data Analysis

When the slides are read, each well is ranked upon the following scale: 4+ (positive cells fluoresce *intensely*); 3+ (positive cells fluoresce brightly); 2+ (positive cells fluoresce to some degree, less than brightly); 1+ (cells fluoresce dully); +/– (varying degrees of fluorescence that may or may not be specific); – (no fluorescence of the cells; cells appear red from the counterstain). Positive wells

must be ranked 2+ or higher. Results are reported as a simple positive or negative by IFA (see Note 4).

3.5. Alphavirus RT-PCR NAAT

3.5.1. RNA Extraction

Any RNA extraction method can be used. However, this method describes the use of Viral RNA extraction kits from Qiagen (Valencia, CA) following the manufacturer's protocol; generic alternatives, such as the one described here, using Trizol reagent (Invitrogen), can also be used as an alternative (see Note 5).

1. Add 100 µl of cell culture supernatant to 1-ml Trizol, followed by 2 µl tRNA (10 mg/ml), and mix by inversion.
2. Let the sample stand for 5 min at room temperature.
3. Add 0.2 ml chloroform, vortex for 15 s, stand at room temperature for 2 min.
4. Centrifuge at 12,000 × *g* for 10 min at 4°C.
5. Transfer the aqueous phase to a clean, RNase free Eppendorf tube. Add 0.5 ml of isopropanol, mix well, then incubate at room temperature for 10 min.
6. Centrifuge at 12,000 × *g* for 10 min at 4°C.
7. Remove the supernatant and air dry briefly.
8. Add 18 µl of depc-treated dH_2O and 2 µl RNasin.

3.5.2. Titan One-Step RT-PCR Reaction (Titan One-Tube RT-PCR Kit; Roche Applied Science, Indianapolis, IN)

1. Prepare the Master Mixes no. 1 and no. 2 as follows:

Master mix no.1			Master mix no. 2	
rxns	**1 rxn**	**Reagent**	**1 rxn**	**rxns**
	12.25	dH_2O	14	
–	–	5× buffer	10	
	2.5	DTT (10 mM)	–	
	1	dNTP mix (10 µM)	–	
	2	Primer 1 (10 µM)	–	
	2	Primer 2 (10 µM)	–	
	0.25	RNasin (40 U/µl)	–	
–	–	Enzyme mix	1	
Totals:	20 µl		25 µl	

2. Transfer all of Master mix no. 1 into Master mix no. 2, and pipette up and down to mix thoroughly.
3. Aliquot 45 µl of combined master mixes per tube, then add 5 µl of RNA per tube.

4. Amplify as follows (note thermocycler used, tube numbers, and program):

Control method

Step	Temp(°C)	Time
1	50	30:00
2	94	2:00
3	92	0:10
4	49	0:30
5	68	1:30
6	Go to 3, 10 times	
7	92	0:10
8	49	0:30
9	68	1:30 + 5 s/cycle
10	Go to 7, 25 times	
11	68	7:00
12	4	Forever
13	End	

3.5.3. Superscript II (Invitrogen) Two-Step RT-PCR Reaction (Useful When Multiple PCR Reactions Utilizing Different Primers Are Desirable)

1. cDNA synthesis – Add 5 µl of RNA to the following mixture:

   2 µl RNasin

   1 µl cDNA primer (100 ng/µl, alternative concentration = 2 pmol primer)

   2 µl DTT (0.1 M)

   4 µl reverse transcription buffer (5×; Superscript II buffer)

   2 µl dNTP's (10 mM)

   6 µl depc dH_2O
2. Incubate at 42°C for 2 min.
3. Add 1 µl (200 U) reverse transcriptase (Superscript II).
4. Incubate at 42°C for at least 1 h.

3.5.4. PCR

1. For a 100 µl reaction, add the following (50 µl reactions also work just fine and save on reagents).

Described here is the 100 µl reaction which provides sufficient products for sequencing at a later date.

Promega Taq (or nonproofreading Taq):	Stratagene PfuTurbo (or any proofreading Taq):
5 µl cDNA	5 µl cDNA
3 µl reverse primer (100 ng/µl)	3 µl reverse primer (100 ng/µl)
3 µl forward primer (100 ng/µl)	3 µl forward primer (100 ng/µl)
8 µl $MgCl_2$ (25 mM)	2 µl dNTP's (10 mM)
2 µl dNTP's (10 mM)	10 µl 10× buffer
10 µl 10× buffer	77 µl dH_2O
69 µl dH_2O	

2. Load on to 80°C on thermocycler (Hot start procedure).
3. Add Taq (0.5 µl; Promega) or Pfu (1.0 µl; Stratagene) polymerase.
4. Amplify for 30 cycles using the following format: 94°C for 30 s, 49°C for 30 s, and 72°C for 1–2 min/kb.
5. Perform the final extension for 10 min at 72°C.

3.5.5. Data Analysis

1. Run 10 µl of product on a 1% agarose gel to visualize the final product. Alphavirus products range from 1.2 to 1.6 kb.

3.5.6. Primers Used

This primer set works on virtually all alphaviruses tested to date. It generates a product beginning in the E1 gene proceeding through the 3′ noncoding region to the end of the genome.

- For cDNA synthesis and reverse primer: 5′-TTTTTTTTTTT TTTTTTTTTTTTTTV-3′ (Poly T with 25 T residues and "V" residue (mix of A, G, C) at 3′ terminus.
- Forward primer: 5′,-TACCCNTTYATGTGGGG-3′, (Designated Alpha 10247 A).

3.6. Alphavirus Real-Time PCR NAAT

This protocol is designed to detect and accurately quantify positive-strand viral RNA from a variety of sources and is based on the Qiagen Quantitech probe (TaqMan) RT-PCR kit. The RNA must be in a purified form, which is usually achieved from a column-based kit extraction system (QiaAmp viral RNA kit or equivalent). This is a one-step protocol (RNA→cDNA) with no separate reverse "transcription" step needed. Prior to analyzing unknown samples, users should optimize each oligonucleotide set according to steps described in Note 6.

3.6.1. Reaction Setup

1. Turn on the fluorescent lamp and instrument base before launching the software. Turn off in reverse order.

2. Allow lamps to warm for 10 min prior to performing the run.
3. Gather materials (plate, sealant tape, tips, pipettes, etc.) and setup in a PCR hood or biosafety cabinet.
4. Thaw the primers, master mix, probe, and place in ice (cover the probe since it is light sensitive).
5. Determine the number of standards, unknown samples, and controls to be run. The controls should consist of a no-template (NTC), a no-reverse transcriptase (NRTC), and a RNA extraction control (extracted with unknown samples).
6. Design the plate map which describes the placement of each sample.
7. Calculate the reaction volumes (use the volumes in the calculation worksheet).

Quantitect probe RT-PCR Kit reaction setup sheet

	Plate setup	No. of samples	Total no.	
	RNA stds	6	6	
	Samples[a]	33	66	
	(−) Control	3	3	
	Overage	1	1	
		T=	76	
Components	**Stock conc.**	**Final conc.**	**µl/rxn**	**Total volume**
RNA	×	×	5–10 µl	×
2× Quant. RT PCR master mix	×	×	25	1,900
Quant. RT mix	×	×	0.5	38
Fwd primer	40 µM	[b]400 nM	0.5	38
Rev primer	40 µM	[b]400 nM	0.5	38
Probe	25 µM	[b]150 nM	0.3	22.8
Water (Rnase-free)	×	×	qs	qs
		T=	50	3,420

[a]Run in duplicate
[b]Optimized concentration; see Table 2 for primer and probe sequences

8. Assemble the reaction components in a 15 ml conical tube or equivalent on ice.
9. Aliquot the mix into each plate well with a repeat pipettor.

10. Add the standard curve and unknown sample RNA to appropriate well (mix with a pipette).
11. Seal the plate with plate sealing tape and place into the cycler.

3.6.2. Software Setup: See the Instruments Resource User Guide

1. Open the iCycler software if not already opened.
2. Choose the correct thermocycler program, for example:

Cycle no.	Temp(°C)	Time
1	50	30 min
2	95	15 min
3	94	15 s
4	60	1 min
5	Go to step 3	45×
6		END

3. Input the sample information into the plate grid and select an appropriate dye layer (FAM, HEX, etc).
4. Input the standard curve information (i.e., 10^{-1} = 20,000 Pfu, etc.) and select an appropriate dye layer.
5. Start the run.

3.6.3. Data Analysis

1. Observe the amplification curves and adjust the threshold line within the log phase of the amplification plot only if the default software setting is too low (Fig. 1).
2. The PCR efficiency of the RNA standards should be between 90 and 100%.
3. The controls should be reading N/A (no amplification) or at an acceptable threshold cycle (C_T) level.
4. Create data reports which can be generated by the iQ software.
5. Export the data into an excel sheet to analyze data.

3.6.4. Standard Curves

1. A standard curve is necessary in order to quantify your unknown RNA sample. Since RNA is being used in these assays instead of DNA, a RNA standard curve must be generated and run with each plate of unknown samples. Usually the RNA standards are derived from a viral stock (Fig. 2).
2. Create an RNA standard by performing a tenfold serial dilution of a known titer viral stock by adding 50 µl of stock virus into 450 µl of media and doing a tenfold dilution series ($10^{-1} \rightarrow 10^{-6}$ series should be sufficient).

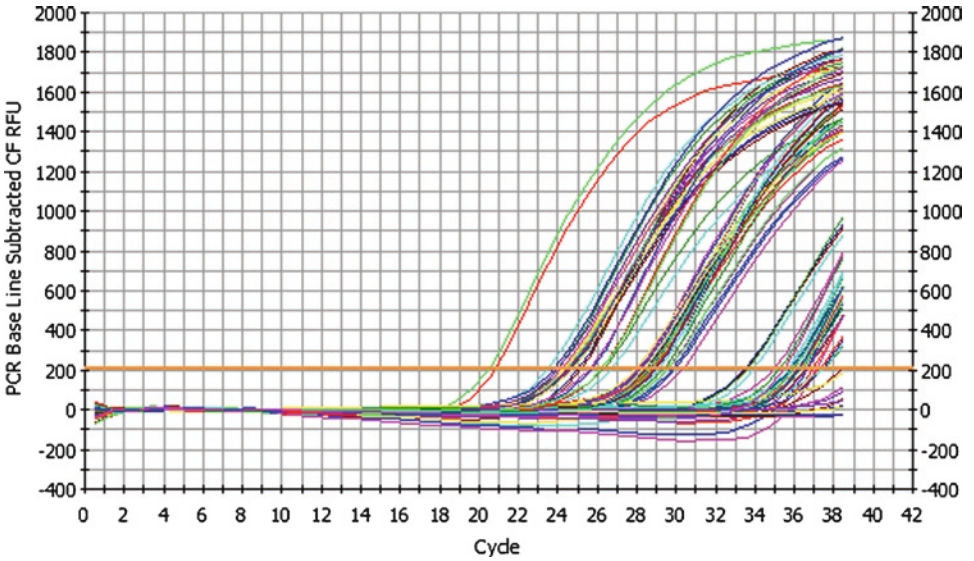

Fig. 1. Sample amplification plot with threshold (*bold gray line*.).

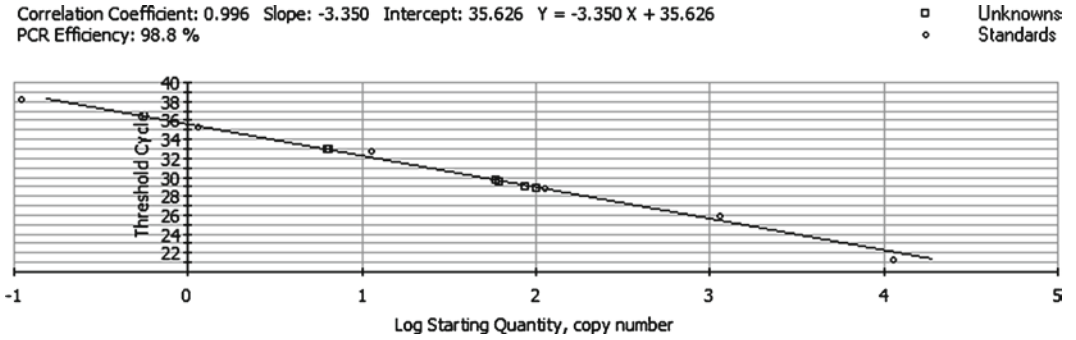

Fig. 2. Sample standard curve plot with PCR efficiency.

3. Perform a plaque assay on each dilution of the series as if each were an individual sample.
4. A series of six standard dilutions will be a total 12 plaque assays run in duplicate.
5. Input the calculated PA values into the real-time software for each standard dilution.
6. "Standard" is sample type and "quantity" equals calculated plaque assay titer.
7. On the same day of the plaque assay, extract RNA (QiaAmp Viral RNA kit or equivalent) from each dilution of the series.
8. The elution volume of the standard curves should be the same as the unknown sample.
9. Aliquot several tubes for each dilution to minimize freeze/thaw and place into −70°C.

4. Notes

1. Antigen-capture ELISA: (1) If you are unsure about the results because absorbance values are close to the negative cutoff, retest the pool, or use a backup test such as the plaque assay in Vero cells. (2) The serologic reactivities of the MAbs used in these assays are shown in Table 1. (3) Occasionally, high backgrounds with uninfected control mosquitoes may be observed. In this case the test should be repeated. (4) For this and all other ELISA assays, MAb reagents are in the form of mouse-ascitic fluids. MAb-enzyme conjugates are commercial preparations using ascitic fluids supplied by our laboratory (Jackson Immunochemicals, West Grove, PA). Reagent potency may vary depending upon preparations and should be independently determined before use.
2. MAC-ELISA: (1) Store all diagnostic specimens at –20°C prior to and after testing. Avoid repeated freeze–thaw cycles, which tend to inactivate IgM. (2) This test is used if serum or CSF samples have been drawn within 45 days of onset. (3) In the event that a *very* early CSF or serum is negative by this test, a convalescent serum specimen must be requested and tested before that patient is reported as negative for serological evidence of recent viral infection. Without testing of a convalescent specimen, a negative result may reflect testing of an acute-phase specimen obtained before antibody response. (4) Occasionally the test serum will be highly positive when tested with the normal SMB antigen. The reason for this is unknown. If this happens, the test should be repeated. If high backgrounds persist, another test must be used.
3. IgG ELISA: (1) We have tried a number of detector antibodies in this test. The IgG (Fc-specific)-AP conjugate gives the best results with the lowest backgrounds. (2) Using the 1A4B-6 MAb as a capture antibody for all alphaviruses allows for easy antigen standardization. (3) Remembering that antialphavirus IgG is in general more cross-reactive that IgM; therefore, the specificity of this test is less than that of MAC-ELISA.
4. IFA assay: (1) If any of the controls do not perform within the expected reaction range, the test must be repeated. (2) Unlike normal polyclonal antiviral antibodies, MAb reagents are of extremely high potency. Be sure to dilute them appropriately. Using MAb reagents at low dilutions can result in false-positive staining. This high activity is why it is imperative to quantitate MAb dilution by endpoint box titration prior to use.
5. Alphavirus RT-PCR NAAT: (1) Clean, size-specific amplicons can be used for follow-up work such as sequencing.

6. Alphavirus real-time PCR NAAT: The user must perform the following steps prior to running unknown samples. These steps will insure that a single amplicon is achieved per oligonucleotide set as well as maximize reaction efficiency. The following is a general guideline. Specifics can be found in the instrument resource guide or in the real-time reagent kit user's manual. (1) Primer design: (a) Always design primers that will be specific for your product and design before the probe. (b) Use the Primer Select or other comparable primer design software. (c) Follow the user's resource manual primer guidelines (i.e., no G's on 5 end, etc). (d) The primer amplicon should be between 75 and 200 bp in length. (e) The primers T_m should be 10°C less than probe T_m. (f) Avoid designing primers in secondary structures (http://www.bioinfo.rpi.edu/applications/mfold). (g) Perform a nucleotide BLAST on the chosen primers in order to assure specificity. (h) Test new primers with SYBR green or other DNA binding kit. This permits you to see if primer dimers are present. (i) Run three concentrations of DNA template with primers. (j) Run a melt-curve analysis with the real-time software at the end of the amplification. (2) Probe Design (TaqMan): (a) Always order the probe after the primer pair has been optimized to minimize cost. (b) Use Primer Select or other comparable design software. (c) Follow the user's resource manual probe guidelines (i.e., no Gs on 5,,end, etc.). (d) Use a 5 -reporter dye (FAM, TET, HEX, etc) and a 3, quencher (Tamra, etc.). (e) Make several working stock (25 μM) aliquots to minimize freeze/thaw. (3) Optimizing oligonucleotide concentrations: This step is done to determine the minimal oligo concentration giving the lowest C_T with maximum fluorescence. These steps will also minimize nonspecific amplification. (a) Run a one-step real-time PCR reaction by varying the primer concentrations (50, 300, 900 nM) while keeping a constant probe concentration and RNA template (tenfold dilution series with three or more concentrations). (b) Repeat with varying probe concentrations (50–250 nM) while using the optimized primer concentration from the previous step and constant RNA template. (c) Perform a run with the optimized oligonucleotide concentrations on a tenfold series of RNA standards. (d) The PCR efficiency should be between 95 and 105%.

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and conclusions in this report are those of the author(s) and do not necessarily represent the views of [the Centers for Disease Control and Prevention/the Agency for Toxic Substances and Disease Registry]."

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Chapter 3

Detection of Human Caliciviruses in Fecal Samples by RT-PCR

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Abstract

Human caliciviruses, noroviruses in particular, are a common cause of gastroenteritis in persons of all age groups. Although both antigen detection and serologic methods for diagnosis of infection with these viruses have been described, the best and most common methods used for diagnosis are molecular assays. Traditional RT-PCR methods are commonly used for diagnosis, but these require the use of a confirmatory test (such as probe hybridization or sequencing of amplicons). More recently, real-time RT-PCR assays have been developed that allow the rapid and accurate identification of caliciviruses in fecal samples. There is no single primer set that allows the detection of all strains within a calicivirus species, and separate primer pairs are generally used to identify strains belonging to different norovirus genogroups. Inhibition of nucleic acid amplification by substances contained within fecal samples is a common problem facing the diagnostician, but protocols to effectively remove the majority of such inhibitors have now been developed. This chapter describes methods for sample collection and processing of fecal specimens for molecular detection of enteric viruses, and it also describes both traditional and real-time RT-PCR assays for norovirus diagnosis.

Key words: Calicivirus, Norovirus, Feces, RT-PCR, Genogroup

1. Introduction

Human caliciviruses (HuCVs) are common causes of gastroenteritis (1). HuCVs belong to the family *Caliciviridae* and are nonenveloped, icosahedral viruses with a single-stranded, positive-sense RNA genome. There are two genera that contain HuCVs: *Norovirus* and *Sapovirus*. Each genus is further subdivided into at least five genogroups (designated GI-GV) based upon sequence similarities across the genome. Genogroups are further subdivided into genotypes based upon the amino acid sequence of the major capsid protein. The prototype human calicivirus, Norwalk virus, is classified as a GI.1 (genogroup I, genotype 1) norovirus (NoV).

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HuCVs cannot be propagated in cell culture at this time, so it is not possible to classify them serotypically. Thus, it is uncertain whether the genotype designation will correspond to serotype when such a classification scheme becomes available.

HuCVs have a genome that is 7.5–7.7 kb in length. The 5′ end is capped by a genome-linked viral protein (VPg), and the 3′ end is polyadenylated. The NoV genome has three open reading frames (ORFs), and the sapovirus (SaV) genome has only two ORFs (Fig. 1). For NoVs, the first ORF encodes a polyprotein that is cleaved to form the nonstructural proteins (including from amino to carboxy terminus an N-terminal protein, an NTPase, a 3A-like protein, the VPg, a protease, and an RNA-dependent RNA polymerase). The second ORF encodes the major capsid protein (VP1) and the third ORF encodes a minor capsid protein (VP2). For SaVs, the VP1 gene is in the first ORF along with the nonstructural proteins. NoVs produce a subgenomic RNA that contains ORF2 and ORF3 (2). Recombination between NoV genotypes (within a genogroup) has been reported to occur near the ORF1–ORF2 overlap, leading to increased viral diversity (3). Similar findings are less well described for SaVs but do occur (4).

Expression of the VP1 protein, with or without the VP2 protein, leads to the formation of virus-like particles (VLPs). The VLPs are morphologically identical to intact virions, which are 28–40 nm in size. Most NoVs have some evidence of structure when viewed with an electron microscope, leading to their previous classification as small round-structured viruses (SRSVs). On the other hand, most SaVs have a more defined structure, with deeper cup-like depressions on the surface that can give a "Star-of-David"

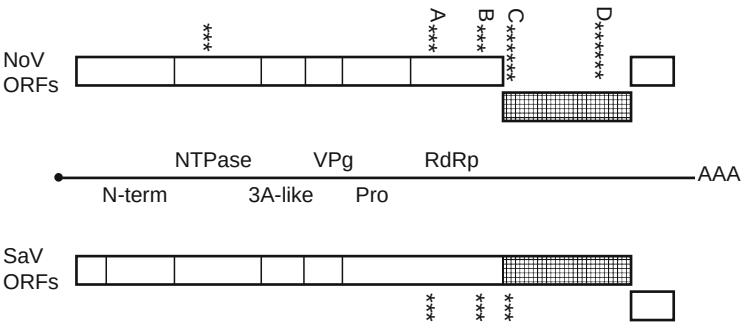

Fig. 1. Genomic organization of human caliciviruses and locations targeted by diagnostic RT-PCR assays. The ORFs for noroviruses (NoV) and sapoviruses (SaV) are shown, with putative nonstructural and structural protein locations noted. Locations of genes encoding putative nonstructural and structural (VP1 and VP2) proteins are shown, using nomenclature NoV proteins. *Asterisks* identify locations targeted by RT-PCR assays. The letters A–D indicate the names of genomic regions, commonly targeted by different NoV-specific RT-PCR assays. The hatched area identifies the location of the major capsid protein (VP1) for each virus. *ORF* open reading frame; *N-term* N-terminal; *Pro* protease; *RdRp* RNA-dependent RNA polymerase.

appearance to the virion. However, some NoVs also have the "Star-of-David" appearance in EM, and some SaVs have the morphology of SRSVs. Therefore, morphologic identification of the virus can result in misclassification of an individual strain (5).

VLPs have been used to develop both monoclonal and polyclonal antibodies against HuCVs. These antibodies have then been used to develop antigen enzyme immunoassays for NoVs and SaVs. All of the antigen detection assays developed to date have lacked sensitivity. Thus, these assays have had the greatest utility in identifying outbreaks (where multiple stool samples can be tested) rather than in identifying the individual patient who is infected with a HuCV (6).

RT-PCR (reverse transcription-polymerase chain reaction) assays are currently the most sensitive diagnostic methods available for the detection of HuCVs. Unfortunately, no single primer pair will detect all NoVs or SaVs, but many assays that detect a broad range of strains are currently available. This chapter will describe sensitive RT-PCR methods for identification of HuCVs in fecal samples. Two methods for obtaining RNA for amplification will be described, providing alternative approaches that provide some flexibility in sample analysis. Similarly, both a standard RT-PCR method as well as a real-time RT-PCR assay will be presented.

2. Materials

2.1. Reagents and Equipment Used for Extraction of Viral RNA from Stools

1. Purified water (e.g., Millipore, Milli-Q UF Plus system).
2. Heat block or water bath at 95°C.
3. Vertrel XF (1,1,1,2,3,4,4,5,5,5-decafluoropentane), Miller-Stephenson MS-782.
4. QIAamp Viral RNA Mini Kit, Qiagen 52904.
5. Yeast tRNA, Invitrogen 15401-029.
6. 100% Ethanol.
7. Microcentrifuge.

2.2. Reagents and Equipment Used for Standard RT-PCR

1. Purified water (e.g., Millipore, Milli-Q UF Plus system).
2. GeneAmp 10× PCR Buffer I, Applied Biosystems (provided with AmpliTaq).
3. Recombinant ribonuclease inhibitor (rRNasin), 40,000 U/mL (Promega N2511).
4. Deoxynucleoside triphosphate mixture (dNTPs) (e.g., Fisher BP2564-4), dATP, dGTP, dCTP, and dTTP, pooled and reconstituted to 10 mM each.
5. Avian myeloblastosis virus reverse transcriptase (AMV-RT), 20 U/μL, Life Sciences AMV 007.

6. AmpliTaq recombinant *Taq* DNA polymerase, Applied Biosystems N8080160.
7. Custom-synthesized oligonucleotides (e.g., Integrated DNA Technologies) suspended as 50 µM in Milli-Q water for use as primers and probes (7):

Target NoV genogroup	Primer[a]	Probe[b]	Sequence[c] (5′–3′)	Sense	Genomic location[d]
Genogroup I	Mon432		tggacIcgYggIccYaaYca	+	5093–5112
	Mon434		gaaScgcatccaRcggaacat	–	5285–5305
		Mon458	atgtatgtRccaggatggcaRgcc	+	5261–5284
Genogroup II	Mon431		tggacIagRggIccYaaYca	+	4820–4839
	Mon433		gaaYctcatccaYctgaacat	–	5012–5032
		Mon459	atggatttttacgtgcccaggcaa	+	4982–5005

[a]Oligonucleotides have to be HPLC purified after synthesis
[b]Probes to be end-labeled with digoxigenin
[c]I: inosine; R: a or g; S: c or g; Y: c or t
[d]Corresponds to the nucleotide position of Norwalk/68 virus (M87661) for genogroup I and Camberwell virus (AF145896) for genogroup II

8. 10× TBE buffer: 108 g Tris base, 55 g boric acid, and 9.3 g disodium EDTA per liter.
9. Programmable thermocycler.
10. Standard agarose (e.g., GenePure LE, ISC BioExpress E3120-500).
11. 10× gel loading buffer (e.g., 10× Blue Juice, Invitrogen 10816-015).
12. Ethidium bromide solution (e.g., AMRESCO X328).
13. Positively-charged nylon membrane (e.g., Immobilon NY+, Millipore INYC000010).
14. VacuGene XL Vacuum Blotting System (GE Healthcare 80-1266-24).
15. Molecular Weight Marker VIII (Roche 11 336 045 001).
16. Molecular Weight Marker VIII, DIG-labeled (Roche 11 449 451 910).
17. Denaturation solution: 87.9 g NaCl, 20.0 g NaOH per liter.
18. Neutralizing solution: 121 g Tris base and 87 g NaCl per liter, adjusted to pH 7.5 using HCl, filtered through a 0.45 µm pore membrane.
19. Transfer solution (i.e., 20× SSC): 175.3 g NaCl, 88.2 g sodium citrate dihydrate per liter, adjusted to pH 7.0 using HCl.
20. ExpressHyb hybridization solution (Clontech Laboratories 636832).

21. DIG Oligonucleotide 3′-End Labeling Kit, 2nd Generation (Roche 03 353 575 910).
22. 0.5× wash solution: 0.5× SSC, 0.1% sodium dodecyl sulfate (SDS).
23. 2× wash solution: 2× SSC, 0.1% SDS.
24. 10× Genius buffer: 121 g Tris base, 87.8 g NaCl per liter of purified water.
25. Blocking solution: 2% Blocking Reagent (Roche 11 096 176 001) in 1× genius buffer.
26. Antibody solution: Anti-Digoxigenin-AP (Roche 11 093 274 910) diluted 1:5,000 in blocking solution.
27. Detection buffer: 12.1 g Tris base and 5.86 g NaCl, per liter of purified water, adjusted to pH 9.5 using HCl, filtered through a 0.45 µm pore membrane.
28. NBT solution (Roche 11 383 213 001).
29. BCIP solution (Roche 11 383 221 001).
30. 10× TE buffer: 12.1 g Tris base and 3.6 g disodium EDTA per liter of purified water, adjusted to pH 8.0 using HCl, filtered through a 0.45 µm pore membrane.

2.3. Reagents and Equipment Used for Real-Time RT-PCR

1. DNase and RNase-free water. Suitable ultrapure water is available commercially.
2. 2× ThermoScript™ Reaction mix (buffer containing 0.4 mM of each dNTP and 3 mM $MgSO_4$) (Platinum Quantitative RT-PCR Thermoscript™ one-Step System, InVitrogen). It is better to use a separate ROX buffer, so the concentration may be adjusted depending on the apparatus used.
3. Primers and probes (8).

Target NoV genogroup	Primer[a]	Probe[b]	Sequence[c] (5′–3′)	Sense	Genomic position[d]
Genogroup I	NVLC1R		ccttagacgccatcatcatttac	–	5351–5375
	QNIF4		cgctggatgcgNttccat	+	5291–5309
		NVGGI	tggacaggagaYcgcRatct	+	5321–5340
Genogroup II	COG2R		tcgacgccatcttcattcaca	–	5080–5100
	QNIF2d		atgttcagRtggatgag RttctcWga	+	5012–5037
		QNIFS	agcacgtgggagggcgatcg	+	5042–5061

[a]Oligonucleotides have to be HPLC purified after synthesis
[b]Taqman probes are labeled with the 5′reporter fluorophore FAM (6-carboxyfluorecein) and the 3′ quencher dye Tamra
[c]N: a, c, g or t; R: a or g, W: a or t, Y: c or t
[d]Corresponds to the nucleotide position of Norwalk/68 virus (M87661) for genogroup I and Camberwell virus (AF145896) for genogroup II

4. Real-time instrument: This assay has been utilized successfully on:
   - ABI prism 7000, 7300 SDS detector (Applied Biosystems, France)
   - MX3000 (Stratagene).
5. Microplates developed for the real-time instruments and adhesive plastic covers to seal the plates.
6. Quantitation standards. We cloned a fragment of Norwalk virus (nucleotides 4487–5671) and Houston virus (nucleotides 4191–5863) into the pCRII TOPO (Invitrogen) vector. Each vector was transformed in *Escherichia coli*, and transformant clones were screened. Plasmids were extracted, linearized, and transcribed in vitro using the Promega riboprobe system. After DNAse treatment, RNA standards were purified and quantified by optical density at 260 nm (OD_{260}).

3. Methods

3.1. Storage of Stool Specimens

Fresh fecal samples can be stored at 4°C for weeks without significant loss in viral RNA titer. Samples that are to be kept for a longer period of time should be stored frozen. We prefer to store the virus at –70°C, although the virus can be stored at –20°C.

Prepare a 10% suspension of fecal material to make a working stock. For example, add approximately 50 mg, or 50 µL of watery stool, to 450 µL of purified water. Vortex (mix) the suspension vigorously for 15 s. The 10% suspension can be stored at 4°C and used in either extraction method described below.

3.2. Extraction of Viral RNA from Stools

A number of methods have been described for extraction of viral RNA from stools. The main purpose of this step is to remove substances present in the fecal sample that may inhibit the enzymatic activity of the reverse transcriptase or DNA polymerase used in the RT-PCR. The simplest approach to accomplish this task is the heat release method, where the sample is simply diluted and the viral RNA is released from the viral capsid by heating to 95°C (9). However, this method does not remove RNases present in the sample, so such samples must be analyzed quickly (same day) to prevent loss of target RNA due to degradation by RNase activity. The second method, the modified Boom method (10), yields purified RNA that can then be stored frozen at –70°C for analysis at a later time. A variety of kits that utilize this approach are commercially available.

The RNA extraction step should be performed in a location separate from that used for PCR amplification and postPCR analysis to prevent carryover contamination. Separate equipment (e.g., pipettors, microfuges) and gowns should also be used. The day's work schedule should be arranged so that postPCR analysis does not occur prior to RNA extraction or PCR set-up.

3.2.1. Heat Release Method

1. Preheat the heat block to 95°C. Dilute the 10% suspension 100-fold in purified water to a final volume of 50 µL. Transfer at least 5 µL at each step of the dilution.
2. Heat the 1:100 dilution of stool suspension for 5 min at 95°C. To avoid long periods during which the RNA is unprotected, heat the samples in staggered groups of 5 or 6, 2.5 min apart.
3. Quench the sample immediately in ice/water mixture, and within 2.5 min, transfer up to 20 µL of sample to the prepared RT mix in a reaction tube.

3.2.2. Modified Boom Method

1. Use 200–500 µL of 10% stool suspension. Add an equal volume of Vertrel and vortex for 30 s followed by centrifugation for 30 s in a microcentrifuge.
2. Prepare the QIAamp Viral RNA Mini Kit according to the manufacturer's instructions (http://www1.qiagen.com/HB/QIAampViralRNAMiniKit_EN) with the following substitution. Omit the poly-A RNA supplied with the kit; instead add an equivalent amount of yeast tRNA to the suspension buffer. This approach is necessary for assays that target the 3′ poly-A site for cDNA synthesis or amplification.
3. Transfer 140 µL of the supernatant resulting from step 1 to a 1.5-mL microcentrifuge tube containing the modified suspension buffer. Any unused Vertrel-extracted sample may continue to be stored at 4°C since the Vertrel forms a separate phase at the bottom of the tube. Proceed using the Spin Protocol provided with the Kit including the recommended centrifugation to prohibit any Buffer AW2 carryover. We perform a double elution using 2×40 µL of Buffer AVE.
4. Use up to 20 µL of the purified viral RNA in RT-PCR. Store the unused portion at –70°C.

3.3. Standard RT-PCR Method (see Note 1)

1. *RT reaction*: Prepare a RT master mix according to the following formula. Increase the total volume as needed to allow for loss. Keep all reagents on ice once thawed. Minimize the time that the enzyme is outside of the freezer. Add 10 µL of the master mix to each reaction tube.

RT-mix component	Per reaction
Purified water	0.25 µL
10× PCR buffer	3.00 µL
dNTPs mixture	2.00 µL
rRNasin	0.50 µL
Primer MON433	2.00 µL
Primer MON434	2.00 µL
AMV-RT	0.25 µL
	10.00 µL

Add up to 20 µL of viral RNA and/or purified water for a final 30 µL for each reaction. Incubate in a thermal cycler for 60 min. at 43°C. Follow this with 5 min. at 94°C to inactivate the reverse transcriptase. Cool to 37°C or lower and place on ice. Add 70 µL of PCR mix to each sample on ice.

2. *PCR*: Prepare a PCR master mix according to the following formula. Increase the total volume as needed to allow for loss. Keep all reagents on ice once thawed (see Note 2).

PCR-mix component	Per tube
Milli-Q water	58.0 µL
10× PCR buffer	7.0 µL
MON 431	2.0 µL
MON 432	2.0 µL
AmpliTaq	1.0 µL
	70.0 µL

Add 70 µL of PCR mix to each sample on ice. Incubate at 94°C for 2 min, followed by 40 cycles of the following: 92°C for 15 s, 50°C for 30 s, and 72°C for 30 s. Finish PCR with 72°C for 7 min.

3. *Gel electrophoresis*: Mix 20 µL of each PCR product with 2 µL of 10× gel loading buffer. Load equal portions of this mixture on two 1.5% agarose gels containing 0.5 µg/mL of ethidium bromide. Load into one lane of each gel, 2 µL of unlabeled Molecular Weight Marker VIII plus 5 µL of Digoxigenin-labeled Molecular Weight Marker VIII and gel loading buffer. Electrophorese the gel in 0.5× TBE until the expected band of 213 bp is well resolved by observation over UV light (see Note 3).

4. *Southern blot*: Transfer the PCR products to positively charged nylon membranes using the VacuGene XL system according to the manufacturer's instructions (http://www.gelifesciences.co.jp/tech_support/manual/pdf/nastain/56113080af.pdf), with the following modifications. Simply, wet the nylon membranes with distilled water prior to placing them on the gel support. Omit the depurination step. Leave the denaturation and neutralizing solutions on the gels for 9 min. Leave the transfer solution for 1–2 h. Following transfer, lay the membranes face down over the UV light for 3 min. Allow the membrane to dry overnight or for about 15 min in a 50°C oven. Drying is necessary for good binding of the nucleic acid.
5. *Probe hybridization*: Prepare digoxigenin-labeled probes MON458 and MON459, using the DIG Oligonucleotide 3′-End Labeling Kit, and hybridize them separately to the membranes according to the manufacturer's instructions (https://www.roche-applied-science.com/pack-insert/3353575a.pdf) with the following considerations. Cut the membranes, if necessary, to fit in hybridization bottles. Do not stack membranes within bottles. We typically label 200 pmol of each oligonucleotide and use half of it in a 15-cm hybridization bottle. Use ExpressHyb instead of DIG Easy Hyb. Conduct prehybridization and hybridization at 50°C. Hybridization for 1 h is sufficient the. Hybridization buffer can be frozen after use and may be used many times. Carry out the posthybridization washes with gentle agitation. The 0.5× SSC washes are conducted at 45°C. Proceed immediately to the next step.
6. *Antibody reaction*: For each of the following steps, place the membranes in a clean tray and incubate in the specified reagent with gentle agitation at room temperature.
   (a) A small amount of 1x Genius Buffer for 1 min.
   (b) Blocking buffer sufficient to allow free flow for 30-60 min.
   (c) Antibody solution for 30 min with gentle agitation.
   (d) 100 mL of Genius Buffer for 15 min. Drain and repeat.
   (e) A small amount of detection buffer for 2 min.
7. *Colorimetric detection*: During step 6.d above, prepare the color substrate solution by adding 50 µL of NBT solution plus 37.5 µL of BCIP solution per 10 mL of detection buffer. Place the membranes face up and separated in a clean dry tray and gently pour the color substrate solution on top so that it covers them completely but runoff is minimal. Cover the tray to exclude light and incubate for 1–16 h until the color reaction has produced bands of the desired intensity. Pour off the

color substrate solution, rinse gently with distilled water, and incubate 5 min in a small amount of 1× TE buffer. Photocopy or photograph the blots immediately or after allowing them to dry in the dark. Documentation is important since the background color development increases over time, especially where the blot is in contact with clear tape, sheet protectors, etc.

3.4. Real-Time RT-PCR Method

1. After NA extractions, prepare the samples to be tested (undiluted and 1:10 dilution), negative, and positive controls (GI and GII reference strains or transcripts).

   In a separate room under a hood:

2. Adjust the primer and probe concentrations to 10 µM in RNAse-free sterile water. *Note: repeated freeze–thaws can damage the labeled probe, so freeze the diluted probe in small aliquots.*
3. Prepare sufficient master mix for the assay:

   Standard curve: four concentrations in duplicate, enough for eight wells

   Samples: undiluted sample and 1:10 dilution of sample, each in duplicate

   Negative controls: include one negative control for every six wells (three samples)

   Calculate the number of wells needed and add one extra.

Reagents	Volume (µL)/ well	Final concentration
Water	2.375	–
2× Thermoscript mix	12.5	1×
Downstream primer (10 µM)	2.25	900 nM
Upstream primer (10 µM)	1.25	500 nM
Probe (10 µM)	0.625	250 nM
Rox reference dye 50×[a]	0.5	1× or 0.1×[a]
ThermosScript Plus/*Taq*Platinum	0.5	5 U

[a]Use 0.1× for MX 3000P apparatus;

4. Mix all reagents in an Eppendorf tube by vortexing; centrifuge briefly and place in the microplate. *Note: avoid the corners and, if possible, the edges when using adhesive cover (evaporation may occur by accident).*
5. Cover the plate and move to a separate room to add NA extracts. Add 5 µL of each sample using filter tips and the negative controls (if possible use the water used for NA suspension).

Close this part of the plate using adhesive of caps. Move to another room or to a dedicated cabinet to add the dilutions of transcript RNA for the standard curve. Begin by adding the highest dilution using filter tips.

6. Cover the plate, tap the plate two or three times to eliminate any bubbles, and centrifuge the plate for 30 s.
7. Add the plate to the real-time PCR machine. The amplification cycle conditions are as follows: reverse transcription at 50°C for 30 min, denaturation at 95°C for 5 min, and then 50 cycles of amplification, with denaturation at 95°C for 15 s and primer annealing and extension at 60°C for 1 min.
8. Interpret the results. The cycle threshold (Ct) is the cycle at which a significant increase in fluorescence occurs in rRT-PCR (i.e., when the fluorescence becomes distinguishable from the background). Check negative controls and standard curve to validate the plates. If a standard curve is used, the quantity of target RNA can be calculated. The method of calculation varies based upon the software available with the real-time thermal cycler.

4. Notes

1. Although both of the extraction methods describe as in Section 3.2 are effective at removing inhibitors of amplification, up to 5–10% of samples may still contain inhibitors sufficient to prevent virus detection. One approach to identify these inhibitors is to add a known number of RNA transcripts that will be amplified by the virus-specific primers but will yield amplicons that can be distinguished from those generated from viral RNA (e.g., different length, different reactivity with a probe) (9).
2. A number of different primers and probes have been described for the detection of NoVs and SaVs, and many different assays are used around the world (4, 5, 11, 12). No single assay is 100% sensitive, and different primers and probes can be substituted for those described above.
3. A variety of methods can be used to confirm the identity of the PCR products in place of Southern blot hybridization, including reverse line blot hybridization (13), reverse line blot hybridization, and direct sequencing of products. Probes used in hybridization assays can be selected not only to identify virus genus but also to classify into genogroup and even genotype.

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Chapter 4

Detection of Dengue Virus

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Abstract

Global incidence of dengue has increased considerably over the past decade. Dengue fever (DF) is a self-limiting disease; however, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are fatal. Since there is no therapy and vaccine against dengue, timely diagnosis is therefore necessary for patient management. Laboratory diagnosis is carried out by virus isolation, demonstration of viral antigen, presence of viral nucleic acid, and antibodies. Further, recombinant dengue envelope protein can be used to detect specific antibodies, both IgG and IgM against all four serotypes of virus using an *E. coli* vector. The purified protein can then be used for detection of dengue specific IgG or IgM antibodies in patient serum with higher sensitivity and specificity, than that of traditional assays. Molecular detection can be accomplished by a one-step, single-tube, rapid, multiplex, RT-PCR for serotype determination. Despite many advantages of the modern techniques, isolation of virus is still considered as "gold standard" in dengue diagnosis.

Key words: Dengue virus, Antibody detection, Recombinant protein, Dipstick ELISA, Multiplex RT-PCR, Virus isolation

1. Introduction

Dengue is an endemic viral disease affecting tropical and subtropical regions around the world, predominantly in urban and semiurban areas. Dengue fever (DF) and its more serious forms, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), are becoming important public health problems. Global prevalence of dengue has grown dramatically in recent decades. An estimated 2.5 billion people in more than 100 countries are at risk of acquiring dengue viral infections with more than 50 million new infections being projected annually, 500,000 cases of DHF that must be hospitalized and 20,000–25,000 deaths,

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mainly among children (1). Dengue viruses (DV) belong to the family *Flaviviridae* and have four serotypes (1–4). Transmission is mainly through the mosquito *Aedes aegypti*, and *Aedes albopictus* is also implicated. Dengue virus is a positive-stranded, encapsulated RNA virus, approximately 11 kb in length. The genomic RNA is composed of three structural genes that encode the nucleocapsid or core protein (C), a membrane-associated protein (M), an envelope protein (E), and seven nonstructural (NS) genes. The gene order is 5′-CprM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′ as for other flaviviruses (2). The proteins are synthesized as a polyprotein of about 3,000 amino acids that is processed cotranslationally and posttranslationally by viral and host proteases.

The diagnosis of dengue virus infections on the basis of clinical syndromes has shown not to be reliable, and should therefore be confirmed by laboratory analysis, because more than half of infected individuals are either asymptomatic or have a mild undifferentiated fever (3). A primary dengue virus infection is characterized by elevation in specific IgM antibody levels, 3–5 days after the onset of symptoms which generally persists for 30–60 days. IgG levels become elevated after 10–14 days and remain detectable for life. During a secondary infection, IgM levels generally rise more slowly and reach lower levels than that demonstrated in the primary infection, while IgG levels rise rapidly from 1 to 2 days after the onset of symptoms.

Laboratory diagnosis of dengue virus infection can be made by the detection of specific virus, viral antigen, genomic sequence, and/or antibodies (4). At present, three basic methods used by most laboratories for the diagnosis of dengue virus infection are; viral isolation and characterization, detection of the genomic sequence by nucleic acid amplification technology, and detection of dengue virus specific antibodies. During the postfebrile stage, which lasts for a few weeks, the IgM and IgG antibodies persist in circulation and can be detected by Enzyme Linked Immunosorbent Assays (ELISA). During the primary phase of infection, viremia and fever coincide, while during a secondary infection with dengue virus, the viremia is present for 2–3 days, and NS1 antigens remain in the blood for a longer period of time. Dengue virus causes a broad spectrum of illnesses, ranging from inapparent infection, flu-like mild undifferentiated fever, and classical DF to the more severe form DHF/DSS, with high rates of morbidity and mortality (4). DF is characterized by fever for 3–5 days, headache, muscle and joint pain, and rashes, which is self-limited, and the patients usually recover completely. At present there is no effective therapeutic agent or licensed vaccine for humans against dengue fever. Consequently, there is a great demand for the rapid detection and differentiation of dengue virus infection in the

acute phase of illness to provide timely clinical treatment and etiologic investigation and disease control.

The whole virus antigens used for the detection of antidengue antibodies in patient's sera are produced in tissue culture or suckling mice brain and are consequently associated with an inherent biohazard risk. Detection kits developed with these antigens are expensive due to the high costs associated with antigen production, making them unaffordable for use in the economically weaker countries, where dengue is mostly prevalent. Apart from this, a major shortcoming of the commercial kits is that they do not differentiate between dengue and other *Flavivirus* infections (such as Japanese encephalitis and yellow fever viruses). Additionally, sera from patients with typhoid, malaria, and leptospirosis can also produce positive results using these kits, due to non specific reactions. There is, therefore, a need to develop cost-effective, safe, and simple diagnostics that combine sensitivity and specificity; this includes using gene technology for the production of recombinant antigens, which has already been used to support the effective diagnosis of a range of viruses.

2. Materials

2.1. Production of Recombinant Antigens

1. Recombinant *Escherichia coli* (SG13009 harboring recombinant plasmid pQE 60 resistant to ampicillin and kanamycin, containing the IgG-specific Dengue Multi Epitope gene for detection of antidengue IgG antibodies/DH5α harboring recombinant plasmid pMAL-c2X resistant to ampicillin, containing the IgM specific dengue multi epitope gene for detection of antidengue IgM antibodies) glycerol stock (30%). The recombinant antigen has been produced at the Defence Research & Development Establishment, Gwalior (India) in collaboration with International Centre for Genetic Engineering and Biotechnology, New Delhi (India).
2. Luria Bertani broth (LB) (Difco, USA).
3. Ampicillin, Kanamycin (Sigma, USA).
4. Isopropyl β-D-thiogalacto pyranoside (IPTG) (Sigma, USA).
5. Incubator-Shaker (New Brunswick, USA).
6. Refrigerated centrifuge (Sorvall, USA).
7. Ultrasonic disintegrator (Sonics, USA).
8. Akta explorer chromatography system (GE Health Care, Sweden).
9. Ni-NTA super flow resin (Qiagen, Germany).
10. Solubilization buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M Urea, pH 7.5).

11. Wash buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M Urea, pH 6.3).
12. Elution buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M Urea, pH 4.3).

2.2. Enzyme Linked Immunosorbent Assays

1. Plastic comb with a nitrocellulose membrane on each of 12 projections (MDI, India) or Polystyrene plate with 96 microwells (Nunc, Denmark).
2. Phosphate buffer saline, pH 7.4 (Hi Media, India).
3. Coating buffer (0.1 M Na_2CO_3, 0.2 M $NaHCO_3$, pH 9.6).
4. Blocking solution – PBS containing 2% Bovine Serum Albumin (Sigma, USA).
5. Wash buffer-PBS containing 0.05% Tween-20 (Sigma, USA).
6. Serum diluent (PBS+ 1% BSA with 0.01% Tween-20).
7. Goat anti-human IgG and IgM horseradish peroxidase conjugate (Sigma, USA).
8. Conjugate diluent (PBS+ 1% BSA).
9. Citrate–Phosphate buffer (0.1 M citric acid, 0.2 M $Na_2HPO_4{\cdot}2H_2O$, pH 5.0) with orthophenylenediamine (OPD)/3,3′-Diaminobenzidine (DAB)-4 mg and hydrogen peroxide 30% (w/v)-15 µl per 10 ml.
10. Stopping solution: 1 M H_2SO_4.
11. ELISA Reader (Bio-Tek, USA).

2.3. Multiplex Reverse-Transcriptase Polymerase Chain Reaction

1. Dengue virus source: Infected tissue culture fluid from $C_{6/36}$ cells, human serum samples from dengue suspected cases.
2. Thermal cycler (Bio-Rad, USA).
3. Bench-top refrigerated microcentrifuge (Sigma, Germany).
4. Oligonucleotide primers (Table 1).
5. Access Quick 2× Master Mix (Promega, USA).
6. Nuclease-free water (Promega, USA).
7. Nuclease-free thin-walled PCR tubes.
8. Avian Myeloblastosis Virus-Reverse Transcriptase (Promega, USA).
9. Agarose (Sigma, USA).
10. Ethidium bromide (Sigma, USA): 10 mg/ml.
11. 10× TBE Buffer (Tris 108 g, Boric acid 55 g, 0.5 M EDTA 18.6 g, Triple distilled water 1 l, pH 8.0).
12. 6× Gel loading dye (bromophenol blue 25 mg, xylene cyanol 25 mg, Ficoll 400 15 mg, and Triple distilled water 100 ml).
13. DNA ladder (100 bp) (#SM0243, Fermentas, USA).

Table 1
Nucleotide sequence of dengue primers

Code	Sequence	Genomic position
D1	5′ TCAATATGCTAAAACGCGCGAGAAACCG 3′	134–161
TS1	5′CGTCTCAGTGATCCGGGGG3′	588–572
TS2	5′CGCCACAAGGGCCATGAACAG3′	252–232
TS3	5′TAACATCATCATGAGACAGAGC3′	421–400
TS4	5′TGTTGTCTTAAACAAGAGAGGTC3′	525–504

D1: Dengue virus group specific consensus forward primer
TS1–4: Dengue virus 1–4 serotype-specific reverse primers

14. Gel casting tray and combs (Bio-Rad, USA).
15. Gel electrophoresis assembly and Gel documentation system (Bio-Rad, USA).

2.4. Virus Isolation

1. Preformed monolayer of $C_{6/36}$ cells.
2. Tissue culture flask: 25 cm^2 (Greiner Bio-one, Germany).
3. Incubator (Narang Scientific, India).
4. Laminar Airflow Unit (Esco, USA).
5. Inverted microscope (Olympus, Japan).
6. Refrigerated centrifuge (Sorvall, USA).
7. Eagle's Minimum Essential Medium (EMEM) (Sigma, USA).
8. Tryptose Phosphate Broth (TPB) (Sigma, USA).
9. Fetal Bovine Serum (FBS) (Sigma, USA).
10. Syringe filter (0.22 μm) (Millipore, USA).
11. Sterile serological pipettes (Greiner Bio-one, Germany).

3. Methods

3.1. Expression and Purification of Recombinant Protein (Antigens)

Most of the recombinant DNA-based strategies focus on the envelope (E) and NS1 proteins of the dengue viruses. The E protein is organized into distinct domains designated as I, II, and III. Of these, domain III (amino acid 300–400) is stabilized by a single disulfide bond and is particularly important from the viewpoint of diagnostic and vaccine development as it contains multiple serotype-specific conformation dependent neutralizing epitopes and the host cell receptor recognition sites (5, 6).

Subsequently, dengue virus recombinant proteins of the envelope including domain III and NS1 could be used as antigens in an ELISA to detect antibody and could also be used for vaccine studies (5–10).

1. Inoculate 1 ml of glycerol stock of *E. coli* (IgG or IgM specific clones) in 50 ml of LB broth (see Note 1) containing ampicillin (100 μg/ml) and kanamycin (25 μg/ml). Grow for 18 h at 37°C with shaking at 200 rpm in an incubator shaker.
2. Inoculate 1,000 ml of LB broth containing antibiotics (see Subheading 3.1, step 1) with 10 ml of overnight (18 h)-grown culture and grow with shaking at 37°C until an absorbance of 0.6–1.0 at OD_{600} is reached.
3. Add 1 ml of 1 M IPTG (final concentration 1 mM) and grow for a further 4 h (see Note 1).
4. Harvest cells by centrifugation at 8,000 × *g* for 20 min at 4°C.
5. Resuspend cell pellet in solubilization buffer (1:40 w/v) and stir for 1 h at room temperature; alternatively, store the pellet at −80°C for further use.
6. Sonicate the above solution on crushed ice for 10 min using a Vibra cell Ultrasonic disintegrator with thermal probe for 9 s on/off pulse at 40% frequency and cool it at 2 min intervals.
7. Centrifuge the above suspension at 15,000 × *g* for 40 min at 4°C and collect supernatant.
8. Arrange a prepacked Ni-NTA superflow cartridge or chromatography column packed with Ni-NTA superflow resin (see Note 2) on an Akta Explorer system. Equilibrate the column with 5–8 column volumes of solubilization buffer.
9. Apply the supernatant (from Subheading 3.1, step 7) to the column and wash with 10 column volumes of wash buffer.
10. Elute the bound protein using 1 column volume of elution buffer. Pool the relevant fractions (peak at absorbance at 280 nm), concentrate, and dialyze against 50 mM phosphate buffer containing 1 M Urea and 250 mM NaCl (pH 5.8) (see Note 3).
11. Store at −20°C for use in ELISA.

3.2. Indirect Enzyme Linked Immunosorbent Assay

This assay is useful for detection of antidengue IgM and IgG antibodies in dengue infected patient serum samples. For this purpose, purified recombinant antigen expressed in *E. coli* (5, 9–12) or insect cells (7) are coated in the wells of a microtiter plate or NC combs. After blocking unbound sites with bovine serum

albumin, the plates or combs are incubated with serum samples containing specific antibodies; any unbound antibodies are then washed away. A suitable anti IgG/IgM conjugate solution is then added and incubated for 1 h. The plates or combs are then washed and a substrate solution is added to reveal any bound antibody. The result is assessed by either optical density or visual inspection, in case of dipstick.

3.2.1. Dipstick ELISA

1. Cut the required number of projections from a NC comb. Coat 0.2 µg (2 µl) or 0.6 µg (2 µl) of purified antigen (see Note 4) per projection separately using a micropipette and tips for detection of antidengue IgG and IgM antibodies respectively.
2. Incubate the NC combs at 37°C for 1 h (in plastic tray with lid).
3. Wash the coated NC combs with PBS three times, for a duration of 5 min at room temperature.
4. Dispense 200 µl per well of blocking buffer in an ELISA plate, add the NC combs, and incubate at 4°C for 18 h.
5. Wash the NC comb with washing buffer three times for 5 min duration, then dry with the help of an air dryer.
6. For the detection of antidengue IgM antibodies, add 2 µl of patient's serum to 198 µl (serum diluent), and for anti-dengue IgG antibodies, add 1 µl of patient's serum to 999 µl of serum diluent; mix thoroughly by inversion and dispense 200 µl per well of an ELISA plate (see Note 4).
7. Dip each projection of the NC comb in 200 µl of diluted sera in the ELISA plate for either IgM or IgG detection. Incubate for 1 h at 37°C.
8. Wash three times with PBS-T.
9. Dip the NC projections in 200 µl of anti-human IgM HRP conjugate (diluted 1:2,000 in conjugate diluent) for detection of antidengue IgM and 200 µl of antihuman IgG HRP conjugate (1:4,000 in conjugate diluent) for detection of anti-dengue IgG antibodies and incubate for 1 h at 37°C (see Note 4).
10. Wash three times with PBS-T.
11. Dip the washed combs in 200 µl of phosphate citrate buffer with DAB substrate in the wells of an ELISA plate and incubate at 37°C for 10 min.
12. Wash with distilled water to remove any excess substrate.
13. Appearance of distinct brown dots (see Note 4) in the projection with an antihuman IgM HRP conjugate indicates a primary or recent infection and that with an antihuman IgG

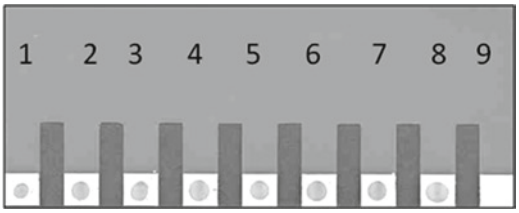

Fig. 1. The results of Dipstick ELISA with recombinant antigen. Projections 1–3: IgM positive; projections 4–8: IgG positive and *lane 9*: negative serum sample.

HRP conjugate indicates secondary infections due to dengue viruses (Fig. 1).

3.2.2. Microwell Plate ELISA

1. Coat 0.3 µg (100 µl) of purified antigen (see Note 4) per well of a microwell plate using a micropipette and tips.
2. Incubate the microwell plate at 37°C for 1 h.
3. Wash the coated wells with PBS three times for 5 min.
4. Dispense 100 µl per well of blocking buffer in to the coated microwell plates and incubate at 4°C for 18 h.
5. Wash the coated wells with PBS three times for 5 min.
6. After the last wash, remove the residual buffer (see Note 4).
7. For the detection of anti-dengue IgM antibodies, add 1 µl of patient's serum to 99 µl of serum diluent, and for antidengue IgG antibodies, add 1 µl of patient's serum to 999 µl serum diluent, mix thoroughly, dispense 100 µl per well in separate ELISA plates, and incubate the plates for 1 h at 37°C (see Note 4).
8. Wash three times with PBS-T.
9. Dispense 100 µl/well of antihuman IgM HRP conjugate (1:2,000 in conjugate diluent) to the anti-dengue IgM plate and 100 µl/well of anti human IgG HRP conjugate (1:4,000 in conjugate diluent) to the antidengue IgG plate and incubate for 1 h at 37°C (see Note 4).
10. Wash three times with PBS-T.
11. Dispense 100 µl, per well, of phosphate citrate buffer with OPD substrate and incubate at 37°C for 10 min.
12. The appearance of a distinct yellow color in the microwells with anti-human IgM HRP conjugate indicates a primary or recent infection and with the anti-human IgG HRP conjugate indicates a secondary infection due to dengue virus.
13. The peroxidase reaction is stopped with 100 µl of 1 M H_2SO_4 and then the ELISA plate read at 450 nm using an ELISA reader.

14. The absorbance ratio of positive to negative samples should be more than 2.0. The ratios in respect of test samples greater than 2.0 are taken as positive and below 2.0 as negative (see Note 4).

3.3. One-Step, Single-Tube, Multiplex RT-PCR

Once a dengue virus infection has been confirmed, it is essential for epidemiological and further clinical investigations to identify the serotype of the virus. This can be done by molecular detection, which is accomplished by Reverse Transcription Polymerase Chain Reaction (RT-PCR). In routine practice, a two-step method with a RT-PCR followed by a nested PCR is used for serotype determination of dengue viruses. However, this method is expensive, time consuming, and also suffers from carryover contamination problems. To improve on this, a one-step, single-tube, rapid, multiplex PCR assay (13) is given here for rapid detection and differentiation of dengue serotypes in acute-phase serum samples.

3.3.1. Extraction of Viral RNA

Extract RNA (see Note 5) from dengue virus infected culture fluid/serum samples using QIAamp Viral RNA mini kit (Qiagen, Germany) according to the manufacturer's protocol. Finally denature the eluted RNA (around 60 µl) at 65°C for 10 min and store at –80°C.

3.3.2. One Step Single Tube Multiplex RT-PCR

The multiplex RT-PCR is carried out in a final volume of 50 µl using RNA as the template, forward primer (D1), and type-specific reverse primers (TS1/TS2/TS3/TS4) (see Note 5). The reaction in each tube is carried out as follows.

1. Take the following reagents in a 0.2-ml, thin-walled PCR tube.
   (a) Nuclease-free water : 18 µl
   (b) 2× master mix : 25 µl
   (c) Dengue-specific Forward primer (D1) (50 pmol/µl): 0.5 µl
   (d) DEN-1-specific Reverse primer (TS1) (50 pmol/µl): 0.5 µl
   (e) DEN-2-specific Reverse primer (TS2) (50 pmol/µl): 0.5 µl
   (f) DEN-3-specific Reverse primer (TS3) (50 pmol/µl): 0.5 µl
   (g) DEN-4-specific Reverse primer (TS4) (50 pmol/µl): 0.5 µl
   (h) AMV-RT: 1 µl
   (i) RNA template: 3.5 µl.
2. Mix the reaction mixture thoroughly by gentle pipetting and keep the tube in the thermal cycler.

3. Set the thermal profile (see Notes 5 and 6) conditions as follows:

Temp	Time	No. of cycles	Remarks
48°C	45 min	1	Reverse transcription
95°C	2 min	1	Initial denaturation
94°C	1 min	35	Denaturation
55°C	1 min		Primer annealing
72°C	2 min		Primer extension
72°C	10 min	1	Final extension
4°C	Infinite	–	Hold

3.3.3. Agarose Gel Electrophoresis

The size and quantity of the PCR amplicon is determined by electrophoretic analysis using a 1.5% agarose gel (see Note 5).

1. Clean the gel casting tray of the horizontal electrophoresis apparatus, air-dry, assemble, and seal the sides with cellophane tape. Place the comb in its position.
2. Prepare 1.5% agarose by boiling 375 mg of agarose in 25 ml of 1× TBE buffer.
3. Cool down to 60°C and add 2.5 µl of ethidium bromide to a final concentration 1 µg/ml and mix thoroughly.
4. Pour it into the gel tray, avoiding entrapment of any air bubbles. Place the gel in the electrophoretic tank. Submerge the gel in the electrophoresis buffer (1× TBE).
5. Mix 10 µl of PCR product with 2 µl of 6× gel loading dye and load into the well using a micropipette. A molecular weight marker (100 bp DNA ladder) should also be loaded in an adjacent well to identify bands.
6. Carry out electrophoresis at 60 V for 2 h, until the dye reaches the end of the gel.
7. After electrophoresis, scan the gel and take a photograph (Fig. 2) with the help of the gel documentation system to preserve the data.

3.4. Virus Isolation

Although the assays described in this chapter are very effective, virus isolation still remains the "gold standard" for definitive diagnosis of dengue infections. However, since the procedure of virus isolation is both time consuming and tedious to perform, this has been replaced by a modern molecular method involving PCR. Isolation of viruses from clinical samples should be carried out using cultured mosquito cells such as $C_{6/36}$ cell lines, or mammalian cells such as Vero and BHK-21 cell lines. At present virus isolation from

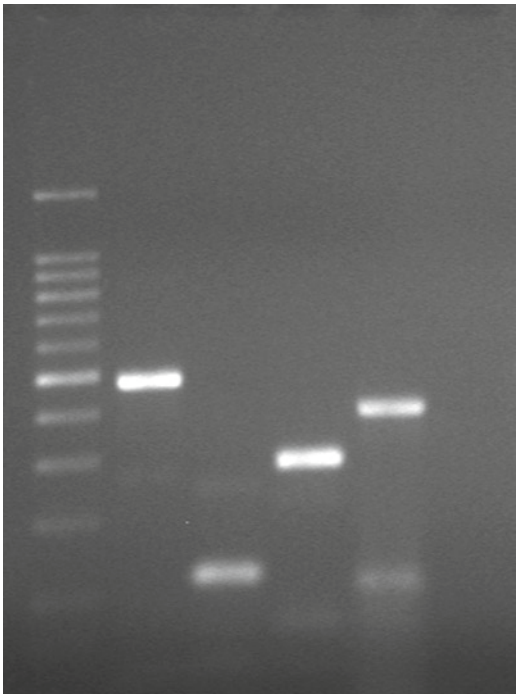

Fig. 2. Agarose gel electrophoresis demonstrating the amplicons generated by the single-tube dengue multiplex RT-PCR. *Lane 1:* 100 bp DNA ladder (Fermentas), *lanes 2–5:* Dengue 1 (482 bp), Dengue 2 (119 bp), Dengue 3 (290 bp), Dengue 4 (389 bp) and *lane 6:* negative control.

acute-phase serum or plasma of patients using the $C_{6/36}$ cell line is the method of choice for routine dengue virus detection (14).

1. Grow $C_{6/36}$ cells in growth medium using EMEM + 10% FBS with an initial seeding density of 2×10^5 cells/ml in a tissue culture flask (T-25), and then incubate at 28°C until 80% confluent in a nonhumidified incubator (see Note 7).
2. When the $C_{6/36}$ cells are confluent (after approximately 72–96 h) discard the growth medium from the TC flask in a laminar airflow biosafety cabinet.
3. Gently wash the cell layers with sterile PBS.
4. Filter the virus/serum sample (see Note 8) using a PVDF membrane filter of 0.22 μm pore diameter and infect the cells with a multiplicity of infection of 0.1 (0.05 ml/25 cm^2 flask or 1 ml/75 cm^2 flask).
5. Keep the cells at 37°C for 2 h with intermittent shaking at 30 min interval and allow the virus to be adsorbed to the cells.
6. Decant the residual inoculum and wash twice with sterile PBS. Replenish with 10 ml of maintenance medium (EMEM containing 2% FBS) in each of the 25 cm^2 flasks.

7. Incubate the flasks at 32°C in an incubator, and observe daily for the appearance of any cytopathic effect (CPE).
8. If any cytopathic effect is observed, harvest the infected culture fluids (ICF) from the infected cells or perform this on the fourth to sixth postinfection day (PID).
9. Centrifuge the fluid at 1,000 × *g* for 10 min at 4°C.
10. Collect the supernatant and store at ultra-low temperature (−80°C) for further confirmation by Multiplex RT-PCR (see Subheading 3.3).

4. Notes

1. The growth medium used for cultivation of recombinant bacteria should be optimized for maximum protein expression. The IPTG concentration, the time of induction, and the time of harvesting should be optimized. The yield of desired protein may vary along with the growth phase. SDS–PAGE analysis should be carried out to check the protein expression level at hourly intervals after induction as well as with varying concentrations of IPTG. Expression of recombinant antigen used for detection of IgG and IgM antibodies against dengue have been described previously (6, 10–12).
2. The cell pellet can be stored at −80°C for further use. Alternatively, proceed for downstream purification immediately. The chromatography buffers should be freshly prepared and pH adjusted just before use. Store all resins in 20% ethanol and wash extensively with deionized water before use. The resin should not be allowed to dry out. The buffer containing 8 M urea will require heating at 37°C in a water bath to dissolve it completely. SDS–PAGE analysis should be carried out to check the protein expression in all the fractions designated as preload, column wash, and elutes.
3. After purification, the protein concentration should be measured spectrophotometrically using the theoretical extinction coefficient of the protein, as well as by employing a protein assay using the BCA method (Pierce, USA). Also, pure antigens should be used to avoid cross reactions with other related diseases. All experimental conditions including incubation time and temperature, washing times, reagent concentration, and temperature should be kept constant to ensure that reproducible results are achieved.
4. The optimal antigen concentration, antibody dilution, and conjugate dilution should be standardized by the checkerboard titration. Serial dilutions of the antigen are coated in wells row-wise and antibody dilution in wells column-wise in

a microtiter plate. Similarly, conjugate dilutions are used to determine the optimal absorbance. The Positive: Negative ratio should be more than 2. While performing the assay, both positive and negative controls should always be used with the test samples. In the case of the plate ELISA, after the last wash remove the residual buffer by wrapping the microwell plate in tissue paper and gently flick it face down onto several paper towels. In the dipstick ELISA the positive controls produce clear brown dots against a white background, while the negative controls should not give any coloration.

5. DNase-free and RNase-free tips and tubes should be used for the RT-PCR. The concentration of each primer is crucial as higher concentrations give false results. Powder-free gloves should be changed in between each sample to avoid carryover produced from contamination. The higher temperature of 48°C for RT reduces nonspecific band formation without affecting the sensitivity of the RT-PCR. The amplification and postamplification manipulations should be physically segregated.
6. A final extension step of 10 min is necessary. While preparing the RT-PCR mixture, add the AMV-RT at length and mix by gentle pipetting. Annealing conditions may be optimized taking into account the T_m of the primers.
7. Further, for virus isolation, the cells should be seeded in the tissue culture flask for 24–48 h, and when the confluency is around 90%, they give better results.
8. The clinical samples should be collected in the acute phase of the infection and must be brought to the laboratory using a suitable cold chain. When used for virus isolation, this should be done as quickly as possible to avoid any viral inactivation, which can interfere with the isolation of the viruses.

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Chapter 5

Detection of Enteroviruses from Clinical Specimens

Chit Laa Poh and Eng Lee Tan

Abstract

Enteroviruses are positive stranded RNA viruses belonging to the genus Enterovirus of the *Picornaviridae* family. Human enteroviruses are transmitted through the fecal–oral route and have been shown to cause mild to life-threatening diseases. Various diagnostic methods have been developed to detect enteroviruses from clinical specimens but many were impeded by requirements for special reagents, lengthy procedures, low sensitivity or cross-reactivity. This chapter describes rapid and highly sensitive methods of enteroviral detection directly from clinical specimens based on a conventional one-step Reverse Transcription polymerase chain reaction (RT-PCR) and a one-step real-time RT-PCR.

Key words: Enteroviruses, Diagnostic methods, Conventional one-step RT-PCR, Real-time one-step RT-PCR

1. Introduction

Enteroviruses are positive stranded RNA viruses with a nonenveloped capsid and a genome size of approximately 7.5 kb (Fig. 1). They belong to the genus enterovirus of the *Picornaviridae* family. The human enteroviruses are further classified into five species: HEV-A, HEV-B, HEV-C, HEV-D, and poliovirus (1). Currently, enteroviruses are represented by 91 serotypes of which 64 serotypes are known to infect humans (2).

The stability of enteroviruses in an acidic environment allows them when ingested to inhabit the alimentary tracts of humans and animals (3). Enteroviruses are therefore transmitted mainly through the fecal–oral route. Human enteroviruses have been shown to cause mild to life-threatening diseases, such as hand, foot and mouth disease, aseptic meningitis, respiratory illness, myocarditis, encephalitis, and acute flaccid paralysis (4).

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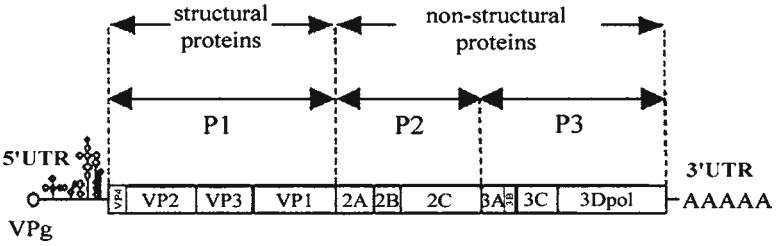

Fig. 1. Genome structure of an enteroviral RNA and the genetic organization of its polyprotein. The 7.51 kb genome serves as a template for both viral protein translation and RNA replication and is translated as a single polyprotein that is subsequently cleaved to yield viral proteins VP1–4, 2A–C, and 3A–D as shown. *VPg* virion protein genome, *5′UTR* 5′ untranslated region, *3′UTR* 3′ untranslated region, *P1–3* precursor proteins, *VP1–4* virus capsid proteins (1).

Table 1
Clinical manifestations of enterovirus serotypes

Clinical manifestations[a]	Enterovirus serotypes
Paralysis	Poliovirus 1–3; Coxsackievirus A7, A9, B2–5; Echovirus 4, 6, 9, 11, 30; Enterovirus 70, 71
Aseptic meningitis	Poliovirus 1–3; Coxsackievirus A2, A4, A7, A9, A10, B1–6; Echovirus 1–11, 13–23, 25, 27, 28, 30, 31; Enterovirus 70, 71
Hand, foot, and mouth disease (HFMD)	Coxsackievirus A5, A10, A16, Enterovirus 71 (EV71)
Herpangina	Coxsackievirus A2–6, A8, A10
Acute hemorrhagic conjunctivitis	Coxsackievirus A24, Enterovirus 70
Encephalitis	Echovirus 2, 6, 9, 19
Meningoencephalitis	Coxsackievirus B1–5; Enterovirus 70, 71
Pericarditis, myocarditis	Coxsackievirus B1–5

[a]One symptom may potentially be caused by more than one enterovirus (1)

Table 1 shows the various clinical symptoms associated with infections caused by some enteroviruses.

1.1. Diagnosis of Enteroviral Infections

Isolation of human enteroviruses in cell culture, followed by serotype identification based on a neutralization assay, is regarded as the gold standard for diagnosing enteroviral infections. Supporting this, enteroviruses have been grown in cell lines such as Vero (African green monkey kidney cells), human Rhabdomyosarcoma (RD; human skeletal muscle cells), MRC-5 (human lung fibroblast cells),

and MDCK (monkey kidney cells) (5, 6). Serotypic identification of enteroviruses could be determined by neutralization with serotype-specific antisera such as the Lim–Benyesh-Melnick (LBM) pool (7), while alternative intersecting pools of antisera were later developed by the National Institute of Public Health and Environment (RIVM) in the Netherlands to replace the LBM pool and allowed identification of enteroviruses 68–71 (8).

Enteroviruses have been isolated from clinical specimens derived from the skin, saliva, stools, urine, cerebral spinal fluid (CSF), serum, throat and rectal swabs (1, 9–12). However, despite being regarded as the gold standard, the tissue culture method has its limitations. Further, the isolation and identification of specific enterovirus serotypes by neutralization requires a long time frame of 1–2 weeks. In addition, the viral titer present in clinical specimens such as in CSF might be too low to be detected using this technique. In some instances, neutralization could be hindered due to aggregation of the virus, antigenic drifts, or the presence of multiple viruses in clinical specimens (8, 13, 14). Several studies have shown very low sensitivity of isolating EV71 directly from clinical specimens such as CSF cultivated in tissue culture (11, 12). Isolating enteroviruses from cell cultures is impractical in outbreak situations where rapid and accurate diagnosis at an early stage of enteroviral infection is needed.

1.1.1. Immunofluorescence Assay

Another approach for enterovirus detection is the indirect immunofluorescence assay (IFA) which involves the use of serotype-specific monoclonal antibodies (Echoviruses, Coxsackievirus A, EV71) (Chemicon International, USA) (15). This method allows serotype-specific identification of enteroviruses grown in cell cultures. For example, to identify presence of EV71, the clinical specimens are first inoculated in Vero, RD, or MRC-5 cell lines (5). Immunohistological staining with a monoclonal anti-EV71 antibody (Chemicon International, USA) was then performed on the inoculated cell lines. The monoclonal antibody bound to the EV71 isolated from the cell culture and the antigen–antibody complex was detected by a secondary FITC (fluorescein isothiocyanate-labeled) antibody observed under a fluorescence microscope. The advantage of this method is that it uses a more specific set of antibodies and does not require the LBM pools for typing. However, this method still requires prior growth of the virus in the cell culture which generally required 1–2 weeks. However, some monoclonal antibodies, for instance, a monoclonal antibody against EV71 was reported to cross-react with CA16 (16) and could lead to misinterpretation of serotypes.

1.1.2. Enzyme-Linked Immunosorbent Assay

The detection of IgM in sera from EV71-infected patients by Enzyme-Linked Immunosorbent Assay (ELISA) was shown to be effective in diagnosing acute EV71 associated infections (17).

A similar approach based on the detection of IgM from immune sera to diagnose EV71 showed a high sensitivity and specificity of 97.7 and 93.3%, respectively (18). Although ELISAs are commonly used in diagnostic laboratories, they have their limitations. In the IgM-capture ELISA, the whole EV71 virion was used as the antigen and the need to prepare large quantities of purified virions which interact with secondary anti-human IgM in the ELISA assay made the manufacturing an expensive, laborious, and lengthy process. As the whole virus is used as the capture antigen in the ELISA, cross-reactions with antibodies against other enteroviruses could produce false-positive reactions. Thus, the specificity of the IgM-based ELISA could be compromised by the presence of common epitopes of other enteroviruses.

1.1.3. Reverse Transcription Polymerase Chain Reaction

The polymerase chain reaction (PCR) has been accepted as the "new" standard for detection of viruses in diagnostic microbiology laboratories. Most of the PCR primers designed for the detection of enteroviruses were targeted at the highly conserved 5′UTR region of the genome (19–21). The advantage of this detection strategy for enteroviruses based on the 5′UTR is that a single pair of primers could be used for the identification of enteroviruses in general. However, there is little correlation between the enterovirus serotype and the highly conserved 5′UTR region. No information could be obtained regarding the identity of the specific enterovirus serotype without additional sequence analysis. Since analysis of the VP1 region was shown to correlate more closely with enterovirus serotypes (22), several studies based on reverse transcription polymerase chain reaction (RT-PCR) have employed primers targeting at the VP1 region. Different enterovirus serotypes were subsequently shown to be identified by specific primers (10, 14, 16, 23, 24). An improvement of the conventional RT-PCR, known as the miniature RT-PCR system was developed by Liao et al. (2005) (25) for rapid diagnosis of RNA viruses such as EV71. The principle was based on a two-step conventional RT-PCR carried out in a miniature PCR chamber which was operated by 9 V batteries. CSF collected from an autopsy specimen was loaded into a valve and the two-step RT-PCR process was carried out in the automated system which reduced cross-contamination. Since a microchip was used to control the temperature, the PCR product was formed within 15 min and a detection limit as low as 6 pg/μL was achieved.

1.1.4. Combination of RT-PCR and Specific Probe Hybridization

The PCR-ELISA detection format for enteroviruses was developed by Rotbart et al. (26, 27) based on their earlier work on identification of enteroviruses using conventional RT-PCR. The method was based on the principle of amplification and detection of the amplified DNA by ELISA. The Light Diagnostics Pan-Enterovirus OligoDetect™ kit (Chemicon International, USA) was first used to

detect enterovirus-specific RT-PCR products amplified from clinical specimens. The detection was completed by using an ELISA to analyze the RT-PCR products via hybridization to an internal biotinylated probe conjugated to a streptavidin-coated microtitre plate. Colorimetric detection of positive specimens is therefore based on the reaction of HRP with the substrate 3, 3′, 5, 5′-tetramethylbenzidine (TMB). As the primers were designed to target at the 5′UTR region of the viral genome, the PCR-ELISA method is applicable for the detection of enteroviruses in general, but is not able to resolve differentiating specific serotypes.

1.1.5. Real-Time RT-PCR

Real-time RT-PCR is an improvement of the classical RT-PCR amplification process in which monitoring of the accumulating amplicons during the course of the PCR process is made in real time. Several studies have reported using the real-time PCR approach by employing specific primers targeting the 5′UTR region. These studies were able to detect and differentiate enteroviruses from other viruses such as herpes simplex virus (HSV) (28), Varicella-Zoster virus (VZV) (28), and rhinoviruses (29) based on melting curve analysis.

The TaqMan probe assay has been shown to be a more reliable approach for the specific detection of enteroviruses from clinical specimens (30, 31). Besides the specificity and rapidity in detection, the real-time RT-PCR assay was also shown to be highly sensitive. Several studies have reported detection of enteroviruses directly from CSF specimens, which have been associated with low viral titres (30, 32, 33).

1.2. Principle of Methodology

This chapter describes in detail both a one-step conventional RT-PCR method and a one-step real-time RT-PCR method for detection of enteroviruses. A convenient and efficient RNA extraction method which is suitable for recovering RNA from a diverse range of clinical specimens prior to RT-PCR amplification is also described.

2. Materials

2.1. RNA Extraction

1. Filtered pipette tips (2–20 µL, 20–200 µL, and 100–1, 000 µL).
2. Sterile pipettes.
3. Sterile 0.2 and 1.5 mL eppendorf tubes.
4. QIAamp Viral RNA Mini kit (Qiagen, Cat. No. 52906).
5. Absolute and 70% Ethanol.
6. 3 M Sodium Acetate.

2.2. Conventional RT-PCR and Real-Time RT-PCR

1. Qiagen one-step RT-PCR kit (Cat. No. 210212).
2. LightCycler real-time PCR machine (Roche Diagnostics, version 2.0).
3. LightCycler RNA Master Hybridization probe kit (Roche Diagnostics, Cat. No. 03018954001).
4. GeneAmp® PCR System 2400 PE (Applied Biosystems, USA).
5. Oligonucleotide primers (prepared to 10 μM) and TaqMan probe (prepared to 2 μM) (Table 2).
6. ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit version 3.1 (Applied Biosystems, Cat. No. 4336917).

2.3. Gel Electrophoresis

1. Electrophoresis tank, gel caster, gel comb, and power pack.
2. UV transilluminator.
3. Agarose powder.
4. 10× Tris–borate EDTA (TBE) (0.089 M Tris base, 0.089 Borate, and 0.002 M EDTA at pH 8.0) buffer. Dilute to 1× with deionized water to a total volume of 1 L.
5. 6× Gel loading buffer (Fermentas, Cat. No. R0611).
6. 10 mg/mL ethidium bromide (dissolved in water).
7. Ready-Load 100 bp DNA size markers (Fermentas, Cat. No. SM0243).
8. DNA Ladder marker (Fermentas, Cat. No. SM0243).

Table 2
Sequences of primers and TaqMan probe used in detection of enteroviruses by conventional RT-PCR and real-time RT-PCR

Primers or probe	Sequence (5′–3′)	Nucleotide position	References
Forward primer (NC1)[a]	CTCCGGCCCCTGAATGCG	449–466	(19, 20)
Forward primer (NC1M)[b]	CCCTGAATGCGGCTAATCC	456–474	(30)
Reverse primer (E2)[a, b]	ATTGTCACCATAAGCAGCCA	582–601	(30)
TaqMan probe	FAM-AACCGACTACTTTGGGTG TCCGTGTTTC-TAMRA	539–566	(30)

[a]Primers used in conventional one-step RT-PCR
[b]Primers used in real-time one-step RT-PCR

3. Methods

3.1. Processing of Viral Isolates and Clinical Specimens

1. Proceed directly to Subheading 3.2 for extraction of RNA.

3.1.1. Viral Isolates, CSF, Saliva, and Urine Specimens

3.1.2. Throat and Rectal Swabs

1. Suspend the swabs in 1× phosphate-buffered saline (PBS).
2. Proceed to Subheading 3.2 for extraction of RNA.

3.1.3. Stool Specimens

1. Suspend 0.5 g of stool (0.5 mL for fluid stools) in 5 mL of 1× PBS.
2. Centrifuge at 12,000 × *g* for 10 min and then filter.
3. Collect the filtrate and proceed to Subheading 3.2 for extraction of RNA.

3.1.4. Serum Specimens

1. Allow the blood to stand for 15–20 min at room temperature.
2. Centrifuge at 12,000 × *g* in a 10 mL centrifuge/blood tube for 10 min and then filter the upper layer using a 0.2 μm Millipore filter.
3. Collect the filtered serum and proceed to Subheading 3.2 for extraction of RNA.

3.2. Extraction of Viral RNA

The extraction of viral RNA is carried out using a QIAamp Viral RNA Mini kit (Qiagen, USA) according to the manufacturer's instructions.

1. Pipette 560 μL of the AVL buffer containing the carrier RNA into a 1.5 mL eppendorf tube.
2. Add 140 μL of the viral isolate or the clinical specimen to the eppendorf tube containing the Buffer AVL-carrier RNA, and mix by pulse-vortexing. Incubate at room temperature for 10 min.
3. Briefly centrifuge the eppendorf tube to remove any fluid from the inside of the tube.
4. Add 560 μL of absolute ethanol to the sample and mix by pulse-vortexing for 15 s.
5. Add 630 μL of the sample from step 4 to the QIAamp Mini spin column (in a 2 mL collection tube) and centrifuge at 6,000 × *g* for 1 min. Place the QIAamp spin column into a clean 2 mL collection tube. Discard the filtrate.
6. Repeat step 5.

7. Carefully open the QIAamp Mini spin column, add 500 µL of Buffer AW1. Close the cap and centrifuge at 6,000 × *g* for 1 min. Place the QIAamp spin column into a clean 2 mL collection tube. Discard the filtrate.
8. Carefully open the QIAamp Mini spin column, add 500 µL of Buffer AW2. Close the cap and centrifuge at 13,500 × *g* for 3 min. Place the QIAamp spin column into a clean 2 mL collection tube. Discard the filtrate.
9. Repeat step 8, but only centrifuge for 1 min.
10. Place the QIAamp spin column in a clean 1.5 mL eppendorf tube. Add 40 µL of Buffer AVE, incubate at room temperature for 1 min then centrifuge at 10,000 × *g* for 1 min.
11. Repeat step 10. Store at –80°C for further analysis.

3.3. One-Step Conventional RT-PCR

1. Determine the number of samples to be analysed. Prepare a master mix as follows:

Reagent	Volume/final concentration
5× Qiagen one-step RT-PCR buffer	5.0 µL
Forward primer – NC1 (10 µM)	0.6 µM
Reverse primer – E2 (10 µM)	0.6 µM
dNTP Mix (10 mM)	2.0 µL
Qiagen one-step RT-PCR Enzyme Mix	2.0 µL
RNase inhibitor	0.5 µL
5× Q-solution	5.0 µL
RNA template	100–500 ng
PCR grade water	variable
Final reaction volume	50.0 µL

2. Perform RT-PCR in a GeneAmp® PCR System 2400 PE (Applied Biosystems) thermal cycler with the following cycling parameters:

Reverse transcription	50°C for 30 min	
Predenaturation	95°C for 5 min	
Denaturation	95°C for 1 min	40 cycles
Annealing	60°C for 30 s	
Extension	72°C for 1 min	
Final extension	72°C for 3 min	

3.4. One-Step Real-Time RT-PCR

1. Determine the number of samples to be analysed. Prepare a master mix as follows:

Reagent	Volume/final concentration
$Mn(OAc)_2$	3.5 mM
Forward primer – NC1M (10 μM)	0.2 μM
Reverse primer – E2 (10 μM)	0.2 μM
TaqMan probe (2 μM)	0.3 μM
LightCycler RNA Master Hybridization probe reaction mix	2.0 μL
RNA template	100–500 ng
PCR grade H_2O	Variable
Final reaction volume	20.0 μL

2. Perform RT-PCR in a LightCycler real-time PCR machine (version 2.0) (Roche Diagnostics, Germany) with the following cycling parameters (Fig. 2):

Reverse transcription	59°C for 20 min	
Pre-denaturation	95°C for 30 s	
Denaturation	95°C for 1 s	45 cycles
Annealing/extension	63°C for 40 s	

3.5. Agarose Gel Electrophoresis

1. Weigh 1.2 g of agarose using a weighing machine.
2. Transfer the agarose to a conical flask containing 100 mL of 1× TBE buffer.
3. Dissolve the agarose in the TBE buffer by heating in a microwave oven for 2 min at 750 W.
4. Add 1.0 μL of ethidium bromide into the molten agar and pour the molten agarose into a gel caster (preprepared with a well comb).
5. Once the gel is set, gently remove the well comb from the agarose gel.
6. Mix 10 μL of the PCR reaction with 2 μL of the loading buffer for each of the samples and load into the wells using a 20 μL pipette tip.
7. Load 10 μL of the marker100 bp DNA ladder into one of the empty wells.

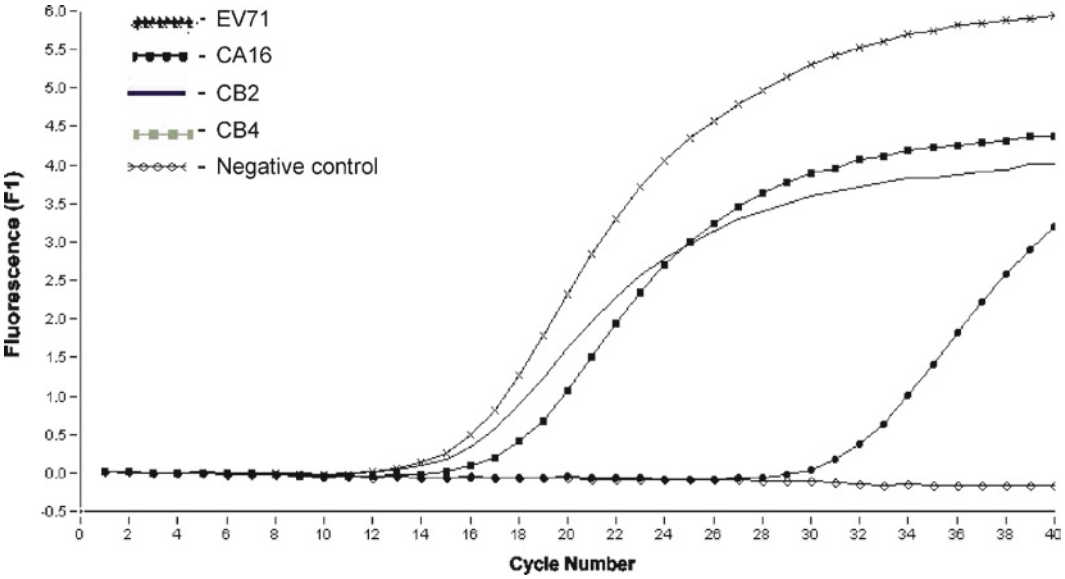

Fig. 2. Real-time TaqMan RT-PCR for specific detection of enteroviruses. The amplification plot is generated from LightCycler real-time PCR machine (version 2.0) (Roche Diagnostics, Germany). The negative control represents all reagents without RNA template.

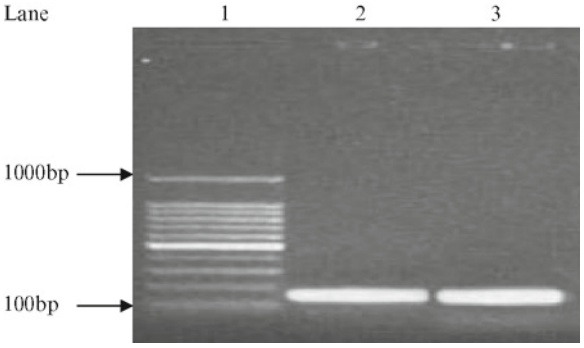

Fig. 3. Agarose gel electrophoresis of PCR products. Expected PCR amplicon size of 153 bp generated from one-step conventional RT-PCR using primer pair NC1 and E2 (*Lane 2*). *Lane 1* represents 100 bp DNA size markers (Fermentas, Cat. No. SM0243). *Lane 3* represents the expected PCR amplicon size of 146 bp generated from one-step real-time RT-PCR using the primer pair NC1M and E2.

8. Run the gel electrophoresis at 90 V for 1 h.
9. Visualize the DNA bands using the UV transilluminator (Fig. 3).

3.6. DNA Sequencing

Sequence the PCR amplicons generated from either the conventional RT-PCR or real-time RT-PCR to determine the enterovirus serotype.

1. Determine the number of samples to be analysed. Prepare a master mix as follows:

Reagent	Quantity
Big Dye Terminator cycle sequencing Big dye	4.0 µL
Forward primer (either NC1 or NC1M) (10 µM)	2.0 µL
PCR products	7.0 µL
5× Reaction buffer	1.0 µL
PCR grade H_2O	6.0 µL
Final reaction volume	20.0 µL

2. Perform RT-PCR in a GeneAmp® PCR System 2400 PE (Applied Biosystems) thermal cycler with the following cycling parameters:

Pre-denaturation	95°C for 5 min	
Denaturation	95°C for 30 s	
Annealing	50°C for 30 s	25–30 cycles
Extension	60°C for 1 min	

3. Add 2 µL of 3 M sodium acetate and 50 µL of ice-cold acetate ethanol to the reaction mix and incubate either at –20°C overnight or –80°C for 1 h.
4. Centrifuge at 13,500 × *g* for 20 min.
5. Remove the supernatant and add 200 µL of 70% ethanol. Centrifuge at 13,500 × *g* for 20 min.
6. Remove the supernatant and air dry the pellet at room temperature.
7. Resuspend the PCR products in 100 µL sequencing buffer.
8. Sequence the PCR products by cycle sequencing using the Big dye sequencing kit (Applied Biosystems, USA).

4. Notes

1. As RNA degrades easily, it is important that gloves must be worn at all times. This is to prevent RNase contamination of the samples. Use RNase-free plastic disposable tubes and pipette tips, and use RNase-free water to prepare all the reagents. Dedicate an area that is only used for RNA extraction.

2. Contamination with previous PCR amplifications can be a major problem. Therefore ensure at least one negative control (contains all reagents but without RNA template) is included in every PCR setup. If a false positive is encountered, it is advisable to discard all reagents and prepare new solutions. It is also recommended that different areas are dedicated for each PCR setup and the analysis of PCR products and that there is no exchange of equipments or reagents.
3. Primers are stable at –20°C and have a shelf life of 4 weeks at 4°C. If there is any problems with contamination, discard the primers being used and prepare fresh aliquots.
4. It is advisable to quantitate the amount of RNA templates used for PCR reactions; high concentrations of nucleic acids can inhibit the PCR process. A suitable amount of RNA recommended for both conventional RT-PCR and real-time RT-PCR is between 100 and 500 ng.
5. Ethidium bromide is a carcinogen which is widely used for staining agarose gels. Thus, it is important that precautions must be taken to prevent contamination of the work environment. Ensure that an area is dedicated wholly for gel electrophoresis and gloves are worn when handling the gels.

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Chapter 6

The Detection of Hepatitis Viruses

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Abstract

The hepatitis viruses, named A–E, cause acute and chronic liver disease depending on the virus. Laboratory-based diagnosis relies on the detection of specific markers of infection for each virus, through the use of appropriate serological tests. In recent years, molecular tests that rely on the amplification of viral nucleic acids have also been employed, not only in confirming active viral replication but also in monitoring antiviral treatment efficacy. Some of these tests that are currently in use in routine diagnostic laboratories are outlined in this chapter.

Key words: Hepatitis virus, Markers of infection, Serological tests, Elisa, Antigen, Antibody, Molecular tests, Hybridisation, Polymerase chain reaction, RT-PCR, Real-time PCR, Nucleic acid extraction, Nucleic acid amplification, Nucleic acid detection

1. Introduction

1.1. General

Hepatitis or liver inflammation can be an autoimmune phenomenon, the result of exposure to hepatotoxic drugs or due to alcohol abuse, but most importantly, due to infection with one of the five well-recognised hepatotropic viruses. These viruses have been cloned and identified in the last 60 years or so, and have been given as names, the letters of the alphabet from A to E. Each one of them belongs to a different virus family. Moreover, their mode of transmission differs depending on the virus as do their geographic distribution and prevalence from country to country. All of them are capable of causing acute hepatitis and three, namely, the hepatitis B, C, and D viruses (HBV, HCV, HDV) can persist by establishing a chronic carrier state. It is estimated that worldwide, there are in excess of 650 million people who are chronic HBV or HCV carriers. The hepatitis viruses are, therefore, a major cause of morbidity and mortality. The latter is the outcome of long-term chronic infection that may result in liver failure and

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development of cirrhosis and hepatocellular carcinoma. Antiviral treatment offers the only practical means for interrupting this progression, whilst prophylaxis through active immunisation is currently available for two of the viruses, namely, the hepatitis A virus (HAV) and the HBV (1).

Over the years, a number of tests have been developed for the diagnosis of infection based on serological or molecular approaches. Such tests have proved invaluable not only in the diagnosis of infection but also in monitoring the natural history of chronic disease and in evaluating the efficacy of antiviral drugs, as well as determining response to vaccine administration. This chapter will attempt to give examples of currently available tests and methodology that can be used for diagnostic or research purposes. It is not intended to be an exhaustive expose of a wide range of methods, but rather a description of those most widely used. Moreover, as many of the tests currently used are commercially available from several sources, the mention of a particular trade mark does not imply that they are endorsed or that they are superior to those not mentioned.

Firstly though, a brief description of each virus will be given with emphasis on those aspects of the infection that aid diagnosis or pose diagnostic challenges.

1.2. Hepatitis A Virus

HAV is enterally transmitted and the only member of the hepatovirus genus within the *picornaviridae* (2). The virion measures 27 nm in diameter and is non-enveloped. Its 7.5-kb RNA genome is single stranded, positive sense, and contains a single open reading frame (ORF) that encodes the viral polyprotein. The latter is post-translationally cleaved by a viral protease to yield the components of the capsid and a range of non-structural proteins, including the viral RNA polymerase and protease. The ORF is flanked on either side by the 5′ and 3′ untranslated regions (UTRs), with roles in the translation of the polyprotein and replication of the virus (negative strand synthesis), respectively. HAV displays a high degree of nucleotide and amino-acid conservation between isolates. However, sequencing studies involving amplification by polymerase chain reaction (PCR) of genomic regions that may include the C terminus of the capsid protein VP3, the N terminus of VP1, the junction between VP1/2A or larger fragments such as the entire VP1 or VP1-2B, have demonstrated the presence of sufficient genetic diversity (at the nucleotide level, not amino-acid) to define different genotypes and sub-genotypes of the virus (3). Of the seven genotypes recognised so far, only genotypes I, II, III, and VII are associated with human infection, the first three being subdivided into sub-genotypes A and B. Antigenically, there is only one serotype, and virus-neutralising antibodies are directed to a conformational epitope formed by capsid proteins VP3 and VP1 (4).

Exposure to the virus leads to acute infection following a mean incubation period of 14–40 (mean 28) days. In children, infection is usually asymptomatic. Shedding of the virus in faeces precedes the symptomatic phase of the disease and consequently the rise in alanine aminotransferase (ALT) levels, and stops with their return to normal. The appearance of antibodies (anti-HAV) of IgM class is diagnostic of acute infection, whilst IgG anti-HAV antibodies associated with full recovery are long lasting. Detection of these antibodies following administration of current HAV vaccines is indicative of successful immunisation.

1.3. Hepatitis B Virus

HBV is a member of the *hepadnaviridae*, and the infectious Dane particle has a diameter of 42 nm with an external envelope and an internal core (5). The latter contains the compact DNA genome of the virus which is 3.2 kb in length, partially double stranded and circular in shape. The genome contains four partially or totally overlapping ORFs, one of which encodes for the three envelope proteins (Pre-S1, Pre-S2, and S) generally referred to as the surface antigen (HBsAg). These are coterminal and bear the "a" antigenic determinant, antibodies against which are virus neutralising (anti-HBs). The pre-core/core ORF encodes two proteins, the pre-core/core protein that is the precursor for the soluble "e" antigen (HBeAg), and in addition, the nucleocapsid antigen (HBcAg) or core protein. The other two ORFs encode for the X protein (HBxAg) and the DNA polymerase/reverse transcriptase.

Diagnostically, the detection of HBsAg denotes current infection, whilst its presence longer than 6 months denotes chronic infection. Active virus replication is associated with positivity for HBeAg, as well as detection of HBV-DNA in serum and liver. High levels of IgM anti-HBc are present during acute infection, whilst these may be detectable at much lower levels during chronic infection. In patients experiencing acute exacerbations during the anti-HBe positive phase of chronic infection and associated with the presence of core promoter/pre-core variants of the virus, IgM anti-HBc may become moderately elevated (6). Recovery from infection is associated with the appearance of anti-HBs, the production of which is also the ultimate goal of active immunisation with existing vaccines.

Of increasing significance in the management of chronically infected patients, particularly in offering treatment and assessing treatment response, are the following: (1) determination of virus load by quantitative PCR before, during, and after treatment (7), (2) the presence of the core promoter or the pre-core variants, (3) the virus genotype (there are eight genotypes A–H), (4) mutations conferring resistance to nucleos(t)ide analogues, and finally, (5) mutations elsewhere in the genome, particularly in the HBsAg ORF, which may evade detection by diagnostic enzyme immunoassays (EIAs). Such variants may arise in the vaccination setting, in

transplant patients treated with anti-HBs, and patients reactivating past infection following cytotoxic therapy (8).

Exposure to the virus leads to acute infection 4–12 weeks later, with possible incubation periods of up to 6 months. Asymptomatic infection is not unusual. Exposure in adults is through sex and intravenous drug use, or percutaneous exposure in general, using contaminated needles or implements (body piercing, acupuncture, etc.). Infants born to HBV carrier mothers are at increased risk of becoming infected perinatally, as are siblings and peers of carrier children, through horizontal transmission.

1.4. Hepatitis C Virus

HCV is an enveloped RNA-containing particle of about 60 nm in diameter. The virus belongs to the *flaviviridae* and has been assigned to its own *hepacivirus* genus (9). The genome of the virus is a single-stranded RNA of positive sense and 9.6 kb in length. It contains a single ORF flanked on either side by the 5′ and 3′ UTRs, which as in the case of HAV control polyprotein translation and replication, respectively. The polyprotein is proteolytically cleaved to yield the structural and non-structural proteins of the virus. The structural proteins, which include the core protein and the two envelope glycoproteins E1 and E2, are released from the amino-terminal one-third of the polyprotein by host signal peptidases. The non-structural (NS) proteins, on the other hand, are processed by virus-encoded proteases (NS2 and primarily NS3).

Infection with the virus is through parenteral exposure and leads, in about 80% of cases, to a chronic carrier state. Diagnosis is based on EIA testing of serum samples for antibodies to various viral proteins and detection of HCV RNA by reverse transcription (RT)-PCR. Quantitative measurement of viral load before, during, and after pegylated interferon alpha treatment in combination with ribavirin is important in determining sustained response, relapse, or non-response (10). In the context of anti-viral treatment, assignment of genotype is also important, as this will dictate the length of treatment (6 months for genotypes 2 and 3, and 12 months for 1 and 4).

The assignment of genotype relies entirely on the nucleotide diversity between the isolates of the virus. There are six genotypes of the virus, each one of which is further subdivided into a number of subtypes that currently number more than 70 (11). In addition, as a result of the error-prone activity of the RNA polymerase, the viral population in an individual at any given time constitutes a mixture of closely related genetic variants known as quasispecies.

The incubation period following exposure to the virus is 1–6 months, with only a minority of patients experiencing recovery. Transmission is through intravenous drug use and other percutaneous exposures as for HBV. Sexual and vertical transmission is not common.

1.5. Hepatitis D Virus

HDV measures 36 nm in diameter, and its envelope consists of HBsAg, which it borrows from HBV (12). It is, therefore, a defective virus that requires the presence of HBV for productive infection. The nucleocapsid of the virus consists of delta antigen (HDAg), the only protein encoded by the viral RNA genome. The latter is single stranded, 1.7 kb in length, and of negative polarity. Although circular in shape, it forms a rod-like structure due to extensive intramolecular base pairing and in addition has features similar to those of viroids and virusoids. As a result the virus has not been assigned to a virus family as yet, but is the sole member of the *deltavirus* genus. There are eight genotypes of the virus identified by phylogenetic tree analysis (13).

The virus is transmitted in a manner similar to HBV and can cause co-infection when both viruses are transmitted together to a naïve person or superinfection when infecting somebody who is already a chronic carrier. Diagnosis is dependent on the detection of HDAg, anti-HD (total) and/or HDV RNA in serum during acute infection, whilst HDV RNA and IgM anti-HD antibodies that persist are diagnostic of chronic infection.

1.6. Hepatitis E Virus

This virus, like HAV, is enterally transmitted and has recently been assigned to a new family of viruses named *Hepeviridae* under the genus *Hepevirus* (14). The virion is a non-enveloped particle, measuring 32–34 nm in diameter. The single-stranded positive sense RNA genome of the virus is polyadenylated and approximately 7.5 kb in length. It contains three ORFs (1–3); ORF 1 encodes the non-structural proteins of the virus including the RNA-dependent RNA polymerase, whilst ORF 2 encodes the 660-amino-acid long nucleocapsid protein and ORF 3 a protein of 122 amino-acids, of unknown function.

Epidemics of HEV infection have been associated with poor hygiene and sanitary conditions, resulting in contamination of drinking water with sewage. Incubation periods range from 14 to 60 days. Recovery from infection is complete. Diagnosis is based on the detection of IgM anti-HEV in serum or HEV RNA in serum and faeces during the early symptomatic phase. If viraemia or virus shedding is missed, an increasing titre in IgG anti-HEV would be diagnostic of HEV. It should be stressed that current in-house EIA tests are not very sensitive and may also be prone to false-positive results. So wherever possible, these should, in addition, be supported by PCR testing. There are four genotypes of the virus (1–4), each one subdivided into a number of subtypes currently numbering 24 (15).

1.7. Specimen Collection, Storage, and Processing

The collection of the appropriate specimen, at the right time (particularly in relation to acute infection), and its storage under optimal conditions until processing are of paramount importance in obtaining reliable results. The most commonly used specimen

for the diagnosis of infection with the hepatitis viruses, even in the case of the enterally transmitted ones, is serum.

1.7.1. Serum Collection

Blood for serum preparation is normally obtained by venepuncture. This is nowadays collected in vacutainers minimising the risk of needlestick accidents. Ten millilitres of blood is normally drawn and allowed to clot at room temperature for an hour. After this time, the clotted blood can be refrigerated for up to 24 h if intended for serological testing, or processed preferably on the same day if for RNA detection by RT-PCR. In either case, the serum is removed and aliquoted into screw-capped vials following centrifugation at 2,000×*g* for 10 min at 4°C. These are then stored at –20°C (serology, DNA detection) or –80°C (RNA detection).

1.7.2. Plasma Collection

Anti-coagulated blood for plasma separation can also be used for the same diagnostic procedures. In this case, blood is drawn into vacutainers containing an anti-coagulant such as ethylenediaminetetraacetic acid (EDTA). The tube is inverted 3–4 times to mix its contents and then processed as described above. If heparin is used as the anti-coagulant (~5 U/ml final concentration), then the plasma cannot be used in PCR reactions as the heparin will inhibit the reaction.

1.7.3. Liver Tissue

Liver biopsies carried out by percutaneous Menghini needle puncture, can be used for viral RNA or DNA extraction. In such cases, liver cores about 1 cm in length are snap frozen in liquid nitrogen and then transferred to a –80°C freezer or kept in liquid nitrogen until use.

1.7.4. Stool Extracts

A 10% (w/v) suspension is normally prepared following vigorous shaking of 1 g of faecal sample in 10 ml of phosphate-buffered saline (PBS, pH 7.4). The suspension is next clarified by centrifugation at 3,000×*g* for 15 min at 4°C. The supernatant is then carefully transferred into the barrel of a syringe fitted with a 0.22-μm filter (Millipore) for removal of bacteria. The fluid is forced through the filter by the insertion of the syringe plunger (see Note 1). The filtrate can be reduced to a desired volume using an Amicon centricon concentrator. The filtrate can then be aliquoted and stored at –80°C.

1.7.5. Peripheral Blood Mononuclear Cells

Peripheral Blood Mononuclear Cells (PBMCs) are normally prepared from anti-coagulated blood within 2 h of collection. Six millilitres of diluted blood (blood mixed with an equal volume of PBS or 0.9% saline) is carefully transferred into a Histopaque^{R}-1077 tube or layered on 3 ml Lymphoprep$^{™}$ using a plastic Pasteur pipette and then centrifuged at 800×*g* for 15–20 min at room temperature. During centrifugation, erythrocytes and granulocytes

are aggregated and rapidly settle to the bottom of the tube, whilst lymphocytes and other mononuclear cells remain at the plasma–histopaque/lymphoprep interface. These cells are carefully removed using a Pasteur pipette and transferred into a universal bottle. The PBMCs are pelleted following dilution in 10 ml of PBS or 0.9% saline at 250 g for 10 min. The supernatant is removed and the pellet frozen as already described or processed immediately (see Subheading 3.1).

In view of their infectious nature, the hepatitis viruses have been categorised by the Advisory Committee on Dangerous Pathogens into Hazard groups 2 (HAV) and 3 (HBV, HCV, HDV, and HEV). Handling of specimens requires the use of biosafety cabinets under containment level II conditions by experienced personnel.

2. Materials

2.1. DNA/RNA Extraction

2.1.1. From Serum

1. Proteinase K stock solution at 2 mg/ml in sterile distilled water.
2. Phenol:Chloroform:Isoamyl alcohol (25:24:1, v/v).
3. Yeast t-RNA at 0.8 mg/ml.
4. Glycogen 20 μg/μl.
5. Ethanol at 100 and 70%.
6. Chloroform.
7. Plasticware (see Note 2).

Instead of the above, one could use one of the following commercially available extraction kits:

1. QIAamp DNA Blood Mini or Midi kit.
2. QIAamp Viral RNA mini kit or Purescript^{R} Cell and Tissue RNA extraction kit (Gentra/Flowgen) or other similar kits.
3. TRIzolR and TRIzolR LS reagent (InVitrogen).

2.1.2. From Tissues or Cells

As above, but in addition:

1. Mortar and pestle mini borosilicate glass homogenisers (Fisher Scientific, see Note 3).
2. Liquid nitrogen.

2.2. PCR and RT-PCR

1. Programmable thermal cycler or LightCycler.
2. Filtered 20, 200, and 1,000 μl pipette tips.
3. 0.5-ml flat-capped PCR tubes.
4. Sterile molecular biology-grade water.

5. 10× PCR buffer without $MgCl_2$ provided with the thermostable polymerase.
6. 25 mM $MgCl_2$ supplied with the thermostable polymerase.
7. Deoxynucleotide triphosphates (dNTPs). Mix 20 µl of each with 120 µl of H_2O to make a stock solution of 10 mM and aliquot in amounts to be thawed once.
8. Primers appropriate for the region to be amplified.
9. Taq polymerase or any other thermostable polymerase, including proof readers, depending on the purpose of the PCR.
10. Moloney murine leukaemia virus (MMLV)-RT for cDNA synthesis.
11. 5× first strand synthesis buffer supplied with the RT.
12. RNAsin 20–40 U/µl (Promega).

If using the LightCycler, then use the reagents supplied with the appropriate kit.

2.3. Agarose Gel Electrophoresis

1. Electrophoresis apparatus consisting of tank, casting mould with combs, and power pack.
2. 10× Tris–borate–EDTA (TBE) buffer pH 8.3 (104 g of Tris base, 55 g of boric acid, 40 ml of EDTA pH 8.0 made to 1 l).
3. Loading buffer nowadays provided with the Taq or incorporated with the enzyme solution. If not, make up as follows (6×): 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol in H_2O and store at 4°C.
4. Molecular biology-grade agarose.
5. 10 mg/ml ethidium bromide solution or SybrSafe™ DNA stain solution (×10,000 concentrate in DMSO, InVitrogen).
6. Molecular weight markers appropriate for the size of DNA expected.
7. UV transilluminator.
8. Polaroid camera or digital imager.

3. Methods

3.1. Extraction of Nucleic Acids

3.1.1. DNA from Serum or Plasma

3.1.1.1. Method 1: Using an Established Protocol (16)

1. Mix in a 1.5-ml Eppendorf tube 50 µl of serum/plasma with 20 µl of proteinase K (2 mg/ml), 2.5 µl of t-RNA (0.8 mg/ml), 1.7 µl of SDS (25%), 100 µl of 2× buffer (50 mM sodium acetate, 5 mM EDTA) and make up to 200 µl with H_2O.
2. Incubate at 65°C for 2 h.
3. Add an equal volume of phenol:chloroform:isoamyl alcohol and invert several times to mix.
4. Centrifuge at 12,000×*g* for 10 min.

5. Transfer the top aqueous phase into a new tube and mix with an equal volume of chloroform.
6. Centrifuge as above and remove the top aqueous layer into a new tube.
7. Add one-tenth volume of 3 M sodium acetate (pH 5.2) and two volumes of ethanol before placing at –80°C for 1 h.
8. Centrifuge as above, but this time at 4°C. Add 500 µl of 70% ethanol and re-spin for 5 min.
9. Air-dry the pellet and re-suspend the DNA in 20 µl of H_2O.
10. Store at –20°C until use.

3.1.1.2. Method 2: Using QIAamp DNA Mini Kit

This kit is suitable for extraction of viral DNA utilising spin columns for the binding of DNA and avoiding the use of phenol and chloroform. Moreover, the whole process is shorter than the one described above.

1. Add 20 µl of protease to 200 µl of sample in a 1.5-ml Eppendorf tube.
2. Add 200 µl of buffer AL and mix by vortexing for 15 s.
3. Incubate at 56°C for 10 min. Microfuge briefly to remove droplets from the lid.
4. Add 200 µl of ethanol, mix by inverting several times, and re-spin for 15 s.
5. Apply mixture to the spin column already placed in a 2-ml collection tube. Cap and spin at 6,000 *g* for 1 min. Transfer the column to a new collection tube.
6. Add to the column 500 µl of buffer AW1 and spin for 1 min. Transfer the spin column to a new collection tube.
7. Add 200 µl of buffer AW2, cap, and spin for 3 min at microfuge top speed.
8. Transfer column to a 1.5-ml Eppendorf tube (with top removed but kept) and add 100 µl of buffer AE to the column. Allow to stand for 1 min and then centrifuge at 6,000 *g* for 1 min.
9. Cap the tube with the lid that was kept and store the DNA at –20°C until use. The collection tubes and eluates from steps 5 and 7 are discarded.

3.1.2. Extraction of RNA from Serum/Plasma

3.1.2.1. Method 1: Using an Established Protocol

The protocol is the same as that described for DNA extraction, except that the t-RNA step is omitted. Instead, after chloroform extraction, 1 µl of glycogen is added to the aqueous layer that is removed to act as carrier. This is followed by ethanol precipitation. The final RNA pellet is re-suspended in 20 µl of RNAse-free water and stored at –80°C until use.

3.1.2.2. Method 2: TRIzol® LS Reagent

1. Mix 250 µl sample with 0.75 ml Trizol (this can be scaled down but maintain sample to reagent ratio at 1:3 and incubate at room temperature for 5 min).
2. Add 200 µl of chloroform, mix vigorously, and allow to stand at room temperature for 2–15 min.
3. Microfuge the sample at 12,000 × *g* for 15 min at 4°C.
4. Remove the upper aqueous layer containing the RNA and transfer to a new tube. Add 0.5 ml of isopropanol per 0.75 ml of Trizol used.
5. Incubate at room temperature for 10 min and then spin as in step 3.
6. Discard the supernatant and wash the RNA pellet with 1 ml of 70% ethanol by inverting several times and then spinning at 7,500 × *g* for 5 min at 4°C.
7. Discard the supernatant, air-dry the pellet, and re-suspend the RNA in 20 µl of water.
8. Store at –80°C until use.

3.1.2.3. Method 3: Using QIAamp Viral RNA Mini Kit

1. Pipette 560 µl of buffer AVL containing carrier RNA into an Eppendorf tube containing 140 µl of sample.
2. Mix by pulse vortexing for 15 s and incubate at room temperature for 10 min.
3. Microfuge briefly to remove drops from lid and add 560 µl of ethanol. Pulse–vortex once more and microfuge briefly.
4. Apply half of the mixture to the column already placed in a 2-ml collection tube. Microfuge at 6,000 × *g* for 1 min, place column in a new collection tube, and discard the previous one with eluate.
5. Repeat step 4 with the remainder of sample.
6. Transfer binding column to a new collection tube and apply 500 µl of buffer AW1. Microfuge as above for 1 min and transfer column to a new collection tube.
7. Add 500 µl of buffer AW2 and microfuge at 20,000 × *g* for 3 min.
8. Transfer the column to a 1.5-ml Eppendorf tube (with lid removed but kept) and add 60 µl of buffer AVE equilibrated at room temperature. Allow to stand for 1 min and then spin at 6,000 × *g* for 1 min.
9. Store the re-capped Eppendorf tube containing the RNA at –80°C until use.

3.1.2.4. Method 4. Using the Purescript Kit

1. Add 500 µl of cell lysis solution to 100 µl of sample in a 1.5-ml Eppendorf tube and mix thoroughly.
2. Incubate at 65°C for 5 min.

3. Add 200 µl of protein-DNA precipitation solution to the lysate, invert several times to mix, and place on ice for 5 min.
4. Centrifuge at 20,000 × *g* for 3 min to precipitate proteins and DNA.
5. Decant the supernatant into an Eppendorf tube containing 600 µl of isopropanol and 1 µl of glycogen, before mixing thoroughly.
6. Centrifuge at 16,000 × *g* for 3 min.
7. Pour off the supernatant taking care not to dislodge the pellet. Add 600 µl of 70% ethanol, invert several times, and microfuge as above for 1 min.
8. Pour off the ethanol, invert the tube over absorbent paper to drain any remaining alcohol, and allow to air-dry.
9. Re-suspend the pellet in 20 µl of rehydration solution and store at −80°C until use.

3.1.3. Extraction of RNA from Faecal Samples

All methods described for the extraction of RNA from serum or plasma can equally be applied to faecal extracts.

3.1.4. Extraction of Nucleic Acid from PBMCs

Use $5–10 \times 10^6$ cells as a pellet. Re-suspend in 0.75 ml Trizol and make up volume to 1 ml with water. The procedure from this point onwards is as described under Subheading 3.1.2 in Method 2.

3.1.4.1. Trizol LS Method

3.1.4.2. Purescript Method

The method uses $1–2 \times 10^6$ cells.

1. Pellet cells in a 1.5-ml Eppendorf tube and remove supernatant except for a residual 20 µl volume. Re-suspend the cells by vortexing.
2. Add 300 µl of cell lysis solution and pipette up and down to lyse the cells.
3. Add 100 µl of protein-DNA precipitation solution to the lysate and invert several times to mix.
4. Place on ice for 5 min and then microfuge at top speed for 3 min.
5. Transfer the supernatant into a new tube containing 300 µl of isopropanol (+1 µl of glycogen) and invert several times to mix thoroughly.
6. Spin at top speed for 3 min to pellet the RNA.
7. Follow steps 7–9 under Subheading 3.1.2.4.

3.2. Nucleic Acid Amplification

For example for HBV-DNA:

3.2.1. By PCR

1. Prepare a multiple of the following quantities depending on the number of samples, including positive and negative controls.

Component	Volume (µl)
5× PCR buffer	10
$MgCl_2$ (25 mM)	3
dNTPs (10 mM)	1
Primer 1 (50 µM)	1
Primer 2 (50 µM)	1
Taq (5 U/µl)	0.2
Water	28.8

Thus, each amplification reaction contains 45 µl of the above master mix to which 5 µl of the appropriate template is added.

2. Cycling conditions in a thermal cycler with heated lid:
   a. 1 cycle: 94°C for 3 min.
   b. 35 cycles: 94°C for 30 s, *X*°C for 1 min (appropriate annealing temp for the primer set, usually around 53°C, Table 1) and 72°C for 2 min.
   c. 1 cycle: 72°C for 7 min; hold at 4°C.
3. For nested PCR, a second set of primers is used in a reaction using 48 µl of the master mix prepared as described above, but adjusting the water volume accordingly and using 2 µl of the first-round PCR product as template. The cycling conditions are as above but once again the annealing temperature would be dependent on the new primer set.

3.2.2. By RT-PCR

For example, the detection of HCV-RNA normally requires the use of nested or hemi-nested PCR. Nevertheless, the same process can be used for the detection of HAV-, HDV-, and HEV-RNA.

3.2.2.1. cDNA Synthesis

1. In a 0.5 ml PCR tube, mix the following:

Component	Volume (µl)
Water	6
Anti-sense primer (50 µM)	1
RNA extract	5
	12

2. Place in a heating block at 80°C for 3 min and then chill on ice.
3. Next, depending on the number of samples and controls, prepare the appropriate amount of master mix. Controls

Table 1
Primer sets that have been used successfully for the amplification of hepatitis virus nucleic acids

Virus	Region	Polarity	Primer sequence	nt positions	Fragment size (bp)
HAV[a]	VP1 (N terminus) (2)	+	2133P-GTGAATGTTTATCTTTCAGCAAT	2133–2155[a]	318
		–	2451N-GATCTGATGTATGTCTGGATTCT	2451–2429	
		+	2172P-GCTCCTCTTTATCATGCTATGGAT	2172–2195	243
		–	2415N-CAGGAAATGTCTCAGGTACTTTCT	2415–2392	
HBV	Pre-C/C (17)	+	M3-CTGGGAGGAGTTGGGGGA	1732–1749[b]	728
		–	3C-CTAACATTGAGATTCCCGAGA	2460–2440	
		+	PC1-GTCTGCGCACCAGCACC	1799–1815	178
		–	BC1-GGAAAGAAGTCAGAAGGCAA	1977–1957	
	Surface (18, 19)	+	S1-GACGTTGGTGAGATCCCCTT	157–177[b]	684
		–	S4-GGGTTTAAATGTATACCCAGAGAC	841–818	
		+	S3-CAAGGTATGTTGCCCGTTTG	457–476	232
		–	BCS3-GGCACTAGTAAACTGAGCCA	689–670	
HCV	5 NCR[c]	+	HCV26-GTCTAGCCATGGCGTTAG	77–94	261
		–	HCV28-GTGTCGTGCAGCCTCCAG	338–321	
		+	HCV27-GCACGGTCTACGAGACCT	100–117	217
		–	HCV29-GGGGCACTCGCAAGCACC	317–300	
HDV	HDAg (20)	+	1164-CCGGCTACTCTTCTTTCCCTTCTCTCGTC	1164–1192[d]	133
		–	1297-CACCGAAGAAGGAAGGCCCTGGAGAACAA	1297–1268	

(continued)

Table 1
(continued)

Virus	Region	Polarity	Primer sequence	nt positions	Fragment size (bp)
HEV	ORF2 (21)	+	TqFwd-CTGTTYAAYCTTGCTGACAC	6309–6328[e]	112
		–	TqRev-GTCGGCTCGCCATTGGCTGAGAC	6399–6421	
		+	Tqprobe-HEX-CCGACAGAATTGATTTCGTCGGC-BHQ 1	6345–6367	

The table is not meant to give an exhaustive range of primers, but just one example in each case. *VP1*, capsid protein; *PreC/C*, pre-core/core; *NCR*, non-coding region; *ORF*, open reading frame; *Tq*, TaqMan; *HEX*, 6-carboxy-2′-4-4′-5′-7-7′-hexachlorofluorescein; *BHQ-1*, Black Hole Quencher 1

[a]Positions relative to the genome of HAV strain HM175 (accession no. M14707)

[b]Accession no. D00329

[c]In-house primers, accession no. M62321

[d]Accession no. X04451

[e]Accession no. AF459438

(see Note 4) should include mock samples from the RNA extraction stage. The master mix contains:

RT buffer (5×)	4
dNTPs (10 mM)	1
RNAsin	1
MMLV	1
Water	1
	8

4. Add 8 µl of the master mix to each tube, mix, and place in a thermal cycler at 42°C for 1 h and then at 95°C for 5 min to denature the RT.

3.2.2.2. First Round PCR

1. Prepare a master mix as described for HBV-DNA (see Subheading 3.2.1) scaled up accordingly depending on the number of samples and controls.
2. Aliquot in 45-µl amounts and add 5 µl of cDNA to the appropriate tube.
3. Perform first-round amplification using the parameters described under Subheading 3.2.1.

3.2.2.3. Nested PCR

1. Aliquot the master mix, which now contains the nested set of primers, in 48 µl amounts using the required number of tubes.
2. Add 2 µl of the first-round PCR product.
3. Thermocycle as described under Subheading 3.2.1, using the appropriate annealing temperature for the new primers.

3.3. Agarose Gel Electrophoresis

The most widely used approach for the qualitative detection of the PCR product (or amplicon) is agarose gel electrophoresis followed by staining of the bands and visualisation under UV transillumination.

1. Add 1 g of agarose to 100 ml of 1× TBE buffer (1% gel, see Note 5).
2. Heat in a microwave oven for 2 min.
3. Allow to cool and when still molten, pour into the gel casting apparatus with the comb in position.
4. When the gel has solidified, add 1× TBE buffer until the gel is fully immersed.
5. Mix 15 µl of PCR product with 3 µl of loading buffer and load into well. Include a well of molecular weight markers.

Table 2
Qualitative tests for the detection of HCV RNA

Test	Method	Detection limit IU/ml
Amplicor HCV	RT-PCR	50
Ultra-Qual	RT-PCR	2–3
Procleix	TMA	5
Versant	TMA	5

AMPLICOR® HCV Test, v2.0, Roche Diagnostics (COBAS® AMPLICOR HCV Test, v2.0, semi-automated version for use with COBAS Amplicor Analyser) (COBAS® AmpliPrep/COBAS® AMPLICOR HCV Test v2.0, for automated extraction and amplification, limit 15 IU/ml)
Ultra-Qual™ 1000, National Genetics Institute
PROCLEIX® HIV-1/HCV Assay, Novartis Diagnostics
Versant HCV RNA Qualitative Assay, Siemens Diagnostics

6. Electrophorese at 125 V until the bottom dye is about 1/3 off the bottom of the gel.
7. Place the gel in 50 ml of 1× TBE buffer containing 2.5 µl of ethidium bromide or 5 µl of Sybr Safe for 10 min.
8. Examine and photograph under UV light.

3.4. PCR Applications

3.4.1. Qualitative Tests

In-house assays or commercial ones can be used for this purpose. The commercially available tests (Table 2) rely on the qualitative detection of HCV-RNA based on the principle of target amplification using RT-PCR (Amplicor) or transcription mediated amplification (TMA, Versant). Detection of the amplicon varies depending on the assay. The Amplicor test generates a biotin-labelled product since the anti-sense primer is so labelled, which then binds a streptavidin–horseradish peroxidase complex that acts on a chromogenic substrate. The TMA assay consists of three steps performed in a single tube: target capture, target amplification, and specific detection by hybridisation protection assay. The final product of the amplification is a single-stranded RNA which is then hybridised to a labelled DNA probe in a chemiluminescent assay (22). Assay performance is monitored by means of an internal control, which is added to each sample with the target capture reagent, and is processed through the assay.

A commercial assay for the qualitative detection of HAV RNA also exists (HAV UltraQual™ PCR, National Genetics Institute), but not for HDV or HEV.

3.4.2. Genotyping

Assignment of genotype is particularly important in the treatment of chronic HCV infection. The duration of treatment determines whether a patient is to be offered 6 months of therapy with pegylated interferon and ribavirin (genotypes 2 and 3)

or 12 months (genotypes 1 and 4) (10). The most widely used test is the newly developed VERSANT^{R} HCV Genotype Assay (LiPA) 2.0 (Siemens Healthcare Diagnostics), which uses an amplicon from the 5′ UTR of the HCV extending to the core region (unlike the version 1.0 kit), allowing distinction between HCV genotype 1 and subtypes c-l of genotype 6, and between subtypes a and b of genotype 1. Genotype/subtype assignment is dependent on the use of specific oligonucleotide probes immobilised on nitrocellulose strips, which through reverse hybridisation bind the appropriate amplicon under very stringent conditions. The latter is generated by employing a 5′-biotinylated primer. Streptavidin labelled with alkaline phosphatase is then added and bound by the biotinylated hybrids. Addition of a chromogenic substrate (BCIP/NBT) leads to the formation of a purple/brown precipitate producing a visible line pattern which is specific for each genotype.

3.4.3. Resistance Mutations

The use of nucleoside/nucleotide analogues for the treatment of chronic HBV infection has resulted, with prolonged treatment, in the appearance of amino-acid substitutions in the polymerase gene which confer resistance to these drugs (23). Characteristic amino-acid substitutions associated with resistance to lamivudine and adefovir as well as known compensatory mutations (codons 80, 173, 180, 181, 204, and 236) can be defined using the INNO-LiPA HBV DR v2 line probe assay, which detects nucleotide substitutions in the relevant codons, using the same principle as described under Subheading 3.4.2 For this purpose, amplicons are generated from the HBV DNA polymerase region.

3.4.4. Other Mutations

INNO-LiPA HBV PreCore is a line probe assay that differentiates between wild-type, and basal core promoter and pre-core mutant nucleotide substitutions in the HBV genome. The assay is designed to identify nucleotide polymorphisms at nt1762 (A to T) and nt1764 (G to A) in the basal core promoter region and at codon 28 (nt1896, G to A) in the precore region.

3.4.5. Quantitative PCR

Quantitative measurements of HBV DNA and HCV RNA are used to monitor the efficacy of current anti-viral treatments, as well as determine viraemia levels in acute and chronic infection. Nucleos(t)ide analogues or pegylated interferon and pegylated interferon in combination with ribavirin are the therapeutic options for the treatment of chronic HBV and HCV carriers respectively. In the case of HCV, viral-load measurements at 4 and 12 weeks of treatment aid decisions as to whether to continue treatment or not. Moreover, assessment of new anti-virals such as the HCV protease and polymerase inhibitors is reliant on how effective they are in reducing viral-load levels (24).

Table 3
Commercially available tests for the quantitation of HBV DNA and HCV RNA

HBV quantitation			HCV quantitation		
Assay	**Method**	**Lower detection limit (IU/L)**	**Assay**	**Method**	**Lower detection limit (IU/L)**
Amplicor (v2.0)	PCR	40	Monitor (v2.0)	RT-PCR	600
Versant (v3.0)	bDNA	360	SuperQuant™	RT-PCR	39
TagMan	PCR (real time)	12	Versant (v3.0)	bDNA	625
			TaqMan	RT-PCR (real time)	10
			Real time HCV	RT-PCR	12–30[a]

COBAS® AMPLICOR HBV MONITOR® Test v2.0 (limit 200 IU/L), a semi-automated test is used with the COBAS Amplicor Analyser and COBAS® AmpliPrep/COBAS® TaqMan® HBV Test (limit 12 IU/L) is fully automated for HBV-DNA extraction and amplification respectively, Roche Diagnostics
Versant HBV DNA v3.0, Siemens Diagnostics
AMPLICOR HCV MONITOR® Test, v2.0, Roche Diagnostics
COBAS® AMPLICOR HCV MONITOR Test v2.0 and COBAS® AmpliPrep/COBAS® TaqMan® HCV Test, Roche Diagnostics
SuperQuant™ HCV, National Genetics Institute
VERSANT® HCV RNA 3.0 Assay (bDNA), Siemens Healthcare Diagnostics. Automated version of test using the Versant 440 Molecular System, Siemens Diagnostics
TaqMan HCV analyte-specific reagent (ASR), Roche Diagnostics
RealTime HCV, Abbott Laboratories
[a]Depending on whether 200 or 500 µl of serum/plasma is used

Most commercially available methods are based on PCR amplification of the target nucleic acid followed by detection of the amplicon, except one. The principles involved utilise either target amplification techniques (competitive template or real-time PCR) or branched DNA (bDNA) technology that involves signal amplification instead. Existing commercially available assays are listed in Table 3, together with the technology involved and the minimum detection level for the method.

The monitor and amplicor tests from Roche use a competitive template in the PCR reaction. The superQuant assay uses an internal control, and amplicons are detected densitometrically following Southern blotting and hybridisation. The intensity of the bands is then compared to that of the internal control for quantitation. Finally, the TaqMan from Roche and the RealTime assay from Abbott utilise real-time PCR technology.

The current practice in reporting quantitative results is in terms of IU/L rather than copies/ml as previously done. As can be seen from Table 3, the sensitivity (lower detection limit) of these tests varies depending on the test, as do the dynamic range which describes the highest and lowest concentrations of nucleic

acid that can be detected (not shown), and the linearity of this range. Samples may need dilution to bring them within this range so that they can be quantitated accurately.

There are no quantitative tests for HAV, HDV, or HEV RNAs, so quantitative measurements for these viruses are based on in-house real time PCR assays.

3.5. Serological Assays

There is a wide choice of commercially available kits for the detection of viral antigens and antibodies for the hepatitis viruses. Nowadays, these tests are EIAs utilising native or recombinant antigens or specific antibodies immobilised on a solid support. Detection of antigen is through a specific antibody labelled with an enzyme such as horseradish peroxidase or phosphatase which act on a chromogenic substrate leading to a colour change, or labelled with a chemiluminescent compound such as acridinium ester, leading to emission of light. If the detecting antibody is bound by an immobilised antigen, then an anti-human antibody is similarly labelled for detection.

Kits for the detection of hepatitis virus antigens and antibodies are available from several manufacturers. A list of hepatitis virus markers for which there are tests is given in Table 4. Most of these tests are offered by companies in a format to comply with their platform instruments and can therefore be fully automated. However, there are still a number of kits that can be used in testing sera manually, and two examples are given below. These involve the use of the Murex HBsAg v3 and Murex anti-HCV v4.0 for antigen (HBV) and antibody (HCV) detection respectively.

Table 4
Hepatitis virus markers for which commercial assays are in existence

Virus	Markers
HAV	Anti-HAV (IgG), anti-HAV (IgM), anti-HAV (total)
HBV	HBsAg, anti-HBs
	HBeAg, anti-HBe
	Anti-HBc (total), anti-HBc (IgM)
HCV	Anti-HCV, HCV core
HDV	HDAg , anti-HD (total), anti-HD (IgM)
HEV	In-house assays

Ortho® trak-C™

3.5.1. HBsAg Testing

1. Select the required number of antibody (mouse monoclonal anti-HBs)-coated wells for samples and controls.
2. Add 25 µl of sample diluent followed by 75 µl of sample, positive, or negative controls.
3. Cover plate with a lid and incubate at 37°C for 1 h.
4. Add 50 µl of conjugate to each well, shake plate for 10 s to mix well contents, and incubate as above for 30 min.
5. Wash with glycine/borate wash fluid diluted as instructed, five times. Tap plate on absorbent paper to drain all fluid.
6. Add 100 µl of substrate solution and incubate as above for 30 min. A purple colour develops in reactive samples.
7. Add 50 µl stop solution to each well and read the absorbance at 450 nm within 15 min.

Thus, in the first step of this test, the sample is pre-incubated in microwells coated with monoclonal antibodies to epitopes of the "a" determinant of HBsAg. The immobilised HBsAg binds, in turn, the HRP conjugated goat anti-HBs antibody. The HRP acts upon the TMB substrate (3,3 ,5,5 -tetramethylbenzidine) which is added next leading to a colour change (purple) in the presence of hydrogen peroxide. The purple colour is converted to orange following the addition of sulphuric acid and this is spectrophotometrically measured. A cut-off value is calculated based on the negative controls and readings above this cut-off are regarded as positive.

3.5.2. Anti-HCV Testing

1. Select the required number of coated wells for samples and controls.
2. Add 180 µl of sample diluent to each well followed by 20 µl of sample or control.
3. Cover the wells and incubate at 37°C for 1 h.
4. Wash plate as described under Subheading 3.3 in step 1.
5. Add 100 µl of conjugate to each well, cover the plate, and incubate as above for 30 min.
6. Wash the plate once again, add 100 µl of substrate solution, and cover the wells before incubating as above for 30 min.
7. Add 50 µl of stop solution (0.5–2 M sulphuric acid per well) and read absorbance at 450 nm within 15 min.

The test, therefore, entails binding of anti-HCV antibodies that may be present in the sample to the recombinant antigens immobilised onto the microwell walls. After washing, captured antibody is incubated with HRP conjugated mouse monoclonal anti-human IgG. The conjugate binds to the immobilised

anti-HCV during this step. After washing, the bound enzyme acts upon the TMB substrate in the presence of hydrogen peroxide leading to a purple colour development in the positive wells. The enzyme reaction is stopped by the addition of sulphuric acid that causes the development of an orange colour that is read spectrophotometrically. A cut-off value is calculated based on the negative controls and readings above this cut-off are regarded as positive.

4. Notes

1. Care must be exercised so that the filter is not ejected from the syringe due to excessive pressure.
2. Molecular biology-grade plastic disposables and reagents that are sterile and free of nucleases should be used for all DNA and RNA extractions, PCR, and RT-PCR.
3. In the case of RNA extraction where a mortar and pestle, or tissue grinders are used, these should be immersed in sterile water containing 0.1% diethylpyrocarbonate (DEPC) for 1 h and then autoclaved.
4. Controls in RT-PCR should include a mock sample from the RNA extraction step, at the cDNA synthesis stage one without RT, first-round PCR one where the template is replaced with water, and a similar control in the second-round PCR.
5. The % agarose used can vary depending on the size of DNA. For small fragments, less than 100 bases a 2% gel is more appropriate.

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Chapter 7

Molecular Detection of Herpesviruses

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Abstract

In routine molecular diagnostics, detection of herpesviruses has made a major impact. Infection with herpesviruses is indicated by demonstrating the presence of the virus in selected specimens. Rapid and reliable detection of herpesvirus DNA helps to decrease the lethality as well as the sequelae of herpesvirus infection in patients at risk. This chapter discusses specimen types and both laboratory-developed and commercially available assays useful for molecular detection of herpesviruses. To meet the need for reliable laboratory results, it is advisable to employ maximum automated and standardized kits based on reagents and standards of reproducible high quality. In the routine diagnostic laboratory, introduction of IVD/CE and/or FDA-labeled tests is preferred.

Key words: Molecular detection, Herpesvirus, Herpesviridae, Automation, Standardization, Specimen, Quantitation

1. Introduction

The herpesvirus group also called *Herpesviridae* is a large family of viruses that produce diseases in animals including humans (1). Members of the *Herpesviridae* family include the cytomegalovirus (CMV), herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), Epstein–Barr virus (EBV), varicella zoster virus (VZV), human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7), and human herpesvirus 8 (HHV-8).

All members of the herpesvirus group share a common structure. The virions are composed of relatively large, double-stranded, linear, DNA genomes encased within an icosahedral capsid, which is itself wrapped in a lipid bilayer envelope. Infections with herpesviruses most commonly occur during the first two decades of life. If immunocompetent, the infected individuals are asymptomatic or may present with benign mostly febrile illness. However, in individuals with compromised immunity, such as

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transplant recipients and patients with human immunodeficiency virus (HIV) infection as well as in newborns with immature immune system, clinical disease with high morbidity may develop and, in some cases, may lead to death. After primary infection, herpesvirus DNA becomes latent in several host cells; reactivation usually emerges with immune suppression or deficit of the host. The actual mechanisms for reactivation are unknown but are apparently initiated through multiple causes, such as periods of stress, traumata, solar radiation, fever, various infections, and immunosuppression.

2. Molecular Detection of Herpesviruses

Rapid laboratory diagnosis is essential for decreasing the lethality as well as the sequelae of herpesvirus infection in patients at risk. Infection with herpesviruses is indicated by demonstrating the presence of the virus in selected specimens. Molecular techniques have become an important tool in routine laboratory diagnostics of herpesvirus infection.

Reliable detection of herpesvirus DNA depends on preanalytical issues, such as choice of the correct specimen type, optimal sampling time with regard to the progress of disease, and both time and conditions of the sample transport to the laboratory. Specimen types appropriate for molecular detection of each relevant member of the herpesvirus family are discussed below.

Prior to amplification, the target nucleic acid must be extracted. Nucleic acid extraction, also called sample preparation, is a crucial step in molecular diagnostics. It includes lysis of the nucleic acid-containing specimen and removal of substances which might inhibit subsequent amplification while protecting herpesvirus DNA from degradation. Furthermore, the risk of contamination and potential hazards caused by toxic reagents must be kept to a minimum during sample preparation. Classic extraction protocols have usually been time-consuming, labor-intensive, and susceptible to contamination. Today, commercially available kits on automated platforms achieve effective recovery of herpesvirus DNA and have largely replaced the classic protocols.

The introduction of real-time PCR has significantly simplified routine molecular diagnostics (2). Compared to the conventional PCR procedure, real-time PCR offers several important advantages (3). Real-time PCR combines amplification of target DNA with detection of amplification products in the same closed vessel. Therefore, the potential for contamination is significantly reduced. With real-time PCR, the analytical turnaround time is significantly shorter than that required for assays utilizing conventional

PCR. In contrast to conventional PCR, real-time PCR allows for log-phase analysis. Therefore, the quantitation range for assays based on real-time PCR is significantly greater (5–6 logs) than that for assays based on conventional PCR (2–3 logs).

To guarantee accurate and reliable results with molecular assays, several issues must be addressed. Major issues include introduction of an internal control, the probe detection format, and an evaluation of the assay. Because amplification may fail in a reaction due to interference from inhibitors, an internal control must be incorporated in every molecular assay to exclude false-negative results. To ensure an accurate control of the entire molecular assay, the internal control should be added to the sample before the start of the nucleic acid extraction procedure. Either a homologous or a heterologous IC can be employed. With regard to the detection format, introduction of a probe detection format is required to guarantee analyte-specific testing. Today, major probe detection formats include hybridization probes, TaqMan probes, molecular beacons, and scorpions. To obtain additional information, e.g., the genotype of the DNA product, the generation of a melting curve may be useful (3, 4). Melting curve analysis can be performed for all detection formats, except for the TaqMan probe format, because signal generation depends on the hydrolysis of the probe. Unexpected melting peaks may indicate either primer:primer or primer:probe dimers or sequence variants (Fig. 1).

When introducing a new molecular assay for detection of a member of the *Herpesviridae* family, it is advisable to employ an IVD/CE and/or FDA-labeled test. For such a test, the manufacturer is responsible that the IVD achieves the performance as stated. Nevertheless, the user must verify that performance characteristics, such as accuracy and imprecision, are achieved in the laboratory (5).

The following sections include general information on specific viruses and the adequate specimen types for their molecular detection and provide an overview on both laboratory-developed and commercially available molecular assays. Currently, frequently used commercial assays in Europe are listed in Tables. It is important to note that ranges of linearity are influenced strongly by both the sample preparation protocol and the amplification and detection system employed. Therefore, data provided herein must always be compared with those provided in the latest version of the manufacturer's package insert. Furthermore, the "real" upper limit of linearity may be unknown because of the lack of extremely high concentrated samples. Finally, it must be observed that different assays may have different units of reporting, making the comparison between values obtained very difficult or even impossible. For serial or sequential specimens obtained from the same patient, the identical molecular assay should thus be employed.

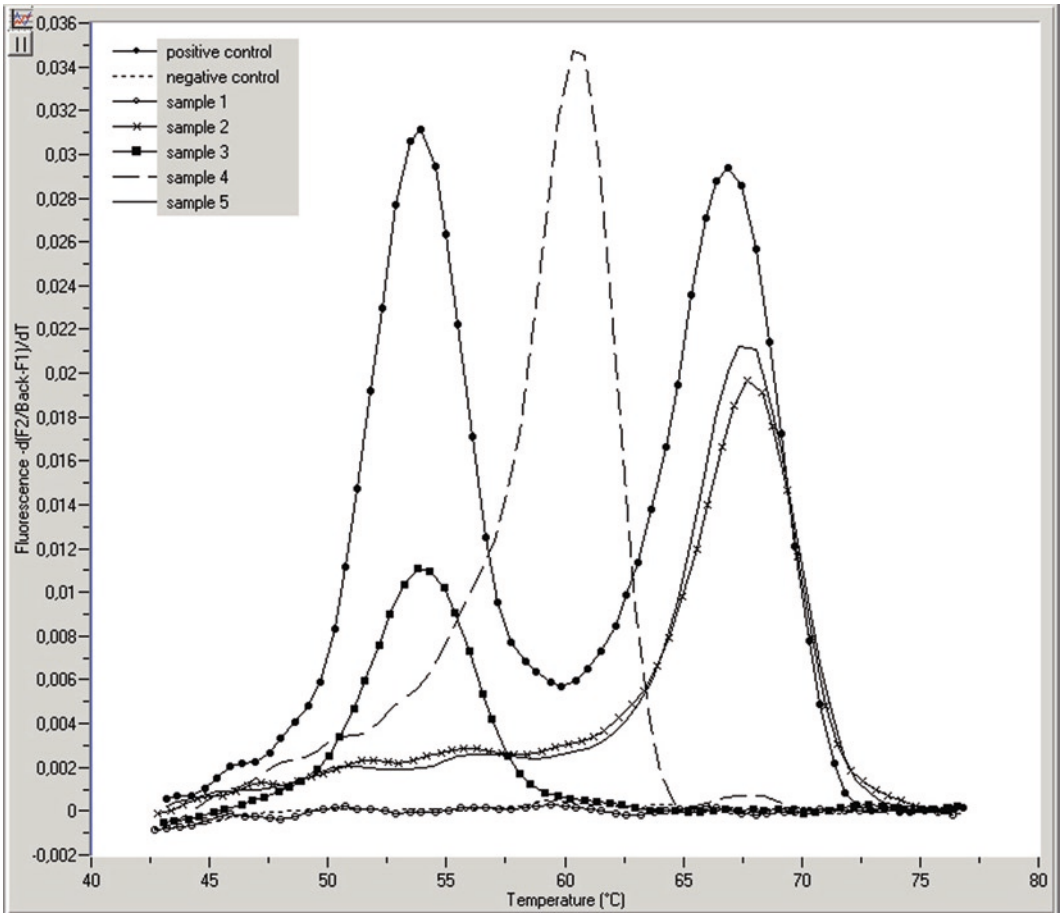

Fig. 1. Detection of herpes simplex virus (HSV) type 1 and type 2 DNA by real-time PCR. Melting curves of clinical samples (genital swabs). In sample 3, HSV-1 was detected; in samples 2 and 5, HSV-2 was found. The positive control contained both HSV-1 and HSV-2. Sample 4 shows an unexpected melting peak at 60.5°C. Sequence variation in the HSV DNA polymerase gene may produce melting peak values that differ from expected values for HSV type 1 or type 2 (modified from ref. 4).

3. Cytomegalovirus

The CMV has the ability to establish lifelong latent infection following primary exposure. Under certain conditions, CMV can reactivate, resulting in asymptomatic viral shedding or development of disease. While in the immunocompetent individual the infection is held in check by the host's immune response, CMV disease is generally restricted to the immunocompromised or immunologically immature host (6). The CMV is thus the most important opportunistic viral pathogen for immunocompromised patients, such as solid organ and bone marrow recipients and AIDS patients.

To avoid lethal outcome of CMV disease, the start of treatment at the earliest stage is of extreme significance (7, 8). The level of CMV DNA has been found to be an important prognostic marker for the ongoing disease (9–11). Detection and quantitation of CMV DNA has been implemented and has replaced the pp65-antigenemia testing in the routine diagnostic laboratory.

3.1. Specimen Types for the Detection of CMV DNA

There is currently no consensus on the optimal blood compartment for routine molecular CMV DNA testing. EDTA whole blood, peripheral blood leukocytes, and plasma have been used for routine diagnosis (12, 13). In several studies, EDTA whole blood has been found to be superior to peripheral blood leukocytes or plasma (8, 13–20). With a high-sensitive molecular assay, latent virus may be detected, but the clinical relevance of low-level CMV DNA in whole blood is unresolved. Nevertheless, the early detection of CMV DNA has the advantage of warning clinicians to be aware of the evolution of infection and to carefully follow up viral load kinetics (21).

Further specimen types for detection of CMV DNA in the immunocompromised patient include cerebrospinal fluid, bronchoalveolar lavage, aqueous humor, and bone marrow. For prenatal issues, amniotic fluid may be an additional specimen type.

3.2. Laboratory-Developed Assays for the Quantitation of CMV DNA

Following the era of laboratory-developed assays based on conventional PCR in the 1990s, several assays based on real-time PCR have been established more recently. Molecular assays based on quantitative real-time PCR have been shown to provide several important advantages to detection of CMV antigen, even though some studies indicated general agreement between the two methods. Advantages of molecular assays based on quantitative real-time PCR include increased sensitivity for early detection of CMV infection or reactivation, utility for patients with neutropenia, wide range of linearity (up to 8 $\log_{10}$), ability to process large number of specimens, and the potential for increased accuracy of results through precision instrumentation (9, 13, 22–24).

However, laboratory-developed molecular assays for the detection of CMV DNA are almost unique to each laboratory usually lacking standardization. In a recent review, more than ten different target regions of the CMV genome and at least three different units of result reports have been described (25). In this context, it must be emphasized that the choice of the target region requires special attention. In several laboratory-developed molecular assays, the glycoprotein B gene (UL55) has been used as the target region. However, CMV variants that could not be quantified using quantitative real-time PCR targeting the glycoprotein B gene have been reported (26). Sequence analysis revealed a single base pair mutation in the target sequence of the

downstream probe. More seriously, several additional sequence differences with the probes used in the glycoprotein B gene assay have been reported recently (27).

3.3. Currently Frequently Used Commercially Available Assays for the Quantitation of CMV DNA

Several commercial assays have been developed for the quantitation of CMV DNA. A summary of currently frequently used commercial assays in Europe is provided in Table 1. The majority of those assays employ the real-time PCR method, while the COBAS Amplicor CMV Monitor Test is based on the conventional PCR technology thus being impaired by a limited range of linearity. The COBAS Amplicor CMV Monitor Test has been evaluated in numerous studies including comparisons with detection of CMV antigen and other molecular assays and has been found to be suitable for routine diagnostic testing of plasma samples obtained from patients at risk of or suffering from CMV infection (28–31). To improve the limit of quantitation, this assay may be performed in an ultrasensitive version by addition of a high-speed precentrifugation step to the standard procedure (32). However, in comparison to molecular assays based on real-time PCR, the COBAS Amplicor CMV Monitor Test was reported to underestimate the viral DNA concentration in plasma samples (33–35).

Currently, the COBAS Amplicor CMV Monitor Test is more and more replaced by alternative commercially available molecular assays based on real-time PCR. The latter assays show a wide range of linearity, and several of them have been evaluated for comparisons with detection of CMV antigen and other molecular assays and have been found to be suitable for the detection and quantitation of CMV DNA in the routine diagnostic laboratory (16, 36–40).

4. Herpes Simplex Virus

HSV causes a wide spectrum of clinical manifestations. HSV type 1 (HSV-1) is commonly associated with oropharyngeal infections, keratoconjunctivitis, and infections of the central nervous system (CNS), whereas the HSV type 2 (HSV-2) commonly produces genital infections. Neonatal HSV infection including HSV encephalitis and retinitis is predominantly caused by HSV-2, whereas an increasing proportion of genital infections are attributable to HSV-1 rather than to HSV-2 (41, 42).

Today, effective therapeutic management exists; however, especially for CNS disease, antiviral drugs must be administered at a very early stage. Rapid laboratory diagnosis is thus essential for decreasing the lethality as well as the sequelae of HSV infection.

Table 1
Comparison of currently frequently used commercially available assays for the quantitation of cytomegalovirus (CMV) DNA

	Manufacturer and details				
Characteristics	**Argene**	**Nanogen Advanced Diagnostics**	**Qiagen GmbH**	**Roche Molecular Diagnostics**	**Roche Molecular Diagnostics**
Kit name	CMV R-gene	CMV Real Time Complete Kit	artus CMV PCR Kit	COBAS Amplicor CMV Monitor Test	LightCycler CMV Quant Kit
Target sequence	UL83 (lower matrix Phosphoprotein 65)	UL123 (major immediate early protein)	UL122 (immediate early protein)	UL54 (DNA polymerase)	UL54 (DNA polymerase)
Amplification method	Real-time PCR	Real-time PCR	Real-time PCR	Conventional PCR	Real-time PCR
Detection method	Fluorescence	Fluorescence	Fluorescence	EIA	Fluorescence
Internal control	Heterologous	Heterologous	Heterologous	Homologous	Heterologous
Standards	Four EQS	Four EQS	Four EQS	One IQS	Four EQS
Range of linearity	$5.0\times10^{2}-2.5\times10^{6}$ copies/mL	$3.2\times10^{2}-1.3\times10^{7}$ genome equivalents/mL	$3.2\times10^{2}-1.0\times10^{7}$ copies/mL	$6.0\times10^{2}-1.0\times10^{5}$ copies/mL	$1.0\times10^{3}-2.0\times10^{7}$ copies/mL

EQS external quantitation standards; *IQS* internal quantitation standard

4.1. Specimen Types for the Detection of HSV DNA

For CNS disease, the detection of HSV DNA in cerebrospinal fluid specimens has been recognized as the gold standard. HSV DNA is detectable in cerebrospinal fluid as early as 1 day after onset of clinical symptoms (43). For suspected dermal or genital HSV infection, detection of HSV DNA in swabs is the standard method. Further specimen types for detection of HSV DNA include aqueous humor (in case of retinitis and/or conjunctivitis) and EDTA whole blood (in case of generalized HSV infection).

4.2. Laboratory-Developed Assays for the Qualitative Detection/Quantitation of HSV DNA

Assays for the qualitative detection/quantitation of HSV DNA based on real-time PCR have been established recently. Several gene targets have been selected including genes coding for glycoproteins B, C, D, and G, thymidine kinase, DNA polymerase, and DNA binding protein (25). Molecular assays based on real-time PCR have shown increased detection rates when compared to cell culture methods for diagnosis of HSV infections.

While quantitation of HSV DNA is usually unnecessary, the recognition of HSV-1 and/or HSV-2 in a specimen must be warranted in the laboratory-developed molecular assay (44–46). However, with real-time PCR assays utilizing the melting curve analysis, amplified DNA of HSV may exhibit atypical melting temperatures that occur between the predicted melting temperatures obtained with HSV-1 and HSV-2 (47, 48). According to different polymorphism patterns, these so-called intermediate strains may be designated type A and type B (48).

4.3. Currently Frequently Used Commercially Available Assays for the Qualitative Detection/Quantitation of HSV DNA

Several commercial assays have been developed for the qualitative detection/quantitation of HSV DNA. A summary of currently frequently used commercial assays in Europe is provided in Table 2. The majority of those assays employ the real-time PCR method, while the NucliSens EasyQ HSV-1/2 test is based on the nucleic acid sequence-based amplification (NASBA) technology. Originally, the NASBA was designed for the amplification of RNA targets. For this qualitative DNA NASBA assay, restriction enzyme digestion has been incorporated into the NASBA reaction allowing efficient amplification of DNA targets (49). Recently, evaluation studies on commercially available molecular assays for the detection of HSV DNA have been published. Investigations include comparisons with alternative molecular assays, and the newly introduced assays have been found to be reliable for the detection and quantitation of HSV DNA in the routine diagnostic laboratory (50–52).

5. Epstein–Barr Virus

EBV has the ability to remain latent in B-cell lymphocytes following primary exposure and thus has major importance in immunocompromised patients, especially in transplant recipients and

Table 2
Comparison of currently frequently used commercially available assays for the qualitative detection/quantitation of herpes simplex virus (HSV) DNA

	Manufacturer and details				
Characteristics	**Argene**	**bioMerieux**	**Cepheid AB**	**Qiagen GmbH**	**Roche Molecular Diagnostics**
Kit name	HSV R-gene	NucliSens EasyQ HSV-1/2	affigene HSV-1/2 tracer	artus HSV-1/2 PCR Kit	LightCycler HSV-Detection Kit
Target sequence	US7 (glycoprotein I, HSV-1); US2 (hypothetical protein, HSV-2)	UL30 (DNA polymerase)	US6 (glycoprotein D, HSV-1); US4 (glycoprotein G, HSV-2)	UL27 (glycoprotein B)	UL30 (DNA polymerase)
Amplification method	Real-time PCR	NASBA	Real-time PCR	Real-time PCR	Real-time PCR
Detection method	Fluorescence	Fluorescence	Fluorescence	Fluorescence	Fluorescence
Internal control	Heterologous	Homologous	Heterologous	Heterologous	Heterologous
Standards	Four EQS	–[a]	–[a]	Four EQS	–[a]
Range of linearity	5.0×10^2 – 5.0×10^5 copies/mL	–[b]	–[c]	NA	–[d]

NASBA nucleic acid sequence-based amplification; *EQS* external quantitation standards; *NA* data not available
[a]Qualitative test
[b]Lower limit of detection 8.4×10^1 copies/isolation (HSV-1), 1.4×10^2 copies/isolation (HSV-2)
[c]Lower limit of detection 1.3×10^2–1.8×10^2 copies/mL (depending on the sample preparation protocol employed)
[d]Lower limit of detection 4×10^2 copies/mL

AIDS patients. Quantitation of EBV DNA in these patients may provide information for initiating treatment and monitoring response to therapy. Information on quantitative viral load may guide a preemptive strategy to reduce the incidence and level of EBV reactivation in transplant recipients. However, EBV is also associated with development of certain malignancies, including posttransplant lymphoproliferative disease (PTLD) and lymphomas. Thus, quantitation of EBV DNA may not only reduce the incidence of EBV reactivation but also the subsequent development of PTLD and lymphomas. Recently, a correlation between the EBV DNA level and the likelihood of development of PTLD was demonstrated (53). In transplant recipients, development of PTLD was found to be associated with a rapid increase of EBV genomes per peripheral blood mononuclear cell (54).

5.1. Specimen Types for the Detection of EBV DNA

There is currently no consensus on the optimal blood compartment for routine molecular EBV DNA testing. The most meaningful results may be obtained by quantitation of EBV DNA in EDTA whole blood including both the cellular and the cell-free compartments from serial or sequential specimens obtained from the same patient (55, 56). For CNS disease, detection of EBV DNA in cerebrospinal fluid specimens of patients with meningitis and encephalitis is the standard method. EBV DNA was successfully detected in cerebrospinal fluid specimens of patients with AIDS-related lymphomas (57).

5.2. Laboratory-Developed Assays for the Quantitation of EBV DNA

Assays for the quantitation of EBV DNA based on real-time PCR have been established recently. Several gene targets have been selected including genes coding for the DNA polymerase, the thymidine kinase, the Epstein–Barr nucleic antigen type 1, the glycoprotein B, the ZEBRA protein, and a nonglycosylated membrane protein (25). Molecular assays based on real-time PCR have shown superior performance when compared to assays based on conventional PCR (58, 59).

Similar to the detection of CMV, the choice of the target region requires attention as EBV sequence variation is a recognized phenomenon. Especially, if the latent membrane protein genes are used as the target region, sequence variation may lead to an underestimation of the EBV viral load or even to a false-negative result (60–62).

5.3. Currently Frequently Used Commercially Available Assays for the Quantitation of EBV DNA

Several commercial assays have been developed for the quantitation of EBV DNA. A summary of currently frequently used commercial assays in Europe is provided in Table 3. All of those assays are based on real-time PCR. Recently, evaluation studies on commercially available molecular assays for the quantitation of EBV DNA have been published. Assays investigated were found to be reliable and suitable for routine EBV-associated disease

Table 3
Comparison of currently frequently used commercially available assays for the quantitation of Epstein–Barr virus (EBV) DNA

	Manufacturer and details				
Characteristics	**Argene**	**Cepheid AB**	**Nanogen Advanced Diagnostics**	**Qiagen GmbH**	**Roche Molecular Diagnostics**
Kit name	EBV R-gene	affigene EBV trender	EBV Q-PCR Alert Kit	artus EBV PCR Kit	LightCycler EBV Quant Kit
Target sequence	BXLF1 (thymidine kinase)	BKRF1 (Epstein–Barr nucleic antigen 1)	BKRF1 (Epstein–Barr nucleic antigen 1)	BKRF1 (Epstein–Barr nucleic antigen 1)	LMP2 (latent membrane protein 2)
Amplification method	Real-time PCR	Real-time PCR	Real-time PCR	Real-time PCR	Real-time PCR
Detection method	Fluorescence	Fluorescence	Fluorescence	Fluorescence	Fluorescence
Internal control	Heterologous	Heterologous	Heterologous	Heterologous	Heterologous
Standards	Four EQS	Two EQS	Four EQS	Four EQS	Four EQS
Range of linearity	$5.0\times10^{2}-5.0\times10^{5}$ copies/mL	$1.0\times10^{3}-2.0\times10^{7}$ copies/mL (EDTA plasma); $3.5\times10^{3}-2\times10^{7}$ copies/mL (EDTA whole blood)	$4.0\times10^{1}-4.0\times10^{6}$ genome equivalents/10^{5} cells	$1.0\times10^{3}-1.0\times10^{6}$ copies/mL	$1.0\times10^{3}-2.0\times10^{7}$ copies/mL (EDTA plasma); $2\times10^{3}-2\times10^{7}$ copies/mL (EDTA whole blood)

NA data not available; *EQS* external quantitation standards

monitoring (63–65). However, it must be mentioned that one of those assays (the LightCycler EBV Quant Kit) amplifies part of the EBV latent membrane protein 2 gene. In presence of the recognized EBV sequence variation (see above), a shifted melting curve may result because of an altered binding of one of the fluorescent hybridization probes. Therefore, careful analysis of the melting curve is of paramount importance to avoid false-negatives when using this assay.

6. Varicella Zoster Virus

After primary infection, VZV persists in latent form and may reactivate in the advanced age producing vesicles that are typically confined to a single dermatome of the skin. However, VZV infections can also produce systemic infections of the central nervous and respiratory systems, mainly in immunocompromised patients, especially in transplant recipients and AIDS patients (66). Because the clinical presentation of VZV dermal disease can be confused with that produced by HSV, laboratory diagnosis is of major importance for distinguishing HSV from VZV infections (67). In immunocompromised patients, quantitation of VZV DNA may provide information for initiating treatment and monitoring response to therapy. Information on quantitative viral load may guide a preemptive strategy to reduce the incidence and level of VZV reactivation in transplant recipients.

6.1. Specimen Types for the Detection of VZV DNA

For suspected dermal VZV infection, detection of VZV DNA in swabs is the standard method. In immunocompromised patients, the most meaningful results may be obtained by quantitation of VZV DNA in EDTA whole blood including both the cellular and the cell-free compartments from serial or sequential specimens obtained from the same patient. For CNS disease, detection of VZV DNA in cerebrospinal fluid specimens is the standard method. Recently, VZV DNA was the most prevalent herpesvirus DNA detected in cerebrospinal fluid specimens (68). For respiratory VZV infection, detection of VZV DNA in bronchoalveolar lavages is meaningful. Recently, detection and quantitation of VZV DNA in oral fluid proved to be useful in patients with Ramsey Hunt Syndrome (69).

6.2. Laboratory-Developed Assays for the Qualitative Detection/Quantitation of VZV DNA

Several laboratory-developed assays for the qualitative detection/quantitation of VZV DNA based on real-time PCR have been established. Gene targets include genes coding for the DNA polymerase, the glycoprotein B, the DNA binding protein, and the immediate-early transactivator (25). Molecular assays based on real-time PCR have shown superior performance when compared to conventional shell vial cell culture for detection of VZV (70–72).

6.3. Currently Frequently Used Commercially Available Assays for the Qualitative Detection/Quantitation of VZV DNA

Several commercial assays have been developed for the qualitative detection/quantitation of VZV DNA. A summary of currently frequently used commercial assays in Europe is provided in Table 4. All of those assays are based on real-time PCR. Currently, no evaluation studies have been published so far. One of the assays, the RealArt VZV LC PCR test, has been employed recently for the quantitation of VZV DNA in patients with Ramsey Hunt Syndrome, and detection rates have been found to be higher compared to those reported recently (69).

7. Human Herpesvirus 6

HHV-6 has the ability to establish lifelong latent infection following primary exposure, which usually occurs in the childhood presenting as unremarkable febrile illness or resulting in roseola infantum, also called exanthem subitum (73). In contrast, primary infection in an adult seems to be a rare event. However, HHV-6 may reactivate and produce systemic infections of the central nervous system including meningitis and encephalitis, mainly in immunocompromised patients, especially in transplant recipients and AIDS patients (74). Furthermore, HHV-6 may produce hepatitis and pneumonia in those patients. Although there are antiviral drugs available that inhibit the replication of HHV-6 in vitro, evaluation of such agents in larger clinical trial has not been done yet.

7.1. Specimen Types for the Detection of HHV-6 DNA

There is currently no consensus on the optimal blood compartment for routine molecular HHV-6 DNA testing. The most meaningful results may be obtained by quantitation of HHV-6 DNA in EDTA whole blood including both the cellular and the cell-free compartments. Similar to other herpesviruses, such as CMV and EBV, the HHV-6 can be detected in peripheral blood mononuclear cells during both active disease and latency (75). When employing a quantitative molecular assay, it must be taken into consideration that a cutoff to distinguish active infection from latent infection has not yet been identified. Furthermore, the comparability of results obtained from serial or sequential specimens may be impaired through fluctuating leukocyte counts. In serum or plasma, HHV-6 may be found only during active disease. However, HHV-6 DNA may be detectable in serum or plasma obtained from individuals with HHV-6 sequences integrated in their cellular chromosomes, even when they are healthy (76). For CNS disease, detection of HHV-6 DNA in cerebrospinal fluid specimens is the standard method. However, false-positive results may be obtained if mononuclear cells are present in the CSF. For respiratory HHV-6 infection, detection of HHV-6 DNA in bronchoalveolar lavages is meaningful.

Table 4
Comparison of currently frequently used commercially available assays for the qualitative detection/quantitation of varicella zoster virus (VZV) DNA

	Manufacturer and details			
Characteristics	**Argene**	**Nanogen Advanced Diagnostics**	**Qiagen GmbH**	**Roche Molecular Diagnostics**
Kit name	HSV1 HSV2 VZV R-gene	VZV Q-PCR Alert Kit	artus VZV PCR Kit	LightCycler VZV Qual Kit
Target sequence	ORF17 (host shutoff virion protein)	ORF29 (major DNA binding protein)	ORF38 (virion protein)	ORF29 (major DNA binding protein)
Amplification method	Real-time PCR	Real-time PCR	Real-time PCR	Real-time PCR
Detection method	Fluorescence	Fluorescence	Fluorescence	Fluorescence
Internal control	Heterologous	Heterologous	Heterologous	Heterologous
Standards	Four EQS	Four EQS	Four EQS	–[a]
Range of linearity	1.0×10^4 – 1.0×10^7 copies/mL	1.3×10^2 – 1.3×10^7 genome equivalents/mL	NA	–[b]

EQS external quantitation standards; *NA* data not available
[a]Qualitative test
[b]Lower limit of detection 3.5×10^2

Table 5
Comparison of currently frequently used commercially available assays for the quantitation of human herpesvirus 6 (HHV-6) DNA

	Manufacturer and details	
Characteristics	**Argene**	**Nanogen Advanced Diagnostics**
Kit name	CMV HHV6,7,8 R-gene	HHV6 Q-PCR Alert Kit
Target sequence	U57 (U42 for HHV7; ORF26 for HHV8)	ORF13R
Amplification method	Real-time PCR	Real-time PCR
Detection method	Fluorescence	Fluorescence
Internal controls	Heterologous	Heterologous
Standards	Four EQS	Four EQS
Range of linearity	$5.0\times10^2-2.5\times10^6$ copies/mL	$1.3\times10^2-1.3\times10^7$ genome equivalents/mL

EQS external quantitation standards

7.2. Laboratory-Developed Assays for the Quantitation of HHV-6 DNA

Molecular assays based on real-time PCR have been established and used for the detection of HHV-6 DNA in plasma obtained from recipients of allogeneic stem-cell transplantation (77, 78). For the quantitation of HHV-6 DNA in cerebrospinal fluid, specimens obtained from patients with CNS disease were investigated with conventional PCR; however, this assay has not been evaluated sufficiently (79).

7.3. Currently Frequently Used Commercially Available Assays for the Quantitation of HHV-6 DNA

Currently, there are two molecular assays based on real-time PCR commercially available (Table 5). Recently, one of the assays, the CMV HHV6,7,8 R-gene test, was found useful for the routine diagnostic laboratory when compared to two HHV-6 in-house quantitative real-time PCR methods (80).

8. Human Herpesvirus 7 and Human Herpesvirus 8

Both, HHV-7 and HHV-8 have the ability to establish lifelong latency. Primary infection may cause exanthem subitum and/or high fever, but in general, HHV-7 is believed to be less pathogenic than HHV-6. Reactivation following liver and bone marrow transplantation has been reported, and HHV-7 has been associated with the development of encephalitis and hepatitis (81). However, the role of HHV-7 in human disease is still not defined clearly. In contrast, infections caused by HHV-8 have

been described and discussed in detail. Activation of HHV-8 replication in the latently infected cells is responsible for viral spread and presumed to contribute to the development of HHV-8 associated diseases including Kaposi's sarcoma, multicentric Castleman's disease, primary effusion lymphoma, and diffuse large B-cell lymphoma (82).

8.1. Specimen Types for the Detection of HHV-7 DNA and HHV-8 DNA

The most meaningful results for routine molecular HHV-7 and HHV-8 DNA testing may be obtained by quantitation of viral DNA in EDTA whole blood. During infection, viral DNA can be detected in both peripheral blood mononuclear cells and plasma. Detection of HHV-8 DNA in peripheral blood mononuclear cells from HIV-1 infected individuals was associated with an increased risk of subsequent development and the clinical stage of Kaposi's sarcoma (83). Recently, it has been suggested that peripheral blood mononuclear cells and plasma are both adequate specimen types for quantitation of HHV-8 DNA (84). In patients with CNS disease, detection of HHV-7 DNA in cerebrospinal fluid specimens may be meaningful.

8.2. Laboratory-Developed Assays for the Quantitation of HHV-7 DNA and HHV-8 DNA

Molecular assays based on real-time PCR have been established and used for the quantitation of HHV-7 DNA (85, 86). Similarly, molecular assays employing the real-time PCR technique were found useful for the quantitation of HHV-8 DNA (87, 88).

8.3. Commercially Available Assay for the Qualitative Detection of HHV-7 DNA and HHV-8 DNA

The CMV HHV6,7,8 R-gene test (Argene) allows for the qualitative detection of HHV-7 and HHV-8 DNAs and was found to be an efficient and reliable tool for the diagnosis of infections produced by HHV-7 and/or HHV-8 (80).

9. Conclusions

For rapid diagnosis of herpesvirus infection, molecular assays have been introduced in the routine molecular diagnostic laboratory. Today, the majority of assays are based on the real-time PCR technique which provides quantitation through addition of standards with calibrated levels of target nucleic acid. To meet the need for reliable laboratory results, it is advisable to use a maximum automated and standardized kit based on reagents and standards of reproducible high quality.

Especially in immunocompromised patients, screening a panel of herpesviruses may be of importance. This requirement can be met by introduction of a set of real-time PCRs, which are performed with one cycling program in parallel in a single run allowing for flexible testing of clinical samples for the presence of

different herpesviruses and providing unaltered limits of detection/quantitation (89). Today, this principle is already included in commercially available real-time PCR kits. For instance, the CMV HHV6,7,8 R-gene test (Argene) allows for single automated extraction of each sample followed by the quantitation of CMV and HHV-6 DNAs and the qualitative detection of HHV-7 and HHV-8 DNAs with one cycling program in parallel in a single run. Furthermore, there are additional kits available for quantitation of HSV1, HSV2, EBV, and VZV, with all of them using the identical cycling program allowing for screening the whole panel of herpesviruses in parallel in a single run. In contrast, assays based on multiplex real-time PCR may be impaired by the worsened limits of detection/quantitation. In future, this problem may be overcome by improved detection through microarray techniques.

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Chapter 8

HIV Drug Resistance Testing

Patricia Cane

Abstract

HIV genotypic resistance testing has become a routine test informing the management of HIV-positive patients. This chapter describes the background of this testing and provides a protocol for undertaking the assay by a home-brew method. Methods for interpretation of the data are also outlined.

Key words: HIV, Drug resistance, Genotypic interpretation, HIV resistance testing, Nucleotide sequencing, Mutation interpretation

1. Introduction

The introduction of combination anti-retroviral therapy (ART) has had a dramatic impact on the course of HIV disease. Classes of anti-retroviral drugs in use include the nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, integrase inhibitors, and CCR-5 antagonists. Therapy is usually initiated with three drugs within two classes, for example two NRTIs with one NNRTI or PI, with the other classes of drug then being used for second-line therapy. However, despite the success of ART on reducing HIV-associated disease, a substantial minority of patients fail their therapy at some time during their treatment due to the development of virus that is resistant to some or all of the components of their therapy (1).

HIV has a rapid rate of replication combined with "error-prone" replicative enzymes. This means that the mutation rate of the virus is very high and that, rather than being a uniform population, the virus within an individual is a mixture of many different minor variants of the virus (a so-called "quasispecies"). A consequence

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of this is that it is likely that there exist virus particles within an individual which carry mutations at sites that can confer resistance to particular drugs. Such viruses are present at only very low or even undetectable levels in untreated patients but can be rapidly selected to grow out when drug pressure is applied. The likelihood of a single virus particle carrying mutations that confer resistance to a combination of drugs is much lower than the likelihood of resistance to a single drug, which is why combination therapy has been so successful.

1.1. Factors Contributing to the Emergence of Resistance in Patients

If virus replication is completely suppressed, then resistance is likely to be very slow to emerge. However, if drug potency or drug levels are inadequate, which can be due to factors such as poor adherence or pharmacokinetic properties, this may allow some low-level replication of the virus, and thus facilitate the development of resistance.

Resistance to some drugs can be caused by single mutations in the virus. For example, high-level lamivudine resistance results from a single amino acid change, M184V, in reverse transcriptase (the "M" in this notation indicates the amino acid present in wild type virus, i.e. methionine; the "184" is the codon position in reverse transcriptase; the "V" is the amino acid present in the mutant virus, i.e. valine). Likewise, resistance to efavirenz can result from the amino acid change K103N. Such drugs are said to have a low genetic barrier. In contrast, resistance to some protease inhibitors, such as lopinavir, requires the accumulation of multiple mutations and can thus be considered to have a high genetic barrier.

Cross-resistance between drugs is common. This occurs when two drugs share the same resistance mutation profile, such as efavirenz and nevirapine where K103N confers high-level resistance to both drugs, or lamivudine and emtricitabine where M184V results in resistance. Alternatively, if a patient remains on a failing therapy such as zidovudine (AZT), the resistant virus may accumulate more mutations with time finally resulting in more extensive classwide cross-resistance; for example, initial resistance to AZT may be extended to include other NRTIs such as tenofovir or abacavir. Overall, it should be borne in mind that resistance to therapy is a continuum, and resistance levels will increase if a patient is kept on a failing therapy.

HIV resistance testing is increasingly being undertaken within routine clinical virology laboratories. It is very important that there is sufficient molecular virology technical expertise within the laboratory to support this highly complex test, particularly where home-brew methods are used. Results should always be interpreted in the context of the patient's treatment history and complex outcomes supported by expert interpretation and guidance for the treating clinician.

1.2. Guidelines

Up-to-date clinical guidelines for resistance testing are provided by the British HIV association (2), the European AIDS Clinical Society (3), and by the American Panel on Clinical Practices for Treatment of HIV infection (4).

2. Materials

There are several commercial kits (Trugene from Siemens and Viroseq from Abbott) for HIV genotypic resistance testing, which are available for use in routine laboratories able to undertake molecular diagnostics. Other laboratories use "home-brew" methods, which are considerably less expensive and can be more flexible but require a high level of local expertise in nucleotide sequencing and sequence interpretation. The assay described below is a home-brew method in routine use in the author's laboratory.

2.1. Equipment

Standard molecular biology equipment is required for this assay, i.e. PCR machines and sequencers. Filtered pipette tips should be used for all stages of the PCR set-up but are not required for post-PCR steps.

2.2. Reagents

1. Sterile, nuclease-free water.
2. One-step RT-PCR kit, Qiagen.
3. dNTPs, 10 mM.
4. Oligonucleotide primers.
5. Platinum *Taq* polymerase kit, Invitrogen.
6. TBE buffer.
7. Ethidium bromide, 5 μg/ml.
8. Gel loading dye and molecular weight DNA marker, 1 kb.
9. Agarose.
10. Ethanol, absolute.
11. GFX PCR clean-up kit, Amersham, or equivalent.
12. Nucleotide sequencing kit suitable for use with available sequencing equipment.

2.3. HIV-1 Resistance Genotyping Primers

Our laboratory has found the following primers (for protease and reverse transcriptase) to be suitable for use with all HIV-1 group M samples tested (5).

1. RT-PCR:

   P1 – TGA ARG AIT GYA CTG ARA GRC AGG CTA AT

   P2 – CCT CIT TYT TGC ATA YTT YCC TGT T

2. Nested-PCR:
   P3 – CTT TAR CTT CCC TCA GAT CAC TCT
   P4 – GGC TCT TGA TAA ATT TGA TAT GTC CAT
3. Sequencing primers:
   P3 – CTT TAR CTT CCC TCA GAT CAC TCT
   P5 – GTT AAA CAA TGG CCA TTG ACA GAA GA
   P6 – TGG AAA GGA TCA CCA GCA ATA TTC CA
   P7 – GGA ACT GTA TCC TTT AGC TTC CC
   P8 – GGG CCA TCC ATT CCT GGC
   P9 – CCA TCC CTG TGG AAG CAC ATT G
   P10 – CTG TAT TTC TGC TAT TAA GTC TTT TGA

3. Methods

There are two main methods for determining resistance of HIV, phenotypic and genotypic. Genotypic resistance assays are the most commonly used routine test. Commercially available kits target the protease and RT regions of the viral genome, while home-brew assays can be adapted to examine any region as required. The assays involve amplification of the relevant part if the viral genome uses reverse transcriptase-polymerase chain reaction (RT-PCR) followed by nucleotide sequencing of the PCR product. Results of genotypic testing are then interpreted through identification of one or more mutations recognised to confer reduced drug susceptibility. More than 200 such mutations have been identified, and these interpretations are regularly updated (6).

In classical phenotypic assays, a virus isolate is obtained from the patient's blood, and the sensitivity of this virus to the drugs in question is then determined in culture. This method is very seldom used owing to the difficulties in obtaining a primary virus isolate and the prolonged time required. Alternative phenotypic methods use recombinant approaches where the appropriate section of the viral genome is amplified by RT-PCR and then incorporated into a standard virus backbone, and the resulting recombinant virus is then assayed either by standard culture methods or by one-step abortive replication with an indicator system. These methods are relatively slow and expensive and also seldom used outside a research or clinical trial context, although they are available commercially.

3.1. Detailed Protocol for Protease and Reverse Transcriptase Sequencing of HIV-1

This protocol uses a one-tube RT-PCR reaction followed by a nested PCR. The PCR products are then purified using columns and sequenced. The sequences are aligned and edited using Sequencher (Gene Codes Corporation), and the sequence is then submitted to the University of Stanford database programme for analysis with respect to drug resistance (7) (see also Notes 1–5).

3.2. Amplification Using Qiagen One Tube RT-PCR

Primary RT-PCR Qiagen one step master mix

1. Make up the volume of mix required for number of samples plus controls with allowance of reagents for one extra sample to allow for pipetting errors.

Per reaction (µl)	Reagent
23	RNase-free water
10	5× buffer (with kit)
2	dNTP, 10 mM (with kit)
1.5	P1 (10 pmol/µl stock)
1.5	R2 (10 pmol/µl stock)
2	Enzyme mix

2. Pipette 40 µl of the master mix into each 0.2 ml thin-walled PCR tube.
3. Add 10 µl of extract to each tube.
4. Cap tubes and place into PCR machine; carry out cycling as follows:

Temp (°C)	Time	Cycles
50	40 min	
95	15 min	
94	30 s	
53	30 s	35
72	1 min	
72	4 min	
10	HOLD	

3.3. Nested Round PCR

Round 2 master mix

1. Make up the volume of mix required for number of samples plus controls with allowance of reagents for one extra sample to allow for pipetting errors.

Per sample (µl)	Reagent
37.5	RNase-free water
5	10× buffer (with kit)
2	Magnesium chloride (with kit)
1	dNTP (10 mM)
1.5	P3 (10 pmol/µl stock)
1.5	P4 (10 pmol/µl stock)
0.5	Platinum *Taq*

2. Thoroughly mix and pipette 49 µl of the master mix into each 0.2-ml PCR tube.
3. Add 1 µl of first round product to each second-round reaction, per sample.
4. Cap tubes and place into PCR machine; carry out cycling as follows:

Temp (°C)	Time	Cycles
95	5 min	
90	30 s	
50.3	30 s	35
72	1 min	
72	2 min	
4	HOLD	

5. Following the nested PCR step, a sample of the PCR should be run on an agarose gel to check that the product is of the correct size and that the negative and positive controls have worked satisfactorily.
6. The PCR product should then be column purified using the manufacturer's instructions.
7. Following purification, 5 µl of the product should be run on an agarose gel with size markers of known concentration so that the concentration of the product can be estimated.

3.4. Preparation for DNA Sequencing

Each template should be sequenced with primers P3, P5–10. The exact protocol will depend on the make of sequencer, and the manufacturer's protocols should be followed for their sequencing kits.

The raw sequence data should be imported into an appropriate editing programme (such as Sequencher from Gene Codes) and the sequences edited and aligned to give a single sequence "contig." Ideally, the entire region under examination should have sequence available in both directions. Special care should be given to the calling of mixed bases, with direct examination of the accompanying chromatograms. Mixtures should be called when the minority peak height is at least 25% of the dominant peak in one direction, with some evidence of the minority population in the opposite direction. Since detection of mixtures is very important clinically, it follows that sequence quality must be high with minimal background noise or "blipping" of peaks and so one should always be guided by the best quality trace.

The edited sequence can then be submitted to an appropriate website for analysis.

3.5. Interpretation of Sequences

Resistance mutation interpretation systems allow mutational data to be described in terms of drug susceptibility, and have been developed to guide treatment in patients on a failing drug regimen. Examples of these programmes include one at Stanford University developed by Dr R Shafer (6) and also geno2pheno (8). The Stanford system identifies drug resistance mutations and confers a score for each mutation with respect to the available drugs. The scores are then summed and an overall prediction of level of resistance to the drugs calculated. An alternative method of interpreting sequences is through the commercially available "Virtual Phenotype" provided by Virco BVBA. This uses the company's extensive database to correlate the presence of mutations with in vitro phenotypic data to predict the in vitro drug susceptibilities of a virus with the particular sequence being analysed.

Although the reports from the various interpretation systems provide an indication of the drug susceptibilities of the virus present in the patient's blood at time of sampling, it should be remembered that the standard methods of HIV genotypic resistance testing only sample the majority population of virus present at the time the blood is drawn. It neither detects mutant virus present at levels lower than 20% in the plasma nor detects resistant virus that has been archived as proviral DNA in lymphocytes, although more sensitive assays are under development. Resistance tests, therefore, must always be considered in the context of the patient's treatment history and any previous resistance tests and expert virological advice sought if available.

3.6. Data Handling

All sequences generated within the laboratory should be stored in a database. In addition, if a laboratory has previously analysed samples from a patient, then the two sequences should be compared. If the sequences are discordant, other than in the drug resistance codons, then the reasons should be explored to exclude the possibility of sample mix-up. Likewise, it is good practice to compare sequences from recent batches using phylogenetics to check for the possibility of PCR contamination.

4. Notes

1. As mentioned above, HIV resistance genotyping involves the extraction of viral RNA from plasma and then amplification of the appropriate region of the genome using a nested PCR, followed by nucleotide sequencing and interpretation of the sequence. Since the process generates large amount of amplicon for sequencing, it is essential that the most stringent separation of the various steps of the process is put in place to prevent erroneous results due to contamination of the PCRs.

Thus, the minimum accommodation required is: (1) a "clean" room for handling of PCR reagents, (2) a room for RNA extraction and addition of RNA to the RT-PCR tubes, (3) a room for addition of first round PCR product to the second round reagents, and (4) a "post-PCR" room for purification of PCR products and nucleotide sequencing. Each of the rooms must have dedicated equipment and the direction of workflow must be strictly controlled.

2. Sample types: (1) The usual sample used for resistance genotyping is EDTA plasma. Avoid heparinised plasma as this may inhibit the PCRs. At least 1 ml of plasma should be requested from the clinic. Since there are very many processing steps to obtain a genotype, one of the main potential errors is sample switching. Thus only a limited number of samples should be handled simultaneously. The author recommends a maximum of ten samples for manual handling and fewer for inexperienced staff. (2) An alternative substrate for resistance testing is dried blood spots, and these are being increasingly used in the developing world as transport and storage are less demanding (5). Plasma samples should be stored at −70°C where possible or at −20°C for shorter periods where there are no −70°C facilities available. Advice with respect to the viral load level required for resistance testing is currently confused. The commercial kits are validated for use at HIV-1 RNA viral loads >1,000 copies/ml and until recently similar levels have been recommended in clinical guidelines. However, patients are considered to be showing virological failure if their viral load becomes detectable, i.e. >50 copies/ml, and clinicians may, therefore, request resistance testing at levels <1,000 copies/ml. Such testing is now frequently carried out by laboratories and yields useful results (9). Nevertheless, it should be remembered that such testing at very low viral loads may not always give representative results due to the very small number of HIV RNA molecules examined and is also more liable to contamination problems and close attention must be paid to controls (see below).
3. Controls: It is of the utmost importance that a negative control sample (e.g. uninfected human plasma or foetal calf serum) is taken through the entire procedure up to and including the detection of PCR product. If any product is seen in the negative control, then the entire run must be discarded. Clinical samples should not be processed until it has been shown that the negative control can be processed without contamination: this may require discarding all the current batches of reagents. The positive control should be chosen with care. It is unusual for the process to fail completely, but a slow deterioration in sensitivity of the assay with respect to

viral load of the samples can often occur. Thus the positive control should have a viral load not too far above the expected limit of sensitivity of the assay such as a viral load of ~1,500 copies/ml. All laboratories undertaking HIV drug-resistance testing for clinical purposes should be accredited and participate in annual external quality assessment (EQA) panels such as the ENVA panel provided by Quality Control for Molecular Diagnostics (QCMD) (10).

4. Reagents: High-quality reagents should be used, and each new batch should be tested using the positive control. Reagents should be dispensed into single-use aliquots, wherever possible, and stored under the manufacturer's recommended conditions. The most common reagent to deteriorate is the reverse transcriptase, and this should be treated with special care and not allowed to warm up, i.e. it should not be removed from the freezer at the same time as the other reagents are taken out to thaw at room temperature.
5. RNA extraction: RNA extraction can be carried out using either manual or automated methods. The choice of automatic extraction machines requires considerable care since many labs have encountered difficulties with their equipment producing greatly reduced sensitivity. In the main, this is thought to be due to shearing of the RNA resulting in poor yields of product of adequate length, which is not observed when the machines are being used for normal molecular diagnostics, where the target is usually very short. In particular, problems have been reported where laboratories have attempted to use extracts already prepared for viral load estimations, though this problem does not occur with all equipment. Thus, it is essential to validate the use of any automatic extraction equipment for use with HIV resistance testing. Manual RNA extraction kits found to work well in the author's laboratory include those manufactured by Qiagen. Use of such kits should follow the manufacturer's instructions. The sensitivity of the assay for use in low viral load samples can be improved by ultracentrifuging the sample to pellet the virus before RNA extraction. This can be done by spinning the sample in a bench-top ultra centrifuge at 23,500 *g* for 1 h at 4°C, removing the supernatant, and then resuspending the pellet in the appropriate volume for the RNA extraction.

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Chapter 9

Detection, Quantification, and Characterisation of HIV/SIV

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Abstract

Selected techniques for the detection, quantification, and characterisation of HIV1, HIV2, and SIV, as applied to diagnostic and research purposes, are described. Representative nucleic acid testing protocols including nested PCR, RT-PCR, and quantitative real-time PCR, as well as protocols based on virus infectivity, are presented.

Key words: HIV, SIV, Nucleic acid testing, PCR, qPCR, Infectivity assay, Colorectal explant cultures

1. Introduction

Infection with human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS), is at pandemic levels with an estimated 25 million people having died as a result and an estimated 33 million people currently living with the virus (www.unaids.org). HIV, a retrovirus belonging to the lentivirus genus, occurs as two species, HIV-1 and HIV-2. HIV-1 is of higher virulence and is largely responsible for the pandemic, whilst HIV-2 is largely confined to West Africa. The fast replicative cycle of HIV, a high mutation rate, and an ability to recombine results in the generation of multiple genetic variants, even within single individuals. HIV-1 has been classified into three groups, M, N, and O, on the basis of sequence diversity. Group M, the most prevalent, has been further subdivided into nine clades, the distribution of which is geographically distinct [reviewed in (1)]. Simian immunodeficiency viruses are, in the main, more closely related to HIV-2 and are not generally associated with disease in their natural hosts; however, when SIV of sooty mangabeys is introduced into rhesus or cynomolgus macaques, a disease remarkably similar to AIDS in man results.

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This model is used extensively for HIV vaccine and microbicide research [reviewed in (2)].

Various techniques may be applied to detect, quantify, and characterise HIV-1, HIV-2, and SIV in biologically relevant materials for diagnostic and research purposes. The direct detection of viral genome using nucleic acid testing (NAT), usually in conjunction with virus-specific antibody, is generally taken as firm evidence of infection. However, a number of factors profoundly influence the ability to confirm the presence of virus, which may only be present in low numbers or in the form of a genetically divergent strain. Many approaches centre around the polymerase chain reaction (PCR), which can theoretically amplify single molecules of target DNA but may be susceptible to fluctuations in gene sequences. Hence, one of the first considerations prior to attempting to amplify HIV/SIV is to take into account the exceptionally high degree of sequence variability, which will particularly impact on sequence-based assays. Since HIV and SIV are both retroviruses, where the virion form of the nucleic acid is RNA, it is frequently both desirable and appropriate to also detect and quantify viral RNA. In certain situations, it may be a requirement to characterise the amplified fragment or develop assays, which will allow characterisation of nucleic acid by sequencing the viral product. The following nucleic acid-based methods describe the detection, quantification, and sequence characterisation of HIV or SIV from complex biological materials, including tissue samples, where the target sequence may be present in very low abundance. The preparation of the nucleic acid (RNA or DNA) is an important first step in many of the procedures described though details of these are not provided as they either conform to established molecular biology procedures (e.g. extraction of DNA using physico-chemical methods) or are now available as kits from recognised manufacturers. The procedures described here have been built on these. Those described are only examples demonstrated to work well, but other commercially available products may be employed to similar effect. In addition to nucleic acid-based methods, we describe several selected methods for the quantification and characterisation of infectious virus that may be applicable in the research context.

Infectious virus can be quantified by various means, and this is, to some extent, dependent upon the origin of the viruses, the availability of suitable cells, and the purpose for which the virus will be used subsequently. T cell line-adapted (TCLA) viruses may be titrated for infectivity on a wide variety of $CD4^+$ cell lines and virus production measured by the formation of cytopathic effects such as syncytia formation (where applicable), production of virus antigen, either cell associated or in cell culture supernatant, the production of reverse transcriptase activity, cell killing etc. When cytopathic effect is used as a read-out, it is advisable to

validate the assay end point using an additional technique. For example, we have routinely used limiting dilution titration of peripheral blood mononuclear cells (PBMC) or tissue-derived mononuclear cells from SIVmac-infected macaques to quantify SIV cell-associated loads by co-cultivation with the human C8166 T cell line. Cells from cultures at and beyond the cytopathic end point are then stained by immuno-fluorescence for the presence of SIV antigen(s).

Primary isolates are frequently titrated on PBMC and the protocol that we use for HIV is detailed here. Although PBMC assays remain the gold standard, they are subject to variability due to donor differences (even when pooled cells are used), difficult to validate between laboratories, and both labour-intensive and costly, particularly when used to measure inhibition of virus infectivity by neutralising antibodies or antiretroviral agents. The development of reporter gene assays utilising genetically engineered adherent cell lines has brought about a well-standardized and versatile alternative approach to virus quantification that can be applied to HIV-1, HIV-2, and SIV TCLA viruses, primary isolates, molecularly cloned HIV chimaeras constructed with heterologous envelopes including SHIV and pseudotype viruses. In general, such cell lines detect the presence of virus by Tat-induced transactivation of reporter genes through binding to HIV LTR sequences and therefore require only single-cycle infection. We, in common with many other laboratories, have found the TZM-bl cell line to be particularly suitable for virus quantification. The parental cell line JC.53 is a CXCR4 constitutively expressing HeLa cell clone that has been engineered to also express CD4 and CCR5 (3). The TZM-bl cell line was created by the introduction of separate integrated copies of the luciferase and β-galactosidase genes under the control of the HIV-1 promoter (4).

The characterisation in vitro of virus isolates and stocks may be of critical importance for the interpretation of experimental findings and techniques for determination of growth characteristics, using cell lines and primary cells, are well described in the literature. In particular, engineered cell lines expressing a variety of co-receptors are available for determining primary tropism (5). For vaccine and microbicide studies, there is increasing interest in how HIV/SIV isolates and chimeric viruses carrying different envelopes behave in vivo, particularly at mucosal surfaces. Direct experimentation in vivo has obvious limitations even when using the SIV-macaque model; however, aspects of primary infection can be modelled by the use of tissue explant cultures (6–10). Such systems provide, at best, only partial replication of conditions in vivo, nonetheless, they offer a powerful extension to cell culture. For example, the segregation of viruses into different component cell types can be assessed, including their segregation into migratory cells that may be responsible for early systemic dissemination

of virus. Explant cultures are amenable to the powerful techniques of transcriptomics and functional molecule array, and they can be used to measure the efficacy of neutralising antibody and antiretroviral agents. Here, we outline some of the technical considerations needed for the use of colorectal explant cultures.

2. Materials

2.1. Detection of Nucleic Acids

1. Aliquoted oligonucleotide primers (Eurofins, MWG) (see Note 1).
2. AmpliTaq Gold kit reagents, including $MgCl_2$ stock buffer and Taq enzyme (Roche Diagnostics).
3. Nuclease-free molecular biology-grade water, stored at 4°C in 2 mL aliquots in screw-capped tubes (Sarstedt).
4. Balanced dNTP stock containing equimolar concentrations of each nucleotide, aliquoted and stored at –20°C in screw-capped tubes (Sarstedt).
5. Basic thermal cycling block (Biometra).
6. 1–20, 20–200, 200–1,000 µL pipettes (Gilson), and compatible disposable filter-tip pipette tips.
7. Thin-walled, 0.2 or 0.5-mL PCR tubes.
8. "O"-ring 2-mL screw-capped tubes (Sarstedt).
9. Source of control DNA for HIV-1, HIV-2, or SIV either through the NIH AIDS Research & Reference Reagent Program (www.aidsreagent.org/) or the Centre for AIDS Reagents repository (CFAR) at NIBSC (www.nibsc.ac.uk). e.g. LAV 8E5 cell line for HIV-1 DNA amplifications.
10. Hot block (optional).
11. Bench-top picofuge (Heraeus).
12. Agarose-gel electrophoresis and transillumination viewing equipment.

2.2. Quantification of Virus-Specific DNA and RNA by Real-Time PCR

1. Real-time PCR genetic analyser (Stratagene MX3000/5P).
2. Aliquoted primers and probe (Eurofins, MWG) (see Note 2).
3. "Taqman" DNA PCR mastermix (Applied Biosystems) stored at 4°C or Ultrasense RT-PCR kit (Invitrogen) stored at –20°C.
4. Nuclease-free molecular biology-grade water.
5. Optical 96-well PCR plates, plate support, and optical strip caps (Stratagene).
6. "O"-ring screw-capped tubes (2 mL) (Sarstedt).
7. Polystyrene bijoux for mastermix.

8. Pipettes and filter tips as above.
9. Bench-top picofuge.
10. Centrifuge for plate spin.

2.3. Quantification of Virus Infectivity

2.3.1. Titration in Activated Peripheral Blood Mononuclear Cells

1. PBMC purified from at least three buffy coats or whole bloods, from different healthy single donors (see Note 3).
2. RosetteSep CD8 depletion cocktail (StemCell Technologies).
3. Ficoll-Paque™ Plus (GE Healthcare).
4. ACK lysing buffer (BioWhittaker).
5. Anti-CD3 monoclonal Ab OKT3 (eBioscience).
6. Leucoagglutinin PHA-L (Sigma-Aldrich).
7. Human recombinant IL-2 (NIH AIDS Research & Reference Reagent Program, catalogue number 136) (www.aidsreagent.org/).
8. RPMI 1640 (Sigma-Aldrich).
9. Foetal calf serum (FCS), L-glutamine, penicillin, and streptomycin (Gibco-Invitrogen).
10. 96-well flat bottom sterile plates with lid.
11. 1.2-mL micro-tubes in 96-tube cluster plate (Abgene).
12. Multi-channel pipettor.
13. Microplate reader.
14. 5, 10, and 25-mL pipettes.

2.3.2. Titration in TZM-bl Cells

1. TZM-bl cells, available from the NIH AIDS Research & Reference Reagent Program (catalogue number 8129) (www.aidsreagent.org/).
2. Dulbecco's Minimal Essential Medium (DMEM) high glucose (Sigma-Aldrich).
3. FCS, L-glutamine, penicillin, and streptomycin (Gibco-Invitrogen).
4. Luciferase assay system (Promega).
5. 96-well flat bottom sterile plates with lid.
6. 96-well U-bottom sterile plates with lid.
7. 96-well high binding non-sterile white plates without lid (Costar).
8. 1.2-mL micro-tubes in 96-tube cluster plate (Abgene).
9. Multi-channel pipettor.
10. Microplate reader/luminometer.
11. 10-mL pipettes.
12. Disposable polystyrene 50-mL reagent reservoirs (Corning).

2.4. Characterisation of PCR-Amplified Products

1. See basic materials as described in Subheading 2.1.
2. Bench-top vortex.
3. Picofuge.

2.5. Characterisation In Vitro: Growth of HIV/SIV in Colorectal Tissue Explants

1. Colorectal specimens can be obtained form surgical resections of intestinal tissue or from biopsies, after receiving signed informed consent from the patients.
2. Sterile scalpels.
3. Sterile forceps.
4. Petri plates.
5. PBS (Sigma-Aldrich).
6. DMEM high glucose (Sigma-Aldrich).
7. FCS, L-glutamine, penicillin, and streptomycin (Gibco-Invitrogen).
8. Gentamicin (Sigma-Aldrich).
9. 96-well U-bottom sterile plates with lid.
10. 24-well bottom sterile plates with lid.
11. Multi-channel pipette.
12. Gelfoam sponges (gelatin sponges) $20 \times 60 \times 7$ mm – four pieces (Pfizer).

3. Methods

3.1. Detection by Nucleic Acid Testing

3.1.1. Strategy

A range of strategies may be employed to determine the presence of the HIV/SIV genome by NAT in biologically relevant materials, though some common themes emerge which have general application. The genetic variation of the HIV/SIV genomes within their broader virus families should not be underestimated with some regions proving more diagnostically secure than others. In general terms, the overall range of genetic variation increases from LTR > *pol* > *gag* > *env* with *env* representing the most variable part of the HIV/SIV genome. However, in all regions, there are both conserved and variable regions which require careful selection, depending on the task in hand. When designing primers, access to the Los Alamos database is essential, either in paper or in electronic format (www.hiv.lanl.gov). At this point, it is important to clearly identify the purpose of amplification, whether it is purely for detection or if some additional, perhaps later, characterisation is required, thereby influencing both the size and location of the amplification target. BLAST searches of short regions (up to 30 nt in length) provide a basis for selecting sequences for primer locations, or a wide range of primer

selection electronic tools are also available. At this stage, a decision should be taken as to whether a conventional PCR amplification approach or the use of real-time PCR technologies is required. For conventional PCR, single-round amplifications may be brought into the visible range by a second or nested amplification with primers that are internal to the outer set. Considerations of oligonucleotide sequence selection will reflect sequence length, depending on the application, which, in turn, will influence the annealing or melting temperature (Tm). Balanced sequences for Tm are generally desirable for either conventional or real-time PCR amplifications, and primer design will influence greatly optimisation parameters and assay performance.

One approach that has proved successful is the development of sets of oligonucleotide primers and amplification conditions, which detect a wide range of sequence variants of HIV-1. Primers located in the 5′ untranslated region of the HIV-1 genome and the U5/R region of the long terminal repeat have been developed to encompass HIV-1 field strain variation, enabling detection of all Group M and Group O viruses (*11–13*) (Table 1). A parallel, balanced set of primers to amplify HIV-2/SIVsm/SIVmac have also been described (*11*) (Table 1). These have been used successfully to detect HIV-2 in a range of clinical study cohorts (*14*) and SIV in biologically relevant materials, including diverse tissue samples such as spleen, lymph nodes, and mucosal tissues.

3.1.2. Detection of HIV-1, HIV-2, or SIV DNA by Nested PCR

1. In clean reagent set-up area, establish a work station (see Note 4). Remove reagents (e.g. AmpliTaq Gold kit), except the enzyme, from a –20°C freezer and thaw at room temperature or in a 37°C hot block. This includes aliquots of 10× reaction buffer, 25 mM $MgCl_2$ stock solution (from the kit), forward and reverse primers, and balanced dNTP stocks at their stored concentrations.
2. Ensure that the reagents are fully thawed and buffers evenly dispersed by inversion or vortexing. Pulse-spin in a picofuge prior to use. For untried primer combinations, primer and magnesium optimisation experiments/titrations should always be performed, perhaps in a chequer-board fashion.
3. Prepare a master mix solution so that there is sufficient mix available to complete the experiment, or optimisation if required, but without wasting reagents. At this stage the volume of the template to be added should be taken into account. So, for a 50-µL final PCR reaction volume to assay 5 µL of DNA sample to be performed for each individual sample (e.g. $n=10$), in duplicate, allow enough total reagent mix for 22 individual samples (i.e. $n+1\times 2$), where each tube will need 45 µL of final reaction mix. Therefore, a final volume

Table 1
Primer and probe sequences for the detection and/or quantification of HIV-1, HIV-2 and SIVmac

Virus and gene	Primer or probe	Sequence	Assay type
HIV-1, LTR	Forward, outer primer	5′-GAACCCACTGCTTAAGCCTCAAT-3′ (507–529)	Nested PCR (13)
	Reverse, outer primer	5′-TGTTCGGGCGCCACTGCTAG-3′ (628–648)	
	Forward, inner primer	5′-CTCAATAAAGCTTGCCTTGA-3′ (524–543)	
	Reverse, inner primer	5′-GCGCCACTGCTAGAGATTTT-3′ (622–641)	
HIV-2/ SIVmac/sm, LTR	Forward, outer primer	5′-ATTGAGCCCTGGGAGGTTCTCTCCA-3′ (26–50)	Nested PCR (11)
	Reverse, outer primer	5′-TTCGGGCGCCAACCTGCTAGGGATTTT-3′ (287–313)	
	Forward, inner primer	5′-AGCAGGTAGAGCCTGGGTGTT-3′ (56–76)	
	Reverse, inner primer	5′-ACCAGGCGGCGACTAGGAGAGAT-3′ (192–214)	
SIVmac, *gag*	Forward primer	5′-AGTGCCAACAGGCTCAGAAAA-3′ (1,253–1,273)	Real-time PCR (15)
	Reverse primer	5′-TGCGTGAATGCACCAGATG-3′ (1,304–1,322)	
	Taqman probe	5′-(6′-FAM-TTAAAAAGCCTTTATAATACTGTCTGCG-BHQ1)-3′ (1,275–1,301)	

HIV-1 LTR sequences are numbered according to HIV-1_{HXB2}, HIV-2/SIV LTR sequences according to HIV-2_{ROD} and SIVmac *gag* sequences according to SIVmac239. Note: SIVmac primer and probe sequences are optimised to detect SIVmac251 and SIVmac239 only with optimal primer and probe concentrations determined at 300 and 100 nM, respectively

of reaction mix of 990 µL (i.e. 22 × 45) will be required. To vary the amount of template input, e.g. to 10 or 2 µL, the optimised concentration of specific reagent will remain constant for each reaction, but the amount of water added will vary (see Table 2). For the maximum volume of template that can be added see the manufacturer's information.

4. Use a combination of pipettes with appropriate filter tips to build up the master reaction mix in an appropriately sized container, typically a 2-mL screw-capped Sarstedt tube. Larger containers may be needed for large PCR runs though it is vital to use only a single container to ensure equal

Table 2
Example of a multi-component, pre-optimised reagent set to establish a master-mix enabling the performance of ten samples PCR, in duplicate (i.e. 20 PCR tubes)

	[Final]	Reaction volume/μL	Reaction number	Final amount
10× buffer		5	22	110
$MgCl_2$ (25 mM)	e.g. 1.5 mM	3	22	66
dNTP mix (1.25 mM)		8	22	176
NF-DW (to 50 μL)		24.25	22	533.5
Primer (For)		2	22	44
Primer (Rev)		2	22	44
Taq polymerase		0.75	22	16.5
Template		5	22	

Calculated for a 50 μL reaction mix/tube containing 45 μL master mix and 5 μL sample. The use of Excel spreadsheets designed to calculate these volumes automatically for a given final reaction run size can simplify this task

distribution of all reagents. Adding reagents in the following order works well: NF-DW, 10× reaction buffer, $MgCl_2$, dNTP mix, primers and finally enzyme.

5. Distribute the calculated amount of PCR master mix to PCR tubes (flip-top mini Sarstedt thin-walled PCR tubes), which have been coded (e.g. 1–20) with a waterproof indelible marker pen on the sides of the tubes. Avoid trapping air at the bottom of the tube by inserting the pipette tip at a 45° angle and running gently down the side. This is less of a problem with larger volume PCR mixes. Secure the lids and transfer to the sample set-up area, if it is a distinct location from the PCR set-up area.
6. Arrange the samples to be tested in order. At this stage, it is important to include carefully characterised specific DNA controls, which should ideally mimic the nature of the sample. Typically, vortex DNA samples and pulse-spin for 15 s to obviate collection of drops on the lid (see Note 5).
7. Pulse-spin the tubes containing the PCR mix prior to template addition. Using a 0–20-μL pipette and filter tips, add 5 μL of sample to PCR tubes by inserting the sample below the level of the PCR mix, pushing the liquid out to the first stop on the pipette, drawing back into the pipette and then pushing all liquid out past the second stop in a final movement. Repeat the same action in an identical fashion for each sample. Push lids of tubes down firmly and flick the tubes to mix before transfer to a location designated for PCR amplifications and downstream handling of amplified products.

8. Perform one final pulse spin and transfer tubes to a pre-programmed PCR block. The optimal thermoprofile should be determined including temperature and length of denaturation, annealing, and extension steps, typically performed for 30–35 cycles.
9. At the end of the reaction run, products of PCR amplification may be visualised by migration of nucleic acid on an appropriate percentage of agarose gel and visualised according to the stain of choice (see Note 6).
10. In practice, distinct bands will be difficult to visualise after only one round of thermal cycling. A second round using inner, nested primers using similar protocols and procedures as detailed above will usually provide good data. Typically, make up a reaction mix for a 48 or 49 μL final volume, exclude the sample/template addition step, and perform a transfer of 1 or 2 μL of first round reaction product into the fresh second round mix containing the inner primers. Re-amplify for a further 25–30 cycles. Re-visualise on a gel or run both first and second round products together, or just second rounds' products.
11. In conjunction with an appropriate molecular weight marker, e.g. PhiX174 Hae III digest, the mobility of bands stained with an intercalating dye and transillumination will provide a robust means by which the gel result could be interpreted. An anticipation of expected gel sizes, purity of amplification with a single bright band, and correlation with expected known positive controls should be part of any analysis.

3.1.3. Detection of Viral RNA by Reverse Transcription PCR

A similar approach may be adopted for amplification of viral RNA, typically from plasma samples. To attain a signal for PCR (a DNA amplification procedure), reverse transcription of the viral RNA into DNA is required. This can be achieved either as a two-step process (i.e. independent RT and amplification steps) or as a single combined RT and PCR process, depending on the application involved. A range of commercially available products facilitate this process. A two-step system will detect multiple messages mediated through oligo (dT), random hexamers, or gene-specific primers. A one-step approach necessitates the use of gene-specific or primer-directed reverse transcription but allows for ease of processing larger numbers and minimises carryover as cDNA syntheses and PCR amplifications are performed simultaneously without the need to re-open tubes. The SuperScript III with platinum *Taq* high fidelity (Hi-Fi) one-step reagent system (Invitrogen) has been employed to good effect, though other reagents will also provide good data. The source of RT, in this case, M-MLV with reduced RNAase H activity, will provide certain features depending on the application. The systems are designed to produce high

yields and long products and are applicable to total and viral RNA applications. For all RNA manipulations, conditions should be as aseptic as possible.

1. Prior to RT-PCR set-up, set up the thermal cycler such that for the reverse transcription step, it is already at the optimal 45–60°C temperature.
2. In a clean set-up area, follow the basic rules as detailed in Subheading 3.1.2 for DNA PCR.
3. As before, calculate the volume of reagents needed for multiple reactions.
4. Using the SuperScript III/Platinum Taq reagent kit, allow for a 45 μL reaction mix with 5 μL of extracted RNA to be added (see below for RNA extraction). Thaw sufficient reagents (2× reaction mix, primers, nuclease-free distilled water) for the multiple reactions and finally add the enzyme. Distribute to pre-labelled PCR tubes.
5. In the template addition area, rapidly thaw RNA extracts, pulse-spin, and add 5 μL to each tube; flick the tube to mix and then pulse-spin.
6. Transfer to a pre-programmed thermal cycling machine with optimal conditions for RT and PCR stages depending on the length and nature of the target template and product.
7. Perform a nested PCR with inner primers as necessary and view products of amplification as in Subheading 3.1.2 (step 11).

3.2. Quantification of Virus-Specific DNA and RNA

Quantitative PCR (qPCR) provides a direct means of determining the viral burden or level in a particular biological sample or tissue. As with detection assays, as HIV/SIV exist in both a proviral and virion form, quantitative data may be obtained as copies of viral DNA (vDNA) or viral RNA (vRNA). The latter is most frequently applied to clinical studies of HIV-1, HIV-2, and SIV, where the level of virion RNA in plasma samples is expressed as copies of RNA equivalents per millilitre of plasma (RNA copies/mL). For HIV-1 RNA quantification and/or detection, independently calibrated International working standards and reagents are available, as well as a panel for the detection of HIV-1 RNA subtypes (CFAR, NIBSC http://www.nibsc.ac.uk). Increasingly, however, there is a need to accurately determine viral DNA levels in a whole range of body compartments and tissues, including biological materials and samples recovered *post-mortem*. vDNA levels may be expressed as DNA copies per a given range of cell equivalents (see Note 7).

3.2.1. Quantification of SIV DNA Levels by Real-Time PCR

1. Unless a fully internally calibrated PCR system is employed, the inclusion of a standard curve representing the assay range should be included along with test samples (see Note 8).

Reactions are typically set up in triplicate. The inclusion of a "house-keeping" gene to determine the presence of a constitutively expressed cellular gene may provide an independent arbiter for sample input or inhibitors (see Note 9). The assay described is reported in (*15*).

2. Make up a PCR template sheet for the primer/probe set to be used, outlining the 96-well PCR plate position, master-mix recipe, and thermoprofile. Assemble a master mix with Taqman reagents according to the manufacturer's instructions, following basic PCR set-up guidelines, including the appropriate primer sets at their optimal working concentration. The probe is the last reagent to be added, immediately returning the foil-covered aliquot to the freezer.
3. In template addition laboratory, set up workstation and defrost DNA standards and samples on the bench.
4. Flick DNA standards and samples gently and spin down in a bench-top picofuge.
5. Add 3 µL DNA sample to each microfuge tube, containing 75 µl master-mix.
6. Flick tube to mix and pulse-spin.
7. Place a 96-well PCR plate in a plate support and set outstrip caps.
8. Aliquot 25 µL into each well of the plate placing the tip at the bottom of the well and drawing it up the side whilst expelling the mix to reduce bubbles.
9. Remove any remaining bubbles from the wells using a pipette tip and cap plate firmly. An additional centrifugation step may be necessary to remove residual air bubbles especially those trapped at the bottom of the tube or beneath the meniscus.
10. Insert plate into thermal cycler and commence run using an optimised thermal cycling profile.

3.2.2. Quantification of SIV RNA by Real-Time RT-PCR

As for vDNA assays, quantitative viral RNA assays by real-time PCR (TaqMan assays employing hydrolysis probes) will only be as successful as the ability of the primer and probe to recognise the target sequence and perform at high efficiency. An assay for the quantitative measurement of SIV RNA in plasma using primers located in conserved regions of *gag* is described (15). This assay utilises a series of externally calibrated reference materials, which are co-processed with test samples. Materials which provide a reliable and consistent means of validating and standardising assays of this nature are required, either in the form of in vitro RNA transcripts, virions spiked into a negative plasma or, in this case, a high titre sample derived in bulk, which has been purposefully titrated and calibrated in negative plasma. This provides a reference

of viral RNA copy number, which may used to interpolate and interpret unknown test samples. It is preferable that standards are included at the start of the assay process, i.e. at the extraction stage.

3.2.2.1. Extraction of Viral RNA Using Mini Spin-Columns

The integrity of the viral RNA extracted from plasma samples is essential, and precautions should be undertaken to prevent the introduction of ribonucleases, which will degrade RNA and impair reverse transcription and/or PCR amplification. Lysis of the virion, releasing the viral RNA, and the general disruption and inactivation of other proteins using chaotropic agents, typically high-molarity guanidinium salts (e.g. 5 M guanidinium isothiocyanate/TriZol reagents) are usually required. This can be performed by preparing from source reagents or by the purchase of a commercial kit. The current method of choice is the QiaAmp viral RNA mini-kits (Qiagen), though other products with a similar performance are available. Following RNA extraction, using QiaAmp mini-columns (refer to manufacturer's instructions only), 30–50 μL of RNA extract is obtained for downstream analyses. This may be stored at −80°C (or −20°C) prior to use.

3.2.2.2. Real-Time RT-PCR

As for any PCR, it is necessary to make up a master mix for multiple reactions. UltraSense reagents with Rox dye (Invitrogen) for real-time qRT-PCR amplifications is described, though numerous other manufacturers are available. As for RNA detection, this is a one-step combined PCR reaction using gene-specific primers, in this case, in the SIV *gag* gene (15). For treatment of primers and probe see Subheading 3.2.1.

1. Make up a PCR protocol sheet for the primer/probe set to be used, outlining the 96-well PCR plate position, master-mix recipe, and thermoprofile.
2. In clean reagent area, defrost primers and master mix components (except for enzyme mix) in the heat block. Set up workstation. For each standard and sample, place one 2-mL screw-capped tube into a rack labelled with the RNA tube number. Use a sterile polystyrene bijou to make up master mix.
3. Spin down defrosted items. Make up the master mix as per the PCR sheet, flick and spin down the probe and enzymes and add them last. Aliquot 140 μL into each screw-capped tube, close, and transport to template addition area.
4. In template addition area, set up workstation and defrost RNA samples on ice or in a refrigerator or rapidly at room temperature for immediate use.
5. Flick RNA tubes gently and spin down. Add 15 μL viral RNA to each microfuge tube. Flick tube to mix and spin. Place a

96-well PCR plate into its support and set out caps. Aliquot 48 µL into each well of the PCR plate. Avoid bubbles by placing the tip in the base of the well and drawing it slowly up the side as you expel the mix. Remove any bubbles, especially those which sit at the bottom of a well. Seal plate firmly and transport to genetic analyser (see Note 10).

3.3. Quantification of Virus Infectivity

3.3.1. Titration on PBMC

3.3.1.1. CD8 Cell Depletion and Fractionation of PBMC

1. Pour buffy coat into a T75 flask.
2. Add 1 mL of RosetteSep CD8 depletion cocktail, mix thoroughly, and incubate 20 min at room temperature.
3. Add PBS with 2% FCS to give a final total volume of 140 mL.
4. Prepare four 50 mL conical tubes each with 15 mL of Ficoll.
5. Transfer 35 mL of buffy coat to each tube by slowly overlaying the blood on the Ficoll layer.
6. Spin at 600 × *g* for 30 min without brake.
7. Discard some of the plasma (which corresponds to the very top layer).
8. Collect the PBMC layer (white layer at interface) avoiding aspirating erythrocytes and transfer to a 50 mL tube (one tube per original). Fill up with PBS to 50-mL.
9. Spin at 300 × *g* for 10 min.
10. Aspirate the supernatant (caution: the cell pellet is not very compact and can be easily disturbed) and resuspend pellet with 50 mL of PBS.
11. Spin down at 170 × *g* for 10 min (to remove platelets). Sometimes, the platelet content is still very high so another wash is then necessary.
12. Aspirate supernatants from all the tubes. Add 5 mL of ACK buffer to the first tube and quickly transfer the pellet to the second tube. Resuspend the second pellet and transfer sequentially to all the other tubes until combining all pellets in one tube. Let it stand for 5 min at room temperature.
13. Add PBS up to a final volume of 50 mL and spin down the cells at 100 × *g* for 10 min.
14. Resuspend in 40 mL of complete RPMI. Count the cells.

3.3.1.2. Activation of PBMC

1. Pooled purified, CD8-depleted PBMCs from different donors, can be activated at 4×10^6 cells/mL with PHA at 5 µg/mL and 10 U IL-2/mL for 72 h at 37°C in an atmosphere containing 5% CO_2.
2. An alternative method reproduces more closely an in vivo activation by dividing the pooled purified PBMCs into three

different flasks at 4×10^6 cells/mL and activating the cells of each flask in a different way: flask 1 is pre-coated, for at least 2 h, with 8 mL of RPMI (for a T 75 flask) containing 2 μg/mL of monoclonal anti-CD3 Ab OKT3, and the cells are seeded with 10 U/mL of IL-2; cells in flask 2 are activated with PHA at 5 μg/mL and 10 U IL-2/mL; in flask 3, cells are activated with 0.5 μg/mL of PHA and 10 U/mL of IL-2. After 72 h, stimulated PBMCs of each flask are counted and pooled in equal number at the required concentration for virus titration (or for other uses such as virus culture, inhibition/neutralisation assays, etc.).

3.3.1.3. Virus Titration

1. Prepare titration (TIT) medium: RPMI 1640 containing 10% FCS, 2 mM l-glutamine, antibiotics (100 U of penicillin/mL, 100 μg of streptomycin/mL), and 100 U IL-2/mL.
2. Adjust 3 day-stimulated PBMCs to 2×10^6 cells/mL in TIT medium.
3. Prepare virus dilutions (one in five series) as follows: with 1.2-mL micro-tubes in 96-tube cluster plate and using a multi-channel pipettor (see Table 3), place 450 μL of TIT medium in tube 1. Place 600 μL of TIT medium in tubes 2–7. Add 300 μL of virus in tube 1 and mix well. Prepare dilution (dil) 1 for all other virus stocks in the same way. Adjust the multi-channel pipettor to 150 μL. Transfer 150 μL from dil 1 to dil 2. Discard tips. Take new tips and mix dil 2. Transfer 150 μL to dil 3 and continue as above.
4. Prepare the titration plate (see Table 4) as follows: in wells of columns 1 and 12 of a 96-well flat bottom plate, place 200 μL of PBS; in columns 2–8, place 100 μL of cell suspension (stock at 2×10^6 cells/mL and therefore, the final amount of cells per well is: 2×10^5 cells) and 50 μL of TIT medium; in columns 9–11, place 150 μL of TIT medium. Transfer 50 μL of the appropriate virus dil to four replicate wells in the titration plate (see Table 4 and Note 11). Use the multi-channel pipettor for this step to transfer dil 1–dil 7 of one virus at once.
5. Incubate at 37°C in an atmosphere containing 5% CO_2 for 6 days.
6. Collect samples for p24 concentration estimation. Mix cultures well by pipetting up and down with a multi-channel pipettor and harvest 50 μL of each well by transfer to a new 96-well plate. Dilute and inactivate virus by adding 200 μL of detergent at 1% final concentration. Use Empigen, Triton-X 100, or Igepal (replacement of NP-40).
7. Perform p24 ELISA (see Note 12). Several p24 ELISA kits are available, ranging from in-house ELISAs to commercial kits.

Table 3
Virus titration on PBMC: virus dilution format in 96-tube cluster plate

Add:	Virus	300 µL	150 µL dil 1	150 µL dil 2	150 µL dil 3	150 µL dil 4	150 µL dil 5	150 µL dil 6
Add:	TIT medium	450 µL	600 µL	600 µL	600 µL	600 µL	600 µL	600 µL
		1	2	3	4	5	6	7
Virus 1	A	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7
Virus 2	B	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7
Virus 3	C	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7
Virus 4	D	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7
Virus 5	E	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7
Virus 6	F	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7
Virus 7	G	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7
Virus 8	H	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7

Table 4
Virus titration on PBMC: format of titration plate

		Titration: Add 100 µL of cell suspension, 50 µL of TIT medium and 50 µL of appropriate virus dil								Control titration: Add 150 µL of TIT medium and 50 µL of appropriate virus dil			
		1	2	3	4	5	6	7	8	9	10	11	12
Virus 1	A	PBS	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7	dil 1	dil 2	dil 3	PBS
	B	PBS	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7	dil 1	dil 2	dil 3	PBS
	C	PBS	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7	dil 1	dil 2	dil 3	PBS
	D	PBS	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7	dil 1	dil 2	dil 3	PBS
Virus 2	E	PBS	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7	dil 1	dil 2	dil 3	PBS
	F	PBS	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7	dil 1	dil 2	dil 3	PBS
	G	PBS	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7	dil 1	dil 2	dil 3	PBS
	H	PBS	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7	dil 1	dil 2	dil 3	PBS

The final volume of inactivated supernatant is enough for any kit chosen. Read the p24 ELISA in a microplate reader following the manufacturer's instructions.

8. Calculate the $TCID_{50}$/mL using the Spearman–Karber equation (see Note 13). Count the number of wells that are "negative" for p24. p24 concentrations <50 pg/mL are considered negative.

3.3.2. Titration on TZM-bl Reporter Gene Cells

Virus titration in TZM-bl cells is performed for 24, 48, or even 72 h depending on the incubation time to be used in subsequent experiments with the titrated virus stock (see Notes 14 and 15).

3.3.2.1. Titration Set-Up

1. Day 0 in the afternoon: in a 96-well flat bottom plate, seed cells at 3×10^3 cells/well if experiment is going to last for 48/72 h. For incubations of only 24 h, the amount of cells seeded on day 0 can be increased to 5×10^4 cells/well. Seed the cells in 100 µL of fresh complete DMEM media/well.
2. Day 1: start the titration. Set up the titration plate (see Table 5) using quadruplicates for the negative control (NC), and for each of the eight dilutions (dil) used in the virus titration. Wells marked with PBS are filled with 200 µL of PBS. Add 100 µL of fresh complete DMEM media in the NC wells. Prepare the virus dilutions in 1.2-mL micro-tube cluster plate using a multi-channel pipettor (see Table 6). Place 450 µL of medium in tubes 2–8. Place 500 µL of undiluted virus supernatant in tube 1 (dil 0). Set up dil 0 and tubes 2–8 with media for all other virus stocks in the same way. Transfer 50 µL from tube 1 to tube 2 (dil 1). Discard tips. Take new tips and mix dil 1. Transfer 50 µL to tube 3 (dil 2) and continue as above. Transfer 100 µL of each virus dilution to four replicate wells of the corresponding dilution in the titration plate (see Table 5). Use the multi-channel pipettor for this step. This way you can transfer dil 0–dil 7 of one virus at once. Incubate for 24, 48, or 72 h.
3. Day 2 (for 24 h), 3 (for 48 h), or 4 (for 72 h titrations): Lysis of the cells.

3.3.2.2. Preparation of Cell Lysates and Luciferase Read Out

1. Prepare cell lysis buffer by dilution 1:5 of the cell lysis buffer (kept in –20°C freezer) in ddH_2O.
2. Aspirate the media from all the wells of one plate (be careful not to aspirate the cells; fit a 200 µL tip to the aspirating pipette).
3. Add 100 µL of sterile PBS.
4. Aspirate the PBS from all the wells of the plate.
5. Add 100 µL of cell lysis buffer.

6. Repeat the same protocol for all the plates.
7. Prepare 96-well U-bottom plates to transfer the samples (in order to take them out of the CL3 laboratory).
8. Transfer 80–100 µL of sample to the U-bottom plate. Avoid the formation of bubbles.
9. Seal the plate(s), spray with ethanol, and store in the –80°C freezer.
10. At least 2 h later (routinely plates are left overnight in the freezer), thaw the plates and the luciferase buffer.
11. A luminescence protocol should be set up taking into account the specificities of the plate reader available. The reader must use a top position for the optics, and the basic protocol is end-point reading type. The sensitivity is specific for each model (see Note 16).
12. When everything is thawed, transfer 50 µL of sample to 96-well white plates, avoiding the formation of bubbles.
13. Remove the luciferase substrate from the freezer and resuspend it in 10 mL of luciferase buffer. Using a reagent reservoir and a multi-channel pipette, add 50 µL of luciferase substrate to the white plates one by one just before reading them.

3.4. Characterisation of PCR-Amplified Products

3.4.1. Single Genome Amplification for Sequence Characterisation

Amplification of nucleic acid sequences, either from an initial RNA or DNA template, has become a powerful tool enabling the ability to recover sequence information from a multitude of sources. These may be in high abundance or at very low frequency, at the limit of assay detection. The sequencing of PCR-amplified products has been described in detail elsewhere. However, one of the key steps in gaining meaningful sequence information from biologically complex materials is the ability to isolate and analyse single sequences. This may be achieved in one of two ways. A bulk amplification may be performed and sequences cloned into a vector (e.g. TOPO TA cloning), and sequence information generated by sequencing the clones. Alternatively, single-genome amplification (SGA) procedures may be performed at the PCR stage which can be sequenced directly, obviating the need for a cloning step. For amplifications of this kind, proof-reading enzymes are desirable to reduce the mis-incorporation of nucleotides by *Taq* polymerase. Recent studies of HIV-1 (16, 17) and earlier studies of Simmonds et al. (18) have demonstrated the power of this technique to analyse multiple species of related sequences in the context of HIV-1 infection. Equally, these principles and practice have been applied to HIV-2/SIV studies. According to Poisson statistics, the frequency of positive amplification amongst an excess of negative amplifications provides statistical evidence of single-molecule amplification. In 96-well

Table 5
Virus titration on TZM-bl cells: format of titration plate

		1	2	3	4	5	6	7	8	9	10	11	12
Virus 1	A	PBS	NC	dil 0	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7	PBS	PBS
	B	PBS	NC	dil 0	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7	PBS	PBS
	C	PBS	NC	dil 0	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7	PBS	PBS
	D	PBS	NC	dil 0	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7	PBS	PBS
Virus 2	E	PBS	NC	dil 0	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7	PBS	PBS
	F	PBS	NC	dil 0	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7	PBS	PBS
	G	PBS	NC	dil 0	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7	PBS	PBS
	H	PBS	NC	dil 0	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7	PBS	PBS

NC = negative control, dil 0 = undiluted virus supernatant

Table 6
Virus titration on TZM-bl cells: virus dilution format in 96-tube cluster plate

Add:	Virus	500 µL	50 µL dil 0	50 µL dil 1	50 µL dil 2	50 µL dil 3	50 µL dil 4	50 µL dil 5	50 µL dil 6
Add:	Medium		450 µL	450 µL	450 µL	450 µL	450 µL	450 µL	450 µL
		1	2	3	4	5	6	7	8
Virus 1	A	dil 0	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7
Virus 2	B	dil 0	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7
Virus 3	C	dil 0	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7
Virus 4	D	dil 0	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7
Virus 5	E	dil 0	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7
Virus 6	F	dil 0	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7
Virus 7	G	dil 0	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7
Virus 8	H	dil 0	dil 1	dil 2	dil 3	dil 4	dil 5	dil 6	dil 7

experiments the dilution that provides PCR products in fewer than 30% of wells, i.e. 29 or less, can be taken as evidence of one amplifiable DNA or cDNA template per positive PCR. The following example is a simple, minimal limiting dilution experiment, which can be applied to both RNA and DNA templates. All normal PCR rules and procedures should be followed.

1. Calculate and prepare an overall master mix sufficient for the total experiment to be distributed in sets of four PCR tubes to make a dilution series of 1, 1/5, 1/25, 1/125, 1/625, 1/3,125, and so on (i.e. 95, 80, 80, 80, …, 60 µL).
2. In the template addition area, into the first tube containing 95-µL master mix (of the chosen reagent for the application), add 5 µL of sample DNA, RNA, or cDNA as required. Vortex for 15 s.
3. Transfer a 20-µL aliquot into 80 µL of master mix and distribute the remainder into each of four PCR tubes. The transferred aliquot represents the next dilution step.
4. Continue this process down the dilution series.
5. In the final set, 20 µL is transferred in 60 µL and distributed.
6. Perform the PCR directly as all reagents and templates are already present.
7. Perform nested PCR and view results on an agarose gel.
8. Wells are scored positive or negative and the number of DNA (or RNA) molecules in the starting material calculated according to the Poisson probability theory (see Note 17).

3.4.2. Sequence Identity and Phylogenetic Analyses

Once an overall approach has been decided, i.e. bulk amplification → direct sequencing; bulk amplification → cloning → sequencing or limiting dilution PCR → direct sequencing, sequence data may be readily generated. Raw DNA sequence from a DNA sequencer/genetic analyser may be manipulated in a number of ways. In the first instance, it is important to determine the basic sequence identity of the newly generated sequence and its relationship with other sequences. For HIV/SIV, this can be quickly performed by running a BLAST search referring back to the Los Alamos sequence database (www.hiv.lanl.gov). This will provide a simple alignment with known sequences in the database and a percentage similarity result. For HIV-1 and HIV-2, the clade or subtype may be rapidly determined and for SIV confirmation of the strain identity. More detailed phylogenetic and evolutionary analyses may be performed as part of bioinformatics exercises and is covered elsewhere.

3.5. Characterisation In Vitro: Growth of HIV/SIV in Colorectal Tissue Explants

3.5.1. Preparation of Explants

1. Specimens are kept in DMEM for transportation to the lab. If transport time is longer than 30 min, keep the specimen in complete medium: DMEM containing 10% FCS, 2 mM L-glutamine, and antibiotics (100 U of penicillin/mL, 100 µg of streptomycin/mL, and 80 µg of gentamicin/mL) and transport with wet ice.
2. Put the specimen in a Petri plate with some medium. Strip the muscle from the resected specimen with a sterile scalpel. Transfer the remaining tissue into a new Petri plate with a minimal amount of medium (to prevent the explants from floating), and cut the tissue into 2–3 mm^3 pieces comprising both epithelial and muscularis mucosae (8).

3.5.2. Infection of Colorectal Explants

1. Experiments with colorectal explants are performed with two explants per well to obtain reproducible concentrations of p24 in the supernatants.
2. Explants are incubated with 200 µL of virus (10^3–10^4 $TCID_{50}$/mL) (see Note 18) for 2 h in 96-well U-bottom sterile plates. Negative controls, without virus (add 200 µL of complete medium), are included to confirm the HIV negative status of the donor (see Note 19).
3. During the last hour of incubation with virus, prepare the gelfoam rafts: put a piece of gelfoam in a Petri plate with complete medium; each piece of gelfoam can be cut with a sterile scalpel into 12 rafts (three pieces of gelfoam can be cut in one Petri plate); let the gelfoam soak with medium for at least 15 min. Also, prepare 24-well plates to where the rafts will be transferred: add 300 µL of complete medium/well. Once the rafts are wet with complete medium, place one raft per well in the 24-well plate.
4. Before transferring the explants onto the rafts, they will need to be washed in PBS; the washes are done in 96-well U-bottom sterile plates with 200 µL of PBS/well (prepare the wash plates ahead).
5. After 2 h of incubation, wash each explant four times in PBS to remove unbound virus. Transfer the explants onto gelfoam rafts (see Fig. 1).
6. Colorectal explants are maintained for up to 15 days in complete medium at 37°C in an atmosphere containing 5% CO_2.
7. Harvest 200 µL of the culture supernatant on days 3, 7, 11, and 15, in 96-well plates and re-feed the cultures with 200 µL of fresh complete medium/well. Freeze the harvest plates at –20°C.
8. Quantify p24 concentration at each harvest point with a p24 ELISA kit (see Notes 20–22).

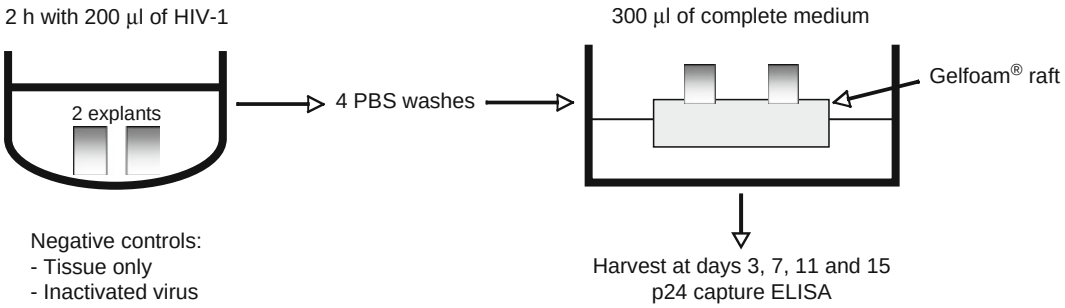

Fig. 1. Colorectal explant culture.

4. Notes

1. Stocks of lyophilised primers obtained from the manufacturer of choice are diluted to 100 µM and 50 µL aliquots and are stored at –20°C. These are diluted to form a working stock of 10 µM.
2. Hydrolysis probes for TaqMan-based assays are usually provided lyophilised. Reconstitute to 100 µM with NF-DW and aliquot (5 µL aliquots are a useful size). Probes are light-sensitive, and tubes should be wrapped in aluminium foil to exclude light and frozen at –20°C in a light-proof box; probe stocks are typically stored in aliquots at 100 µM. On making up a working stock at 2 µM, do not repeatedly freeze/thaw, but store at 4°C, which leaves it stable for 1–2 weeks.
3. Local blood donor centres can also provide multiple-donor buffy coat residues but before using these, check the protocol of filtration they use as recently some centres have introduced additional filtration steps making the multiple-donor residues unsuitable for virus replication.
4. The basic principles of how to establish a PCR assay without compromising further investigations should always be observed (19, 20). PCR-clean reagents which are kept isolated from products of previous PCR amplifications should always be used in a designated "clean" set-up area.
5. DNA samples should be stored and handled in screw-capped tubes with an "O"-ring seal but *never* using the flip-top kind owing to cross-contamination and health risks.
6. Alternatives to ethidium bromide and transillumination are available.
7. A simple way of determining cellular input is the use of fluorimetry or other spectrophotometric determinations such as

the Nanodrop system. In this manner, the number of input viral DNA copies calculated by PCR may be expressed typically as copies per 10^5 or 10^6 cellular equivalents.

8. The derivation of high copy-number template DNA to provide a standard control series can be achieved either by constructing a plasmid containing the key genomic target inserts, including *gag*, as performed for SIV (21), or by extracting DNA from a cell line subjected to continuous co-culture of the virus strain of interest. The LAV 8E5 cell line has been used for HIV-1 determinations. Both approaches have their advantages and disadvantages, but both must be titrated out in some unrelated, biologically inert carrier DNA. Herring sperm or calf-thymus DNA is readily available for this purpose from commercial sources at pre-determined concentrations. At a working concentration, typically 60 μg/mL, the plasmid or high-titre cellular extract may be diluted out to an extinction end point. Ideally, DNA samples should enter a PCR process at a final amount of approximately 0.6 μg. This serves to minimise the possibility of false negative reactions not only in samples with low levels of overall genomic DNA harbouring low levels of specific target sequence but also in samples with very high levels of genomic DNA which would be potentially inhibitory to the PCR process. The inclusion of a cellular gene or an internal calibrator amplified with the same primer set run in parallel to the test sample has also proved effective in many systems.
9. "House-keeping" genes are typically β-globin or phage controls. However, a degree of caution should be exercised since these are typically present in high copy numbers, whereas HIV/SIV copies are frequently present in low numbers, requiring an adjustment for this.
10. A range of genetic analyser platforms are now available and optimisation reactions and conditions should be performed accordingly. The Stratagene Mx3000/5P thermal profilers and allied software have been used to great effect by this group.
11. Columns 9–11 do not contain cells but just the first three dilutions of the virus stock representing the controls of p24 in the supernatant at day 1 of the titration.
12. Store the inactivated harvest plate at 4°C if the p24 assay is not performed immediately.
13. Spearman-Karber equation: $M = \mathrm{xk} + d[0.5 - ((1/n)(r))]$, where:

    xk = dose of the highest dilution (with the above set up of the titration plate, xk = 7)

r = number of p24 "negative" wells

d = spacing between dilutions (in this set up, $d = 1$)

n = wells per dilution (for quadruplicates, $n = 4$)

This equation gives the 50% endpoint: 5^{-M}, which can be converted to 10^{x}. The formula calculates the $TCID_{50}$/mL for the first dilution which is a 1:5 dilution of the virus stock; therefore, this dilution factor needs to be included: add $10^{0.70}$ to the value calculated.

14. The length of the experiment, and therefore of the titration, depends on the assay (neutralisation, inhibition) and the molecules tested (antibodies (Ab), inhibitory compounds). In general, compounds like reverse transcriptase inhibitors (RTI) require at least 48 h. If the compounds tested are entry/fusion inhibitors or neutralising Ab, 24 h will be enough to assess the level of inhibition/neutralisation at the viral entry/fusion step. Sometimes, it is recommendable to test a new compound/Ab at all the three time points (24, 48, and 72 h), and therefore, having a titration done at 48 h ("intermediate" time point) allows to adjust the amount of virus to start with for the three different conditions.
15. For inhibition/neutralisation experiments, normally 50 μL of compound/Ab is added per well for 1 h before infecting the cells with 50 μL of virus/well. Then, the cells are incubated for 24, 48, or 72 h. If the experiment requires a PBS wash, it should be done as quickly and gently as possible, not to disturb the cellular monolayer.
16. The relative light unit (rlu) value, chosen as a normalized read-out for the positive controls of infection, depends on the sensitivity of the machine and therefore needs to be set up based on the titration curves, where you can define a negative cut-off and a value where the titration curve is linear and the luciferase signal is not saturated. Titrations on TZM-bl cells can also give a $TCID_{50}$, using the Spearman–Karber equation.
17. The zero term of the Poisson equation is used where the conversion of the Poisson formula from its logarithmic form is expressed as: $\mu = -\ln Fo$ where the natural logarithm (ln) of the fraction of negative values (Fo) is proportional to the mean of the number of positive reactions (μ) for each group. The number of DNA or RNA molecules per microlitre of sample is calculated for each dilution series and the mean number determined. Four is the minimum number of replicate sets which may be used for this type of experiment (i.e. 25%) but yields informative data on viral copy number and sequence information derived from PCR clones. Once an approximate end-point has been established, for detailed informative characterisation of viral sequences, additional

dilutions of template, to derive sporadic positives in a highly statistically significant excess of negatives, will be required. The 96-well format particularly lends itself to this application (17). For an RNA template, the generation of cDNA only for limiting experiments will be much more cost effective than RNA dilution per se.

18. Due to the limited availability of tissue specimens, virus isolates are first titrated in activated PBMCs to determine a suitable dilution range for the explants.
19. Further negative controls can be added to the experiment, such as heat-inactivated and/or AT-2-inactivated virus.
20. The concentrations of p24 in explant culture supernatants are generally lower than those obtained in PBMC culture supernatants. Therefore, a highly sensitive p24 ELISA kit needs to be used.
21. A transient increase of p24 between day 0 (medium only) and day 3 might be due to leaching of input virus that was not removed sufficiently during washing.
22. The rectal explant system has also been applied to rhesus macaques using SIV (10). Substitute SIV p27 assay for the read-out.

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Chapter 10

Simultaneous Molecular Detection and Confirmation of Influenza AH5, with Internal Control

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Abstract

Influenza viruses continue to be a major cause of respiratory tract infection, resulting in substantial morbidity and mortality throughout the world. Accurate and rapid differential diagnosis of influenza virus infections, particularly associated with zoonotic infections, is important for public health actions, patient management, and treatment. Real-time PCR is widely considered the gold standard for molecular detection of influenza viruses owing to its high assay specificity, extreme detection sensitivity, and wide linear dynamic range. This protocol describes the use of a real-time RT-PCR assay for identification of influenza A and B viruses, detection of H5 subtype viruses, and an internal control, in a multiplexed, single-tube format. The inclusion of an internal bacteriophage control allows the efficiency of the extraction and amplification process to be monitored, so that false-negative results may be avoided. The primers and probe sets in this multiplex assay have been validated with a panel of influenza A viruses of different subtypes (including swine influenza viruses), and influenza B viruses, and specificity further confirmed with non-related respiratory viruses.

Key words: Influenza, Real-time RT-PCR, Diagnosis, Pandemic

1. Introduction

1.1. Influenza Viruses

Influenza viruses are enveloped, negative-sense RNA viruses, which are classified into types A, B, and C according to the serological reactivity of their internal proteins. Influenza A viruses are further classified into subtypes on the basis of the antigenic properties of their two surface glycoproteins, the haemagglutinin (HA) and neuraminidase (NA). To date, 16 HA and 9 NA subtypes have been identified. All influenza A subtypes have been found in aquatic and domestic birds, and only a few subtypes have been recovered from mammals and humans. In contrast, the natural

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host for influenza B and C viruses is man. Influenza B has also been found to infect seals (1) and influenza C, pigs (2). The ability of influenza viruses to undergo antigenic change in the HA and NA (antigenic drift) enables the virus to evade the host immune response and cause repeated infections and epidemics.

Transmission of wholly avian influenza (AI) viruses directly to humans was thought to be restricted by human cell receptor specificity. However, sporadic infection of humans with avian influenza H7N7 and H5N1 highly pathogenic (HP) viruses, and H9N2, H7N3, and H7N2 low pathogenic (LP) viruses have occurred (3–8). In particular, since 1997, AI H5N1 virus infections in poultry have taken on new significance, with increasing numbers of human cases involving poultry-to-human or domestic bird-to-human transmission, and the resulting production of clinically severe and fatal human infections.

A pandemic influenza virus could emerge directly from birds, following adaptation in humans to allow efficient human-to-human transmission. The 1918 "Spanish" influenza H1N1 pandemic virus is thought to have originated in this way (9). Pandemic viruses may also arise following simultaneous infection of a host cell by two different influenza viruses. Subsequent reassortment of the viral RNA segments could lead to the emergence of a virus with a novel combination of HA and NA genes (termed antigenic shift), to which the population has no existing immunity. It is, therefore, important to remain vigilant with respect to zoonotic infections, with H5 currently the highest threat.

1.2. Influenza Infection

Influenza viruses continue to be a major cause of respiratory tract infection, resulting in substantial morbidity and mortality throughout the world. Each annual influenza epidemic is associated with a significant economic and public health impact, with increased hospitalisation rates (10) and excess deaths (11) attributable to influenza infections. Seasonal influenza viruses are easily spread by breathing in the tiny droplets from the breath of infected people. The incubation period before the appearance of symptoms is 2–3 days. The virus infects the epithelial cells of the respiratory tract, with peak virus titres reached 24–48 h after the onset of symptoms (12). Influenza infection ranges from mild respiratory illness in healthy persons to fatal primary viral or secondary pneumonia in certain risk groups, such as the elderly and individuals with chronic medical conditions. Typical symptoms are abrupt onset of fever, malaise, myalgia, sore throat, and dry cough, lasting for 3–7 days in total, followed by a period of convalescence.

Human infection with avian influenza viruses predominantly occurs through close contact with live infected birds, although evidence to support limited human-to-human transmission of H5N1 and H7N7 viruses has also been documented (13–15). Initial symptoms in most H5N1 infected patients are those of

influenza-like illness, with high fever and lower respiratory tract symptoms (16–18). Other symptoms, including watery diarrhoea and conjunctivitis have also been reported (19, 20). The disease typically progresses rapidly from a severe pneumonia to the acute respiratory distress syndrome. The incubation period generally appears to be 7 days or less, and in many cases, this period is 2–5 days. The time from the onset of illness to hospitalisation is usually 3–5 days. The overall case fatality rate is high (approximately 60%), with the time from illness to death in fatal cases between 9 and 10 days. High titres of virus are detectable in specimens from the throat and tracheal aspirates from humans infected with H5N1 virus, indicating initial infection may occur in the upper or lower respiratory tract (21). Limited data show that H5N1 viral RNA may be detectable in the respiratory tract for up to 3 weeks. Plasma viral RNA is detected more often in patients with a fatal disease than in those who survive (21). It has been reported that diagnostic yields are higher with throat specimens than with nasal swabs due to higher viral loads in the throat, and tracheal aspirates have higher viral titres and yields than specimens from the upper respiratory tract (21, 22) Studies have also shown that H5N1 virus can spread beyond the respiratory tract to other organs, including the brain (23).

Avian influenza H7N7, H7N3, and H7N2 virus infection in humans causes conjunctivitis and a mild respiratory infection, although in some cases, the respiratory infection can become severe. One fatal case associated with H7N7 infection has been reported to date (3). In contrast, H9N2 viruses cause a mild, self-limiting respiratory illness in humans.

1.3. Laboratory Diagnosis

Rapid, sensitive, and specific diagnosis of influenza is important, as this can reduce the inappropriate use of antibiotics and guide timely and appropriate treatment with antivirals. Early and accurate diagnosis of H5N1 infection is especially critical for effective clinical management, disease containment, and infection control measures. However, a precise diagnosis of influenza by a physician is difficult since several different pathogens can produce respiratory illnesses with similar clinical symptoms. Laboratory diagnostic tests for verification of the clinical diagnosis of influenza include viral culture, rapid antigen testing, reverse transcription polymerase chain reaction (RT-PCR), immunofluorescence assays, and serology (24), although serological analysis is not helpful for patient management, as influenza infection is confirmed retrospectively. The sensitivity and specificity of these tests varies, as does the time taken to perform each test. Influenza viruses can be detected by isolation of the virus from respiratory specimens using either shell-vial or standard cell culture methods. Although sensitive, virus isolation requires viable virus, takes 2–10 days, and a second step must be performed to specifically

identify the influenza virus. Furthermore, because of biosafety concerns, isolation in culture of highly pathogenic H5N1 viruses is usually performed only in containment laboratories. More rapid diagnostic tests based on antigen detection, such as immunofluorescence and enzyme immunoassay (EIA), are simple and convenient to use. However, such tests are at present directed at conserved viral antigens (e.g. virus nucleoprotein, matrix protein) and detect all subtypes of influenza A viruses, and will not differentiate human virus subtypes from avian influenza H5N1. Additionally, current viral antigen detection rapid tests appear to have low sensitivity for the diagnosis of avian influenza H5N1, and therefore have limited use for the diagnosis of H5N1 disease in humans. Nucleic acid amplification methods for detection of influenza viruses in clinical material include nucleic acid sequence-based amplification (NASBA) (25, 26) and Loop-mediated Isothermal Amplification (LoopAmp) (27, 28), both performed using isothermic conditions, and more usually, reverse transcription polymerase chain reaction (RT-PCR) assays (24, 29–31). In addition to the high sensitivity and specificity of these assays, a further advantage of molecular detection methodology is that for diagnosis of suspect H5N1 infections, such assays can be performed under biosafety level 2 (BSL2) conditions.

1.4. RT-PCR Assays for Influenza

RT-PCR methods have been shown to be useful for both diagnosis and surveillance of seasonal influenza viruses (32–34). RT-PCR assays based on traditional agarose gel detection of amplified products have been described for the identification of human influenza A and B viruses (35–37), and as multiplexed assays, for typing and subtyping of influenza A H1 and H3, and influenza B viruses (32, 38, 39). Real-time RT-PCR assays for typing and subtyping of human influenza viruses have been reported (40), and multiplexing to allow detection of a range of respiratory pathogens in clinical material, including influenza A and B viruses (41–43), has been described. Real-time RT-PCR assays are particularly advantageous in outbreak investigations, providing results within 4–6 h of receipt of sample, and are the method of choice for diagnosis during outbreaks of H5N1 infection (21, 44). Several real-time RT-PCR assays have been reported for the detection of avian influenza viruses, including assays specific for detection of H5 only (45–47), a triplex assay for detection of all influenza A viruses and subtyping of H5 and N1 (48), and assays which can detect all influenza A subtypes with inclusion of an internal control (IC) (49, 50). The advantage of the addition of an IC is the simultaneous monitoring of the efficiency of nucleic acid extraction, reverse transcription, and amplification. Efficient diagnostic testing requires multiplex assays that can detect all influenza A subtypes (H1–H16) and influenza B, as well as specifically identify the presence of H5, with an IC in a single reaction. The quadriplex real-time RT-PCR assay described

in this chapter fulfills these criteria and also allows differential diagnosis of H5. Generic PCR primers and Taqman probes targeting regions of the highly conserved matrix and nucleoprotein genes of influenza A and B viruses, respectively, enables detection of H5 and all other influenza infections, and reduces false negative results that might arise due to mutations in the H5 HA gene. The H5 specific subtyping primers and probe were designed following analysis of HA gene sequences of H5 viruses belonging to described H5 HA clades and subclades (51), and MS2 was chosen as an IC due to its ease of propagation and since it represents encapsidated RNA, with specific primers and Taqman probe designed near the 5′ end of the genome. The quadriplex assay has been extensively optimised and validated against a range of influenza A H5 viruses, including H5 strains associated with outbreaks of H5N1 infections in humans in Turkey and China (45, 52, 53), and H5 viruses isolated from birds in Asia and Europe (45, 51). The specificity of this assay has been evaluated against a panel of influenza A viruses of different subtypes (H1–H15), influenza B viruses, and a panel of related and unrelated respiratory viruses, to ensure specificity of the assay for influenza A, H5, and influenza B. Since its introduction into routine weekly influenza surveillance, and diagnosis, in the Clinical Microbiology and Public Health Laboratory, Addenbrooke's Hospital, Cambridge, the quadriplex assay has proved an invaluable and robust diagnostic tool in the investigation of three suspect human-associated poultry outbreaks that have occurred in poultry farms in the East of England during 2006–07 (8, 54, 55), as well as in investigation of returning travellers from H5N1-affected areas and >2,500 respiratory clinical specimens during routine respiratory surveillance.

The quadriplex assay protocol has been disseminated to 18 laboratories that comprise the UK National Influenza H5 Laboratory Network (56) and is presently the UK's most commonly used frontline, H5 screening assay for assessment of human clinical cases with influenza-like illness and exposure to poultry, or returning travellers from H5N1-affected areas.

The low incidence setting of human H5N1 infections limits the predictive value of a single positive test to <70%. It is crucial that a confirmatory strategy exists to improve the certainty of diagnosis. We, therefore, also describe a second real-time assay, based on Fluorescent Resonance Energy Transfer (FRET) hybridisation probes, for the detection of influenza A H5 viruses. This assay was principally designed and developed to provide a real-time RT-PCR confirmatory assay, targeting a different region of the H5 HA gene sequence to the quadriplex assay, and capable of distinguishing positive control material (A/Duck/Singapore-Q/F119-3/1997 H5N3 virus) from recent H5N1 isolates (see Note 1). This assay has also been subjected to a vigorous optimisation and validation process and is specific only for influenza H5 viruses,

detecting all H5 isolates available to the reference laboratory, with a detection limit equal to or greater than the H5 component of the quadriplex assay (<1 PFU). Recommended laboratory practice is to run both the quadriplex and confirmatory H5 FRET assays in parallel when screening suspect H5 infections, enabling a confirmed result in ≤4–6 h after receipt of specimen.

1.5. Safety Considerations

All handling of clinical material from suspected avian influenza patients must be carried out in a containment level 3 (CL3) facility within a class I/III bio-safety cabinet. Once the clinical material has been added to the lysis buffer employed in the extraction procedure, subsequent extraction of viral nucleic acid can be carried out under bio-safety level 2 conditions (see Note 2).

1.6. Specimens for Diagnosis

Suitable specimen types are respiratory secretions, such as nose and throat swabs (in virus transport medium), naso-pharyngeal aspirates (NPA), broncho-alveolar lavage (BAL), endo-tracheal aspirates (ETA), and sputum (see Note 3). Analysis of blood and faecal material using the methods described below has not been fully validated as yet.

A minimum of 300 μl of sample is required for the extraction of viral nucleic acid for analysis in the quadriplex A/H5/B RT-PCR assay. If H5N1 virus infection is suspected, it is essential that duplicate samples are taken, since if the sample is found to be H5 positive, further analysis will be required. Duplicates may also be required for the exclusion of other microbial pathogens.

2. Materials

2.1. Equipment

1. Rotor-Gene 3000™ (6000) Real-time thermal cycler (Corbett Life Science).
2. Microcentrifuge.
3. Vortex Genie 2.
4. 0.2 ml (36 well rotor) or 0.1 ml (72 well rotor) tubes for the Rotor-Gene.
5. Pipettes with disposable filter tips.

2.2. Reagents

To ensure against contamination with ribonucleases, gloves should be worn when preparing reagents and during all manipulations in the subsequent method sections. To avoid contamination between specimens, pipette tips with filters should be used and standard PCR anti-contamination procedures followed (57–59).

2.2.1. Viral RNA Extraction

1. QIAamp Viral RNA Kit: Cat no: 52906 (250 preps) (see Note 4).

2.2.2. Quadriplex RT-PCR Assay

1. SuperScript III Platinum one-step qRT-PCR system (Invitrogen Cat no:11732-088).
2. RNase-free water (Invitrogen, Cat no:10977035).
3. Control virus=Influenza A H5N3 A/Duck/Singapore/3/97 virus, available from Health Protection Agency (http://www.hpa.org.uk/webw/HPAweb&Page&HPAwebAutoListName/Page/1160994272854?p=1160994272854) (see Note 5).
4. Forward and reverse primers (Metabion):

   AM-F (20 pmol/µl) 5′-GAG TCT TCT AAC MGA GGT CGA AAC GTA -3′

   AM-R (20 pmol/µl) 5′- GGG CAC GGT GAG CGT RAA -3′

   BNP-F (20 pmol/µl) 5′- GCA GCT CTG ATG TCC ATC AAG CT -3′

   BNP-R (20 pmol/µl) 5′-CAG CTT GCT TGC TTA RAG CAA TAG GTC T -3′

   H5PCR-7F (10 pmol/µl) 5′- GCC GAA TGA TGC MAT MAA YT -3′

   H5PCR-7R (10 pmol/µl) 5′- CGC ACC CAT TGG AGT TTG AC -3′

   MS2 F1 (20 pmol/µl) 5′- TGG CAC TAC CCC TCT CCG TAT TCA CG -3′

   MS2 R1 (20 pmol/µl) 5′- GTA CGG GCG ACC CCA CGA TGA C -3′.
5. Dual-labelled probes

   Influenza A: AM Probe (10 pmol/µl);

   5′ – VIC –TCC TGT CAC CTC TGA C – MGBNFQ-3′ (Applied Biosystems)

   Influenza B: BNP-Probe (3.3 pmol/µl);

   5′ – Cy5 – CCA GAT CTG GTC ATT GGR GCC CAR AAC TG – BHQ3-3′ (Metabion)

   Influenza H5: PCR7 probe (5 pmol/µl);

   5′ –FAM– CAT TGC TCC AGA AWA T –MGBNFQ-3′ (Applied Biosystems)

   Internal control MS2 (10 pmol/µl);

   5′ Rox– CAC ATC GAT AGA TCA AGG TGC CTA CAA GC– BHQ2 3′ (Metabion).
6. Bacteriophage MS2 (ATCC 15597-B1) and its *E. coli* Host (ATCC 15597) available from the American Type Culture Collection. Propagate as detailed in the product information sheets provided (see Note 6). Pandemic H1N1 2009 influenza viruses have emerged since the assay was developed, and it should be noted that inclusion of a degenerate base (Y) at nucleotide position 3 in the AM probe improves the amplification

curve with this subtype, although the use of the degenerate probe in the assay has not yet been fully validated against other subtypes.

2.2.3. Confirmatory H5 FRET RT-PCR Assay

1. SuperScript III Platinum one-step qRT-PCR system (Invitrogen cat no:11732-088).
2. RNase-free water (Invitrogen, cat no:10977035).
3. A/Duck/Singapore-Q/F119-3/1997 (as above).
4. Forward and reverse primers (Metabion):

   H5-F (20 pmol/µl) 5′- AGT GGR TAC GCT GCA GAC -3′

   H5-R (20 pmol/µl) 5′- CAG CAT TAT AAG TCC AGA CAT CTA G -3′.
5. FRET hybridisation probes (Metabion)

   LC-H5 (10 pmol/µl);

   5′ LC Red 640- T**C**G AGT TGA CCT TAT T**G**G TGA **C**TC-Phosphate-3′ (mismatches with H5N3 control are in bold font)

   FL-H5 (10 pmol/µl);

   5′- GCC TCA AAC TGA GTG TTC ATT TTG TCA ATG-Fluorescein-3′.

3. Methods

3.1. Specimen Transportation and Storage

Specimens should be processed as soon as possible after collection. Ideally, transportation systems should ensure that samples arrive in the laboratory within 24 h from the time of collection. Specimens that may be delayed should be refrigerated prior to transportation. They should also be refrigerated at 4°C if there is likely to be a delay in processing. If the delay is likely to exceed 24 h, the sample should be stored at –70°C and thawed prior to processing; however, repeated freezing and thawing should be avoided as this may result in degradation of the sample and a reduction in sensitivity of the assay.

3.2. Isolation of Viral RNA

1. Perform extraction in duplicate if sufficient sample volume is available. To a clean labelled 1.5-ml micro-centrifuge tube, add the following in order:

   560 µl of AVL Buffer (ensure carrier RNA is added as per the manufacturer's instructions)

   20 µl of MS2 internal control (10^{-4}) i.e. ~4,600 PFU per extraction (see Note 7)

   Add 140 µl of specimen and controls to their respective tubes. For suspect H5 samples, this should be performed in a CL3 laboratory, in a class I/III cabinet. Close the tubes and vortex for 5 s.

2. In a CL2 laboratory, place the tubes on a rotary mixer (Genie 2) at RT for 10 min on a setting of 5. During the incubation stage, remove individual QIAamp columns from their packaging and place in a rack and label the tops with the corresponding sample number.
3. Pulse spin and add 560 µl of ethanol (97–100%) to the sample, mix thoroughly by vortexing, and pulse-spin.
4. Carefully apply half of the mixture from step 3 to the QIAamp spin column without wetting the rim, close the cap, and centrifuge at 6,000 *g* for 1 min. Discard the waste from the collection tube into a suitable guanidinium waste bottle and discard the used collection tube. Place QIAamp spin column in a clean 2-ml collecting tube.
5. Repeat step 4.
6. Carefully add 500 µl of buffer AW-1 to the column (without wetting the rim) and centrifuge at 6,000 *g* for 1 min. Discard the waste from the collection tube into a suitable container for solvent waste and discard the used collection tube. Place QIAamp spin column in a clean 2-ml collecting tube.
7. Repeat step 6 with wash buffer AW-2 follow by a further centrifugation at 20,000 *g* for 1 min to remove any residual buffer.
8. Place the QIAamp spin column in a labelled clean 1.5-ml flip-top tube (cut the tops out with scissors and keep for later use).
9. Add 60 µl AVE buffer/RNase-free water dropwise at the centre of the column and close the lid. Incubate for 1 min.
10. Elute the RNA by centrifugation at 6,000 *g* for 1 min.
11. Discard the spin columns and recap the tubes.
12. Store on ice if analysing immediately by RT-PCR, otherwise store at –80°C.

3.3. Taqman Flu A, B, H5 & IC RT-PCR on the Rotor Gene

1. Prepare the following Quadriplex real-time master mix (MMX):

Reagent for ×1 reaction	Volume for one tube (µl)
RNase free water	1.38
2× Reaction mix	12.5
10 pmol/µl H5PCR-7F primer	1.0
10 pmol/µl H5PCR-7R primer	1.0
5 pmol/µl H5PCR-7 probe	0.5
20 pmol/µl AM-F primer	0.5
20 pmol/µl AM-R primer	1.0

(continued)

(continued)

Reagent for ×1 reaction	Volume for one tube (μl)
10 pmol/μl AM probe	0.4
20 pmol/μl BNP-F primer	0.16
20 pmol/μl BNP-R primer	0.16
3.3 pmol/μl BNP probe	0.2
20 pmol/μl MS2 F1 primer	0.1
20 pmol/μl MS2 R1 primer	0.1
10 pmol/μl MS2 Taq probe	0.2
Superscript III Platinum one-step enzyme	0.8
Total volume per tube	20.0

The RT-PCR MMX should be $N+2$ (N = No of tests including controls) (see Note 8).

2. Vortex and pulse spin.
3. Load 0.2-ml flat-top clear tubes on an appropriate cooled metal block beginning at position 1.
4. Pipette 20 μl of reaction mix into the 0.2-ml tubes.
5. Add 5 μl of RNA extract to respective tubes, close the tube after each addition.
6. Transfer the tubes into a 36-well carousel at their corresponding locations and load it into the Rotor-Gene. Apply the locking ring (for 36-well Rotor only).
7. Open a Quadriplex Flu folder. Click "NEW" from the menu and "NEW" again from the tool box.
8. Select the Rotor type as 36, tick the "No domed", and click "Next".
9. Type in "operator Name" in the operator box – set reaction volume at 25 μl and click "Next".
10. A "Quadriplex Flu-Taqman" run profile should be displayed as outline below:

Programme	Temp (°C)	Hold time (min)	Acquisition mode	No. of cycles
RT	50	30	Not acquiring	×1
Denature	95	2	Not acquiring	×1
Cycling	95	15 (s)	Not acquiring	×45
	60	1	Acquiring on Fam, Joe, Cy5 & Rox	

11. Select "calibration" and this will display Auto gain calibration set-up menu; ensure that Fam, Joe, Cy5, and Rox channels are displayed. Set temperature to 60°C and click "Start" – this will adjust the gains for the four channels selected, when completed click on "close" followed by "close" on the calibration menu.
12. Click "Next", followed by "Start Run". Save the current run as "Flu ddmmyy" in the Flu runs folder.
13. The run will start and after few seconds will display the run worklist to edit appropriately.
14. Enter sample ID from the worklist including positive controls and leave type as unknown. For water negative control, select type as NTC.

No	Colour	Name	Type	Group	Given Conc	Selected
1		Sample number	Unknown			Yes
2		Duck H5N3	Unknown			Yes
3		Water	NTC			Yes

15. Click on "Finish" and the Rotor-Gene will display the run screen.

3.4. Data Analysis

1. Following completion of the run select "Analysis" in the view menu. An analysis tool box will appear, click on the following sequence: "Quantification" – "Cycling Channel Fam" – "show". Click OK on the new box informing you about the auto-find threshold feature and close the analysis box. In the new quantitative analysis – cycling A Fam window, click linear scale which will change it to log scale (this will display amplification of Flu-H5 in the samples).
2. In the menu box; Click on "Dynamic Tube", "Slope correction" and set the NTC threshold to 10% and ensure the enabled box in the reaction efficiency threshold is left blank in the "More settings" tool box.
3. At the "Eliminate cycles box" (right side of window), enter a value of 10, and in the "Threshold" box, enter a value between 0.01 and 0.05 until the threshold value line just clears the base line.
4. Click on "Report", select full report format, and print a hard copy for record.
5. Each Channel must be analysed separately by repeating steps 1–4 for the remaining "Cycling Channel's" – "Joe for Flu A" – "Cy5 for Flu-B" and "Rox for internal MS2 control".

3.5. Run Validation

Check that the threshold value is within the range 0.01–0.05. Ensure that positive control samples and the MS2 IC have amplified and have signals in the correct channels, and that negative controls show no amplification, before accepting results (see Fig. 1 for a typical example of a quadriplex assay run). If a signal is detected in the negative controls, then contamination must be suspected, and the samples should be re-extracted and re-analysed by the quadriplex assay. Where positive and negative controls pass the validation criteria, but a sample(s) shows a higher than expected Ct value for the IC, that sample(s) should be re-extracted and re-analysed. If a sample(s) is found to be positive for influenza H5, further verification of that result is required, and can be achieved by analysis in a second real-time H5 specific assay, amplifying a different region of the haemagglutinin gene, such as the confirmatory H5 FRET assay. If a sample is found to be positive for influenza A, but negative for influenza B, further subtyping assays are required. Examples of other possible assay results and interpretations are given below:

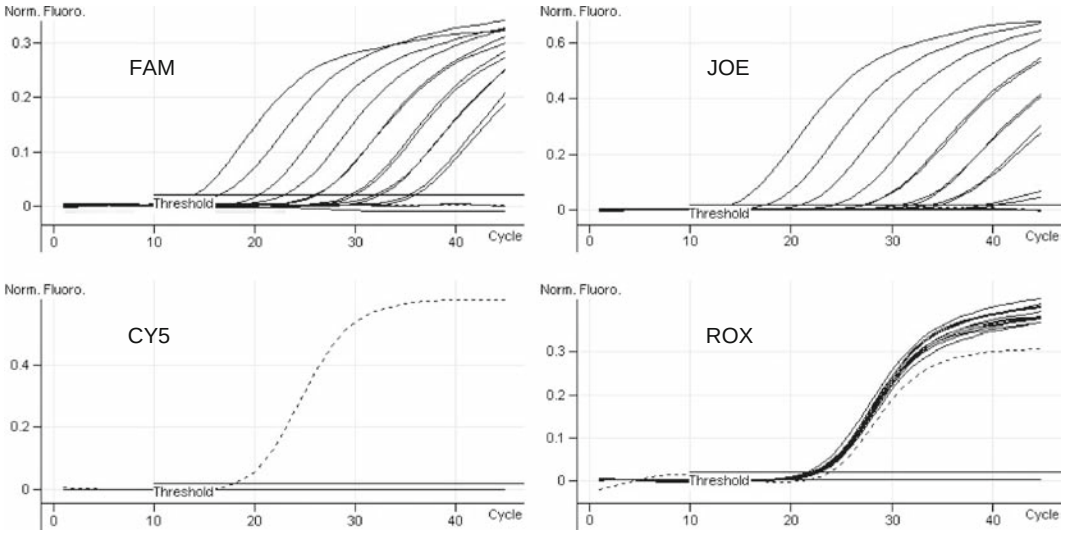

Fig. 1. A representative example of the quadriplex assay. Tenfold serial dilutions of the positive H5N3 control A/Duck/Singapore-Q/3/97 (–2 to –9) were extracted with MS2 and analysed (–6 to –9 in duplicate). A –3 dilution of Flu B (B/Panama/45/90) was also included (*dotted line*). As clearly demonstrated all dilutions of H5N3 control were detected in the H5 and Flu A specific channels (FAM and JOE, respectively). Flu B was clearly only detected in the CY5 channel and the MS2 internal control was detected in all samples in the ROX channel.

Quadriplex target detected

IC	A	H5	B	Result/interpretation
√	√	–	–	Valid/influenza A
√	√	√	–	Valid/influenza A H5 Perform confirmatory H5 assay
√	–	–	√	Valid/influenza B
√	–	√	–	Not valid/repeat quadriplex or perform confirmatory H5 assay
–	–	–	–	Not valid/repeat quadriplex
–	√	–	–	Not valid/repeat quadriplex
–	–	√		Not valid/repeat quadriplex
–	–		√	Not valid/repeat quadriplex

3.6. Confirmatory H5 FRET RT-PCR on the Rotorgene (See Note 9)

1. Prepare the following H5 FRET real-time master mix (MMX):

Reagent for ×1 reaction	Volume for one tube (µl)
RNase-free water	5.1
2× Reaction mix	12.5
20 pmol/µl H5-F primer	0.5
20 pmol/µl H5-R primer	0.5
10 pmol/µl LC-H5 probe	0.3
10 pmol/µl FL-H5 probe	0.3
Superscript III Platinum one-step enzyme	0.8
Total volume per tube	20.0

   The RT-PCR MMX should be $N+2$ (N= No of tests including controls) (see Note 8).
2. Follow steps 2–6 exactly as outlined above for the quadriplex assay in Subheading 3.3.
3. Open a H5 FRET folder. Click "NEW" from the menu and "NEW" again from the tool box.
4. Select the Rotor type as 36, tick the "No domed", and click "Next".
5. Type in "operator Name" in the operator box – set reaction volume at 25 µl and click "Next".

6. A "H5 FRET" run profile should be displayed as outline below:

Programme	Temp (°C)	Hold time (s)	Acquisition mode	No. of cycles
RT	50	1,800	Not acquiring	×1
Denature	95	120	Not acquiring	×1
Cycling	95 50 72	5 20 30	Not acquiring Acquiring on LC 640 Not acquiring	×45
Melt	95 40 40–80	60 30 2/1°	Not acquiring Not acquiring Continuous acquiring on LC 640	×1

7. Select "calibration", and this will display auto-gain calibration set-up menu; ensure that the LC 640 channel is displayed (this must be created as a new channel in the Rotor-Gene software, adjusting the source to 470 nm and the detector to 610 hp). Set temperature to 50°C and Click "Start" – this will adjust the gain LC 640; when completed, click on "close", followed by "close" on the calibration menu.
8. Click "Next", followed by "Start Run". Save the current run as "Flu ddmmyy" in the H5 FRET Flu runs folder.
9. Follow steps 13–15 exactly as outlined above for the quadriplex assay in Subheading 3.3.

3.7. Data Analysis for the FRET

1. Follow steps 1–4 exactly as outlined above in the data analysis Subheading 3.4 for the quadriplex assay, selecting the cycling LC 640 channel.
2. For Melt Curve analysis, return to the analysis box and click on the following sequence: "Melt" – "Melt A LC640" – "show". The mismatches in the LC H5 probe allow differentiation of H5N3 control from H5 wild-type isolates – the control material should melt at a significantly lower temperature i.e. ~51°C.
3. Click on "New Bin" and position the pointer on the apex of the lower melt peak; this will display the melting temperature of the H5 control under Bin A. If additional peaks are present to the right of the positive H5N3 control, then select a New Bin and this time position the pointer on the apex of the second peak. This will display the melting temperature of the H5 wild type. Ensure the threshold line is above the baseline traversing the melting peaks only (see Fig. 2b).

4. Click on "Report", select full report format, and print a hard copy for record.

3.8. Confirmatory H5 FRET Assay Run Validation

1. Ensure that the positive control sample has been amplified and negative controls show no amplification, before accepting results (see Fig. 2 for a typical example of a H5 FRET RT-PCR assay run).
2. Any positive clinical specimens with a melting peak corresponding to the H5N3 control material should be considered as a possible contaminant and not reported. The appropriate steps should be taken to address the problem. All recent H5N1 isolates held by the Reference Laboratory have been examined by the FRET assay and shown, with the exception of A/Indonesia/5/2005, to have a melting temperature of ~ 67°C. (Bin C in Fig. 2). Sequence analysis of the amplicon derived from A/Indonesia/5/2005 isolate revealed two nucleotide changes in the LC-H5 probe target site sequence, namely, a C-T change at position 2 and a C-Y change at position 11 (heterogeneous nucleotide) of the LC-H5 sequence, explaining the lower melting peak (~56°C) and the heterozygous nature of the melting profile (second smaller melting peak at 65°C).

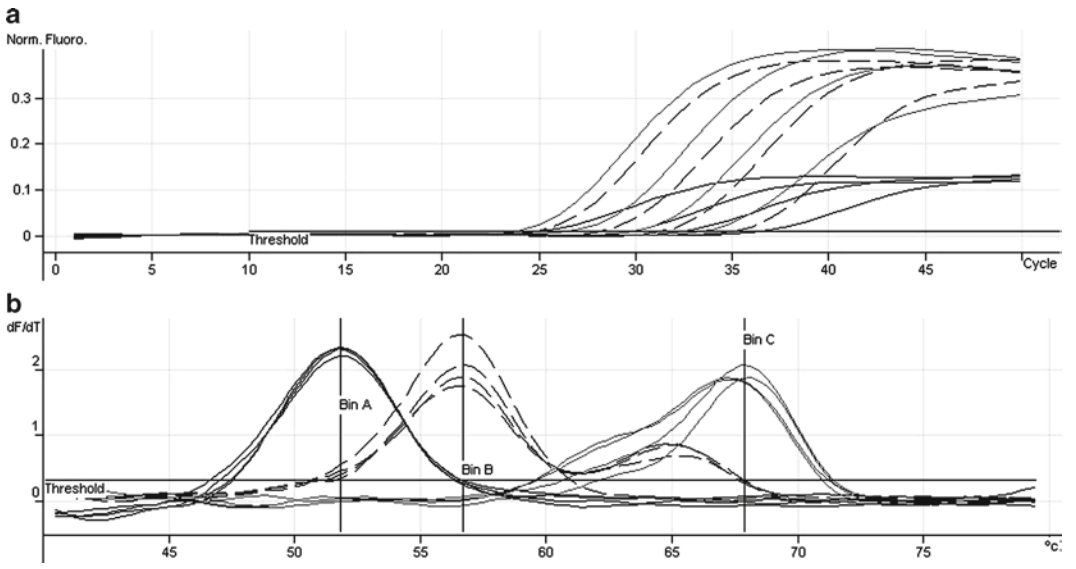

Fig. 2. A representative example of the H5 specific real-time FRET RT-PCR assay. (**a**) Amplification *curves* for tenfold serial dilutions of the H5N3 control A/Duck/singapore/3/97 (*solid black lines*, −4 to −7), and recent H5N1 isolates A/Indonesia/5/2005 (*dashed black lines*, −5 to −8) and A/Turkey/Turkey/2005 (*solid grey lines*, −4 to −7). Note the shallower amplification *curves* for the control H5N3 isolate, emphasising the impact of the mismatched probe. (**b**) The associated melting *curve* plots for the dilution series of all three isolates. Note the lowest melting temperature (~51°C) of the control H5N3 isolate (Bin A). All recent H5N1 isolates normally map to Bin C with the exception of A/Indonesia/5/2005, which has a slightly lower temperature at 56°C (Bin B), yet still clearly distinct from the control material.

4. Notes

1. Discrimination is based on melting temperature analysis of the amplicons, with an inbuilt bias against the A/Duck/Singapore-Q/F119-3/1997 H5N3 isolate, due to three nucleotide mismatches with one (LCH5) of the FRET probes. As a consequence, the A/Duck/Singapore-Q/F119-3/1997 H5N3 isolate displays a melting peak of ~51°C compared with ~67°C for most recent H5N1 isolates, which from the available sequence alignment data are predicted to have no mismatches with this probe (the only exception to date being A/Indonesia/5/2005, with a melting peak of ~56°C).
2. Good laboratory practice must be employed and personal protective equipment worn at all times. In addition, local COSHH and risk assessments should be followed.
3. It should be noted that based on published *data* (21, 23, 60–62), analysis of blood and faecal specimens during the acute presentation phase of illness has been shown to be clinically useful. While the quadriplex assay, in this chapter, has been validated using respiratory secretions, analysis of plasma/serum should not pose any difficulties with the nucleic extraction protocol described. However, a reliable extraction method for the purification of viral nucleic acid from faecal material for use in the RT-PCR assay has not as yet been fully validated. However, the extraction protocol currently used successfully in our laboratory for norovirus RNA detection in faeces could easily be adapted to work in the CL3 facility and may well provide high-quality influenza nucleic acid for analysis (63).
4. A low ratio of lysis buffer to sample may not be sufficient to inactivate high-titred influenza A H5 virus. Qiagen AVL buffer has been shown to render clinical samples non infectious at the volumes used in this protocol (64). If a different nucleic acid extraction procedure is to be used, then each laboratory should perform a risk assessment of the likely risk of the samples to be tested containing influenza A H5 virus, prior to aliquotting of the sample(s) into lysis buffer.
5. Aliquots of 600 µl of influenza A H5N3 A/Duck/Singapore-Q/F119-3/1997 virus (2.5×10^8 PFU/ml) mixed with 200 µl guanidinium isothiocyanate lysis buffer (Severn Biotech Ltd, Worcester, UK) have been prepared and stored at −80°C until use. This control material has been quality assured (CE marked), following standard stability and stress testing. The positive control material and negative controls (RNase-free water) should be extracted and processed at the same time as the test samples, and tested in duplicate in the quadriplex RT-PCR assay.

6. Purified MS2 RNA is available from Roche (cat no: 165 948) at a concentration of 0.8 μg/μl. When used as a standard in a quantitative MS2 assay, we determined the genome copy number to be 9 per plaque-forming units (PFU) of our stock. For those who would prefer to add purified RNA to the master mix, we suggest 0.5 μl of a 10^{-6} dilution of the MS2 RNA (RNase-free water containing 0.1 ng/μl of PolyA carrier RNA) per PCR as a starting point.
7. Optimisation experiments indicated 20 μl of a 10^{-4} dilution (PBS) of our stock (2.3×10^9 PFU/ml) was the most reliable input per extraction for both reproducible MS2 detection and non interference with other routine amplification targets. This equates to 4,600 PFU per extraction and therefore in the case of a 60 μl elution, followed by addition of 5 μl of extract, 383 PFU per PCR.
8. Perform both test samples and control reactions in duplicate or triplicate, depending on the material available.
9. The H5 FRET assay has also been successfully transferred to the Roche LightCycler® 1.0/2.0, maintaining the assay's specificity, sensitivity, and melting profiles. The master mix is modified to accommodate the lower volume (20 μl) capacity of the glass capillaries and the need for bovine serum albumin (BSA). While the primers (0.5 μl), probes (0.3 μl), and enzyme (0.8 μl) additions are the same per tube reaction, the 2× Reaction Mix is dropped to 10 μl and the RNase-free water to 1.6 μl and 1.0 μl of a 10% BSA (Sigma P/N B4287-5G) solution (made up in water) is added. Final volume is 15, and 5 μl of extract RNA is then added. The run profile contains the same 30 min RT step and initial denaturation of 2 min, but the cycling times are shortened to 1 s at 95°C, 15 s at 50°C (acquiring on F2) and 15 s at 72°C, all with a slope of 20°C/s and 45 cycles in total. The melt analysis contains 20 s at 95°C (slope 20°C/s), 20 s at 40°C (slope 20°C/s), ramping to 85°C (slope 0.1°C/s) with continuous acquisition on the F2 channel, followed by a cooling to 30°C (slope 20°C/s) for 10 s. Analysis is performed using F2/F1 Channel and second derivative maximum.

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Chapter 11

Detection of Measles, Mumps, and Rubella Viruses

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Abstract

Measles, mumps, and rubella are infections caused by RNA viruses of the same name and are vaccine preventable. The vaccines are frequently administered in a trivalent form. Laboratory diagnostic methods can include indirect detection via antibody (IgM and IgG) detection methods and direct detection by viral culture or viral genome detection. There are challenges for the laboratory in areas with low prevalence due to high vaccine uptake. In those areas, routine serological methods such as IgM detection may have a reduced positive predictive value and thus require confirmation by other methods. Direct detection of viral genomic material using reverse transcription polymerase chain reaction (RT-PCR) methodologies can play an important role for laboratory confirmation of acute infections. Furthermore, genotyping of these three viruses provides useful molecular epidemiological data for differentiating vaccine from wild-type strains, linking cases and outbreaks, and tracking geographic spread and elimination. The purpose of this chapter is to provide guidance for the laboratory diagnosis of measles, mumps, and rubella virus infections. Where assays are commercially available or previously published, the appropriate references are provided as well as brief comments on the interpretation of results. Detailed protocols are provided for the molecular assays which have been developed and more commonly applied in recent years.

Key words: Measles, Mumps, Rubella, RT-PCR, Serology, Avidity, SSPE, CRS

1. Introduction

Measles virus belongs to the family *Paramyxoviridae* and genus *Morbillivirus*. Measles virus is a 120–250 nm enveloped virus that contains a 15,894 nucleotide single-stranded, nonsegmented, negative-sense RNA genome in a helical nucleocapsid. Measles is highly infectious with clinical symptoms including fever, maculopapular rash, cough, coryza, conjunctivitis, and pathognomonic Koplik spots (lesions with a bluish-white center appearing on the buccal mucosa). Rare complications can include acute disseminated encephalomyelitis, measles inclusion body encephalitis, and subacute sclerosing panencephalitis (SSPE). There is a highly

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effective live-attenuated vaccine for measles. Measles incidence has been greatly reduced compared with the pre-vaccine era and some geographic regions have documented the elimination of endemic measles as a result of high coverage immunization campaigns. The laboratory diagnosis of measles presents certain challenges in areas with low measles prevalence.

Mumps virus, like measles, also belongs to the family *Paramyxoviridae*, but belongs to the *Rubulavirus* genus. Like measles, mumps is a 150–250 nm enveloped virus that contains a single-stranded, non-segmented, negative-sense RNA genome in a helical nucleocapsid. The mumps genome contains 15,384 nucleotides. Clinical symptoms of mumps include mild fever and acute onset of unilateral or bilateral swelling of the parotid or salivary gland. In 25–30% of mumps infections, clinical symptoms may not be apparent. Complications can include meningoencephalitis, orchitis in males or oophoritis in females. The live-attenuated mumps vaccine is often administered along with measles and rubella in the MMR vaccine. There are unique challenges for the laboratory diagnosis of mumps in previously immunized individuals whose immunity may have waned either in the absence of a second booster dose of vaccine or in areas where mumps disease, and thus exposure, is minimal.

Rubella virus belongs to the family *Togaviridae*, genus *Rubivirus*. Rubella virions are enveloped and are approximately 70 nm in diameter. The rubella virus genome is an approximately 10,000 nucleotide, single-stranded, non-segmented, positive-sense RNA genome in a helical nucleocapsid. Postnatally acquired rubella is usually mild and is characterized by a low fever and generalized maculopapular rash. Other symptoms may include lympadenopathy, conjunctivitis, and arthritis or arthralgia in females. However, primary rubella virus infection in pregnant women is highly teratogenic, most notably in the first trimester of pregnancy, and can result in severe congenital abnormalities in the children (congenital rubella syndrome, CRS) including deafness, cataracts, glaucoma, cardiovascular abnormalities, and meningoencephalitis. As such, a live-attenuated vaccine was developed and is typically administered along with measles as MR or with measles and mumps as MMR. Successful rubella immunization programs have resulted in a significant reduction in CRS cases in some countries. Similar to measles, rubella laboratory diagnosis presents some challenges in areas with low prevalence. The laboratory diagnosis of rubella presents particular challenges for diagnosing rubella infection in pregnant women.

For all three of measles, mumps, and rubella, the laboratory diagnosis of an acute infection can be done by (1) the detection of virus-specific IgM antibody in a single serum sample, (2) the detection of viral RNA in a suitable clinical specimen, (3) the isolation of the virus in cell culture from a suitable clinical specimen, or (4) the demonstration of IgG seroconversion or a significant (greater than

or equal to fourfold rise) rise in antibody titer between acute and convalescent paired sera.

In addition to the laboratory testing for the diagnosis of these diseases, virus genotyping for measles, mumps and rubella is useful for molecular epidemiologic purposes.

The purpose of this chapter is to provide guidance for the laboratory diagnosis of measles, mumps, and rubella virus infections. Where assays are commercially available or previously published, the appropriate references are provided as well as brief comments on the interpretation of results. Detailed protocols are provided for the molecular assays which have been developed and more commonly applied in recent years. The methods referenced and described in this chapter are not meant to be comprehensive with respect to encompassing all the methods used for the laboratory diagnosis of these viruses, but reflect the basic approach used by the National Microbiology Laboratory of the Public Health Agency of Canada. It should also be recognized that the methods for diagnosing measles, mumps, and rubella viruses will continue to evolve and that any method used by a laboratory must be validated and/or verified in that laboratory prior to implementation as a diagnostic test. Lastly, it should be recognized that the successful implementation of a non-commercially available assay (e.g. the molecular assays for measles, mumps, and rubella) will likely require some adaptation/modification such that the assay is optimized for the particular laboratory as a consequence of slight differences in the assay components (e.g. buffers, enzymes, instruments) in different laboratories. Thus, these protocols should be considered as guidance rather than as definitive protocols.

2. Materials

Commercial kits for measles, mumps, and rubella-specific IgM and IgG antibodies are widely available, typically in the Enzyme Immunoassay (EIA) format. Many of these IgM assays have been evaluated and there is some variability in the performance characteristics (sensitivity, specificity) between these assays (1, 2). In addition, commercial real-time PCR kits are available from Roche as described by Hummel et al. (3).

3. Methods

3.1. Serologic Methods

3.1.1. IgM Serology

The presence of measles, mumps or rubella-specific IgM in serum is indicative of an acute infection. IgM serology is the most common and practical method for the laboratory confirmation of

measles, mumps, and rubella. The detection of IgM in cord blood or serum of an infant is one method for the diagnosis of congenitally acquired rubella.

It is important for a laboratory to carefully consider such evaluation data prior to implementation of the IgM assay.

The results of IgM assays for measles, mumps, and rubella need to be interpreted carefully. When the disease prevalence is low in a particular geographic region as a result of successful immunization programs, the positive predictive value of the IgM assay is significantly decreased and false-positive results can be problematic. As such, for sporadic cases (i.e. single suspected cases) having no travel history to an area with known endemic disease, nor having an epidemiologic link to a laboratory confirmed case, additional laboratory testing such as paired IgG serology or virus detection should be considered for the laboratory confirmation of such cases. An additional complication for mumps, where outbreaks in previously immunized individuals having waning immunity have been shown to occur, the usefulness of mumps IgM serology is further diminished since such cases may or may not elicit an IgM response upon mumps virus reinfection.

Another important consideration for IgM serology interpretation is the timing of the serum collection relative to the onset of rash. For measles cases, most cases have developed IgM antibodies by 3 days post-rash onset, whereas for rubella cases, the timing of IgM seroconversion is slightly longer at 5 days post-rash onset. Thus, negative IgM results in clinically suspected measles or rubella cases should be interpreted with caution for sera collected before 3 or 5 days, respectively, and retesting of sera collected after this time should be considered prior to ruling out the measles or rubella case. In practice, such collection of second samples at a later time is often logistically difficult. Testing using alternative methods (i.e. molecular assays) of samples collected at first contact with the patient is therefore beneficial.

3.1.2. IgG Serology

Commercial assays are widely available for the detection of measles, mumps, and rubella IgG antibodies. IgG serology can be used for two purposes: (1) to aid in the diagnosis of an acute infection; and (2) to assess the immune status of an individual with respect to measles, mumps, or rubella.

3.1.2.1. IgG for the Confirmation of an Acute Infection

For measles, mumps, and rubella, the qualitative demonstration of a seroconversion between an IgG negative early ("acute") serum and an IgG positive late ("convalescent") serum is confirmatory for an acute infection in the absence of recent MMR vaccination. When both the acute and convalescent sera are positive, a relative significant (greater than or equal to fourfold) rise in titer is also indicative of an acute infection. Although many commercial assays give quantitative results for the IgG, we have found

that performing serial dilutions of both the acute and convalescent sera in parallel is a useful approach for determining the relative difference in titers.

Timing of the collection of sera is important relative to onset of disease for the interpretation of the IgG results. The acute and convalescent sera should be collected at least 10–14 days apart. In addition, late collection of the first serum sample may affect the ability to demonstrate a greater than or equal to fourfold rise in titer.

For rubella, the detection of persistent rubella IgG in an infant, beyond approximately 6 months at which time maternally acquired antibodies usually wane, can be used for the laboratory confirmation of congenitally acquired rubella.

3.1.2.2. Immunity Screening

Although the focus of this chapter is on the diagnosis of acute measles, mumps, and rubella infections, immunity screening will only briefly be mentioned here. It should be recognized that the commercial measles, mumps, and rubella IgG assays detect anti-measles, mumps, and rubella IgG antibodies, which may or may not reflect protective immunity. Thus, the detection of virus-specific neutralizing antibodies would be ideal for assessing immunity status. However, such gold-standard assays are not easily implemented in a routine clinical virology laboratory for high throughput immunity screening. Refer to (4–6) for published reference protocols for measles, mumps and rubella antibody testing, respectively.

3.1.2.3. Measles SSPE

Measles SSPE is a consequence of a rare persistent measles virus infection in the central nervous system resulting in late neurological complications. Although this is a measles virus brain infection, detection of measles virus RNA in the cerebral spinal fluid (CSF) is not typically possible. However, a relative increase in the level of measles-specific antibody in the CSF compared with the levels in the serum can be used to provide some further evidence in addition to the extensive clinical symptoms used to diagnose SSPE. There is a commercial assay available for measles-specific antibody detection in the CSF and serum.

3.1.2.4. Rubella IgG Avidity Assay

The risk of an infant acquiring CRS is dependent both on the timing when the rubella infection occurs during the pregnancy, and whether or not it is a primary infection (7, 8). When a primary rubella infection occurs within the first 11 weeks of pregnancy there is approximately 90% risk of CRS. However, rubella virus infection after 20 weeks gestation has a minimal risk of the infant developing CRS. For asymptomatic rubella virus reinfection, there was an 8% risk of fetal infection in first 16 weeks of pregnancy, but malformations were rare. In regions where rubella prevalence is low, the risk of a false-positive IgM result is important to consider.

Thus confirmatory testing of IgM positive results in pregnant women is imperative considering the important clinical management implications.

Rubella IgG avidity is a useful assay that allows for the differentiation between primary infections (high CRS risk) and past exposure/reinfections (minimal CRS risk) for the investigation of rubella infections in pregnant women (9). Briefly, there is a weaker antigen–antibody interaction (low avidity) immediately following primary infection or first exposure to an antigen, but a stronger antigen–antibody interaction (high avidity) for a more distant past infection. There are previously published methods (10–12) as well as commercial rubella IgG avidity assays available and the performance characteristics of some of these have been compared (12). The timing of sera collection relative to rubella virus exposure or onset of symptoms is important for the interpretation of the rubella IgG avidity results since the avidity maturation occurs over the first few months following infection.

3.2. Virus Detection

Virus detection either through virus isolation or direct detection of the viral RNA by reverse transcription (RT) polymerase chain reaction (PCR) is confirmatory for acute measles, mumps, or rubella infections. The detection of rubella virus in urine, nasopharyngeal or throat swab samples collected during first few months of life is confirmatory for congenitally acquired rubella.

3.2.1. Specimen Collection and Processing

Suitable samples for measles virus isolation or detection are nasopharyngeal or throat specimens or urine collected as soon as possible after onset (<4–5 days postonset). Similarly, the ideal specimens for rubella virus isolation or detection are nasopharyngeal or throat swab specimens collected as soon as possible after onset. The preferred samples for mumps virus isolation or detection are buccal or saliva swabs collected within the first 3–5 days of parotitis or symptom onset.

A minimum volume of 50 mL urine samples are processed by centrifuging at 2,500 × *g* for 15 min at 4°C within 48 h of collection. The sediment is then resuspended in 2 mL of viral transport media (VTM).

Nasopharyngeal, throat, buccal, and saliva swab samples are placed in VTM for a minimum of 1 h to elute the virus. The sample can then be transferred to an appropriate cryovial for transport and storage.

All specimens should be kept at 4°C and shipped to the laboratory on wet ice within 24 h for arrival within 48 h. Beyond that, all specimens should be frozen at −70°C or lower and shipped on dry ice to maintain viral integrity.

3.2.2. Virus Isolation

Measles, mumps and rubella viruses can be isolated in cell culture as described in detail elsewhere (13–15). The isolation of measles,

mumps, and rubella viruses is being increasingly replaced by direct molecular detection of these viruses as a more practical and timely diagnostic approach. However, obtaining representative isolates of these viruses is very important for virus strain characterization purposes.

3.2.3. Measles RT-PCR

The measles RT-PCR method described here has been adapted from the real-time PCR method published by Hummel et al. (3). Processed clinical samples are extracted (200 µl) using Qiagen's QIAamp Viral RNA kit. Automated extraction (200 µl) can be done with a MagNA Pure LC 2.0 system (Roche) using a MPLC Total Nucleic Acid High Performance Isolation kit (Roche). Real-time one-step RT-PCR is performed for both the measles nucleoprotein (N) and hemagglutinin (H) genes in singleplex reactions using 2 µl extracted nucleic acid and Qiagen's QuantiTect Probe RT-PCR kit (10 µl 2× master mix and 0.2 µl of RT enzyme mix per 20 µl reaction). All primers are used at a final concentration of 0.3 µM and probes at 0.25 µM (3). The primer and probe sequences are given in Table 1. All oligos are manufactured by TIB MOLBIOL (http://www.tib-molbiol.com/). The assays are run on either a LightCycler 2.0 (capillary) or 480 (96 well plate) instrument (Roche). The cycling parameters are provided in Table 2 and are the same for both instruments.

After amplification is complete, curves are generated using the default second derivative method of the LightCycler 2.0 or 480 software (Roche). The presence of a curve consistent with amplification denotes a positive sample.

3.2.4. Mumps RT-PCR

The mumps RT-PCR protocol described here is an adaptation of the real-time PCR method published by Uchida et al. (16). Processed clinical samples are extracted (200 µl) using Qiagen's

Table 1
Primer and probe sequences for the two measles real-time RT-PCR assays

Gene/target	Oligo name	Primer/probe	Oligo sequence (5′ to 3′)	Reference
N gene	MVN1139f	F primer	TGGCATCTGAACTCGGTATCAC	(3)
	MVN1213r	R primer	TGTCCTCAGTAGTATGCATTGCAA	
	MVN1163p	Probe	FAM-CCGAGGATGCAAGGCTTGTTT CAGA-BBQ[a]	
H gene	MVH126f	F primer	GTTTGTCATGTTTCTGAGCTTGATC	(3)
	MVH193r	R primer	TGGCTGCCCGATGAAGTC	
	MVH154p	Probe	FAM-TTGCTAGCCATTGCAGGC ATT-BBQ[a]	

[a] *BBQ* Blackberry non-fluorescent quencher

Table 2
Thermocycling conditions for the measles H and N assays on either a LightCycler 2.0 or 480

Step	Temperature	Time	Cycles
Reverse transcription	50°C	20 min	1
Activation	95°C	15 min	1
Amplification	95°C	1 s	40
	60°C[a]	1 min	
Cooling	40°C	30 s	1

[a]Fluorescence data acquisition occurs at this step

QIAamp Viral RNA kit. Automated extraction (200 µl) can be done with a MagNA Pure LC 2.0 system (Roche) using a MPLC Total Nucleic Acid High Performance Isolation kit (Roche). Real-time one-step RT-PCR is performed for the mumps fusion (F) gene in a 20 µl reaction with Qiagen's QuantiTect Probe RT-PCR reagents (10 µl 2× master mix and 0.2 µl of RT enzyme mix), 0.3 µM each of primers Mps5618f and Mps5696r (16), 0.1 µM probe Mps5644p (16) and 2 µl extracted nucleic acid. All oligos are manufactured by TIB MOLBIOL (http://www.tib-molbiol.com/) and their sequences are provided in Table 3. The assays are run on either a LightCycler 2.0 (capillary) or 480 (96-well plate) instrument (Roche). The cycling parameters are the same as the conditions for the measles real-time RT-PCR listed in Table 2 and are the same for both instruments.

After amplification is complete, curves are generated using the default second derivative method of the LightCycler 2.0 or 480 software (Roche). The presence of a curve consistent with amplification denotes a positive sample.

3.2.5. Rubella RT-PCR

Total nucleic acid is extracted from 200 µl of clinical sample using the QIAamp Viral RNA Mini Kit (Qiagen). Automated extraction (200 µl) can be done with a MagNA Pure LC 2.0 system (Roche) using a MPLC Total Nucleic Acid High Performance Isolation kit (Roche). One-step RT-PCR for rubella virus is performed in a 50 µl reaction with 0.2 µM dNTP mix, 10 µl 5× Qiagen OneStep RT-PCR Buffer, 2 µl Qiagen OneStep RT-PCR Enzyme Mix, 0.6 µM each of primers 2E1 (5′-CCCCACCGACACCGTGAT GAG-3′) and 3E1 (5′-GGCGAACACGCTCATCACGGT), 0.4 units of Protector RNase Inhibitor (Roche) and 5 µl of extracted

Table 3
Primer and probe sequences for the mumps real-time RT-PCR assay

Gene/target	Oligo name	Primer/probe	Oligo sequence (5′ to 3′)	Reference
F gene	Mps5618f	F primer	TCTCACCCATAGCAGGGAGTTATAT	(16)
	Mps5696r	R primer	GTTAGACTTCGACAGTTTGCAACAA	
	Mps5644p	Probe	FAM-AGGCGATTTGTAGCACTGGAT GGAACA-BBQ[a]	

[a] *BBQ* Blackberry non-fluorescent quencher

nucleic acid. The reaction is then placed in a thermal cycler set to the following parameters: reverse transcription at 50°C for 30 min, PCR activation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s and extension at 72°C for 1 min. This is followed by an incubation at 72°C for 10 min for final extension and finally, cooling and storage at 4°C. Subsequently, 5 µl of the RT-PCR reactions are analyzed by agarose (1.5%) gel electrophoresis using ethidium bromide detection for the presence of the 321 base pair amplicon.

3.2.6. Virus Genotyping

Measles, mumps, and rubella virus genotyping is useful for molecular epidemiologic purposes such as differentiating vaccine and wild-type strains, linking cases, linking outbreaks, tracking importations, and documenting the elimination of a particular strain from a geographic area. Such molecular epidemiologic activities are most useful for regions where disease incidence has been reduced to low levels and close monitoring of the effectiveness of immunization programs is required.

It is important that approaches for measles, mumps, and rubella genotyping be standardized with respect to: (1) the genomic region for the analysis, (2) the reference strains, (3) the defining of new strains, and (4) a meaningful nomenclature of the strains to ensure both public health laboratory and epidemiology professionals can understand and interpret the molecular epidemiologic data. In this regard, standard genotyping approaches and nomenclature have been developed for measles (17, 18), mumps (19) and rubella (20, 21).

Although, genotyping information can be derived from sequencing of RT-PCR products amplified directly from clinical specimens, it is important that representative virus isolates are obtained for more complete characterization purposes. This is particularly useful when new virus strains are discovered.

4. Notes

The use of external reference standards are becoming increasingly recognized as an important quality control procedure in the clinical virology laboratory. Such standards contain a known titer (IU) of virus-specific antibody and are run in addition to the commercial kit controls to monitor the performance of the assay. Using rules such as those proposed by Westgard (http://www.westgard.com/), helps to monitor the intra-laboratory performance of the assay. By combining similar data with other laboratories using the same external reference standards and the same assay platform further improves the ability to monitor both intra- and inter-laboratory test performances.

In addition to the serologic and molecular methods described above, there has been considerable work to improve the logistics and practicality of testing for measles and rubella infections through alternative sampling methods and detection of both antibodies and viral RNA in oral fluids or dried blood spots (13). The decision to implement such approaches in a particular country should consider factors including but not limited to sampling logistics, specimen transport logistics, and structure of the laboratory network in that country.

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Chapter 12

Detection of High-Risk Mucosal Human Papillomavirus DNA in Human Specimens by a Novel and Sensitive Multiplex PCR Method Combined with DNA Microarray

Tarik Gheit and Massimo Tommasino

Abstract

Epidemiological and functional studies have clearly demonstrated that certain types of human papillomavirus (HPV) from the genus alpha of the HPV phylogenetic tree, referred to as high-risk (HR) types, are the etiological cause of cervical cancer. Several methods for HPV detection and typing have been developed, and their importance in clinical and epidemiological studies has been well demonstrated. However, comparative studies have shown that several assays have different sensitivities for the detection of specific HPV types, particularly in the case of multiple infections.

In this chapter, we describe a novel one-shot method for the detection and typing of 19 mucosal HR HPV types (types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82). The assay combines the advantages of the multiplex PCR methods, i.e., high sensitivity and the possibility to perform multiple amplifications in a single reaction, with an array primer extension (APEX) assay. The latter method offers the benefits of Sanger dideoxy sequencing with the high-throughput potential of the microarray. Initial studies have revealed that the assay is very sensitive in detecting multiple HPV infections.

Key words: Cervical cancer, High-Risk human papillomaviruses, Multiplex PCR, Arrayed primer extension, Multiple HPV infections

1. Introduction

1.1. Human Papillomaviruses and Their Associated Diseases

Papillomaviruses are small, double-stranded DNA viruses with an icosahedral capsid that infect the stratified epithelium of mucosal or cutaneous surfaces. They are widespread in nature and have been isolated in human and many animals, including cattle, rabbits, horses, elk, dogs, sheep, deer, and elephants as well as reptiles and birds.

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More than 90 HPV types have been isolated and fully sequenced, but several independent studies indicate that many more exist. An HPV phylogenetic tree has been designed based on the homologous nucleotide sequence of the major capsid protein L1. In this tree, the different HPV types are grouped in genera (1). The Alpha genus comprises approximately 30 HPV types that are preferentially detected in the female reproductive tract and several benign cutaneous HPV types, e.g., HPV2, that cause common skin warts.

The mucosal HPV types are divided into two groups: low-risk HPVs (e.g., types 6 and 11) that are mainly associated with benign genital warts and high-risk HPVs that are the etiological agents of cervical cancer (2). A recent meta-analysis indicates that 15 different HPVs, namely, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82, are associated with cervical cancer, while three additional HPV types of the same genus can be classified as probable high-risk types, i.e., 26, 53, and 66 (3). In addition, a Monograph of the International Agency for Research on Cancer has recently evaluated the carcinogeneity of different HPV types. This study showed that sufficient evidence was available for HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66 to be considered as class I carcinogens for humans (4). HPV16 and 18 are the most frequently found HPV types in cervical cancers worldwide, being detected in approximately 50% and 20% of cases, respectively (3, 5). In addition, HPV16 and to a much lower extent, HPV18 have a causal role for a subset of cancers of the anus (80%) and of the vulva, vagina, penis, and oropharynx (approximately 30% in all latter cases).

The mucosal high-risk HPV types preferentially infect the cervical transformation zone, which is the junction point of the endocervix columnar cells and the ectocervix stratified squamous epithelial cells. The transformation zone epithelium is subject to continuous changes during a woman's life, and the specific features of this region are believed to facilitate the viral infection. However, the majority of HPV infections do not lead to cytological anomalies or cancer, but they are cleared by the immune system in a relatively short time (6–12 months). A small percentage of infections persist and promote the development of low- and/or high-grade cervical intraepithelial neoplasia (CIN), which may regress or progress to an invasive cervical carcinoma after a period of latency (6). Additional risk factors appear to play a role in progression of HPV-induced disease, most likely influencing immune surveillance or acting as additional carcinogens. These include sexual habits, cigarette smoking, oral contraceptives, parity, and host genetic predisposition (7–12).

In addition to the mucosal high-risk HPV types, emerging lines of evidence indicate that another group of HPVs belonging to the genus beta may be involved in human carcinogenesis, i.e., nonmelanoma skin cancer (NMSC) (reviewed in (13)). These

were first isolated in skin cancer-prone patients suffering from a rare autosomal recessive genetic disorder called *Epidermodysplasia verruciformis* (EV), but it is now clear that they are very common in the skin of healthy individuals (13). Although it is well accepted that these HPVs are responsible for NMSC development in EV patients, their direct role in skin carcinogenesis in normal populations is still a matter of debate. It has been suggested that the cutaneous HPV types may promote the formation of malignant lesions acting as cocarcinogens together with UV that is considered the main risk factor in skin carcinogenesis (13).

1.2. Genomic Organization of HPV, Viral Gene Products and Life Cycle

The molecular cloning and sequencing of the HPV types revealed a genome of approximately 8,000 bp with a highly conserved organization. Three different regions have been identified: (1) a coding region containing the early genes, E1, E2, E4, E5, E6, and E7, (2) a region containing the late genes encoding the major (L1) and minor (L2) capsid proteins, and (3) a noncoding region, termed long control region (LCR), localized between open reading frames (ORF) L1 and E6 and containing most of the regulatory elements involved in viral DNA replication and transcription. The length of LCR can vary in different HPV genomes, ranging from 650 to 900 nucleotides.

The products of E6 and E7 are considered the main HPV oncoproteins, being able to associate with several cellular proteins and inactivate/alter their normal functions. HPV16 E6 and E7 are able to bind the products of tumor suppressor gene, p53 and retinoblastoma (pRb), respectively, leading to loss of control of cell cycle and apoptosis.

The HPV cycle is tightly linked to the differentiation program of stratified epithelia. HPV infects cells of the basal layer, where it is maintained at relatively low copy number. When cells leave the basal layer of the epithelium, HPV initiates the productive phase of the life cycle, characterized by vegetative viral DNA replication. During this phase, the HPV genome is amplified up to more than 1,000 copies per cell. Immediately after, the expression of late genes starts. Finally, viral particles are produced and released. In most high-grade premalignant and malignant lesions, HPV DNA is found integrated in the host genome. This event results in the disruption of several viral genes, with preservation of only E6 and E7 that are efficiently expressed.

1.3. Screening Methods for HPV-Associated Cervical Diseases

Cervical disease screening is currently based on the Papanicolaou (Pap) smear, a technique that was introduced in the 1920s (14). This screening method has been modified and improved over the years, and its profound impact on the reduction in the incidence of cervical disease in developed countries is now well documented (15). After the demonstration of the association between HPV and cervical cancer, many efforts have been made to develop

HPV DNA detection methods. Over the past few years, constant progress has been made on HPV typing based on polymerase chain reaction (PCR) methods. The majority of available protocols use degenerate and/or consensus primers, followed by an additional assay that allows the identification of specific HPV types (16–20). The most commonly used PCR assays amplify the L1, the E1 (21), or the E6/E7 regions (16, 22). The use of degenerate and/or consensus primers offers the advantage of detecting a large spectrum of HPV types by a single PCR. However, they may be less efficient in detecting specific HPVs leading to some under detection, particularly in cases of multiple infections (23–25).

In this chapter, we describe the development of a novel, reliable E7 PCR-based assay to detect a large spectrum of high-risk HPV types. The assay combines the advantages of the multiplex PCR methods, i.e., high sensitivity and the possibility to perform multiple amplifications in a single reaction, with an array primer extension (APEX) assay. The latter method offers the benefits of Sanger dideoxy sequencing with the high-throughput potential of the microarray (26–28).

Initial studies have revealed that the assay is very sensitive in detecting multiple HPV infections. This feature is most likely due to the use of HPV type-specific primers.

2. Materials

2.1. Isolation of DNA from Cervical Specimens in Paraffin Blocks, Fixative Solution (ThinPrep®, Cytyc), Frozen Physiological Solution (PBS)

2.1.1. Equipment

1. Vortexer.
2. Microcentrifuge.
3. Qiagen BioRobot EZ1 (Qiagen).
4. EZ1 DNA tissue card (Qiagen).
5. Microtome.
6. Heating block.

2.1.2. Materials

1. 1.5-mL microcentrifuge tubes.
2. Filter pipette tips.
3. Scalpel.

2.1.3. Reagents

All reagents used should be of molecular biology grade.

1. Ethanol (96–100%).
2. Proteinase K (20 mg/mL). The enzyme is supplied in 50 mM Tris–HCl (pH 8.0), 5 mM $CaCl_2$, and 50% (v/v) glycerol (Fermentas).

3. Triton X-100.
4. QIAamp DNA mini kit (Qiagen).
5. Tween 20.
6. 10 mM Tris–HCl, pH 7.4.
7. Xylen.
8. EZ1 DNA tissue kit (Qiagen).
9. DNA cleaner (Diagnostics Medical International).

2.2. Multiplex PCR Amplification

2.2.1. Equipment

1. Electrophoresis apparatus, including gel trays and combs.
2. Power supply.
3. PCR thermocycler.
4. Vortexer.

2.2.2. Materials

1. 0.2 mL microcentrifuge tubes or 96-well plates, e.g., ABgene® PCR plates (Thermo scientific).
2. Filter pipette tips.
3. Erlenmeyer flask.

2.2.3. Reagents

All reagents used should be of molecular biology grade

1. UltraPure™ Agarose gels (Invitrogen) (see Note 1).
2. Size marker: MassRuler™ DNA ladder mix (100–10,000 bp) (Fermentas).
3. 6× DNA loading dye (Fermentas).
4. Sterile MilliQ water.
5. QIAGEN Multiplex PCR kit (Qiagen).
6. Primers should be purchased desalted or purified, for example, using HPLC, and dissolved in TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA) to obtain a 100 µM stock solution (MWG Biotech) and stored at –20°C (Table 1) (see Note 2).
7. dUTP (Fermentas).
8. HPV genome clones as standard reference (see Note 3).
9. Ethidium bromide (10 mg/mL).
10. DNA cleaner (Diagnostics Medical International).
11. 1× TAE (Eppendorf).

2.3. APEX Assay

2.3.1. Equipment

1. Vortexer.
2. Genorama-003 four-color detector (Asper Biotech).
3. Genorama image analysis software (Asper Biotech).
4. Hybridization block, e.g., Hybaid OmniSlide Thermal Cycler System (Hybaid).

Table 1
Sequences of forward and reverse HPV-type specific primers for the multiplex PCR and sizes of the PCR fragments (see Note 4)

HPV type	Primer sequence[a] (see Note 5)	PCR fragment size (bp)
16	F. 5′-TTATGAGCAATTAAATGACAGCTCAG-3′ R. 5′-TGAGAACAGATGGGGCACACAAT-3′	215
18	F. 5′-GACCTTCTATGTCACGAGCAATTA-3′ R. 5′-TGCACACCACGGACACACAAAG-3′	236
26	F. 5′-CGAAATTGACCTACGCTGCTACG-3′ R. 5′-TGGCACACCAAGGACACGTCTTC-3′	239
31	F. 5′-AGCAATTACCCGACAGCTCAGAT-3′ R. 5′-GTAGAACAGTTGGGGCACACGA-3′	210
33	F. 5′-ACTGACCTAYACTGCTATGAGCAA-3′ R. 5′-TGTGCACAGSTAGGGCACACAAT-3′	229
35	F. 5′-CAACTGACCTATACTGTTATGAGC-3′ R. 5′-TGTGAACAGCCGGGGCACACTA-3′	234
39	F. 5′-TTGTATGTCACGAGCAATTAGGAG-3′ R. 5′-GACACTGTGTCGCCTGTTTGTTTA-3′	357
45	F. 5′-GACCTGTTGTGTTACGAGCAATTA-3′ R. 5′-TGCACACCACGGACACACAAAG-3′	236
51	F. 5′-GCTACGAGCAATTTGACAGCTCAG-3′ R. 5′-ATCGCCGTTGCTAGTTGTTCGCA-3′	242
52	F. 5′-ACTGACCTAYACTGCTATGAGCAA-3′ R. 5′-CAGCCGGGGCACACAACTTGTAA-3′	229
53	F. 5′-ACCTGCAATGCCATGAGCAATTGAA-3′ R. 5′-TTATCGCCTTGTTGCGCAGAGG-3′	253
56	F. 5′-ACCTACARTGCAATGAGCAATTGG-3′ R. 5′-TGATGCGCAGAGTGGGCACGTTA-3′	244
58	F. 5′-GCTATGAGCAATTATGTGACAGCT-3′ R. 5′-TGTGCACAGSTAGGGCACACAAT-3′	219
59	F. 5′-ACCTTGTGTGCTACGAGCAATTAC-3′ R. 5′-GCTGCACACAAAGGACACACAAA-3′	243
66	F. 5′-ACCTACARTGCAATGAGCAATTGG-3′ R. 5′-TGATGCGCAGAGTGGGCACGTTA-3′	244
68	F. 5′-TTGTATGTCACGAGCAATTAGGAG-3′ R. 5′-GATTACTGGGTTTCCGTTGCACAC-3′	258

(continued)

Table 1 (continued)

HPV type	Primer sequence[a] (see Note 5)	PCR fragment size (bp)
70	F. 5′-CACGAGCAATTAGAAGATTCAGACA-3′ R. 5′-TTCCCGATGCACACCAGGGACA-3′	237
73	F. 5′-CTTACATGTTACGAGTCATTGGAC-3′ R. 5′-GTTTCTGGAACAGTTGGGGCAC-3′	221
82	F. 5′-GCTACGAGCAATTTGACAGCTCAG-3′ R. 5′-CATTGCCGATGTTAGTTGGTCGCA-3′	240
β-Globin	F. 5′-CCAGAAGAGCCAAGGACAGGTAC-3′ R. 5′-CCACCAACTTCATCCACGTTCACC -3′	274

[a]*F* forward, *R* reverse

2.3.2. Materials

1. Chip with the probes for 19 different HPV types and β-Globin (Table 2) (see Note 6).
2. Pipette tips.
3. Glass coverslips.
4. Microcon Y30 column (Millipore).
5. 0.2-mL and 1-mL microcentrifuge tubes.

2.3.3. Reagents

1. Shrimp alkaline phosphatase (Amersham Biosciences).
2. HK-UNG Thermolabile uracyl-*N*-glycosylase (Epicentre).
3. Cyanine 5-ddUTP (Perkin-Elmer).
4. Thermo Sequenase (Amersham Biosciences).
5. SlowFade® Light Antifade Reagent (Molecular Probes).
6. 100 mM NaOH solution.

3. Methods

3.1. DNA Extraction from Cervical Specimens

Different methods have been used in our laboratory according to the type of specimen.

3.1.1. DNA Isolation from Cervical Scrapes in PBS (Method 1)

1. Vigorously shake the cell suspension.
2. Transfer 120 µl of the cell suspension to a new 1.5-mL microcentrifuge tube.

Table 2
Sequences of each of the oligonucleotides spotted on the chip

HPV type	Primer sequence
16	1. 5′-ACACACGTAGACATTCGTACTTTGGAAGACC-3′ 2. 5′-AAGCAGAACCGGACAGAGCCCATTAC AATA-3′
18	1. 5′-GTAGAAAGCTCAGCAGACGACCTTCG AGCA-3′ 2. 5′-GCCGAACCACAACGTCACACAATGTTG TGTA-3′
26	1. 5′-ATTGGACTATGAACAATTTGACAGCTCAGA-3′ 2. 5′-TTGAAGCACAATGTTGTATGTGTAATAGT ATAG-3′
31	1. 5′-GTGTACAGAGCACACAAGTAGATATTCGCA-3′ 2. 5′-ACCGGACACATCCAATTACAATATCGTTA CCTT-3′
33	1. 5′-AGTTCGTTTATGTGTCAACAGTACAGCAAG-3′ 2. 5′-GCCACAGCTGATTACTACATTGTAACCTGT-3′
35	1. 5′-ACACACATTGACATACGTAAATTGGAAGAT-3′ 2. 5′-AGACACCTCCAATTATAATATTGTAACGTCC-3′
39	1. 5′-GCAGCTGGTAGTAGAAGCCTCACGGGATAC-3′ 2. 5′-GATGAACCACAGCGTCACACAATACAGTGT-3′
45	1. 5′-GTAGAGAGCTCGGCAGAGGACCTTAGAA CAC-3′ 2. 5′-CCGAACCACAGCGTCACAAAATTTTGTGTG-3′
51	1. 5′-AACTGGCAGTGGAAAGCAGTGGAGACACCC-3′ 2. 5′-GACAGGCTACGTGTTACAGAATTGAAGCT CCG-3′
52	1. 5′-CATTCATAGCACTGCGACGGACCTTCGTAC-3′ 2. 5′-AGCCACAAGCAATTACTACATTGTGACATA-3′
53	1. 5′-ATTGAAACACAGTGTTGTAGGTGTGAGTCGT TGG-3′ 2. 5′-TGTGAGTCGTTGGTGCAGTTGGCTGTTCAG AGT-3′
56	1. 5′-CATTCAGAGTACCAAAGAGGACCTGCGTGT-3′ 2.5′-CACGTACCTTGTTGTGAGTGTAAGTTTGTGG-3′
58	1. 5′-CACCACGGTTCGTTTGTGTATCAACAG-3′ 2. 5′-GACAGCTCAGACGAGGATGAAATAGGCT-3′

(continued)

Table 2 (continued)

HPV type	Primer sequence
59	1. 5′-CAGCTAGTAGTAGAAACCTCGCAAGACGGA-3′ 2. 5′-ACCTGACTCCGACTCCGAGAATGAAAAAGA-3′
66	1. 5′-CATTCAGAGTACCAAAGAGGAGCTACGTG TGG-3′ 2. 5′-ACAACATAAGTGTTACCTAATTCACGTACC-3′
68	1. 5′-ACTACTAGCCAGACGGGACGAACAACAGCG-3′ 2. 5′-AACCCGACCATGCAGTTAATCACCACCAACA-3′
70	1. 5′-ACAAAATACAGTGTATGTGTTGTAAGTGTAA TAC-3′ 2. 5′-TGCACTTAGTAGTAGAAGCCTCACAAGAGA ACC-3′
73	1. 5′-CACGAAGTGTCAGTGCACAGTATGCCTTGC CAT-3′ 2. 5′-GACAAGCTGAACGAGAGTGTTACAGAATAGT TAC-3′
82	1. 5′-CTCGCAGTGGAAAGCAGTGGAGACAGCCTTC GCA-3′ 2. 5′-TTCAGCAAATGTTACTGGGCGACCTAAGCC TGG-3′
β-Globin	1. 5′-ATCACTTAGACCTCACCCTGTGGAGCCATA CCC-3′ 2. 5′-TCCTGAGGAGAAGTCTGCCGTTACTGCCC-3′

3. Treat the suspension by addition of 40 µl of proteinase K solution (200 µg/mL proteinase K in 3% Triton X-100).
4. Inactivate the proteinase K at 95°C for 10 min.
5. Take 10 µl aliquots for PCR purposes (see Note 7).

3.1.2. DNA Isolation from Cervical Scrapes in PBS (Method 2)

1. Vigorously shake the cell suspension.
2. Transfer 200 µl of cell suspension to a new 1.5-mL microcentrifuge tube.
3. Extract the DNA by using the QIAamp DNA mini kit according to the manufacturer's instructions.

4. Elute the DNA in 0.2 mL of elution buffer provided in the kit (10 mM Tris–HCl, 0.5 mM EDTA) and store at –20°C until further use.
5. Use 10 µl of DNA template per PCR reaction (see Note 7).

3.1.3. DNA Extraction from Cervical Specimens in PreserveCyt™ Solution (Automatic Procedure)

We perform DNA extraction from the cervical scrapings by using the Qiagen BioRobot EZ1 with the EZ1 DNA tissue kit and EZ1 DNA tissue card according to the manufacturer's instructions (see Note 8).

1. Transfer 1 mL of cervical cells to a 1.5-mL microcentrifuge tube.
2. Centrifuge at 6,000 rpm for 10 min.
3. Carefully remove the supernatant.
4. Add 200 µl of a mixture containing 10 µl proteinase K solution and 190 µl buffer G2 provided in the Qiagen kit.
5. Resuspend by pipetting the pellet.
6. Incubate at 56°C for 3 h.
7. Transfer the mixture in a sample tube provided in the kit and follow the manufacturer's instructions.
8. Elute the DNA in 50 µl of elution buffer and store at –20°C until further use.
9. Use 10 µl of DNA template per PCR reaction (see Note 7).

3.1.4. DNA Extraction from Formalin-Fixed and Paraffin-Embedded Biopsies (Method 1) (See Note 9)

1. Cut one section of 10 µm from each paraffin-embedded tissue block and collect them in 1.5-ml microcentrifuge tubes (see Note 10).
2. Add 1 mL of xylene to the tissue section and mix by inversion for 5 min at room temperature.
3. Centrifuge at maximum speed (12,000–14,000 rpm) for 5 min at room temperature.
4. Discard the supernatant.
5. Add 800 µl of xilen and mix by inversion for 5 min at room temperature.
6. Add 400 µl of absolute ethanol, and then mix by inversion for 5 min.
7. Centrifuge at maximum speed (12,000–14,000 rpm) for 5 min at room temperature.
8. Discard the supernatant.
9. Add 1 ml of absolute ethanol, and then mix by inversion for 5 min.
10. Centrifuge in a bench centrifuge at maximum speed for 2 min.

11. Remove the ethanol phase.
12. Briefly keep the tube upside-down on filter paper to remove any ethanol residue.
13. Dry the tissue pellet at 55°C for 20 min in a heating block.
14. Add 250 µl of digestion mixture: 10 mM Tris–HCl pH 7.4, proteinase K 0.5 mg/ml, and Tween 20 0.4% (see Notes 8 and 11).
15. Incubate at 56°C overnight with continuous mixing.
16. Centrifuge the digestion mix for 2 min at maximum speed and collect the supernatant in a new 1.5-mL microcentrifuge tube.
17. Incubate at 95°C for 10 min to inactivate the Proteinase K.
18. Store at –20°C; the DNA is ready for the PCR reaction.
19. Use 10 µl of DNA template per PCR reaction (see Note 7).

3.1.5. DNA Extraction from Formalin-Fixed and Paraffin-Embedded Biopsies (Method 2) (See Note 8)

1. Cut three sections of 10 µm from each paraffin-embedded tissue block and collect them in 1.5-ml microcentrifuge tubes (see Note 10).
2. Incubate the paraffin tissue sections in digestion buffer (10 mM Tris–HCl pH 7.4, proteinase K 0.5 mg/ml and Tween 20 0.4%) overnight at 56°C with continuous mixing (see Notes 8 and 11).
3. Keep the tubes at 95°C for 10 min to inactivate the proteinase K.
4. Rapidly centrifuge the tubes for 2 min in a bench centrifuge at 13,000 rpm to separate the paraffin (*top*) and aqueous phases (*bottom*).
5. Place the tubes on ice for a few minutes.
6. Using a clean tip, break the top solidified paraffin layer and transfer the aqueous phase to a new tube.
7. Store at –20°C; the DNA is ready for the PCR reaction.
8. Use 10 µl of DNA template per PCR reaction (see Note 7).

3.1.6. DNA Extraction from Frozen Tissue

The DNA is extracted from fresh biopsies after storage at –80°C.

1. Cut the tissue into very small pieces and place into 1.5-mL tube (see Note 12).
2. Use the QIAamp DNA mini kit to purify the DNA from frozen tissue according to the manufacturer's instructions.
3. Elute the DNA in 0.2 mL of elution buffer provided in the kit (10 mM Tris.Cl, 0.5 mM EDTA) and store at –20°C until further use.
4. Use 1–2 µl or 100 ng of DNA per PCR reaction (see Note 7).

3.2. HPV Typing with Multiplex PCR/APEX Method

3.2.1. Multiplex PCR Conditions (*See Note 13*)

3.2.1.1. Preparation of 10× Primer Mix (Containing Each Primer at 2 μM)

1. Thaw the 33 primers (100 μM stock) for HPV and β-globin gene amplification.
2. Take 10 μl of each primer at 100 μM (100 pmol/μl) and bring the final volume to 500 μl with TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA).
3. Store at −20°C in small aliquots to avoid repeated freezing and thawing.

3.2.1.2. Multiplex PCR Amplification

1. Perform the multiplex PCRs with 25 μl of 2× QIAGEN Multiplex PCR Master Mix containing HotStar Taq® DNA polymerase and Multiplex PCR Buffer, which contains 6 mM $MgCl_2$ and dNTP mix.
2. Add 5 μl of 10× primer mix (final concentration of 0.2 μM).
3. Add 0.5 μl of dUTP 5 mM (final concentration of 50 μM) (see Note 14).
4. Add purified DNA and RNase-free water (negative control) to bring the final volume of the mixture to 50 μl (see Note 15).
5. Denature at 95°C for 15 min and amplify through 45 cycles of 30 s at 94°C, 3 min at 63°C, and 1.30 min at 72°C. Add a final elongation step of 10 min at 72°C.
6. The presence and the sizes of PCR products can be checked on 2% agarose gels (see Notes 1 and 16).

3.2.2. APEX Assay (*See Fig. 1*)

The layout of the chip is shown in Fig. 2 (see Note 17)

1. Purify and concentrate the PCR products with the Millipore Y30 columns.
2. Dilute the PCR products by filling up the column with 300 μl milliQ water.
3. Centrifuge at 12,000 rcf for 14 min.
4. Discard the filtrate and refill the column with 500 μl milliQ water to insure that the PCR products are well cleaned.

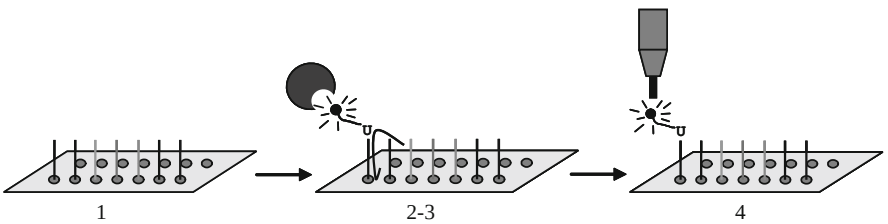

Fig. 1. Schematic representation of arrayed primer extension (APEX) used for HPV typing. (1) HPV type-specific oligonucleotides arrayed onto silanized slides (2) Hybridize the multiplex PCR product with the arrayed oligonucleotides (3) Extend oligonucleotide with labelled cyanine 5-ddUTP (4) Read with Genorama-003 four-color detector: the HPV types are defined by the position of the signals on the chip.

N	HPV39_1	HPV68_1	N
A	HPV39_2	HPV68_2	C
HPV59_1	HPV18_1	HPV45_1	HPV51_1
HPV59_2	HPV18_2	HPV45_2	HPV51_2
HPV16_1	HPV35_1	HPV31_1	HPV33_1
HPV16_2	HPV35_2	HPV31_2	HPV33_2
HPV58_1	HPV52_1	HPV56_1	HPV66_1
HPV58_2	HPV52_2	HPV56_2	HPV66_2
HPV73_1	HPV82_1	HPV26_1	HPV70_1
HPV73_2	HPV82_2	HPV26_2	HPV70_2
G	HPV53_1	BGlo_1	U
N	HPV53_2	BGlo_2	N

Fig. 2. Layout of the APEX chip. The oligonucleotides printed on the array are indicated by their names in the *boxes*. Their sequences are given in Table 2. Each oligonucleotide has been spotted in duplicate. The four corners contain a mixture of four self-elongating marker oligonucleotides that give signals in all dideoxy nucleotide channels (named *N*) and one self-elongation marker oligonucleotide that gives a signal only in one dideoxy nucleotide channels (*A*, *G*, *C*, *U*.).

5. Centrifuge at 12,000 rcf for 14 min.
6. Invert the column in a clean 1.5-mL microcentrifuge tube and discard the tube containing the filtrate.
7. To elute the DNA, carefully apply 30 µl of milliQ water to the membrane of the column.
8. Centrifuge at 1,000 rcf for 3 min.
9. Transfer 15 µl of the eluate to a new 0.2-mL microcentrifuge tube (see Note 18).
10. Perform the fragmentation by adding 1 µl HK™-UNG Thermolabile uracyl-*N*-glycosylase (1 U) and 2 µl UNG Dilution Buffer to the eluate (see Note 14).
11. In the same reaction mixture, add 1 µl Shrimp Alkaline Phosphatase (1 U) to simultaneously inactivate the unincorporated dNTPs.
12. Incubate the mixture at 37°C for 1.5 h and at 95°C for 30 min in a thermocycler and immediately cool it on ice.
13. Put the chip in a 50-mL falcon tube containing 100 mM NaOH for denaturation.

14. Pipette 9 µl of the eluate in a 0.2-mL microcentrifuge tube.
15. Add a reaction mixture containing 0.95 µl of cyanine 5-ddUTP (50 µM), 4 µl of Reaction buffer, 0.875 µl of Dilution buffer, 0.125 of Thermo Sequenase (4 U) and complete with 2.85 µl of milliQ sterile water.
16. Homogenize by pipetting up and down.
17. Wash the chip three times in water at 95°C and remove it slowly from the falcon to avoid drops of water on the surface. Then, put the chip on a hybridization block previously warmed at 58°C.
18. Put the mixture onto the chip with a coverslip on the top and incubate for 10 min. After hybridization of the PCR products to the chip, the extension reaction is performed to allow incorporation of cyanine 5-ddUTP.
19. Wash the slides in water at 95°C to remove the traces of nonhybridized PCR products and cyanine 5-ddUTP not incorporated.
20. Add a droplet of SlowFade® Light Antifade Reagent (Molecular Probes) to limit the bleaching of fluorescein and cover carefully with a coverslip.
21. Analyze the slides with a Genorama-003 four-color detector equipped with Genorama image analysis software.

Fluorescence intensities are measured according to the Genorama image analysis. Consider the signal for specific HPV types positive only if both APEX probes give a signal in the cyanine-5 channel (see Note 19).

4. Notes

1. Two percent agarose gel is prepared following a standard protocol. Briefly, two grams of agarose powder are mixed with 100 mL of 1× TAE buffer (Tris–Acetate 40 mM pH 8.3, EDTA 1 mM) in a 250-mL Erlenmeyer flask. Agarose is melted by boiling the solution for a few minutes. While the agarose solution is cooling, prepare the gel tray with the appropriate comb. After the agarose solution has reached an approximate temperature of 60°C, add 5 µl of Ethidium Bromide (EtBr) and swirl to mix. Pour the gel in the tray, avoiding air bubbles; when they do appear, remove them with a pipette tip. Let the gel solidify before use.
2. Store in small aliquots at –20°C to avoid repeated freezing and thawing.

3. Take precautionary measures in handling this material to avoid risk of HPV DNA contamination. Do not handle the HPV constructs in laboratories where the solutions for the PCR are prepared (pre-PCR room) or where the DNA extracted from human specimens is added to the PCR mix. To avoid the formation of aerosols, briefly centrifuge the tube before carefully opening the lid.
4. Primers were designed taking into consideration the following HPV genome sequences reported in the GenBank with the following accession numbers: K02718 (HPV-16), X05015 (HPV-18), X74472 (HPV-26), J04353 (HPV-31), M12732 (HPV-33), M74117 (HPV-35), M62849 (HPV-39), X74479 (HPV-45), NC_001533 (HPV-51), NC_001592 (HPV-52), X74482 (HPV-53), NC_001594 (HPV-56), NC_001443 (HPV-58), NC_001635 (HPV-59), NC_001695 (HPV-66), Y14591 (HPV-68), U21941 (HPV-70), X94165 (HPV-73), and AB027021 (HPV-82).
5. Due to the homology in the E7 gene of different HPV types, the following primers have identical sequences: HPV18R/HPV45R, HPV33F/HPV52F, HPV33R/HPV58R, HPV 39F/HPV68F, HPV51F/HPV82F, HPV56F/HPV66F, HPV56R/HPV66R.
6. We designed two five-prime C-6 amino-linker-modified oligonucleotides covering two 30-bp regions of each E7 gene (*For probe sequence see* Table 2) (MWG Biotech). The oligonucleotides were spotted onto silanized slides by Asperbio (Tartu), as reported elsewhere (29, 30).
7. The quality of the DNA extracted from human specimens can be determined by amplifying a fragment of the β-globin gene using Hot Start PCR, performed according to standard protocols.
8. To monitor the possible occurrence of cross-contamination between the different specimens during DNA extraction, include tubes containing buffer only (one tube with buffer for every five specimens).
9. Methods 1 and 2 are very similar. The only difference is the use of xylene in Method 1 to remove the paraffin. Xylene is toxic and in absence of adequate equipment, e.g., aspirating hood, only Method 2 should be used.
10. To prevent the risk of cross-contamination between different specimens during cutting, change the blades frequently and wash the blades and microtome extensively with DNA cleaner. In addition, to monitor possible cross-contamination, cut empty paraffin blocks every ten specimens and process the cut section in the same way as the specimen sections.

Tubes containing only paraffin sections should be blindly processed/analyzed together with specimen sections.

11. Due to the high density and viscosity of Tween 20, cut the filter tip with a sterile scalpel to facilitate the withdrawal of the liquid.
12. Do not use more than 25 mg.
13. Respect all the conditions of pre- and post-PCR separation. During the procedure, sterile tubes, filter pipette tips, and gloves must be used. Use a workstation equipped with UV light.
14. The incorporation of dUTP will lead to the reduction in size by fragmentation of the PCR products after the uracyl-*N*-glycosylase treatment. This will allow a better hybridization of the PCR products with the arrayed oligonucleotides.
15. As optional step, after HPV DNA transformation with standard protocols, a bacterium colony could be picked with a sterile tip and directly resuspended in the PCR amplification mixture. This sample represents a positive control. The use of a bacterium colony, instead of purified HPV DNA, will avoid unnecessary manipulation of a large amount of HPV DNA and will reduce the risk of PCR contaminations.
16. Add 5 μl of template DNA to 2 μl 6× loading buffer and 3 μl MilliQ water per sample. The solution is mixed by pipetting up and down several times. Load the samples on the gel. Load 5 μl of the size marker. This step is optional. The PCR product can be directly processed for the APEX assay.
17. Two 5′-C-6 amino-linker-modified oligonucleotides (C-6 oligonucleotides) covering two 30-bp regions of each HPV E7-region gene were designed within polymorphic sequences. In order to have all immobilized C-6 oligonucleotides labeled with only one dye-labeled dideoxynucleoside triphosphate terminator after the primer extension, we designed each oligonucleotide in a E7 region immediately adjacent to a T at the 3′ end. In this way, after hybridization of the PCR products on the chip, only uracyl (cyanine 5-ddUTP) is incorporated during the sequence-specific extension reaction of the 3′ ends of the oligonucleotides. Thus, the analysis of the chip can be performed on very simple instruments equipped with only one laser.
18. Continue the procedure even if the volume of the eluate is smaller than 15 μl.
19. If one sample reacts with one probe only, a HPV type-Specific PCR should be performed to sequence the amplicon to report a potential new variant.

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Chapter 13

The Detection of Parvoviruses

Sean Doyle

Abstract

Parvovirus B19 is a single-stranded DNA virus which causes severe disease in immunocompromised patients and foetal loss in pregnant women. It is classified as an *Erythrovirus* and this genus also comprises two related viral genotypes (so-called LaLi/A6 (genotype 2) and V9 (genotype 3)) which appear to be immunologically indistinguishable from Parvovirus B19. Serological and nucleic acid test (NAT) systems to detect Parvovirus B19-mediated infection are commercially available; however, some NAT systems are genotype-specific. International standard preparations of Parvovirus B19 IgG and DNA have been produced for assay standardisation purposes, and to ensure consistency of assay manufacture and performance. Immunological assays, such as B-cell ELISpot, T-cell stimulation, and cytokine detection can also be used to confirm exposure to Parvovirus B19. Immunohistochemical techniques, employing commercially available monoclonal antibodies, are used to localise the virus in infected tissue and Parvovirus B19 viral antigen can also be detected in serum and plasma using antigen-specific ELISA. NAT systems have also been described to detect newly identified parvoviruses such as human bocavirus (HBoV), PARV4, and PARV5, although absolute confirmation of clinical diseases associated with these agents is required. This chapter describes the current status of detection systems for all the aforementioned parvoviruses, with particular emphasis on *Erythrovirus* detection by serological, NAT, and immunological approaches.

Key words: Parvovirus, Erythrovirus, ELISA, B-cell ELISpot, Cytokines, NAT testing, Human bocavirus, Recombinant protein

1. Introduction

Human parvovirus B19 (B19) is a non-enveloped, 5.5 kb single-stranded DNA virus (1). It belongs to the family *Parvoviridae*, sub-family *Parvovirinae*, genus *Erythrovirus*. Although initially identified as related genotypes of B19, so-called genotypes 2 (K71/LaLi/A6) and 3 (known as V9) have been reclassified as human parvovirus B19-A6, -LaLi, and -V9, respectively (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_parvo.htm).

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However, for clarity and familiarity, occasionally erythrovirus will be referred to as B19, genotype 2, and genotype 3 (or V9), respectively, throughout this chapter. These strains are immunologically indistinguishable from B19, although they exhibit up to 10–12% sequence divergence at the DNA level (2–4). B19 is a human pathogen, and infection is primarily a concern in both immunocompromised patients (e.g. cancer patients undergoing chemotherapy) and pregnant women, where B19 infection in the first two trimesters of pregnancy can lead to foetal loss (5). Emerging roles for B19 in the pathogenesis of certain kidney diseases and myocardial damage are also emerging (6–9).

B19 transmission may be by respiratory droplets, vertical transmission of mother to foetus or via the receipt of B19-contaminated blood or blood products (e.g. packed red cells, plasma, or platelets) (10). Upon infection with B19, high levels of virus appear in the circulatory system within days to weeks and may exceed 10^{13} viral particles/mL, followed by the appearance of virus-specific IgM and ultimately high-titre IgG (11). B19 is composed of two distinct viral proteins, namely VP1 (83 kDa) and VP2 (58 kDa), which form the 20 nm diameter viral capsid (1). These proteins are co-linearly encoded by the viral genome and are present in the viral capsid in the ratio 5:95 (VP1:VP2) (12). The recombinant expression of B19 VP1 and VP2 capsid proteins in the baculovirus expression system and their application in immunodiagnostic assays, to detect B19 exposure, have been extensively described (12, 13).

B19 DNA detection by PCR is now routinely used to complement patient serology profile and viral DNA can be detected in serum, plasma, tissue extracts, and amniotic fluid (14). The latter has proven to be particularly useful for diagnosing materno-foetal B19 transmission (15). However, B19 viraemia may not be associated with symptoms of infection, so in a blood donor context, an infected person could potentially donate blood within this time window, with obvious implications for blood or blood product recipient exposure to the virus. As a consequence, many blood collection organisations and blood product manufacturers have implemented screening protocols to detect B19 DNA by PCR using minipool screening (i.e. making pools of aliquots of blood serum/plasma and screening same for B19 DNA) (16). Minipool, as opposed to individual specimen screening, is performed to reduce the cost associated with detection of B19 presence. However, despite the high sensitivity of detection associated with B19 detection via PCR, the associated cost, allied to (a) the potential high levels of viraemia leading to cross-contamination during screening and (b) the possibility of false negativity of detection during PCR due to sequence differences between genotypes 1 and 3, means that alternative strategies need to be consider for blood screening (17).

Although a haemagglutination assay has been proposed to detect B19 in plasma (18, 19), this system is apparently too insensitive to detect clinically relevant virus levels, moreover, B19 IgG presence in plasma may lead to virus occlusion and resultant false negativity of detection (2, 17). Consequently, two groups have proposed the use of an ELISA system, utilising anti-B19 IgG to detect the virus in human serum and plasma (17, 20). In addition, Corcoran et al. (17) have demonstrated that viral antigen detection can be enhanced by the use of a specialised diluent which significantly improves virus capture by the IgG[anti-B19] used in their immunoassay system. However, although both assays appear to work satisfactorily, the sensitivity of viral detection is limited to greater than 10^7 particles/mL. Thus, the potential to use emerging technologies (21) to improve the sensitivity of detection of B19 in plasma and serum merits consideration.

In order to standardise B19 serological (IgG) and molecular (DNA) diagnostic test systems and to aid B19 IgG and DNA assay performance, a number of WHO International Standards are available to both assay manufacturers and diagnostic laboratories (22, 23).

A number of parvoviruses, distantly related to the erythrovirus genus have been recently identified. Human bocavirus (HBoV), discovered in 2005, is classified as a member of the family *Parvoviridae* (sub-family *Parvovirinae*, genus *Bocavirus*). Four HBoV proteins are likely to be encoded by the compact viral genome, namely NS1, NP-1, VP1, and VP2 with the latter two proteins co-linearly encoded as is the case for the *Erythrovirus* genus (24). HBoV infection appears to be strongly associated with acute respiratory tract infections (ARTI) with preliminary evidence emerging that the virus may also be a potential cause of gastroenteritis (25). Molecular and serological tests have been developed which allow the sensitive and specific detection of HBoV nucleic acid (26, 27) and HBoV IgG and/or IgM (28–30). Indeed, 17 PCR protocols for HBoV DNA detection, deploying a range of primer pairs and detection strategies, have recently been described (26). HBoV recombinant VP2 protein has been generated by two groups utilising the baculovirus expression system (29, 30), while another group has expressed both HBoV VP1 and VP2 proteins in *E. coli* (28). At present, no cell culture systems for HBoV or virus-specific monoclonal antibody preparations are available to enable direct viral detection in vitro, although high-titre rabbit polyclonal antibodies have been produced which can immunoprecipitate HBoV virions from respiratory specimens (29).

A distinct parvovirus, termed PARV4 (parvovirus 4), was identified in plasma obtained from an intravenous drug user in 2005 (31). This virus has been shown to contaminate approximately 5% of plasma pools, with PARV4 viral loads less than

10^6/mL (31). PARV4 is presently unclassified but is likely to form part of a new genus within the sub-family, *Parvovirinae*. In addition, a related viral genotype, termed either PARV4 genotype 2 or PARV5 has also been identified (32). Although yet to be associated with any specific disease, the potential parenteral transmission route of PARV4 has led to intensive investigations and it appears that tissue persistence of PARV4 is associated with parenteral exposure. Tissue persistence was initially thought to be associated with immunosuppression or HIV/HCV co-infection; however, PARV4 has also been detected, at a low rate, in liver and bone marrow specimens from HIV- and HCV-negative individuals (33). No serological assay systems are presently available for PARV Ig detection.

This chapter will describe detailed serological, cellular, and molecular assay systems for B19 detection and introduce some of the nascent molecular, and serological assays for HBoV and PARV4 detection.

2. Materials

1. WHO Parvovirus B19 IgG International Standard (01/602), contains 77 International units (IU) of B19 IgG per ampoule (NIBSC, Potters Bar, Herts, UK). http://www.nibsc.ac.uk. No B19 IgM standard is currently available.
2. WHO Parvovirus B19 DNA International Standard (99/800), contains 5×10^5 IU of B19 DNA per vial (NIBSC, Potters Bar, Herts, UK). http://www.nibsc.ac.uk.
3. WHO Parvovirus B19 DNA working Reagent (99/736), contains 1,000 IU of B19 DNA per mL (NIBSC, Potters Bar, Herts, UK). http://www.nibsc.ac.uk.
4. Histopaque-1077 (Sigma-Aldrich, Dorset, UK).
5. Nitrocellulose-lined plates (96 well) (Millipore, Bedford, MA, USA).
6. *Staphylococcus aureus* cells (Cowan I strain) SAC (Calbiochem, Darmstadt, Germany).
7. Interleukin-2 (IL-2) (Serotec Ltd, Oxford, UK).
8. RPMI medium and foetal calf serum (http://www.invitrogen.com).
9. [^{3}H]-Thymidine (GE Healthcare, Amersham, UK).
10. Liquid scintillation fluid (GE Healthcare, Amersham, UK.).
11. Streptavidin-conjugated alkaline phosphatase (Sigma-Aldrich, Dorset, UK).

12. Tetramethylbenzidine substrate (BioFX Laboratories Inc., Owings Mills, Maryland).
13. Proteinase K (Sigma-Aldrich, Dorset, UK).
14. Qiagen QiAmp Blood kit (Qiagen, Hilden, Germany).
15. Ethidium bromide (Sigma-Aldrich, Dorset, UK).
16. Polyclonal rabbit IgG [anti-parvovirus B19 VP2] (Code: B-0091) (DakoCytomation, Glostrup, Denmark).
17. *Taq* polymerase and dNTP preparations (Promega, WI, USA).
18. Betaine (Sigma-Aldrich, Dorset, UK).

3. Methods

3.1. Detection of B19 IgG and IgM

Detection of B19 IgG and/or IgM is now routinely carried out either by commercially available ELISA, Western blot, or immunofluorescent in vitro diagnostic tests (http://www.biotrin.com/; http://www.ibl-america.com; http://www.mikrogen.de). Indeed, application of these validated and reliable test systems has removed much, if not all, of the variability associated with B19 antibody testing which was evident in the early 1990s. Two FDA-approved immunoassays are available to detect B19 IgM as a marker of recent infection (34), and IgG as a marker of past infection (35), respectively. These microplate immunoassays utilise B19-capsid VP2 and can detect genotype 1, 2, and 3 IgG (4, 36–39).

All commercial B19 IgG detection systems capture B19-specific IgG onto an immobilised, recombinant B19 protein. After B19 IgG binding, removal of unwanted serum proteins and irrelevant IgG is achieved by a wash step. B19-specific IgG is then detected using either an enzyme- (generally horseradish peroxidase) or fluorophore-labelled anti-human IgG antibody (Fig. 1). For microplate ELISA systems 3,3′,5,5′-tetramethylbenzidine (TMB) is now the substrate of choice. Precipitating substrates such as 3,3′-diaminobenzidine (DAB) are used in Western blot assay systems. Detection of B19 IgM by ELISA systems from most commercial manufacturers is also enabled by the aforementioned assay format; however, IgG or rheumatoid factor (RF) removal is essential to avoid assay interference. IgM-capture (μ-capture) technology is utilised in the FDA-approved B19 IgM assay system which appears to confer high assay specificity and avoids the requirement to use additional reagents such as anti-human IgG or RF adsorbents (34).

Thus, accurate laboratory serodiagnosis of recent B19 infection or past exposure relies on testing serum or plasma specimens

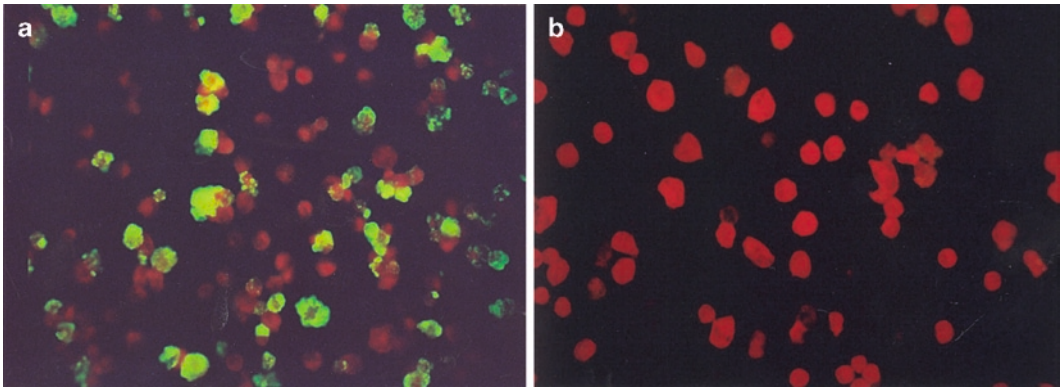

Fig. 1. Immunofluorescent detection of parvovirus B19 IgG using B19 VP1 recombinant baculovirus-infected *Spodoptera frugiperda* cell line (Sf_9). (**a**) B19 IgG reactivity with B19 VP1 protein is detected using FITC-labelled murine anti-human IgG (intense *green* fluorescence against a background of *red* counterstained Sf_9 cells). (**b**) No reactivity observed with B19 IgG seronegative specimen. Courtesy of Biotrin (Dublin, Ireland).

for either specific antibody reactivity against viral capsid proteins, VP2 or VP1, expressed in eukaryotic expression systems (e.g. baculovirus expression system) by ELISA. A specific advantage of the eukaryotic baculovirus expression system is its ability to enable the post-translational protein-folding necessary for the generation of soluble VP2 capsids (12). Unlike B19 VP2, VP1 does not appear to form soluble capsid structures; however, VP1 has been produced as a "conformationally intact" protein which has been shown to retain conformational epitopes present in the native virion (12, 13) (Fig. 1). Co-expression of VP1 and VP2 in eukaryotic expression systems has been proposed to result in the formation of empty capsids, which are antigenically indistinguishable from native B19 virions.

Exposure to B19 in immunocompetent individuals results in the appearance of high-titre B19 IgM. The appearance of B19 IgG then coincides with a diminishing IgM antibody response, and B19 IgG reactivity against conformational epitopes of VP1 and VP2 persists post-infection. However, for both capsid proteins, reactivity against linear epitopes disappears abruptly against VP2, but more slowly against VP1 (13, 40–42) – an observation which has significant consequences for diagnosis. Antibody reactivity against linear VP2 epitopes, predominantly directed against a heptapeptide (amino acids 344–350; KYVTGIN) identified by analysis of acute-phase sera (40), usually disappears within 6 months of B19 infection (41). This heptapeptide sequence is also present in V9 (genotype 3). Thus, detection of B19-specific IgG, directed against linear epitopes of VP2, may assist in timing B19 exposure to within a 6-month period. Although the antibody response wanes against linear epitopes on B19 capsid proteins it persists against conformational epitopes of both capsid proteins. Indeed, this observation has been extended to a so-called second

generation epitope type-specific (ETS) EIA which determines the ratio of B19 IgG reactivity against capsid VP2 and aforementioned heptapeptide (an ETS index). According to the authors, a cut-off value of 10 will distinguish between acute infection (ETS index ≤10) or past infection (ETS index ≥10) (43).

The V9 VP1 and VP2 open reading frames have been cloned and expressed in the baculovirus expression system (37). These authors observed self-assembly of V9 VP1/VP2 and VP2 capsids into parvovirus-like particles (diameter approximately 23 nm). A panel of 270 clinical specimens were screened for the presence of V9 IgM and IgG antibodies in ELISA and showed 100% serologic cross-reactivity between B19 and V9 when comparing V9 VP2 capsids to the B19 VP2 ELISA. Thus, V9 and B19 antibody responses may be diagnosed equally well by ELISA using either V9 or B19 recombinant capsids as antigen source (37). Moreover, Heegaard et al. noted that most genetic variation between genotypes 1 and 3 (Table 1) resulted in silent mutations resulting in 96–97% identity at the protein sequence level.

Controversial data was presented in 2004 which suggested that enzyme immunoassays utilising B19 VP2 capsids derived from genotype 1 did not detect a subset of V9-derived IgG (36). This preliminary finding was based on the use of an unvalidated in-house IgG ELISA which used a V9 VP1 protein as a diagnostic antigen. Candotti et al. (36) also noted that the small numbers of amino acid substitutions in VP1u and NS1 regions of genotype 3 strain, compared to genotype 1, did not modify

Table 1
Parvovirus nomenclature

Name	Genotype	Additional name
Parvovirus B19-Au	1	Parvovirus B19
Parvovirus B19-A6	2	K71, LaLi, A6
Parvovirus B19-V9	3	V9
Human Bocavirus	–	HBoV
PARV4	–	–
PARV5	–	PARV4, genotype 2

Parvoviruses belong to the family *Parvoviridae* (sub-family *Parvovirinae*, genus *Erythrovirus*). Genotypes of B19 (genotype 2 (K71/LaLi/A6) and 3 (V9) are now classified as human parvovirus B19-A6, -LaLi, and -V9, respectively (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_parvo.htm). Parvovirus B19 has been officially classified as B19 virus but this nomenclature has failed to gain widespread use. A number of companies manufacture in vitro diagnostic tests, based on B19 capsid proteins, which detect human antibodies produced upon infection with each of the parvovirus B19 genotypes (1–3). HBoV falls within the genus *Bocavirus* and classification of PARV4 and 5 is outstanding

protein hydrophilicity or antigenicity. This again underlines the fundamental identity and indistinguishable immunological nature of both B19 and V9-derived proteins. The findings of Candotti et al. relating to proposed differences between B19 and V9 IgG detection were disputed (38) and work was completed to resolve this significant issue (39). Resultant data, from analysis of blinded specimens using microplates coated with various combinations of parvovirus antigens (again blinded), confirmed that B19 VP2 capsids can indeed detect all V9-derived IgG, a finding which confirms the diagnostic utility of this validated immunoassay system for all B19 IgG detection (35). Moreover, the findings of Parsyan et al. (39) made it clear that the use of the VP2 capsid facilitates optimal antibody detection and that the additional presence of the VP1 protein did not improve diagnostic accuracy. In separate studies, all three erythrovirus genotypes were shown to both haemagglutinate human red cells and infect myeloid cells (KU812Ep6 or UT7/EpoS1 cells) with equal efficiency leading to the conclusion that the three virus genotypes belong to the same species – parvovirus B19 (4). Moreover, utilisation of erythrovirus genotype 1 capsids enabled detection of IgG from genotype 2-infected individuals ($n = 25$) and erythrovirus genotype 2 capsids detected IgG from genotype 1-infected individuals ($n = 24$) (4).

Thus, it can be concluded that present data support the utility of B19 immunoassay systems to efficiently detect both genotype 2 and genotype 3 IgM and IgG, respectively.

3.2. B-Cell Memory

In the absence of robust cell culture methods to detect B19 in test specimens, alternative techniques can be used to both confirm B19 IgG detection and immune status. One such technique is determination of the B19-specific memory B-cell population in the patient and a memory B-cell ELISpot technique has been developed for this application (44–46) (Fig. 2).

3.2.1. Isolation of PBMC and Stimulation of Cells

Isolate peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation as follows:

1. Pipette Histopaque-1077 (20 mL) into each of three sterile 50 mL conical centrifuge tubes.
2. After collecting 60 mL blood in heparinised tubes, slowly layer 20 mL blood on top of each Histopaque layer. Maintain centrifuge tubes at a 45° angle and allow specimens to run down side of tube until it fills to the 40 mL mark.
3. Centrifuge specimens at $1{,}000 \times g$ for exactly 30 min at room temperature (25°C), then carefully remove the tubes from the centrifuge so that the gradient is not disturbed.
4. Using a sterile pipette, slowly aspirate the upper layer (plasma) to within 0.5–1 cm from the opaque interface containing the PBMC.

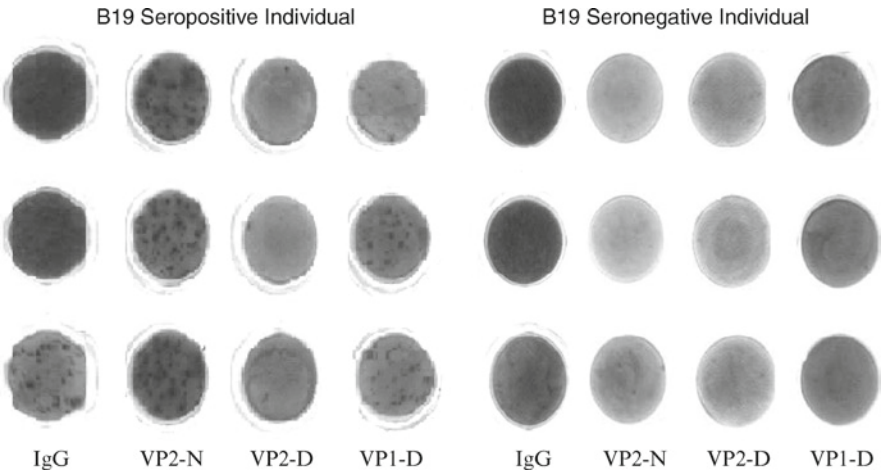

Fig. 2. B-cell ELISpot using PBMC from a B19 seropositive and seronegative blood donor. Plasma from the seropositive donor was strongly reactive against conformational epitopes of capsid VP2 (VP2-N) and linear epitopes of VP1 (VP1-D). No memory B-cells were detectable against linear epitopes of VP2 (VP2-D). Distinct ELISpot wells were coated with anti-human IgG as a positive control to verify B-cell stimulation. All incubations with PBMC at 6×10^6 cells/mL (in triplicate).

5. With a sterile transfer pipette, carefully transfer the opaque interface (Buffy layer) to a fresh 50 mL conical tube, avoiding carryover of the lower clear layer (Histopaque). The interface (containing PBMC) from all three tubes can be pooled into one 50 mL conical tube for washing.
6. Wash PBMC with RPMI medium at room temperature and mix gently by inversion. Following centrifugation at $250 \times g$ at room temperature for exactly 10 min, remove the supernatant and discard (leaving small amount of liquid at bottom). Using a pipette to aid in dispersion, gently resuspend the lymphocyte pellets in complete RPMI (12 mL). (Complete RPMI is prepared made up with 500 mL RPMI 1640 Medium, 8%(v/v) heat-inactivated FCS, 100 U/mL penicillin, 100 U/mL streptomycin, 2 mM l-glutamine, and 50 μM 2-mercaptoethanol.)
7. Conduct a cell count using a haemocytometer with approximately 10 μL of cell suspension and using a 10× microscope lens. A good yield is about 2.4×10^6 cells/mL (representing 1 million cells/mL whole blood).
8. Culture cells using a minimum of 5×10^6 cells/mL in 2 mL per well complete RPMI in 24-well tissue culture plates.
9. Culture PBMC (5×10^6 cells/mL) for 5 days in complete RPMI in the presence of heat-killed *Staphylococcus aureus* cells (Cowan I strain) (SAC) diluted 1:5,000 and 10 ng/mL interleukin-2 (IL-2). SAC and IL-2 jointly function to induce generalised antibody production in resting memory B cells (46).

10. Coat nitrocellulose-lined plates (96 well) with either native B19 VP2 (10 μg/mL), denatured VP2 (10 μg/mL), denatured VP1 (10 μg/mL), or rabbit anti-human IgG (10 μg/mL) in 50 mM sodium carbonate buffer pH 9.6, overnight at 4°C.
11. Wash ELISpot plates with sterile phosphate-buffered saline (PBS) and block with sterile 20%(v/v) FCS in RPMI medium.
12. Add PBMC (post-stimulation) at concentrations of 1×10^6, 1×10^5, and 1×10^4 cells/well to the plates and incubate in complete RPMI for 18 h at 37°C (100 μL per well).
13. Wash ELISpot plates with PBS (4 × 200 μL per well) to remove unbound cells.
14. Detect memory B cells (secreted IgG) by incubation with biotinylated rabbit anti-human IgG (1 μg/mL in (1% (w/v) BSA in PBS)), for 1 h at room temperature, followed by PBS washing (4 × 200 μL per well).
15. Add streptavidin-conjugated alkaline phosphatase at a dilution of 1/1,000 (1% (w/v) BSA in PBS) and incubate for 1 h at room temperature, followed by PBS washing (4 × 200 μL per well).
16. Addition of 5-bromo-4-chloro-3-indolyl phosphate/(nitro blue tetrazolium (BCIP/NBT) substrate (100 μL per well) facilitates memory B cell detection as spots. This development step proceeds for 15 min after which spots can be counted under light microscopy (Fig. 2).
17. The number of spots are averaged from triplicate wells and resultant data reported as the mean number of spot-forming cells (SFC)/million starting cells (44).

3.3. B19 T-Cell Proliferation Assays and Cytokine Detection

1. Isolate peripheral blood mononuclear cells (PBMC) from individuals by density gradient centrifugation as described in Subheading 3.2.1.
2. Culture PBMC (2×10^6 cells/mL) in triplicate with purified recombinant VP1 (10 μg/mL) or VP2 (10 μg/mL) for 72 h (previously 0.2 μm filtered). Optional: parallel cultures can be set up for (a) proliferation assays and (b) cytokine analysis from cell culture supernatants.
3. Cells cultured with medium alone or with a combination of phytohemagglutinin (PHA; 2 μg/mL) serve as negative and positive controls, respectively.
4. Add 0.5–1 μCi of [^{3}H]-Thymidine for the final 4 h of culture, to facilitate labelling of cells, before harvesting onto glass fibre filters using automatic cell harvesting.
5. Liquid scintillation counting (LCS) is carried out using 5 mL LCS fluid per glass fibre filter. Background values for negative

control samples are typically between 200 and 600 cpm, and always less than 1,500 cpm. Results are expressed as stimulation indices (S.I.) representing the proliferative response for test samples divided by the response obtained from the negative controls.

6. IL-2, IL-10, IL-13, IFN-γ, and other cytokines can, in parallel cell culture supernatants, be assayed by commercially available ELISA with pg/mL sensitivity of detection or IFN-γ ELISpot (35, 47–50).

3.4. Nucleic Acid Testing

The main technique used for B19 detection is PCR and many groups have described both qualitative and quantitative PCR strategies for detection of individual, or all three, B19 genotypes (51–54). Apart from total genotype detection, the balance between PCR assay sensitivity and specificity to facilitate detection of acute infection only is important (2).

3.4.1. Specimen Preparation and B19 DNA Amplification (See Note 1)

1. Obtain stock proteinase K and dilute to 20 mg/mL, or prepare stock proteinase K (20 mg/mL) by resuspending in either 25 mM Tris–HCl or sterile high purity water.
2. Obtain clinical specimens (ideally serum or plasma) and add 2 µL proteinase K (20 mg/mL) per 100 µL, mix by gentle vortexing and incubate at 56°C for 1 h. Specimens can be pre-diluted (10^{-4}–10^{-10}) in phosphate-buffered saline (PBS) prior to proteinase K digestion to facilitate semi-quantitative B19 DNA titre evaluation.
3. After incubation, boil all treated specimens at 100°C for 5 min to inactivate proteinase K and centrifuge (13,000 × *g* for 30 min at 4°C) to remove precipitated protein. It should be noted that biological specimens (plasma, amniotic fluid or soft tissue) can also be extracted using the Qiagen QiAmp Blood kit, or similar DNA extraction kits, as described by the relevant manufacturer.
4. Add template DNA (5 µL), present in each specimen supernatant, to the following mixture: 10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1%(v/v) Triton X-100, 2.0 mM $MgCl_2$, 200 µM of each dNTP, 1 M betaine, 1.0 µM of each oligonucleotide primer (Table 2 (54)), specific for regions within the NS1 coding region of the B19 genome, and 1.25 U of *Taq* polymerase in a total volume of 50 µL.
5. PCR amplification uses the following programme: 95°C for 6 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min terminating in 72°C for 5 min.
6. 10 µL of each PCR product is then subjected to 1% (w/v) agarose gel electrophoresis containing 0.5 µg/mL of ethidium bromide for 30 min at 100 V. Amplicon (202 bp) visualisation

Table 2
PCR primers for high sensitivity, single-round amplification of parvovirus B19 (53, 54)

Primers	Gene	Position	Sequence 5′–3′
F1	NS1	1399–1422	AATACACTGTGGTTTTATGGGCCG
R1	NS1	1600–1576	CTAAAATGGCTTTTGCAGCTTCTAC

These primers do not efficiently amplify either genotype 2 or 3 due to base-pair mismatch

can be performed using an Eagle-Eye II gel documentation system (Stratagene, CA, USA) or similar instrument.

7. This assay can detect at least 200 IU B19 DNA (4×10^4 IU/mL) (23) and sensitivity can be further improved by use of PCR-ELISA detection (54).

3.4.2. Quantitative Detection of Genotypes 1–3

At present, two commercial quantitative B19 PCR assays are available, namely the Artus Parvo B19 PC Kit (http://www.qiagen.com) and the Lightcycler® Parvovirus B19 Quantification Kit (http://molecular.roche.com). According to the manufacturers, both systems operate at high sensitivity and specificity of detection and the Artus Parvo B19 PC Kit is capable of detecting all three genotypes of parvovirus B19. However, at least one non-commercial, quantitative B19 PCR assay has been extensively validated for detection of B19 DNA (51) and further evaluated and optimised to detect all three genotypes (52). Details of these non-commercial assays, which amplify a 113-bp fragment in the VP1 region, are presented herewith:

1. Primers: 5′-GACAGTTATCTGACCACCCCCA-3′ (forward) and 5′-GCTAACTTGCCCAGGCTTGT-3′ (reverse).
2. Target specific probe (5′-FAM (6-carboxyfluorescein)-labelled and 3′-TAMRA (6-carboxytetramethylrhodamine) (quencher)): 5′-CCAGTAGCAGTCATGCAGAACCTAGAGGAGA-3′.
3. PCR conditions: 7 mM $MgCl_2$; 200 µM dATP, dCTP and dGTP; 400 µM dUTP; 300 nM each primer; 100 nM probe; 0.01 U Amperase and 0.025 U AmpliTaq Gold DNA polymerase.
4. Template, 10 µL extracted DNA, resulting in a total volume of 50 µL.
5. Amplification: 50°C for 2 min, followed by 95°C for 10 min and subsequently 45 cycles at 95°C for 15 s and annealing and extension at 58°C for 1 min (51).

   An internal control probe can also be used (51)

   or

Amplification: 95°C for 15 min and subsequently 45 cycles at 95°C for 15 s and annealing and extension at 60°C for 1 min (52). These authors further showed that reduction of annealing/extension temperature to 56°C contributed to an improvement in the detection of genotype 3 isolates.

6. Amplification and detection can be carried out using the ABI Prism 7700 Sequence Detection System (51) or Lightcycler® instrument (52). In addition, Baylis et al. (52) modified the original B19 PCR assay to function with Sybr Green I (60°C (using a commercial Lightcycler Faststart DNA Master^{PLUS} SYBR green I kit) and a Lightcycler instrument and found concordance between genotype detection using the above primers.

3.5. Detection of B19 Viral Antigen

A limited number of reports have appeared in the literature which describe cell culture conditions that facilitate B19 replication and the ability of B19 IgG to diminish virus infectivity (55–57). Although excellent research methodologies, to date, these methods have not gained widespread applicability for B19 antigen detection in routine diagnostic laboratories. Consequently, two alternative technologies have been proposed to detect B19 antigen in human serum and plasma, namely (a) B19 antigen detection immunoassays (17, 20) and (b) a red blood cell agglutination assay termed receptor-mediated haemagglutination assay (RHA) (18, 19). Since the latter system (i.e. RHA) occasionally fails to detect B19 antigen in the presence of B19 IgG, thereby producing false negativity, it will not be further discussed.

However, B19 antigen assays have been shown to be easy to perform and, in addition, one assay is capable of detecting all three genotypes of human parvovirus B19. Moreover, combined B19 antigen and IgM detection in clinical specimens by a B19 antigen assay and a B19 IgM assay facilitated the identification of 91% of acute B19 infections in a test population (17). This B19 antigen detection strategy is presented in Subheading 3.5.1.

3.5.1. B19 Antigen ELISA

1. Dilute test plasma and control specimens (1 in 5) in a low pH proprietary commercial diluent (Biotrin International Ltd., Dublin, Ireland) and add to IgG [anti-B19 VP2] sensitised microwells (100 µL/well) for 1 h.
2. Following a wash step (4× PBST), rabbit IgG [anti-B19 VP2]-horseradish peroxidase conjugate is incubated in the wells for 30 min.
3. Following a further wash step (4× PBST), add TMB substrate (100 µL/well) to the microwells for 30 min.
4. Terminate the reaction using 1 N sulphuric acid (100 µL/well) and measure absorbance at 450/630 nm.

5. The presence of B19 antigen in a specimen is determined by the absorbance ratio of specimen sample to cut-off calibrator specimen (Index value). Specimens which yield index values ≥1.0 are classed positive while those ≤1.0 are deemed negative.

3.6. Immunohistochemical Detection of B19

Immunohistochemistry is perhaps the least frequently used technique to detect parvovirus B19 as it requires extensive optimisation. It is also dependent on many factors such as tissue type or source, previous tissue treatment, available instrumentation and most significantly, the quality of anti-parvovirus B19 antibody utilised for virus detection. Consequently, the procedure presented below reflects the strategies deployed by numerous authors to detect parvovirus B19 in paraffin-embedded tissue sections using immunohistochemical techniques (58–64).

1. Paraffin-embedded tissue sections can be dewaxed as follows: Immerse in xylene (60°C, 10 min, twice) followed by 5 min sequential immersions, two per treatment, in absolute ethanol, 95%(v/v) ethanol and distilled water (30 s) at room temperature.
2. Block endogenous peroxidase activity by immersion of slides in 3% (v/v) hydrogen peroxide in distilled water, with gentle stirring for 30 min at room temperature.
3. Remove residual hydrogen peroxide by gentle rinsing in distilled water.
4. To facilitate antigen retrieval, heat slides at 96°C for 30 min in 10 mM sodium citrate pH 6.0 and then cool to room temperature for 20 min.
5. Immerse in TBST (0.1 M Tris–HCl, 0.2 M NaCl, and 0.05% (v/v) Tween 20, pH 7.4), twice, and cover with blocking solution (1% (w/v) skim milk powder, 1% (w/v) BSA in TBS) and incubate in a humidified chamber for 1 h at 37°C.
6. Immerse slides in optimised polyclonal rabbit IgG [anti-B19] antibody solution (DAKO; 1/50–1/300) (58–60) and incubate in a humidified chamber for 16 h at 4°C.
7. Wash slides twice in TBS, for 5 min on each occasion and probe using the appropriate secondary antibody–enzyme conjugate and precipitating substrate (e.g. 3,3′-diaminobenzidine (DAB)) detection system.
8. Ensure appropriate using of negative control sera to ensure validity of observed results.

Other anti-parvovirus B19 antibody preparations have been used for immunohistochemistry and include: (a) mouse monoclonal IgG [anti-VP1/VP2] from Dade Behring, Marburg, Germany) (61), (b) mouse monoclonal IgG [anti-VP2] from Chemicon International, USA (62) and (c) parvovirus B19

primary antibody (code R92F6; NCL-Parvo, Novocastra, Newcastle upon Tyne, UK) (63).

3.7. HBoV and PARV4 Detection

3.7.1. HBoV PCR

Seventeen PCR protocols for HBoV DNA detection, deploying a range of primer pairs and detection strategies, have recently been reviewed and described (26). More recently, the relative sensitivities of conventional vs. quantitative PCR have been compared (27). These authors describe a primer set comprising: Fwd primer 5′-TGGCTACACGTCCTTTTGAACC-3′ and Rev primer 5′-GACTTCGTTATCTAGGGTTGCG-3′ using the following PCR reaction mixture and amplification conditions:

DNA extract (4 µL), 5 µL 10× PCR buffer, 400 µM of each dNTP, 1 µL Taq polymerase, primers (10 pM each) in nuclease-free water to 50 µL.

PCR: 95°C for 15 min, 35 cycles of amplification (94°C for 30 s; 55°C for 30 s and 72°C for 30 s) plus a final extension step at 72°C for 10 min.

Choi et al. (27) demonstrated that this PCR assay yielded a PCR product of 384 bp and was approximately two logs less sensitive than a real-time PCR assay described in the same publication, nonetheless, the aforementioned conventional HBoV PCR assay represents a useful starting point for initial forays in HBoV detection.

3.7.2. PARV4 PCR

A quantitative PCR assay utilising consensus primers to amplify a region of ORF2 of PARV4 and PARV5, which is highly conserved between the two genotypes, has been developed (31, 32).

Fwd: 5′-CTAAGGAAACTGTTGGTGATATTGCT-3′ located between nucleotides 3285 and 3310 of ORF2 (GenBank Accession Number AY622943).

Rev: 5′-GGCTCTCCTGCGGAATAAGC-3′ located between nucleotides 3368 and 3387 of ORF2.

These primers amplify a 103-bp product.

Probe5′-(FAM)TGTTCAACTTTCTCAGGTCCTACCGCCC (TAMRA)-3′) which hybridises to nucleotides 3313–3340 of ORF2.

Amplification reaction mixture: 1× QuantiTect Probe (Qiagen, Germany) PCR master mix, 10 pmol of each primer, 0.05 µM of probe, and 5 µL of template DNA. Final volume: 20 µL. Amplification conditions: 95°C for 15 min and then 45 cycles of 95°C for 15 s and 60°C for 1 min.

Extensive primer details and PCR conditions for PARV4 and 5 detection have also been presented elsewhere (64, 65).

3.8. Concluding Remarks

Standardised and validated ELISA and immunofluorescent detection systems are now commercially available to detect parvovirus B19 IgM and IgG resulting from exposure to all erythrovirus

genotypes. Validated molecular assays are also available to accurately detect viral DNA. In addition, WHO International Standards are now in place and enable inter-laboratory standardisation of B19 IgG and DNA detection. Furthermore, a battery of cellular, immunohistochemical and infectivity assays can facilitate ongoing research into B19 pathogenicity. Although numerous validated research assays have been developed to detect HBoV, PARV4, or 5 DNA or antibody responses (HBoV only), no commercial tests are yet available; however, it is clear that many of the lessons learned from B19 assay development will find resonance in future studies on, and detection systems for, these emerging pathogens.

4. Note

1. DNA extraction should always be performed in separate laboratory to that of PCR reagent preparation and nucleic acid amplification.

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Chapter 14

Detection and Characterization of Polioviruses

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Abstract

Poliovirus has been the subject of research for many virologists during the last 80 years. Research on the poliovirus biology has helped to understand the molecular basis of many biological processes, particularly RNA replication and virus–host cell interactions. Laboratory research has also been instrumental in the development of effective and safe vaccines against poliomyelitis and continues to be critical for the assessment of the quality of vaccines used in humans. The work carried out in diagnostic laboratories located in most countries of the world to isolate and characterize poliovirus from clinical samples is also essential to support the Global Polio Eradication Initiative, the most ambitious public health effort ever attempted by man. This chapter describes the most common techniques used in laboratories to isolate, identify, and characterize poliovirus isolates. They include cell culture isolation and typing techniques, virus purification methods, and molecular analyses of viral nucleic acids.

Key words: Poliovirus, Oral polio vaccine, Global Polio Eradication Initiative, AFP surveillance, CPE, Sucrose gradient, Infectious dose, VP1 capsid protein

1. Introduction

The earliest documentary evidence of disease caused by a virus infection is believed to be a funerary stele from 18th dynasty Egypt (1580–1350 BC). It shows a priest with the withered limb and dropped foot typical of paralytic poliomyelitis, the most serious and evident consequence of infection with poliovirus. However, clear evidence of poliomyelitis cases is scarce before the late 19th and early twentieth century when large epidemics occurred. As a response to the increasing number of cases, two vaccines of very high safety and efficacy were developed in the 1950s. In May 1988, and due to the early success of the live-attenuated vaccine version (as opposed to the "killed" inactivated polio vaccine), the World Health Assembly launched the Global

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Polio Eradication Initiative (GPEI) with an aim to eliminate poliomyelitis by the year 2000. The GPEI is the largest public health effort in history, and although it was not completed by the year 2000, very significant progress has been made during the last 20 years. The number of polio cases worldwide decreased drastically from an estimated 350,000 in 125 countries in 1988 to 483 cases in 10 countries in 2001. Key strategies for polio eradication have been the conduction of extensive vaccination campaigns with live-attenuated oral polio vaccine (OPV) and the implementation of high-quality surveillance for cases of acute flaccid paralysis, (AFP) which is the main clinical manifestation of the disease. The fact that only a fraction of poliovirus infections leads to the paralytic disease reinforced the need to devise very sensitive and reliable laboratory techniques to isolate and identify poliovirus from samples of AFP cases, which is also associated with several other syndromes and diseases. This led to the establishment of strict quality criteria for AFP surveillance that included the detection of a minimal number of paralytic cases in children of less than 15 years of age due to other causes than polio, the timely sampling of at least 80% of AFP cases, and the analysis of AFP samples in a fully accredited laboratory using standardized protocols. Poliovirus has also been and continues to be one of the most widely used virus in research, and work in many laboratories worldwide has helped understanding many viral and biological processes such as virus cell entry, RNA replication and translation, viral antigenicity, etc.

Polioviruses belong to the genus *Enterovirus* in the family Picornaviridae. Enteroviruses have been traditionally distinguished within the Picornaviridae family on the basis of their physical properties such as buoyant density in caesium chloride and stability in weak acid. However, recent advances in molecular and cell biology have changed the focus to the analysis of the genomic structure and nucleotide sequence, details of the viral replication cycle, and antigenic/immunogenic properties. The viral genome of poliovirus consists of a single RNA strand of positive polarity of about 7,500 nucleotides in length containing the coding sequences for structural and nonstructural viral proteins flanked by 5 and 3 noncoding sequences that modulate RNA replication and translation. The virus particle consists of 60 protomers each containing a single copy of each of the four capsid proteins (VP1 to VP4) arranged in icosahedral symmetry. Poliovirus exists in three serotypes based on specific neutralization reactions with immune sera. Each serotype is defined by the inability of antisera raised against the other two serotypes to completely neutralize infectivity.

Polioviruses are also distinguished from other enteroviruses by neutralization with serotype-specific sera. However, the main distinctive properties of poliovirus are its ability to bind CD155,

a member of the immunoglobulin superfamily, as a receptor for cell entry and the propensity to cause paralysis in humans. The most important route of transmission is fecal–oral, and the virus replicates efficiently in the intestinal tract with shedding in feces typically lasting for 2–4 weeks. The examination of stool samples from AFP patients for the presence of poliovirus has been the driving force of the GPEI, allowing to link poliovirus isolates to specific individuals and to focus the investigations and public health interventions to particular communities. Large numbers of excreted poliovirus particles remain infectious in the environment for varying lengths of time depending on the immediate conditions. The virus presence in samples from the wastewater system may be detected by a variety of laboratory methods for concentration, separation, and identification. Environmental surveillance has indeed proven to be very successful in detecting poliovirus circulation in specific populations even in the absence of associated AFP cases and is becoming a very useful supplementary tool for the surveillance for poliovirus.

The present chapter describes the most common laboratory procedures for isolation, identification, and characterization of polioviruses used in clinical and research laboratories.

2. Materials

1. Cell lines: Polioviruses are able to infect most cultured cells of human or nonhuman primate origin. The most commonly used cell lines are:
   (a) L20B: L mouse connective cells genetically engineered to express the human poliovirus receptor. These cells are highly selective for polioviruses. Some nonpolioviruses that are capable of producing cytopathic effect (CPE) in L cells (e.g., adenoviruses and reoviruses) are also likely to produce CPE in L20B cells, but their CPE is usually noticeably different from poliovirus-induced CPE. A small number of nonpolio enteroviruses (e.g., Coxsackie A viruses) may also grow in L20B cells, and they can produce characteristic enterovirus CPE.
   (b) RD(A): Human rhabdomyosarcoma cells. Highly susceptible to polioviruses, many ECHO viruses, and some other enteroviruses, all of which produce a characteristic enterovirus CPE.
   (c) HEp-2c cells: Human Caucasian larynx carcinoma epithelial cells. Highly susceptible to polioviruses, many Coxsackie B viruses, and some other enteroviruses, all of which produce a characteristic enterovirus CPE.

2. Serotype-specific antisera against type 1, 2, and 3 poliovirus raised in animals. They can be prepared in the laboratory or purchased commercially.
3. Sabin-specific monoclonal antibodies against the individual type 1, 2, and 3 strains. They can be prepared in the laboratory or purchased commercially.
4. Standard reference Sabin poliovirus strains can be obtained from an accredited laboratory.
5. Sterile 96-well flat-bottomed cell culture microtiter plates with lids.
6. Sterile 6-well flat-bottomed cell culture plates with lids.
7. Sterile cell culture flasks (25, 75, and 150 cm^2).
8. Sterile pressure-sensitive film to seal cell culture plates if no CO_2 incubator is used.
9. Sterile externally threaded screw-capped storage vials (2 and 5 ml).
10. Sterile polythene chloroform-resistant centrifuge tubes (15 and 50 ml).
11. Sterile ultracentrifuge tubes.
12. Sterile universal dilution tubes (10 and 20 ml).
13. Sterile disposable pipets (1, 5, 10, and 25 ml).
14. Sterile glass pipets for aliquoting of chloroform (1 and 5 ml).
15. Sterile glass Pasteur pipets.
16. Sterile glass Erlenmeyer flask (1–2 L).
17. Sterile medical syringes and needles (1 and 2 ml).
18. Sterile 0.2-μm disposable filters.
19. Sterile aerosol-resistant precision pipette tips.
20. Sterile wooden or metal spatula.
21. Sterile glass balls, approximately 2.5–3.5 mm in diameter.
22. pH (5–10) indicator strips with 0.5 U scale.
23. Sterile Pyrex glass separation funnels with stopcock (1–2 L).
24. Equipment:
    (a) Fridge/freezer.
    (b) Class II Biological Safety Cabinet.
    (c) Inverted microscope.
    (d) Spectrophotometer.
    (e) Thermal cycler.
    (f) Precision balance.

(g) Vacuum pump with waste trap.

(h) Refrigerated bench centrifuge with buckets.

(i) Ultra centrifuge with rotors and buckets.

(j) Bench centrifuge for Eppendorf tubes.

(k) Mechanical shaker.

(l) CO_2 incubator at 35°C (alternatively a microbiological incubator or a temperature-controlled room with restricted access can be used).

(m) Retort stand, boss heads, and clamps.

(n) Temperature-controlled water baths.

(o) Gradient mixer.

(p) Magnetic stirrer and sterile magnetic bars.

(q) Electronic pipet aid.

(r) Variable volume pipettes (2–20, 10–200, and 20–1,000 μl).

25. Solutions and media:

(a) Cell media (for 100 ml of Eagle's minimum essential medium).

1-ml Penicillin (10,000 U/ml) and streptomycin (10 mg/ml) solution.

1-ml Amphotericin B (250 μg/ml).

2 or 10-ml fetal calf serum for maintenance or growth medium, respectively.

1-ml L-Glutamine (200 mM).

4-ml NaHCO solution (7.5%).

1-ml HEPES (1 M).

0.2-ml phenol red (0.4%).

(b) Microsol or equivalent disinfectant solution.

(c) Phosphate-buffered saline (PBS) solution.

(d) Tris/Borate/EDTA (TBE) buffer solution.

(e) Chloroform biotech-grade solution (≥99.9% purity).

(f) Dextran solution containing 22% (w/w) Dextran T40.

(g) Polyethylene glycol (PEG) solution containing 29% (w/w) PEG6000 molecular biology grade.

(h) 5 N NaCl and 4 M solutions.2.25.9 $NaCH_3COO$ pH 5.2 3 M solution.

(i) 100 mM EDTA pH 8.0 solution.

(j) Phenol:chloroform:isoamyl alcohol 25:24:1 solution.

(k) Sodium dodecyl sulphate (SDS) 20% solution.

(l) Ethanol.

(m) 1 N NaOH (1 N HCl) solution for pH adjustment.

(n) Naphthalene Black stain solution containing 0.1% naphthalene black, 1.36% sodium acetate, and 6% acetic acid.

(o) Sucrose solution containing 33% normal saline, 10 mM Tris–HCl pH 7.5, and 15–45% RNase-free sucrose.

(p) Noble agar solution containing 2% agar in PBS.

(q) Agarose.

3. Methods (Note 1)

3.1. Isolation of Poliovirus

3.1.1. Processing of Stool Samples for Poliovirus Isolation

1. Label centrifuge tubes with sample numbers or identification codes.
2. Add 10 ml PBS, 1-g glass beads, and 1-ml chloroform to each tube.
3. Transfer approximately 2 g of each fecal sample to a labeled centrifuge tube.
4. Retain the remaining of the original sample and store at ≤–20°C.
5. Close centrifuge tubes securely and mix for 5 min using a mechanical shaker.
6. Centrifuge for 20 min at 1,500 × *g* (*g* is the relative centrifugal force; to convert to RPM use the following formula: $g = (11.7 \times 10^{-7})\ RN \times 2$, where R is the radius in mm from centrifuge spindle to extreme point on the tube and N is the speed of centrifuge spindle in RPM) in a refrigerated bench centrifuge, ensuring that centrifuge caps are securely in place and centrifuge buckets are sealed.
7. Transfer supernatant from each sample into labeled externally threaded screw-capped storage vials.
8. Store samples at ≤–20°C.

3.1.2. Concentration of Polioviruses in Environmental Specimens

1. Label separation funnels and tubes with sample numbers or identification codes.
2. Centrifuge the entire sewage sample (1 L), in several portions if necessary, for 10 min at 1,000 × *g* using a refrigerated bench centrifuge.
3. Pool supernatants in a 2-L Erlenmeyer flask (or 2 × 1 L). Keep the pellets at 4°C.
4. Adjust the pH of the supernatant to neutral (pH 7.0–7.5), usually a few ml 1 N NaOH are needed. Measure the volume of the supernatant.

5. Add 79-ml dextran solution, 575-ml PEG solution, and 70-ml 5 N NaCl solution to 1,000 ml of supernatant.
6. Mix thoroughly and keep in constant agitation for 1 h at 4°C using a horizontal shaker or magnetic stirring.
7. Prepare two sterile 1 L (or 1×2 L) separation funnels each attached to a stand. Check water tightness with a small volume of sterile water.
8. Pour the mixture into the funnels and leave for 12–16 h at 4°C.
9. Open the valve with caution. Collect the entire lower phase and the interphase slowly, dropwise, into a sterile universal tube (usually 10–20 ml/L of original sample).
10. Resuspend the pellets stored at 4°C with the collected sample.
11. Extract with 20% volume of chloroform by shaking for 1 min using a mechanical shaker.
12. Centrifuge and proceed as with fecal suspensions (see previous section).
13. Store samples at ≤–20°C.
14. Isolation and propagation of virus in cell culture: The above procedures can be used to isolate poliovirus from processed biological or environmental samples or for the propagation of poliovirus from laboratory viral stocks and reference virus strains. Depending on the purpose of the study, one or a combination of the three cell lines mentioned above can be used. For example, the combination of L20B and RD cells for extracts of stool samples has proven to be of great sensitivity and specificity in detecting poliovirus while maintaining the ability to detect some enteroviruses as an assurance of good technique. A detailed process algorithm including L20B and RD(A) separate arms and cross-passages between cell line supernatants is currently used as a standard method for poliovirus isolation in WHO laboratories involved in poliomyelitis surveillance.

3.2. Isolation/ Propagation of Polioviruses in Cell Culture

1. Use 25 cm^2 cell culture flasks for routine poliovirus isolation. Larger 75 cm^2 or 150 cm^2 can be used when preparing larger stocks particularly for research purposes.
2. Check by microscopy that the cell monolayers are confluent and healthy. A suitable monolayer would be one formed within 1–2 days of seeding. A good guide is to use 1×10^6 total cells per plate, although this may vary according to cell line, media, etc.
3. Label the flasks using an informative code, including country, year of sampling, ID number of sample, etc. or name of the

reference virus. Label one flask as a negative control. Handle the flasks one by one.

4. Remove the culture medium.
5. Pipet 3 ml of fresh maintenance medium on the upper wall (without the cells) of the flask. Then, rinse the monolayer by turning the flask, and remove the fluid by pipet.
6. Inoculate the flask with 0.5 ml of the sample inoculum by pipeting on cell-free surface and then spreading the inoculum evenly over the monolayer by turning the flask.
7. Close the flask and keep at 35°C for 1 h in an incubator or temperature-controlled room to allow virus cell adsorption.
8. Respread the inoculum over the cells by tilting the flask several times every 20 min.
9. After 1 h adsorption, remove the inoculum and add 5 ml of maintenance medium. Again, treat the flasks one by one. Cell medium without fetal calf serum can be used when preparing virus stocks. This is particularly important when preparing virus stocks for animal tests, virus and RNA purification, etc.
10. Incubate at 35°C and monitor possible virus replication by microscopy. Examine and record cultures daily for the appearance of CPE.
11. If characteristic enterovirus CPE appears, i.e., rounded, refractive cells detaching from the surface of the flask, allow to develop until ≥90% of the cells are affected; harvest the culture by freezing/thawing three times.
12. Centrifuge for 20 min at 1,500 × *g* in a refrigerated bench centrifuge ensuring that centrifuge caps are securely in place and centrifuge buckets are sealed.
13. Transfer supernatant from each sample into two labeled externally threaded screw-capped storage vials.
14. If no CPE appears after 7 days, collect cell culture fluid after freezing/thawing three times, eliminate cell debris by centrifugation at 1,500 × *g* as above then perform a blind passage by inoculating fresh cells and continue examination for a further 7–10 days. Each flask should be repassaged separately. If still CPE is not observed, record as a negative sample.
15. If CPE is seen after the repassage, collect and store the virus supernatant as described above.
16. In some cases, toxicity or microbial contamination may cause cell death. In most cases, particularly with bacterial or fungal contamination, this should be distinguishable from enterovirus CPE. If contamination is confirmed, reprocess original sample. If toxicity is suspected, dilute the original sample (between 1:5 and 1:20) and repeat the process of isolation.

3.3. Concentration and Purification of Poliovirus Using Sucrose Gradients

The possibility to obtain highly concentrated poliovirus stocks is of great interest for some laboratory applications such as physicochemical analyses, electron microscopy, crystallographic studies, etc. The following protocol describes how to concentrate poliovirus from large volumes of virus solutions followed by purification using sucrose-buffered gradients.

1. Cool SW28 Beckmann rotor and buckets to 4°C. Alternative rotors can be used if required volumes or ultracentrifuge brand name is different.
2. Pour 20 ml of virus supernatant into an SW28 UltraClear centrifuge tube. Carefully pipette 8–10 ml of 30% sucrose solution onto the bottom of the tube, forming a cushion.
3. Balance centrifuge buckets containing tubes.
4. Centrifuge in SW28 Beckmann rotor for 16 h at 100,000 *g* and 4°C.
5. Carefully remove and discard supernatant and sucrose into a beaker with Microsol (or any suitable viral disinfectant) to decontaminate any remaining poliovirus.
6. Resuspend pellet with 0.5–2 ml of cell medium (with no serum) using a mechanical shaker.
7. Filter the virus using a 1-ml syringe and a sterile 0.2-µm disposable filter.
8. Prepare sucrose gradients in SW28 UltraClear centrifuge tubes by mixing equal volumes of 15 and 45% sucrose solutions in a gradient mixer.
9. Load virus concentrate carefully onto the sucrose gradient.
10. Centrifuge in SW28 Beckmann rotor for 4 h at 100,000 *g* and 4°C.
11. Carefully collect 1-ml fractions by bottom puncture of centrifuge tubes with a syringe needle. Routinely, poliovirus will be found between the 5th and the 16th ml of the gradient. This can be checked by optical density or protein gel analysis.
12. Pool selected fractions in a T50 ultracentrifuge tube.
13. Centrifuge in T50 Beckmann rotor for 4 h at 100,000 *g* and 4°C.
14. Carefully remove and discard supernatant and resuspend pellet with 0.5–2 ml of cell medium (with no serum) using a mechanical shaker.
15. Transfer each virus suspension into labeled externally threaded screw-capped storage vials.
16. Store samples at ≤–20°C.

3.4. Characterization of Polioviruses Using Cell Culture Assays

3.4.1. Identification and Typing of Poliovirus by Neutralization Assay

Stool and environmental samples often contain enteroviruses other than polioviruses and also other human viruses that may replicate in cells used for poliovirus isolation. To confirm the presence of poliovirus in RD(A) and Hep-2c cells, virus harvests can be passaged in L20B cells. L20B are cells of mouse origin (see Subheading 2), and human viruses other than polioviruses rarely generate productive infection in these cells. However, some reoviruses and adenoviruses are known to be able to infect L20B cells. In most cases, the CPE produced is clearly distinct from enterovirus-characteristic CPE. In order to identify the presence of poliovirus in virus harvests, neutralization tests that use polyclonal sera raised in animals against poliovirus type 1, 2, and 3 have been devised. The protocol shown here also includes the use of monoclonal antibodies against Sabin 1, 2, and 3 strains. In this manner, the test will determine whether the agent causing CPE in cells is poliovirus, what serotype/s it is, and if the virus is related to a Sabin OPV strain or not. This method is extremely useful for the rapid detection/identification of poliovirus isolates during polio surveillance but can be used for other clinical or scientific analyses (modifying the assay format if necessary).

1. Label 10-ml universal tubes 1–6 for dilutions 10^{-1}–10^{-6}.
2. Dispense 4.5 ml of cell maintenance medium to tubes 1–6 using a sterile 5-ml pipet and pipet aid.
3. Add 0.5 ml of virus to the first tube using a pipette with sterile aerosol-resistant tip. This is the 10^{-1} dilution.
4. Cover the tube and mix gently using a mechanical shaker.
5. Take another pipette tip, transfer 0.5 ml to the second tube, and discard pipette tip.
6. Cover the tube and mix gently using a mechanical shaker.
7. Repeat dilution steps by transferring 0.5 ml each time and always changing pipette tip between dilutions, up until tube 6 (dilution 10^{-6}).
8. Four arbitrarily selected dilutions (10^{-2}, 10^{-3}, 10^{-4}, and 10^{-6}) have been found in practice to yield results with most cell-derived poliovirus isolates. This series of dilutions enables detection of virus mixtures in which one component is present in considerable excess.
9. Prepare polyclonal antisera and monoclonal antibody mixtures using sterile universal tubes. Use validated animal polyclonal sera and monoclonal antibodies (at dilutions known to neutralize $\geq 10^4$ infectious units of virus) as follows:
   (a) Mixture 1 + 2: polyclonal serum against type 1 + polyclonal serum against type 2.
   (b) Mixture 2 + 3: polyclonal serum against type 2 + polyclonal serum against type 3.
   (c) Mixture 1 + 3: polyclonal serum against type 1 + polyclonal serum against type 3.

(d) Mixture 1 + 2 + 3: polyclonal serum against type 1 + polyclonal serum against type + polyclonal serum against type 3.

(e) Mixture Sabin-MAbs: Sabin-specific neutralizing monoclonal antibodies to poliovirus type 1, 2, and 3.

10. Label 96-well plate with virus isolate number or identification code. Include information on location of virus dilutions and antibody mixtures as shown in Fig. 1. Use one plate per virus isolate. Sabin reference virus strains can be used to validate the assay.
11. Add 50 µl of each antibody mixture to the appropriate wells using a pipette and sterile aerosol-resistant tips (a multichannel pipette can be used to ease the process).
12. Add 50 µl of virus dilution to the appropriate wells using a pipette and sterile aerosol-resistant tips (a multichannel pipette can be used to ease the process).
13. Incubate plates for 2 h at 35°C.
14. Add 100 µl of maintenance medium to wells A5 to H5 in row 5 and A11 to H11 in row 11. These are wells with no antibody.

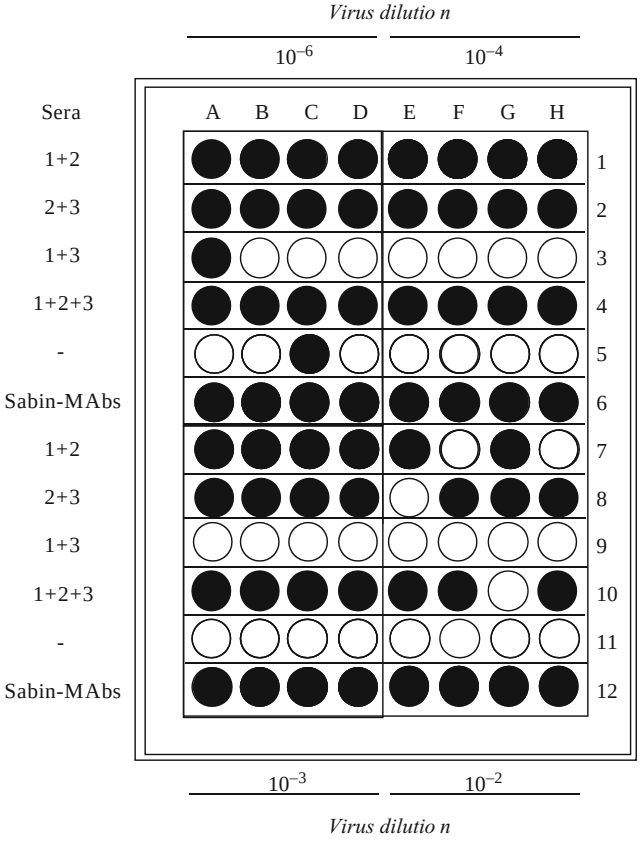

Fig. 1. Identification and typing of poliovirus by neutralization assay. The *plate layout* is shown indicating the location of antiserum mixtures and virus dilutions. A *black well* indicates stained cells and therefore cell survival and absence of virus infection.

15. Prepare a cell suspension of approximately $1–2 \times 10^5$ cells/ml in maintenance medium by calculating at least 10 ml/plate and using standard cell culture procedures. L20B cells are ideally used in this procedure since they are very restrictive for human viruses other than poliovirus.
16. Add 100 µl of cells from the cell suspension to all wells in rows 1–12 on the plate.
17. Cover the plate with sterile pressure sensitive film to seal cell culture plates if no CO_2 incubator is used and incubate at 35°C.
18. Examine for development of CPE, using an inverted microscope, and record daily readings for 5–7 days.
19. Analysis of wells A5 to H5 in row 5 and A11 to H11 in row 11 will determine the presence of virus and the approximate titer. This should be followed by detailed analysis of the rest of the wells which will help typing the poliovirus isolate/s. In the example shown in Fig. 1, a Sabin-like type 2 poliovirus isolate was identified because the virus was neutralized by $1+2$, $2+3$, and $1+2+3$ serum mixtures and also by the Sabin-MAbs mixture. The decision should be based on the highest dilutions at which virus infection is observed since lower dilutions may contain an excess of virus.
20. If a mixture of serotypes are detected, viruses of a single serotype should be collected from appropriate wells and store at ≤–20°C for future characterization.

3.5. Quantification of Poliovirus Titer by Estimation of the Cell Culture 50% Infectious Dose

Determining the virus titer of different virus preparations with high precision can be very useful for different applications. The main test for the quality control of OPV is to establish if the virus titers of the three serotype components are within specification. It is also critical to know the titer of virus inocula to prepare the experimental doses to be used in animal tests, particularly when testing the levels of neurovirulence of vaccine lots. The use of virus reference standards of known virus content has also proven to be very useful in testing the sensibility to poliovirus infection of cell culture systems used in clinical laboratories working for polio surveillance.

1. Label 10-ml universal tubes 1–8 for dilutions 10^{-1}–10^{-8}.
2. Dispense 4.5-ml cell maintenance medium to tubes 1–8 using a sterile 5-ml pipette and pipet aid.
3. Add 0.5 ml of virus to the first tube using a pipette with a sterile aerosol-resistant tip. This is the 10^{-1} dilution.
4. Cover the tube and mix gently using a mechanical shaker.
5. Take another pipette tip, transfer 0.5 ml to the second tube, and discard pipette tip.
6. Cover the tube and mix gently using a mechanical shaker.

7. Repeat dilution steps by transferring 0.5 ml each time and always changing pipette tip between dilutions, up until tube 8 (dilution 10^{-8}).
8. Label a 96-well plate with a virus isolate number or identification code. Include information on location of virus dilutions and antibody mixtures as shown in Fig. 2. Use one plate per virus isolate. Sabin reference virus strains with assigned virus titer can be used to validate the assay.
9. Add 100 µl of virus dilutions to wells 1–10 in rows A to H – that is, ten wells per dilution as shown in Fig. 2.
10. Add 100 µl of maintenance medium to wells A11 to H12 in rows A to H for the cell controls.
11. Prepare a cell suspension of approximately $1–2 \times 10^5$ cells/ml in maintenance medium by calculating at least 10 ml per plate and using standard cell culture procedures. L20B cells are ideally used in this procedure since they are very restrictive for human viruses other than poliovirus.
12. Add 100 µl of cells from the cell suspension to all wells in rows A to H on the plate.
13. Cover the plate with sterile pressure-sensitive film to seal cell culture plates if no CO_2 incubator is used and incubate at 35°C.
14. Examine for development of CPE, using an inverted microscope, and record daily readings for 5–7 days.
15. At day 7, add Naphthalene Black solution to cover wells and stain cells for about 1 h at room temperature. The presence of

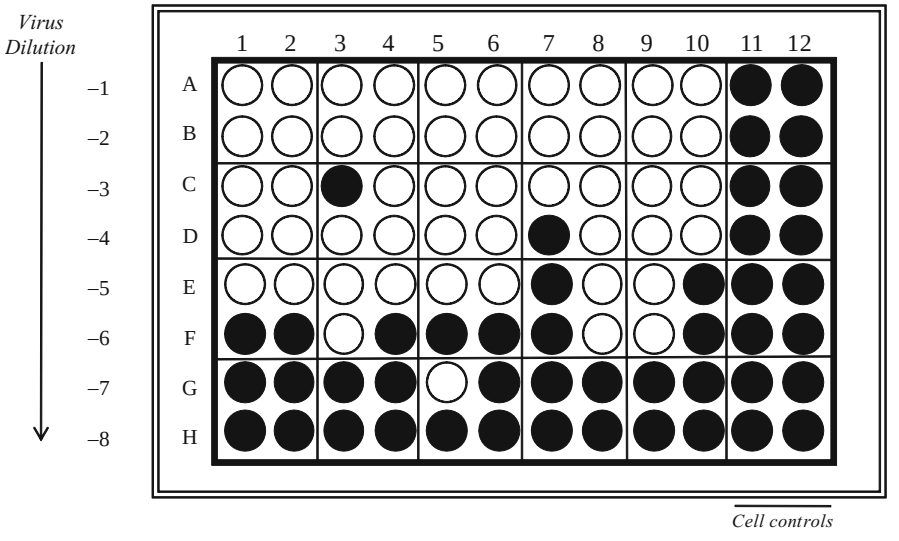

Fig. 2. Quantification of poliovirus titer by estimation of the cell culture 50% infectious dose ($CCID_{50}$). The *plate layout* is shown indicating the location of virus dilutions and cell controls. A *black well* indicates stained cells and therefore cell survival and absence of virus infection.

a stained cell sheet indicates cell survival and absence of poliovirus infection.

16. Remove stain and allow plates to air-dry.
17. For a valid test, the cell control should have a complete monolayer of healthy cells.
18. Calculate the virus titer using the Karber formula as follows:

$$\text{Log CCID}_{50} = L - d(S - 0.5)$$

where L is the log of lowest dilution used in the test, d is the difference between log dilution steps, and S is the sum of proportion of "positive" tests (i.e. cultures showing CPE).

In the example shown in Fig. 2:

$L = -1.0$; $d = 1.0$; $S = 1 + 1 + 0.9 + 0.8 + 0.8 + 0.3 + 0.1 + 0 = 4.9$

Log $\text{CCID}_{50} = -5.4$; Virus titer $= 10^{5.4}$ $\text{CCID}_{50}/0.1$ ml

19. Ideally three titer determinations should be performed and the average calculated.

3.6. Quantification of Poliovirus Titer by the Plaque Assay

An alternative to the cell culture 50% infectious dose (CCID_{50}) method for measuring the poliovirus titer is to use the classical plaque assay. Although less favored for quality control testing, it can be very useful for different applications such as testing virus growth at different temperatures or purification of single virus progenies in virus mixtures.

1. Use 6-well plates with lids (individual 35 cm^2 cell culture dishes can be used).
2. Check by microscopy that the cell monolayers are confluent and healthy. A suitable monolayer would be one formed within 1–2 days of seeding. A good guide is to use 3×10^6 total cells per plate, although this may vary according to cell line, media, etc.
3. Proceed with preparation of 10^{-1}–10^{-8} virus dilutions as in the previous section.
4. Label plates with name and dilution of virus. Use at least two duplicate wells per virus dilution.
5. Carefully remove medium from plate using a Pasteur pipette attached to a vacuum pump.
6. Immediately inoculate each well with 100 µl of corresponding virus dilution, replace lid, and move plate gently to ensure that the inoculum covers the cell sheet.
7. Repeat with next sets of virus dilutions.
8. Incubate plates at 35°C for 1 h to allow virus to adsorb to cells, with occasional gentle agitation to avoid drying of cell sheets.
9. During incubation prepare overlay by mixing equal volumes of 2% agar (previously melted and kept at 55°C) and

2× MEM, 20 ml of overlay mix per 6-well plate. Warm to 37°C in water bath until use.

10. Carefully remove the inocula from plates using a Pasteur pipette attached to vacuum pump and add 3 ml of overlay mix per well.
11. Allow agar to set at room temperature.
12. Cover the plates with sterile pressure-sensitive film to seal cell culture plates if no CO_2 incubator is used.
13. Incubate upside down at the desired temperature for 72 h. An incubator with or without CO_2 or a temperature-controlled room or water bath can be used.
14. Remove agar carefully using a spatula and add 3 ml of Naphthalene Black solution per well to stain cells for about 1 h at room temperature.
15. Remove stain and allow plates to air-dry.
16. Count plaques and assign a virus titer as plaque-forming units (PFU) per ml of virus. In the example shown in Fig. 3, the virus titer is approximately 1×10^8 PFU/0.1 ml.
17. Ideally, three titer determinations should be performed and the average calculated.

3.7. Molecular Characterization of Poliovirus Strains

The molecular characterization of poliovirus genomic RNA provides the ultimate information for the identification of poliovirus strains. It allows establishing epidemiological links between poliovirus isolates and reconstructing the natural history and geographical

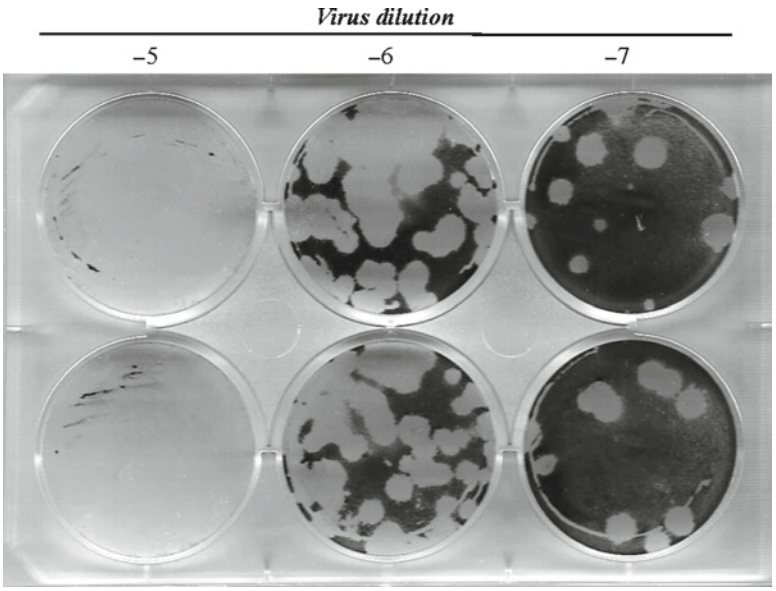

Fig. 3. Quantification of poliovirus titer by the plaque assay. The *plate layout* is shown with two duplicate wells for each virus dilution. Viral plaques are shown as *unstained white circles* due to cell lysis.

spread of viral genotypes. These data provide invaluable information to help assess the quality of AFP surveillance and to monitor the progress of global eradication providing guidance for public health interventions. The molecular analysis of poliovirus sequences is also very useful in research laboratories for the characterization of laboratory mutants, recombinant cDNA clones, etc. There are many protocols available for viral RNA isolation, PCR amplification, and nucleotide sequencing, including a number of commercial kits which require a minimum setup. Typical procedures are described below.

3.7.1. RNA Extraction

1. Take 500 µl of tissue culture fluid containing the virus sample (>10^7 PFU/ml) and add to an appropriately labeled 1.5-ml Eppendorf tube.
2. Add 8 µl of 3 M sodium acetate pH 5.2, 25 µl 100 mM EDTA pH 8.0, and 25 µl 20% SDS to each tube and mix using a mechanical shaker.
3. Add 550 µl of phenol:chloroform:isoamyl alcohol (25:24:1) and mix using a mechanical shaker.
4. Centrifuge for 5 min at 15,000 *g* on a bench centrifuge.
5. Transfer supernatant to another Eppendorf tube.
6. Repeat phenol:chloroform:isoamyl alcohol extraction.
7. Transfer supernatant to another labeled Eppendorf tube.
8. Add 1-µl glycogen, 15 µl of 4 M NaCl, and 1 ml of ethanol (stored at –20°C) to each tube and mix using a mechanical shaker.
9. Incubate tubes overnight at –20°C.
10. Centrifuge for 10 min at 15,000 *g* on a bench centrifuge.
11. Remove ethanol with a Pasteur pipette.
12. Add 150 µl of 75% ethanol (stored at –20°C) to each tube.
13. Centrifuge for 10 min at 15,000 *g*.
14. Carefully remove 75% ethanol with pipette tip.
15. Air-dry at 55°C in heat block for 30 min.
16. Resuspend pellet in 20 µl of nuclease-free water.
17. Store at –20°C until use.

3.7.2. RT-PCR

Amplification of poliovirus RNA can be carried out in two successive enzymatic reactions: cDNA synthesis using reverse transcriptase followed by PCR using Taq DNA polymerase; or combining both steps in a single reaction as it is described in the method below (OneStep RT-PCR, Qiagen). Examples of universal primers that can be used to obtain DNA fragments specific to the 5′NCR, VP1, and 3C/3D regions of poliovirus genome are given in Table 1, and a typical agarose gel is shown in Fig. 4.

Table 1
Properties of oligonucleotides used to amplify poliovirus sequences

Name	Region	Polarity	Sequence
M13-PCRF2	5′NCRNucleotides 32-554	Sense	CAGGAAACAGCTATGACCGAGGCCCACGYGGCGGC
M13-5NTR3R		Antisense	TGTAAAACGACGGCCAGTCACCCAAAGTAGTCGGTTCCGC
M13-WAG2VP1	5′-VP1Nucleotides 403-3014	Sense	CAGGAAACAGCTATGACCTTTGTRTCRGCNTGYAAYGA
M13-VP13050R		Antisense	TGTAAAACGACGGCCAGTGANGTTTGCCANGTGTARTC
M13-VP13000F	3′-VP1Nucleotides 2937-3503	Sense	CAGGAAACAGCTATGACCCAGGTNTAYCARATNATGTA
M13-2A3500R		Antisense	TGTAAAACGACGGCCAGTAGGTCTCTGNYCCACATR
M13-PV3D5800T	3C/3DNucleotides 5748-6557	Sense	CAGGAAACAGCTATGACCGTGAACACTAGYAAGTACCCYAATATGT
M13-AM18		Antisense	TGTAAAACGACGGCCAGTGCCATTCTCATKGCCACTGA

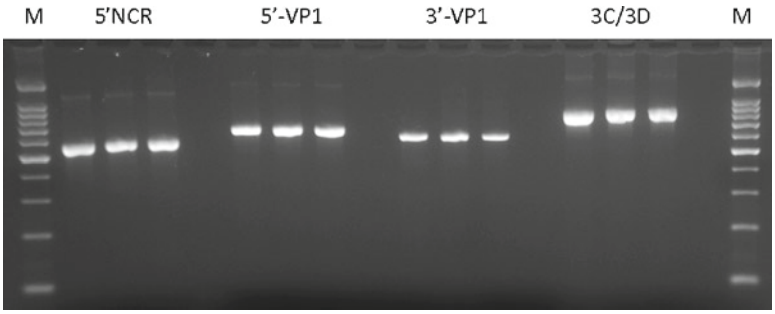

Fig. 4. PCR. Ethidium bromide-stained 1.5 % agarose gel showing the results of a PCR analysis of the genome of three poliovirus strains using primers corresponding to different regions in the poliovirus genome (5′NCR, 5′-VP1, 3′-VP1, and 3C/3D) as indicated. Negative control lanes containing no DNA template are shown after the three *positive lanes* for each primer pair. *Lane M* corresponds to the 100-bp DNA molecular marker (New England Biolabs).

1. For each RT-PCR reaction, prepare a mixture in a thin-walled tube containing 10 µl of 5× reaction buffer, 10 µl of 5× Q-Solution, 2 µl 10 mM dNTPs, 2-µl enzyme, 2-µl sense primer, 2-µl antisense primer, 20-µl H_2O, and 2-µl viral RNA.
2. Incubate tubes in a Thermal Cycler machine using the following conditions:
   (a) 30 min at 50°C
   (b) 10 min at 94°C
   (c) 30 cycles including the following segments:
      1 min at 94°C
      1 min at 50°C
      2 min at 72°C
3. Analyze the amplified DNA by agarose gel electrophoresis using appropriate DNA molecular markers (commercially available) as follows.
4. Prepare an agarose gel by adding 0.5 g of agarose (GTG Seakem, Lonza) to 50 ml of TBE buffer and boil for 2 min to dissolve the agarose. Add 25 µl of ethidium bromide solution at 1 mg/ml to stain the DNA.
5. Cool the agarose to 55°C and pour into an 80 mm×105 mm gel tray with 5-mm well formers. Allow the gel to set firmly.
6. Mix 10 µl of the PCR product from the reaction and mix with 2 µl of loading buffer provided by the manufacturer.
7. Load the mix into the well of the gel.
8. Load a DNA marker onto the gel in loading buffer.
9. Run the gel for 20 min at room temperature at 100 mA (125 V, 12 W) in an electrophoresis tank containing 1× TBE buffer.

10. Observe the gel under a UV transilluminator to visualize the DNA fragments and take a photograph to a permanent keep record.
11. DNA PCR products can be purified for further analysis. A protocol using the High Purification kit (Roche) is as follows.
12. Add RNAse-free H_2O to the PCR product to make a total volume of 100 µl in a 1.5-ml Eppendorf tube.
13. Add 50 µl of binding buffer (Roche) containing guanidine hydrochloride, which provides the adequate concentration and pH for adsorption of DNA and mix using a mechanical shaker.
14. Transfer mix onto a filter tube placed on the collection tube and centrifuge for 1 min at 15,000 *g*.
15. Discard filtrate and add 500 µl of washing buffer (Roche) containing ethanol to wash away salts.
16. Centrifuge for 1 min at 15,000 *g*.
17. Repeat washing step with 200 µl of washing buffer.
18. Elute DNA by adding 50 µl of elution buffer (Roche) or RNase-free H_2O to the tube; centrifuge for 1 min at 15,000 *g* and transfer to a fresh Eppendorf tube.

3.7.3. RFLP Analysis

Restriction fragment length polymorphism is a DNA analysis technique based on the use of restriction endonucleases that digest double-stranded DNA at specific short nucleotide sequence targets generating a DNA fragment pattern that can be analyzed by agarose gel electrophoresis. RFLP can be very useful as an alternative method to nucleotide sequencing when comparing known related sequences that differ at a few nucleotide positions. The method described below was designed to compare MEF-1 poliovirus strains available at different laboratories. MEF-1 is a reference laboratory strain originally isolated from a polio case in Egypt in 1942 and commonly used for the production of inactivated polio vaccine. There are at least three different versions of MEF-1 used by vaccine manufacturers that differ in only 1–2 nucleotides between them. A RFLP protocol was devised for the quick identification of MEF-1 variants to assess the possible association of MEF-1 strains used by vaccine producers with a number of polio cases due to MEF-1 that were identified in India in 2004.

1. Run a OneStep RT-PCR reaction as described in Subheading 3.7.2 using primers *MEF-1 Sense* (CAGGAAAC AGCTATGACCCACGAGAAATGCCTTGACA) + *MEF-1 Antisense* (TGTAAAACGACGGCCAGTCTCCAACTTA CGTCTTAAC) and viral RNA from the samples to be analyzed. The primers locate in the 5′end region of the MEF-1 VP1 genomic region and result in a 356 nucleotide-long DNA PCR fragment.

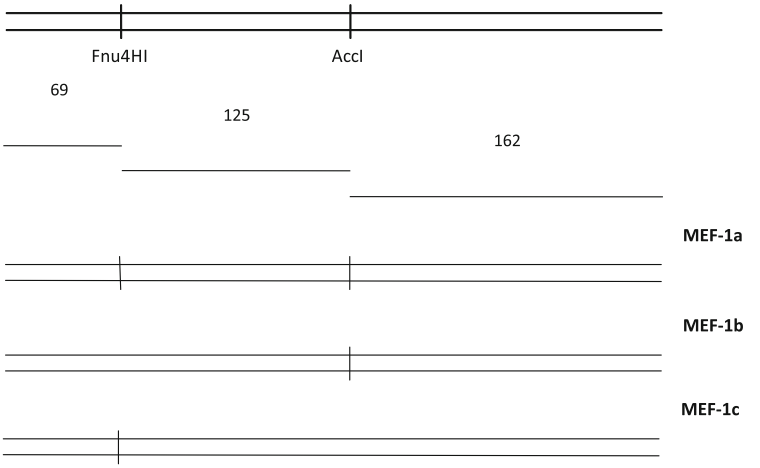

Fig. 5. PCR-RFLP. Schematic representation of a PCR-RFLP analysis designed to identify the three known versions of MEF-1 poliovirus. A double restriction enzyme digestion with Fnu4HI and AccI would result in DNA fragment patterns of [69, 125, 162], [162, 194], and [69, 287] nucleotides in length for MEF-1a, MEF-1b, and MAF-1c, respectively. These DNA fragments can be easily resolved by agarose gel electrophoresis.

2. For each restriction enzyme reaction, prepare in an Eppendorf tube a mix containing 14 µl of H_2O, 2 µl of 10× buffer 4 (New England Biolabs), 1-µl Fnu4HI enzyme (New England Biolabs), 1-µl AccI (NEB), and 2 µl of DNA RT-PCR product (~300 ng/µl). The location of restriction sites for each of the three MEF-1 poliovirus variants is shown in Fig. 5.
3. Incubate for 16 h at 37°C.
4. Analyze the DNA fragments by agarose gel electrophoresis as described above.

3.7.4. Nucleotide Sequencing and Phylogenetic Analysis

As stated above, molecular characterization and nucleotide sequencing in particular provides the ultimate identification of poliovirus isolates. Nucleotide sequencing gives the final confirmation of any suspect polio case, providing a unique case identifier that can be contrasted with any available epidemiological information. At present, the entire genomic region coding for the capsid protein VP1 (900–906 nucleotides depending on serotype) is sequenced. VP1 is the most exposed protein in the virion and contains antigenic sites that are known to change during poliovirus evolution. Also, nucleotide VP1 synonymous changes appear to accumulate uniformly at a predicted rate, so it is possible to determine the relative age of two or more sequence-related poliovirus strains. The VP1 sequence of any newly identified poliovirus strain is compared to thousands of sequences in WHO databases so as to determine the virus origin and detect possible importations from endemic countries. The sequence heterogeneity among related

poliovirus strains gives a good indication of the quality of AFP surveillance. For example, orphan poliovirus strains defined as those containing more than 1% VP1 sequence divergence to contemporary poliovirus isolates have identified important gaps in surveillance in some regions in Africa. Extensive nucleotide analyses have also confirmed the association of evolved vaccine-derived poliovirus with polio outbreaks which has given a new perspective to the GPEI in terms of planning current and future vaccination strategies. Sequence analyses covering other genomic regions have shown that recombination between polioviruses and other polio and nonpolio enterovirus C viruses is a very common phenomenon during poliovirus evolution in humans, which has resulted in the reclassification of polioviruses within *Enterovirus* species C of the *Enterovirus* genus. Today, nucleotide sequencing is mostly preformed by specialized laboratories using high-throughput automated systems. The most common strategy is to use universal sequence tags, such as those from the M13 bacteriophage, incorporated as part of the PCR primers. The purified DNA PCR material is sent to a specialized laboratory indicating the sequencing primers to be used. Most of the current sequencing protocols are based on the dideoxy chain-termination method developed by Frederick Sanger in 1977. Essentially, the sequencing reaction consists of a polymerase reaction using a DNA template (usually a PCR product), a specific primer (usually a universal tag), a mixture of labeled deoxy/dideoxy nucleotides, an adequate buffer, and DNA polymerase. Nucleotides are incorporated specifically in a newly synthesized chain as a copy of the DNA template strand. However, the incorporation of a dideoxy nucleotide prevents further extension by the DNA polymerase. Using the adequate mixture of deoxy/dideoxy nucleotides, the reaction results in a mixture of DNA fragments covering all sizes from one nucleotide to the full length of the PCR fragment. The DNA fragments can be separated by size in a polyacrylamide gel, and the sequence can be read, as each nucleotide (A, G, T, C) is specifically labeled with a different fluorescence dye. A protocol using the Big Dye Termination Kit (Applied Biosystems) is shown below (see Note 2).

1. Prepare reactions in PCR tubes containing Termination Mix (containing the appropriate deoxy/dideoxy nucleotide mixtures, enzyme and reaction buffer), primer (10 pmol), and DNA template (10–20 ng) in a total of 10 µl.
2. Incubate tubes in a Thermal Cycler machine using the following conditions:
   (a) 25 cycles including the following segments:
   30 s at 95°C
   15 s at 45°C
   4 min at 60°C

3. Sequencing reactions are purified using the Dye^{EX} 2.0 spin kit (Qiagen) as follows.
4. Resuspend the resin gel in the column by mixing in mechanical shaker.
5. Place the spin column in collection tube and centrifuge for 3 min at 10,000 *g* using a bench centrifuge.
6. Load ten of the sequencing reactions onto the gel bed and centrifuge for 3 min at 15,000 *g*. Collect the purified DNA and air-dry for 30 min at 50°C using a heating block.
7. Resuspend the pellet in ten of the template suspension reagents (Applied Biosystems), denature for 8 min at 96°C, and load onto an ABI sequencer.

4. Notes

1. Methods: The above protocols describe procedures for isolation and characterization of polioviruses. They detail the processing of stool and environmental samples, but the methods can be easily adapted to other less common types of samples susceptible to contain poliovirus such as rectal and nasopharyngeal swabs, cerebrospinal fluid, tissues from laboratory animals, etc. As with all biological materials, it is important to follow the principles of Good Laboratory Practice and Biosafety regulations when working with poliovirus. Poliovirus is currently classified as a Risk Group 2 microorganism requiring the use of a Biosafety level 2 laboratory. This includes the use of standardized protocols, implementing adequate procedures and protective measures, immunization and training of personnel, regular calibration and maintenance of equipment, keeping complete documentation, restricting laboratory access, etc. As a general rule, for all procedures described here, handling of all virus materials and cell cultures should be carried out in a Class II Biological Safety Cabinet and using sterile materials.
2. Nucleotide sequencing: The sequencing machine will return data including a detailed chromatogram and information on the quality (reliability) of the sequence at each nucleotide point. PCR fragments are usually sequenced at least twice (plus and minus strands), and sequences are compared and edited, correcting any mismatches/inconsistencies. Once the sequence is established, it can be used to search sequence databases for similar sequences and to perform phylogenetic analysis. There are numerous programs and software packages to edit and analyze nucleotide sequences. A few Internet

resources as can be found here: http://evolution.genetics.washington.edu/phylip/software.html; http://en.bio-soft.net/tree.html; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome. Phylogenetic analysis is a very complex subject and would require a dedicated chapter. Nucleotide sequences can be compared using different alignment algorithms, tree-building methods, estimation of genetic distances, and testing models of evolution. Phylogenetic relationships among sequences can be analyzed using various inference models (distance, maximum likelihood and maximum parsimony), postphylogenetic information (such as molecular clocks and selection), and useful subsidiary statistical techniques (such as bootstrapping and likelihood ratio tests). An example showing the genetic relationships between 14 type 2 vaccine-derived poliovirus strains isolated from sequential stool samples (covering a period of 5 years) from an immunodeficient patient can be found in Fig. 6. The analysis was carried out using parameters known to be adequate for poliovirus sequences. Phylogenetic relationships between strains were established by comparing the sequences determined and aligning them using the alignment program CLUSTAL X. The degree of nucleotide sequence identity and of protein similarity between strains was determined using the default scoring matrices. Phylogenetic relationships between sequences were inferred by the maximum likelihood method with DNADIST/NEIGHBOUR of Phylogeny Inference Package (PHYLIP) version 3.6 and a

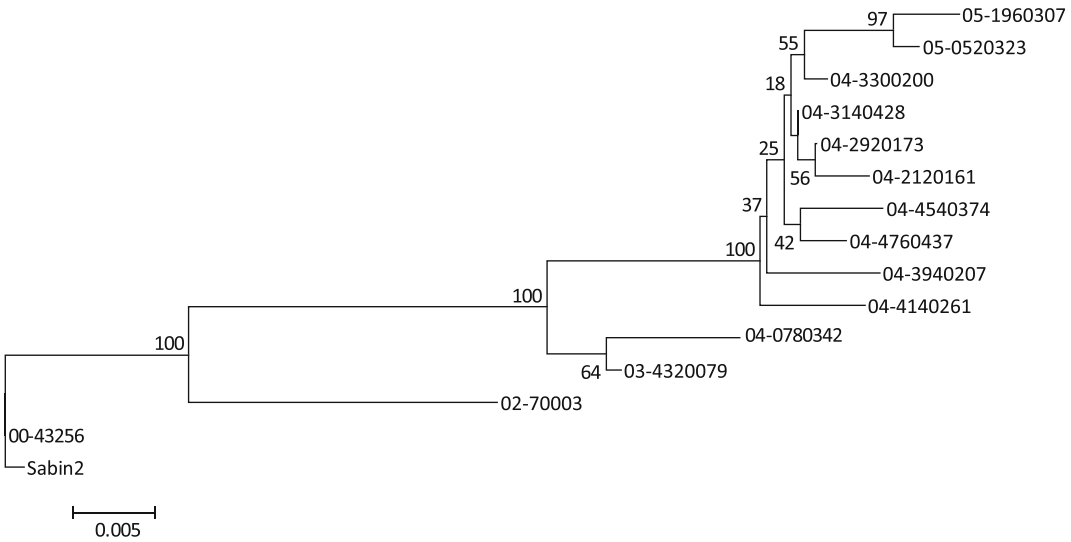

Fig. 6. Phylogenetic analysis. Neighbor-joining tree representing phylogenetic relationships between 14 strains from an immunodeficient patient and Sabin 2. The numbers at the nodes indicate the percentages of 1,000 bootstrapping pseudoreplicates supporting the cluster.

distance matrix was calculated using the F84 model of nucleotide substitution with a transition/transversion ratio (Ts/Tv) of 10.0. The robustness of phylogenies was estimated by bootstrap analyses with 1,000 pseudoreplicate data sets generated with the SEQBOOT program of PHYLIP. Phylogenetic trees were constructed using neighbor joining of PHYLIP and drawn using TREEVIEW or NJ Plot software.

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Chapter 15

Detection of Human-Pathogenic Poxviruses

Andreas Kurth and Andreas Nitsche

Abstract

With the eradication of smallpox about 30 years ago, the identification and differentiation of other poxviruses with varying pathogenicity in humans present a challenge for diagnostic facilities. While a clinical differentiation can be demanding, electron microscopy is the fastest approach to identify poxviruses. Molecular techniques, based on specific genomic sequences, are routinely applied to identify poxvirus species and distinguish between individual virus variants. In this chapter, we present detailed protocols for both techniques and discuss questions relevant to fast and reliable diagnostics of poxviruses.

Key words: Smallpox, Poxviruses, Electron microscopy, Real-time PCR

1. Introduction

1.1. Poxviruses

The earliest classification of the family Poxviridae was based on disease signs or symptoms characterized by pocks on the skin of vertebrates. Of the eight genera within the subfamily Chordopoxvirinae, only four contain human-pathogenic poxviruses, namely, *Orthopoxvirus*, *Parapoxvirus*, *Molluscipoxvirus*, and *Yatapoxvirus* (1) (Table 1). The most dangerous infections were caused by orthopoxvirus (OPV) variola, which was eradicated in the last century by vaccination (2). However, current infections with monkeypox (3, 4), vaccinia (5–7), or cowpox (8, 9) virus can be fatal, depending on the immunological state of the infected individual. Infections with parapoxviruses (10, 11), molluscipoxviruses (12), and yatapoxviruses (13) are usually self-limiting and remain mostly localized. While vaccination and administration of vaccinia immunoglobulins were the only options in the past for prevention and therapy of variola virus, nowadays other antipoxviral drugs are available, e.g., the FDA-approved drug cidofovir (14, 15) and the new small compound ST-246 (16, 17), which proved to be effective in a recent

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Table 1
Human-pathogenic poxviruses [a]

Genus	Species	Abbreviation	Nonhuman hosts
Orthopoxviruses	Variola virus	VARV	
	Monkeypox virus	MPXV	Primates
	Cowpox virus	CPXV	Cat, cattle, exotic animals, rodents
	Vaccinia virus	VACV	Rabbit, cattle, water buffalo
Parapoxvirus	Orf virus	ORFV	Sheep, goat, ruminants, artiodactyls
	Pseudocowpox virus	PCPV	Cattle
	Bovine papular stomatitis virus	BPSV	Cattle
Molluscipoxvirus	Molluscum contagiosum virus	MOCV	
Yatapoxvirus	Tanapox virus	TANV	Rodents
	Yaba monkey tumorvirus	YMTV	Primates

[a]Modified from ICTVdb (http://www.ncbi.nlm.nih.gov/ICTVdb/)

vaccinia virus (VACV) transmission from a vaccinee to an infant (6). To enable a timely treatment of patients and a reliable risk assessment of suspected cases, the most important tasks in poxvirus diagnostics are the identification of the causative agent of poxvirus-like lesions (18). This chapter summarizes diagnostic approaches for the detection of human-pathogenic poxvirus species and a comparison of methods in regard to their speed and reliability. Straightforward protocols for the analysis of clinical specimens are provided for the classically applied electron microscopy and the highly sensitive real-time PCR.

1.2. Orthopoxvirus

Variola Virus (VARV, smallpox) is nowadays the most relevant virus in the context of potential biological crime and bioterrorism (19). Although it was eradicated from nature by vaccination with vaccinia virus, the virus is still stored in two selected high-security WHO depositories, but illegal possession of VARV is suspected. A deliberate release of VARV could have catastrophic consequences in today's increasingly unvaccinated population (20). The natural infection route for smallpox was transmission by direct contact with a diseased individual or contaminated material. In the last century, the mortality was estimated to be 30–40% (21). So far, no natural reservoir for VARV is known, a fact that rendered eradication possible by immunization of the human population. The disease produced skin eruptions after 3–4 days post-infection through several stages of macule, papule, vesicle,

and pustules with a characteristic distribution involving the face and the extremities.

Human infections by monkeypox virus (MPXV) have been found in the rainforest areas of west and central Africa (22, 23), with rodents being the natural reservoir of the pathogen. One outbreak has been described with a zoonotic transmission involving 72 patients in the USA in 2003 (24). Clinical features of human monkeypox are similar to those of smallpox and therefore difficult to differentiate. Hence, after the eradication of smallpox, an MPXV infection is considered to be the most severe human infection with poxviruses.

VACV infection-related complications in humans have been reported since the beginning of vaccination (25). Although, as for smallpox, no natural reservoir for VACV has been identified so far (1); rare but possibly serious complications following vaccination or contact with a recent vaccinee has been documented. These complications include erythema multiforme, congenital vaccinia, generalized vaccinia, progressive vaccinia, postvaccinal encephalitis, and eczema vaccinatum (26). Recently, natural human infections with VACV were observed in Brazil (7). These viruses were closely related to VACV strains formerly used for vaccination and must have survived in unknown animal reservoirs for decades.

The name cowpox virus (CPXV) is derived from its association with pustular lesions on the teats of cows and the hands of milker's, although infections in cows are not common. CPXV infections display a broad host range and are restricted to the Old World with wild rodents as their natural reservoir (27, 28). Over the past 10 years, increasing numbers of human CPXV infections have been recognized due to an increased awareness of physicians and/or increased susceptibility to OPV infections after abandoning smallpox vaccination (2, 29). Human cases with CPXV infections are commonly described after transmission of poxviruses from domestic cats and, in a few rare cases, directly from rats (30). Infections in humans usually remain localized and self-limited on fingers, hands, and face but can become fatal, particularly in immunosuppressed patients (31, 32).

1.3. Parapoxvirus

Orf virus (ORFV) infections, usually named contagious ecthyma, contagious pustular dermatitis, contagious pustular stomatitis, or sore mouth, occurs in sheep and goats and is transmissible to humans by direct contact (33). The virus of pseudocowpox (PCPV) is also called paravaccinia and causes lesions on teats and udders of milking cows (34). The disease is transmissible to humans by direct contact and is called milker's nodule. The bovine popular stomatitis virus (BPSV) causes a mild disease in calves, producing lesions in and around the mouth (35). Recently, parapoxvirus (PPV) from seals infecting humans were also described (36, 37). In humans, all PPV infections caused lesions on fingers, hands and arms and were

usually resolved in 3–4 weeks without the need for a specific therapy.

1.4. Molluscum Contagiosum

Molluscum contagiosum (MOCV) disease occurs in two forms either in children or in young adults (12). Infected children develop lesions on the face, trunk, and limbs and can transmit the virus by direct contact from skin to skin and from vomit to skin. During young adulthood, the lesions are mostly found in the lower abdominal wall, pubis, inner thighs, and genitalia, and the infection is transmissible by sexual contact. Frequently, immunosuppressed individuals are involved with this viral infection (38).

1.5. Yatapoxvirus

The genus *Yatapoxvirus* comprises two species, yaba monkey tumor virus (YMTV) and tanapox virus (TANV) together with the genetically closely related yaba-like disease virus (YLDV) (1). They are transmitted by insects and affect different species of Asian and African monkeys, causing benign epidermal monkeypox. In rare cases of human infections, tumor-like masses in the subcutis are developed (39).

1.6. Poxvirus Infection

Whenever a patient presents with an illness associated with a vesicular or pustular rash, depending on the case history, attention should be directed to poxvirus infections. Assuming that smallpox no longer occurs naturally, the direct contact with pets and farm animals known to harbor zoonotic poxviruses is at least evidence of poxvirus infections (11). However, since human poxvirus infections are usually a rare occurrence, possible differential diagnosis must include viral infections caused by varicella-zoster virus (VZV, chickenpox), herpes simplex virus 1 and 2 (HSV-1, HSV2), and also bacterial infections with *Bacillus anthracis* (cutaneous anthrax), *Bartonella henselae* (cat scratch disease), or *Staphylococcus* (Impetigo). Also, in the early stages of presentation, drug eruptions and insect bites have to be considered.

1.7. Laboratory Diagnosis

With the eradication of smallpox, comprehensive virological and serological poxvirus testing is nowadays available only on a case-by-case basis in specialized laboratories (18).

Investigations of poxvirus infections are done either by indirect or by direct detection methods (40). Indirect poxvirus detection methods (e.g., immunofluorescence assay, plaque reduction test, or ELISA) are used to demonstrate the specific immunological response of an infected human or animal to a poxvirus infection. However, OPV-specific antibodies are not developed in patients until the onset of symptoms, and only a significant greater-than-fourfold increase in convalescent antibody titer over a 2- to 3-week period (postinfection) would result in a positive diagnosis. Therefore, serological methods are usually performed for retrospective studies, but are not commonly used for primary

diagnosis of acute infections. Direct poxvirus detection implies the proof of characteristic poxvirus compounds, such as complete poxvirus particles that can be identified by electron microscopy, poxvirus proteins that are visualized in infected tissues by staining with specific antibodies, or the poxviral genome that can be proven by PCR. In addition, antigen-capture ELISA can also directly show poxvirus particles in specimens with sufficient viral load. The propagation of poxviruses in cell culture or on chicken embryo chorioallantoic membrane (CAM) gives clear evidence of some poxviruses producing specific cytopathogenic effects (CPE) or pocks, respectively.

The inoculation of OPV on the CAM and subsequent examination and harvesting of these membranes was a standard procedure and is nowadays only performed in a few specialized laboratories (41, 42). Nevertheless, CAM inoculation is a sensitive and reliable method for the laboratory confirmation of an OPV diagnosis but takes at least 3 days when 11–13-day-old embryos are available. PPV, TANV, MOCV, and VZV do not produce pocks on the CAM, while other viruses, such as human herpes viruses (HSV-2), may cause confusion as they can produce pocks similar to VARV and VACV.

Cell culture is a useful alternative virus isolation method, and owing to its ability to detect down to levels of one infectious virus particle under optimized conditions, it is regarded as the diagnostic gold standard. OPV generally grow well in most human and nonhuman primate cell lines, although in contrast yatapoxviruses do so very slowly. PPV also cause genus-unspecific CPE in primary ovine or bovine cells, and MOCV are difficult to grow routinely in cell culture. Depending on the poxvirus isolated, characteristic CPEs might be visualized after more than 12 h. Nevertheless, differentiation of poxviruses using either CAM or cell culture will only be able to be distinguished by experienced staff, with considerable training. A direct immunofluorescence assay (DIFA) with poxvirus-specific antibodies can be applied after cell culture propagation even before any signs of CPE are visible, making a diagnostic result possible after a minimum of 6 h. However, the visualization depends on the growth rate of the individual poxvirus. DIFA must be used with caution for any primary diagnosis as the reporting of false-positive results can occur due to problems with background signals in clinical specimens. Only a CPXV infection can be safely diagnosed with specifically stained intracytoplasmic acidophilic-type inclusions (ATI, Fig. 1), which are not observed for other human-pathogenic OPVs. Also, individual CPXV-forming plaques can be morphologically distinguished from other poxviruses by classic plaque assays on the basis of their characteristics. A positive IFA result in combination with indistinctive CPE morphology indicates a possible poxvirus infection, as often seen during the primary isolation of poxviruses from natural hosts. This lack of clarity emphasizes the need for additional confirmatory diagnostic tests such as PCR.

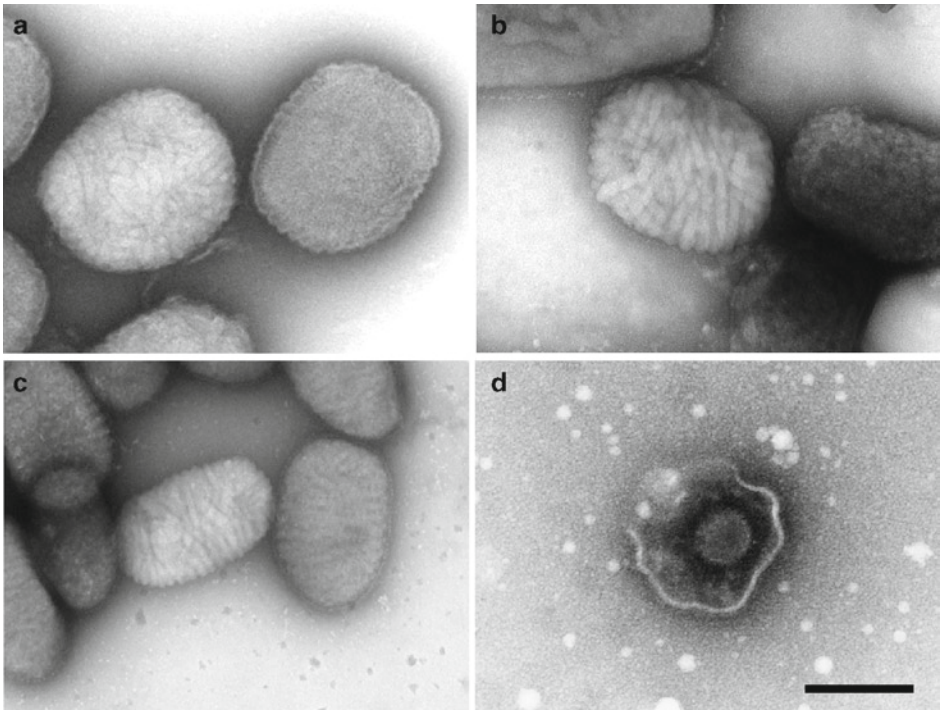

Fig. 1. Diagnostic electron microscopy of distinguishable viruses causing skin lesions in humans. Negative staining with 1% uranyl acetate of OPV (**a**), MOCV (**b**), PPV (**c**), and herpes virus (**d**). Bar = 200 nm.

The use of serological tests to detect poxvirus or poxvirus antigens requires the use of suitable antisera or antibodies. With the introduction of monoclonal antibodies and their use in antigen-capture ELISAs (43), the sensitivity and specificity has increased dramatically with an assay time of just over 3 h to obtain a final result. As of yet, no commercial tests are available, resulting in diagnostic laboratories establishing their own test protocols, which has resulted in variations of sensitivity and specificity. Other less commonly applied methods of detecting poxvirus or poxvirus antigens include immunocytochemistry (44) and the shell vial culture assay (45).

Indirect poxvirus detection methods are used to demonstrate the specific immunological response of an infected human or animal to a poxvirus infection. However, poxvirus-specific antibodies are not developed in patients prior to the onset of symptoms, and only a significant increase in convalescent antibody titer over a 2–3-week period postinfection would produce a positive diagnosis. Therefore, serological methods are rarely used for the primary diagnosis in acute patients.

Although all methods have their relevance for the detailed biological characterization of poxviruses, the primary diagnosis of acute human infections needs to be primarily rapid and unambiguous. Keeping this requirement in mind, the comparison of all available diagnostic methods shows the evident advantages of a combined

EM and PCR diagnosis with respect to speed and reliability. Indirect detection methods proving poxvirus-specific antibodies are present are more useful for retrospective studies. Further, the combined use of EM and PCR techniques enable the detection of all poxviruses and are explained in detail in this chapter.

1.8. Safety Considerations, Specimen Transportation, and Storage

Poxvirus specimens and aseptically drawn sera must be packaged and shipped in a break-proof container in such a way that the materials will not be able to leak in transit. Specimen details and clinical information should be enclosed in the shipping container and must comply with all governmental and airline regulations. Most specimens need no refrigeration if they are shipped to the laboratory by the quickest mode to ensure viability of the virus, otherwise cooling with ice would be sufficient; freezing is not necessary. All handling of clinical material from suspected poxvirus patients must be carried out under biosafety level two conditions. Detailed information regarding specimen collection and transport are provided by the Robert Koch Institute, Germany (http://www.rki.de/cln_091/nn_494682/DE/Content/Infekt/Biosicherheit/Vorsorge/Pockenrahmenkonzept/Ausbildungsmaterialien/ausbildungsmaterialien__node.html?__nnn=true) and the Centers for Disease Control and Prevention, USA (http://www.bt.cdc.gov/agent/smallpox/response-plan/index.asp).

1.9. Specimen Types for Diagnosis

Suitable specimen types for direct detection are vesicular fluid, skin scrapings, dried smears, crusts, lesion swabs, skin and other organ biopsies, and bronchoalveolar lavage (BAL). No transport medium is required. Wherever possible, several lesions should be sampled.

1.10. Preanalytical Specimen Preparation

Lesion crusts and biopsy material should be soaked in three volumes of buffer (e.g., PBS) and homogenized by 10–12 pestle strokes in a dounce homogenizer or an automatic homogenizer for 30 s, e.g., FastPrep®-System. The cell debris and bacteria are removed by low-speed centrifugation (2,000–5,000 × *g* for 10 min). Swab samples may be transferred into 1 ml of buffer (e.g., PBS, Tris-HCl, or HEPES 0.05 M) by vortexing thoroughly, followed by low-speed centrifugation. The supernatants are used directly or stored at –20°C. Aliquots of the obtained sample can now be tested simultaneously using EM, PCR, and cell culture.

2. Materials

2.1. Detection of Poxviruses by Transmission Electron Microscopy

Negative-stain transmission electron microscopy (TEM) is probably the fastest diagnostic frontline method for the confirmation of poxvirus diseases and benefits from its "open view" regarding other distinguishable agents (18, 46–50). Poxvirus infections usually produce skin lesions that contain a substantial number of

viral particles, more than 10^6/ml. Thus, a 5–10% suspension of a clinical specimen usually contains enough particles to allow a positive EM evaluation within 20–30 min. Detailed preparation procedures and the use of direct negative contrast EM of the specimen allow a rapid and reliable virus family-specific detection of the causative virus. However, this method of detection requires considerable investment in EM equipment and the training of specialized staff. Further detailed information on specific aspects of diagnostic electron microscopy is also available on the website of the Consultant Laboratory for Diagnostic Electron Microscopy in Infectious Diseases at the Robert Koch Institute (http://www.rki.de/cln_091/nn_203794/EN/Content/Institute/DepartmentsUnits/NRC/CONSULAB/consulab__node.html?__nnn=true).

2.1.1. Equipment for Transmission Electron Microscopy

1. EM microscope (e.g., Philips Tecnai G2).
2. Copper grids (400 mesh; Plano, Germany), covered with a 20–40 nm layer of plastic (formvar or pioloform), reinforced by a 10–20 nm carbon layer and increased hydrophilicity by glow-discharge (alternatively or in addition with 1% alcian blue, 10 nM poly-l-lysine, UV light; see Note 1).
3. Fine pointed forceps appropriate for grid handling.
4. Petri dish, laid out with filter paper for stained EM grids, Parafilm, and filter paper wedges.

2.1.2. Reagents for Transmission Electron Microscopy

1. Washing reagent, e.g., double-distilled water or 0.05 M HEPES buffer pH 7.2.
2. Negative-staining solution, e.g., uranyl acetate (UAc, 1%), phosphotungstic acid (PTA, 2%), ammonium molybdate (AmMo, 1%), or methylamine tungstate (MAT, 1%).

2.2. Detection of Poxviruses by Polymerase Chain Reaction

The polymerase chain reaction (PCR) is well accepted as the most sensitive molecular method for the detection of viruses (51, 52). Bearing in mind that the mere detection of the virus' nucleic acid usually does not prove replication competence and infectivity of a pathogen, in clinical specimens it can be a significant marker for poxvirus infections.

Depending on the medical history of the patient, the presence of different poxvirus genera and species should be analyzed. Here, we describe detection protocols for the most common human poxvirus infections: OPV, PPV, and MCV. Although diagnosed very rarely in humans, an additional protocol for the detection of yatapoxviruses is presented. For further virus typing, sequence-based techniques are usually applied, including sequencing of the PCR product, digestion with defined restriction enzymes, or identification of single nucleotide polymorphisms by melting curve analysis.

The protocols described include the recognition and differentiation of human-pathogenic poxvirus genera. Approaches to species differentiation are described referring to the respective references for detailed protocols.

The most exact molecular virus typing can be performed by the sequencing of gene stretches amplified by PCR. Crucial genes for OPV differentiation are the hemagglutinin (HA) (53), the acidophilic-type inclusion body (ATI) (54), and the crmB gene (55). Similarly, PCR assays that amplify OPV with species-specific amplicon lengths have been established (56). Recently, a pangenomic approach to OPV classification was published (57). However, for diagnostic purposes, these assays are too laborious and time consuming. For other human poxviruses, few conventional PCR assays have been published (58).

To date, real-time PCR approaches as an advancement of PCR combine several benefits a diagnostic tool should provide: real-time PCR is fast, requires no post-PCR handling, and is therefore safe, enables high throughput analysis, and the option of quantification and genotyping of the detected nucleic acid is available in one reaction tube (52, 59).

For OPV, combinations of OPV-specific amplifications with a subsequent virus type-specific detection by LightCycler hybridization probes to determine VARV or VACV presence by melting temperature have been published (60). Similarly, the application of OPV-generic primers and a VARV-specific Minor-Groove-Binding 5 nuclease probe (MGB probe) for the HA gene resulted in the specific detection of VARVs, as shown by analyzing several OPV isolates (61). Only VARV displayed amplification curves in real-time PCR, while other OPVs showed no curves. These assays produced easily interpretable results. Likewise, in other assays based on the application of specific 5 nuclease probes, the generated fluorescence signal height reflects the perfect match of the 5 nuclease probe, which was utilized as an indication of VARV amplification. This approach can be difficult to interpret in situations where signal height shows minor differences from the controls (62).

Also based on generic amplification of OPV, VARV-specific SNPs have frequently been characterized by real-time PCR with variola-specific hybridization probes using a fluorescence melting curve analysis (FMCA), which takes approximately 10 min subsequent to the PCR run. Well-designed assays can clearly differentiate the virus species exactly matching the hybridization probe from others displaying mismatches in the probe-binding region (51). The latter are represented by a decreased melting temperature that differs from the melting temperature of the perfectly matching virus type by at least 3–5°C, depending on the number and kind of mismatches. In the last few years, a couple of FMCA-based assays have been

published for VARV. Due to the high number of unknown OPV sequences, this created a number of problems; however, the situation was greatly improved by the recent publication of several VARV and further OPV genomic sequences in Genbank. Even so, some of these assays proved unable to distinguish VARV from other *Orthopoxvirus* species (63, 64). However, there are other VARV-specific assays that have been carefully established utilizing various gene regions from several virus isolates. To detect all OPV species, more conserved regions of the OPV genome in comparison with HA and crmB were chosen, e.g., RNA polymerase gene (60), the gene coding for the early transcription factor (60) or the 14-kDa fusion protein of OPV (65). While the first assays used hybridization probes which perfectly matched VARV, further assays identified variola by a variola-specific melting temperature (see Table 2).

For poxvirus-differential diagnosis of VARV infection, several assays have been developed, reliably identifying further human-pathogenic poxviruses like VACV (66), MPXV and nonvariola OPV (67), TANV (68), and PPV (69, 70). The methods described below cover the identification of orthopoxviruses, parapoxviruses, and molluscipoxviruses.

2.2.1. Equipment for Polymerase Chain Reaction

1. Real-time PCR cycler based on 96-well plates. The methods described have been proven to work reliably with AB7500 (Applied Biosystems), LightCycler 480 (Roche Applied Science), MX3005 (Stratagene).
2. Heat-sealing device.
3. Vortex.
4. Heating block.
5. Centrifuge for Eppendorf tubes.
6. Centrifuge for 96-well plates.
7. Pipettes with disposable tips.

2.2.2. Reagents for Polymerase Chain Reaction

1. DNA extraction from the homogenate is subsequently performed using the QIAamp DNA Mini-Kit (250) #51 306, Qiagen.
2. PCR Reaction Mix uses the Platinum Quantitative PCR SuperMix, Cat. No. 11730-017, Fa. Invitrogen.
3. Oligonucleotides used were stored as stock solutions at –20°C in a concentration of 10 mM (Table 3). Ideally, aliquots are adjusted in volume to be thawed only once. Repeated freeze–thawing may impair oligonucleotide quality.

Table 2
Published real-time PCR assays for poxvirus detection

	Target gene	LOD	Chemistry	Specificity	Determined by	T_m/°C
Orthopoxvirus						
Espy	HA	5–10	Hybridization probes	VARV	FMCA	VARV 62.5, OPV 56.7
Ibrahim et al. (72)	HA	12–25	5 nuclease	VARV	Signal generation	–
Kulesh	HA DNA pol HA B9R B10R	12–23 12–23 12–23 12–23 12–23	5′ nuclease MGB 5′ nuclease MGB 5′ nuclease MGB 5′ nuclease MGB 5′ nuclease MGB	OPV OPV VARV VARV VARV	Signal generation Signal generation Signal generation Signal generation Signal generation	– – – – –
Nitsche	rpo18 VETF A13L	5–10 5–10 5–10	Hybridization probes Hybridization probes Hybridization probes	VARV VARV VARV/AMP, FMCA	FMCA FMCA Amplification, FMCA	VARV 62.0, OPV 59.0VARV 63.0, OPV <58.0 VARV 65.0
Panning	HA	3–10	Hybridization probes	VARV	FMCA	VACV/CPXV/MPXV >63.0 VARV 54.9 CMLV 47.0
Olson	14 kDa	4	5′ nuclease/1 hybridization probes	VARV	FMCA	OPV>61.0, VARV 56–58
Aitichou et al. (73)	HA	50–100	LUX	VARV Non-VARV OPV	TET signal generation FAM signal generation	–
Scaramozzino	14 kDa	<25	5′ nuclease	VARV	Signal magnitude	–

(continued)

Table 2
(continued)

	Target gene	LOD	Chemistry	Specificity	Determined by	T_m/°C
Fedele et al. (74)	crmB	100	5′ nuclease MGB	VARV Non-VARV OPV	FAM signal generation VIC signal generation	– –
Li	E9LB6R	1010	MGB Eclipse probe MGB Eclipse probe	NVAR-OPV MPXV	Signal generation	– –
Nitsche	IFNγ	10	Hybridization probes	VACV	Signal generation and FMCA	VACV 59.0 Non-VACV OPV <50.0°C
Parapoxvirus						
Nitsche	Major evelope protein	10	5′ nuclease MGB	ORFV BPSV PsCPXV SPV	Signal generation	–
Gallina	Major evelope protein	10	5′ nuclease MGB	ORFV	Signal generation	–
Yatapox						
Zimmermann	13L	8	5′ nuclease	TPV YLDV	Signal generation	–
Molluscipox						
Nitsche, unpublished	MC001R	10	5′ nuclease	MOCV	Signal generation	–

LOD limit of detection in genome equivalents per reaction, T_m melting temperature as observed in FMCA, *LUX* light upon extension, *NVAR-OPV* CMLV, CPXV, VACV, MPXV, TATERAPOX, ECTV

Table 3
Oligonucleotides recommended for poxvirus detection by real-time PCR[a]

	Orthopoxvirus		**X69198[b]**
OPV rpo F1	CTgTAgTTATAAACgTTCCgTgTg	S	93663-86
OPV rpo R1	TTATCATACgCATTACCATTTCgA	A	93867-44
OPV rpo TM	F-ATCgCTAAATgATACAgTACCCgAATCTCTACT q	A	93826-94
	Parapoxvirus		**AY278208**
Ppox up	TCgATgCggTgCARCAC	S	455-471
Ppox do neu	gCggCgTNTTCTTCTCggAC	A	539-520
Ppox TMGB	F-TgCggTAgAAgCC MGB	S	489-501
	Molluscum contagiosum virus		**M98814**
MC F	CAAgTACAACTTCgTggCgTTT	S	189-210
MC R1	CCTACACggAACAgAATgCTTTg	A	257-279
MC TM	F-CCACACggAACgAgAACATCTTTgg q	A	254-230
	GAPDH		**J02642**
GAPDH F	gAAggTgAAggTCggAgTC	S	7-25
GAPDH R	gAAgATggTgATgggATTTC	A	232-213
GAPDH TM	F-CAAgCTTCCCgTTCTCAgCCT q	A	203-183

[a]In 5′→3′ direction, *S* sense orientation, *A* antisense orientation
[b]Position in GenBank entry/F:FAM/q: dark quencher

3. Methods

3.1. Detection of Poxviruses by Transmission Electron Microscopy: Negative Staining

1. Place 20–50 µl of the cleared, transparent specimen supernatant onto a sheet of Parafilm.
2. Place a hydrophilic grid onto the drop's surface for 5 min adsorption time (see Note 2).
3. Add several washing drops and one drop of staining solution in line onto the Parafilm (see Note 3).
4. Wash the grid with adhering virus by touching the grid surface onto a washing drop and drain the fluid by carefully dabbing the grid from vertically above onto a dry filter wedge.
5. Repeat step 4 with each washing drop (see Note 4).
6. Float the grid on the drop of staining solution for 3 s (UAc, AmMo, MAT) or 30 s (PTA) and remove the fluid by dabbing the grid on the damp edge of a filter paper wedge and place the grid into a Petri dish laid out with filter paper numbered according to the samples used. The stain thickness

depends on the dabbing angle. Allow the grid to air-dry for 3 min before evaluating by TEM (see Note 5).

7. Clean and disinfect used forceps (see Note 6).

3.2. PCR: Isolation of Viral and Control DNA

1. To avoid carryover contamination with PCR products, the DNA extraction is performed in a separate room by personnel wearing lab coats exclusively used there. Extraction of DNA from skin lesions or scabs requires homogenization in a Tissue Lyser (e.g., FastPrep, MP Biomedicals) according to the manufacturer's instructions.
2. Prepare 200 µl of a homogenized clinical sample and use virus-negative material as a control (see Note 7).
3. Use the QIAamp DNA Mini-Kit according to the manufacturer's instructions.
4. Briefly, digest 200 µl homogenized specimen with Proteinase K, to release the DNA from cells.
5. Add the digest to the silica matrix of the preparation columns to bind the DNA.
6. Elute the DNA in 100 µL (e.g., QIAamp elution buffer, TE, water PCR grade) after repeated washing steps (QIAamp washing buffer).
7. Use 5 µL of the eluted DNA for one PCR reaction, store the remainder at 4°C for repeat testing.

3.3. Real-Time PCR Mix

1. Prepare the real-time PCR master mix for each poxvirus-specific assay and the DNA control assay in a template DNA-free box or room, according to Table 4.
2. Prepare enough master mix for two positive controls, two negative controls, and duplicates of each clinical specimen.
4. The formula given in Table 4 can be used for all PCR assays presented here.
5. Transfer exactly 20 µL of the master mix to each required well of a 96-well PCR plate suitable for the real-time PCR cycler used.
6. It is important to then work in an area (bench, box, or room) which is DNA-free and where DNA handling is allowed.
7. Add 5 µL of the poxvirus DNA samples to the master mixes for poxvirus detection and the DNA control assay.
8. Add 5 µL of DNA of the positive control(s) and negative control(s) to the master mixes.
9. Heat-seal the plate according to the plate brand and heat-sealing machine.

Table 4
Preparation protocol for the real-time PCR master mix

	Volume (μL)
2× Platinum Supermix	12.5
$MgCl_2$ (50 mM)	1.0
Primer F (10 mM)	0.75
Primer R (10 mM)	0.75
Probe TM (10 mM)	0.25
ROX (1 mM)	0.5
PCR water	Add 20.0
Template DNA	5.0
Total volume	25.0

It is recommended to multiply the volumes by the number of reactions and to add 10% to factor in pipetting inaccuracies. This protocol can be used for all poxvirus detection assays presented here

Table 5
Real-time PCR cycling conditions

Temperature (°C)	Time (s)	Cycles
50	120	1×
95	600	1×
95	15	40×
60	30	40×

10. Centrifuge the plate at 1,000 rpm for 5 min to remove air bubbles that may influence fluorescence signal detection.
11. Directly place the plate into the real-time PCR cycler.

3.4. Cycling Conditions

1. Set the real-time PCR cycler as described in its respective manual to the cycling conditions given in Table 5. These cycling conditions are valid for all PCR assays presented here, meaning that all assays can be performed in parallel in the same run on the same cycler.
2. For the presented FAM-labeled probes, choose the channel that detects fluorescein. Other fluorescent labels will also

work, depending on the excitation and detection options of the real-time PCR cycler.

3. Detection of fluorescence signals should be set to the combined annealing/elongation step at 60°C (underlined in Table 5).

3.5. Analysis

3.5.1. Transmission Electron Microscopy: Negative Staining

The time and primary magnification used for TEM-evaluation should be standardized, using samples of known virus concentration. Use at least 15 min/grid and two different magnifications (e.g., 10,000× and 40,000×) to enable detection of also low-titered agents or double infections. If "negative", particle enrichment techniques can also be used, e.g., ultracentrifugation or airfuge.

Typical poxviruses can be visualized as a capsulated (C) or mulberry (M) form, depending on the penetration of the staining material into the virion. When examining grids of specimens, one must learn to recognize nonviral particles and artifacts that resemble poxviruses to avoid false-positive results, which can be a common problem when examining clinical samples or staining artifacts in the support film of the grid (10).

Although all OPV are similar in size and morphology and cannot be differentiated by EM, they are distinguishable from PPV, MOCV, and herpes viruses (Fig. 1). OPV are brick-shaped with a size of 300–350 nm × 140–170 nm showing short surface ridges. PPV are ovoid with a size of 220–300 nm × 140–170 nm showing surrounding bundles of spirals. MOCV are very similar to OPV, distinguishable only by their more prominent surface tubules like those on the surface of yatapoxviruses. VZV and other human herpesviruses are clearly distinguishable with their enveloped icosahedral capsid of about 100 nm in diameter.

3.5.2. Analysis of Polymerase Chain Reaction

1. After the run is finished, use the software to analyze the data.
2. Evaluate the performance of the run by checking the controls.
3. All negative controls must not show a fluorescence increase above the threshold value until the last cycle (cycle 40). If the negative controls produce a positive result, the run must be repeated.
4. All positive controls should show the expected CT values (see Note 8) with a nonsignificant variation (±0.5 CT values are acceptable). When positive controls remain negative and the clinical specimens are also negative, again the run should be repeated.
5. If the negative DNA control PCR (GAPDH) is positive, only the run has to be repeated.

6. A negative DNA positive control indicates a failure in DNA preparation. In this case, the complete procedure has to be repeated, starting with the DNA extraction.
7. Providing that the controls work as expected, any clinical specimens are declared positive if the fluorescence signal is greater than the threshold in both duplicate samples.

4. Notes

1. Particle adsorption to the grid depends widely on the grid's surface charges. More detailed descriptions of grid preparation techniques can be found in ref. 48 and Nakano et al. (42). Also, plastic and carbon-coated grids are readily available through most electron microscopy suppliers.
2. Alternatively, if the amount of specimen volume is insufficient, place 3–5 μL on top of a grid secured in forceps with reverse action.
3. The number of washing drops varies from 1 to 5, depending on the purity o the clinical sample.
4. Washing will inevitably reduce the number of particles on the grid. Therefore, a compromise has to be found between particle content, purity, and stain distribution on the grid.
5. While checking the stained grid on the TEM, keep the specimen drop on the Parafilm protected from dust and drying. This helps to shorten preparation time, in case you need additional grids for EM inspections.
6. Always use different forceps for each specimen to avoid cross-contamination.
7. Each analytical PCR run requires the parallel amplification of at least two positive controls and at least two negative controls. The positive control usually consists of DNA from a positive clinical specimen or a plasmid containing the target region of the PCR (plasmids for the presented assays can be obtained from the authors on request). The concentration of the positive control should be low and adjusted to higher CT values, which reduces the risk of cross-contamination by the control DNA. Positive controls should be prepared so that they result in CT values of ~30–33 (between 100 and 1,000 plasmid copies). These concentrations generate reproducible positive amplification curves while reducing cross-contamination. Ideally, virus-negative tissue is used as a negative control, for example, DNA prepared from noninfected human cells. The DNA from the control should be prepared in parallel to

the clinical specimen to check for contamination during the preparation process. To ensure the successful isolation of DNA from the clinical material, apply a PCR assay that amplifies human DNA. Several of these assays are published and can be applied for that purpose. Traditionally, DNA of the GAPDH gene was used as reference for confirmation of amplifiable DNA in the specimen (71). The relevant primers are given in Table 1.

8. One of the most prominent advantages of PCR is the very effective amplification of the target DNA. This process comes with the risk of carryover contamination. Each PCR product can naturally serve as start molecule for a new PCR run using the same primers. Since the described assays are able to detect as little as ten copies per reaction and a successful run of 40 cycles produces about 10^{12} copies of the respective DNA fragment, it becomes obvious that a broken tube containing a completed PCR reaction can rule out the application of this PCR in the same laboratory for quite a long time due to the risk of producing false positive results. Therefore, the application of different countermeasures to contamination is highly recommended in a diagnostic facility. First of all, there should be separate and defined benches or, in the best case, rooms equipped with boxes that are used for: (1) DNA extraction, (2) Preparation of the reaction master mix, (3) Addition of the DNA to the mix, and (4) Performance of the PCR reaction. The workflow should be considered as one-way workflow only once a day. Secondly, performing real-time PCR means an online generation of results. Therefore, reaction tubes or plates containing completed reactions should never be opened. To assure that accidentally opened or broken reaction vessels cannot lead to contaminations, one could include the use of didesoxy uracil triphosphate (dUTP) instead of the thymidine triphosphate (dTTP) in the reaction master mix. In subsequent PCR reactions, the incorporation of UTP during the amplification allows the use of the enzyme uracil-N-glycosylase prior to the amplification cycles, which digests exclusively DNA fragments containing Uracil, but not thymidine. By this simple process, artificially amplified DNA fragments can easily be distinguished from naturally occurring thymidine-containing DNA. Finally, all diagnostic laboratories should be working to good laboratory practice.

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Chapter 16

Lyssaviruses: Special Emphasis on Rabies Virus and Other Members of the Lyssavirus Genus

Graeme Harkess and Anthony R. Fooks

Abstract

Rabies is routinely diagnosed based on the clinical description and history of exposure in a rabies-endemic country. A negative diagnostic test for rabies virus or a related lyssavirus does not exclude the clinical diagnosis. Diagnostic tests are never optimal and are entirely dependent on the nature and quality of the sample supplied. Often, only a sample from a single time point is investigated reducing the overall sensitivity of any diagnosis. With the advent of molecular biology, tests have been developed that are rapid, robust, and sensitive in support of the rapid detection and strain identification of rabies virus from clinical specimens. These molecular tests complement conventional tests in rabies diagnosis, particularly for human cases, for which an early laboratory diagnosis is critical and may decrease the number of unnecessary contacts with the patient, reduce the requirement for invasive and costly interventions, and enable the appropriate medical treatment regimen to be administered for the patient. The barrier to success is in transferring the technology for the latest techniques in rabies diagnosis to rabies-endemic countries. These barriers are not insurmountable and in liaison with international organisations, especially OIE, FAO, and WHO, these diagnostic tests will be validated for rabies diagnosis and surveillance, and implemented in modern and well-equipped diagnostic laboratories throughout the world.

Key words: Rabies, Lyssavirus, RNA virus, Zoonosis, Diagnostic assay

1. Introduction

Rabies is a fatal zoonotic disease that infects all warm-blooded mammals, including humans. (1, 2) Greater than 50,000 people die each year of rabies; however, this figure is thought to be highly conservative given the number of cases that currently go undiagnosed (3). Classical rabies virus is one of the seven species within the Lyssavirus genus, all of which cause clinically indistinguishable disease in various mammal species. There are known animal reservoir species, specific for the different genotypes of lyssaviruses,

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Table 1
Lyssavirus genotypes

Genotype	Virus	Host species
1	Classical Rabies virus	Carnivores almost worldwide, bats in the Americas
2	Lagos Bat virus	Frugivorous and insectivorous bats in Africa
3	Mokola virus	Shrews/cats and small rodents in Africa
4	Duvenhage virus	Insectivorous bats in southern Africa
5	European Bat Lyssavirus type-1	Insectivorous bats in Europe (*Eptesicus serotinus*)
6	European Bat Lyssavirus type-2	Insectivorous bats in Europe (Myotis sp.)
7	Australian Bat Lyssavirus	Frugivorous and insectivorous bats in Australia

in which the virus is maintained and circulated in the wild (Table 1).

Infection from a reservoir host into another species is referred to as a "spillover" event and is ultimately a dead-end for the infecting virus, as propagation into further hosts is unlikely.

Infection starts via transmission from a bite of an infected animal; the virus then accesses the peripheral or central nervous system from infected saliva, and the virus particles make their way up into the brain via the spinal column. Symptoms of infection in humans usually develop within 30–50 days post-exposure (although longer incubation times have been recorded). Symptoms in patients are numerous and can include malaise, loss of appetite, fatigue, headache, and fever. Although less common, numbness at the site of inoculation, insomnia, or depression can also be reported. Between 2 and 10 days after the initial symptoms develop, damage to the nervous system becomes evident, and symptoms such as hyperactivity, hypersensitivity, disorientation, seizures, or paralysis can occur at various degrees. Death always follows and is mostly caused by cardiac or respiratory arrest.

1.1. Importance of Diagnosis

Once an infected patient develops clinical signs of rabies, the course of disease is always fatal. Rabies is the only disease with a 100% fatality rate after the onset of clinical symptoms. Owing to the severity of the disease and the large number of symptoms, it is important to make an initial clinical diagnosis followed by a laboratory-based confirmation, to distinguish the infection from other forms of viral encephalitis (4). For this reason, a number of diagnostic techniques have been standardised internationally for laboratory diagnosis of rabies viruses by the World Health Organisation (WHO) and the World Organisation for Animal Health (OIE). In human cases, it is essential that post-exposure prophylaxis (PEP) is administered at the earliest opportunity after

an exposure, as prophylaxis prior to the clinical disease appearing is the only effective treatment against the disease. The correct diagnosis of the disease is also important for epidemiological reasons, as well as for patient isolation and the medical management of individual cases (5).

1.2. Principle of Techniques Used for Rabies Diagnosis

There are a number of different technologies used in the diagnosis of rabies infection (6, 7). Rabies virus antigen detection techniques such as the fluorescent antibody test (FAT) or the rabies tissue culture infection test (RTCIT) are used to detect virus particles either directly in tissue samples (FAT) or indirectly in tissue culture (RTCIT). Both these tests rely on the ability to bind a detector molecule (usually fluorescein isothiocyanate [FITC]) to a rabies virus specific-antibody to form a conjugate that will bind to and allow the visualisation of rabies antigen using fluorescent microscopy techniques. The fluorescent antibody virus neutralisation (FAVN) test uses a similar principle but is used to measure the level of virus neutralising antibody (VNA) levels in vaccinated/exposed individuals. In this test, the serum is first incubated with a known titre of virus, allowing the VNA within the serum to neutralise this virus. Baby hamster kidney (BHK) cells, which any virus that was not neutralised will infect, are added; the virus can then be visualised using an FITC conjugate. The amount of virus remaining corresponds inversely to the amount of neutralising antibody present in the sample. The development of pseudotype viruses (8) has allowed non-infectious virus particles to be used as a substitute for live virus in FAVN-related tests (see Subheading 1.5.1). These particles are produced by assembling a lyssavirus protein coat with a lentiviral core and have been shown to produce a comparable antigenicity to live viruses, with the advantage that they are non-infectious and the particles can be handled at lower containment levels. The in-situ hybridisation (ISH) method (Subheading 1.5.2) is adapted from the principle that a specific antibody will bind to a specific antigen. This technique uses digoxigenin (DIG)-labelled probes to enable the visualisation of virus within fixed tissue and allows studies of the locality of the virus within tissues.

The use of monoclonal antibodies for characterisation of rabies viruses is a useful tool to distinguish rabies species. Once the monoclonal antibody is raised against a particular strain or virus, it can be used to probe other related strains to see how specifically it binds (9). An antigenicity profile can be generated using a panel of monoclonal antibodies raised against different viruses.

More recently, the advent of molecular biology has changed the face of diagnostic virology, with regard to the detection of lyssaviruses. Molecular techniques based on the detection and manipulation of the genetic information of the virus allow for the high-throughput, low turnaround-time analysis of samples. Once viral RNA has been extracted from a sample, there are a number

of techniques available for its detection. The polymerase chain reaction (PCR) allows the detection of specific rabies virus RNA. Specifically, total RNA is extracted from suspect tissue (using a suitable method such as phenol/chloroform extraction), and once a reverse transcriptase step has been performed to create double-stranded complementary DNA (cDNA), it is possible to amplify virus cDNA by using the PCR technique. The development over the last few years of "real-time" PCR allows the quantification of the RNA in "real-time," giving a relatively quick and reliable method for the quantitative detection of viral RNA.

1.3. Specimen Collection and Test Choice

When deciding on which test is going to be used, it is important that an assessment is made on the available clinical information with regard to its interpretation. The FAT will detect viral antigen only. The RTCIT, however, is designed to detect replicating "live" virus. A FAVN test will detect antibody raised against rabies viruses, but it cannot determine the nature of the antibody (i.e. vaccine or acquired immunity) – an accurate medical history is, therefore, essential to support the interpretation of results. The molecular techniques involving PCR also need to be used carefully. The PCR is an extremely sensitive process, and appropriate controls must be used if results are to be relied upon. It should be noted that PCR tests detect viral RNA, and this alone is not indicative of an active infection, or of replicating live virus within the host tissue (Table 2).

As with the choice of test, the choice of sample used in rabies diagnosis is equally important. Owing to the pathology of lyssavirus infections, samples of brain tissue are the most reliable ones

Table 2
Comparison of the common tests currently used in rabies diagnostic testing

Test	Use	Unit cost	Turn-around time	Sensitivity %	Specificity %
FAT	Confirmatory test	Cheap	2–4 h	Medium	High
RTCIT	Confirmatory test	Moderate	5 days	High	Medium
FAVN	Measuring antibody levels	Moderate	3 days	High	High
MIT	"Gold Standard" confirmatory test	Expensive	28 days	High	Medium
RT-PCR	Screening	Cheap	2 days (inc RNA extraction)	High	High
Real-time PCR	Screening	Cheap	1 day (inc RNA extraction)	High	High

for detecting live virus. With the obvious difficulties associated with sampling from this area, especially in human ante-mortem patients, it is not always possible or advised. At post-mortem, the brain offers the most suitable sample for diagnostic testing. Brain sampling methods vary from animal to animal, and in small mammals such as bats and mice, removing the brain and testing a homogenate using FAT, RTCIT, MIT, or PCR is highly sensitive and reliable. In larger mammals such as dogs, it is possible to be more selective in areas of the brain that are used in obtaining a diagnosis. The pons, medulla, and thalamus are the most reliable areas to sample, but the hippocampus and cerebellum are also acceptable and easier to distinguish morphologically. The brain should be removed from the cranium as carefully as possible, ideally in a biological safety cabinet to contain any aerosols that may be generated. Using a scalpel, the brain should be sectioned and each part tested individually or pooled. In human cases, the nature of ante-mortem brain sampling is extremely difficult. Specific samples must be used for the detection of rabies virus depending on the clinical situation (2).

1.3.1. Oral Secretions/ Saliva

Although virus will only be present in saliva in the latter stages of infection (generally once clinical symptoms appear), in conjunction with other tests, saliva can be a reliable diagnostic sample (4). It is important that the sampling is accurate, as sputum or respiratory secretions are not appropriate. Testing is limited to RTCIT, MIT, or PCR, as the FAT is unreliable in this sample type.

1.3.2. Nuchal Region Neck Biopsy

In man, neck biopsies – if taken correctly – can be an excellent indicator of a rabies infection (10). Viral antigen can be detected by FAT staining on frozen sections. PCR can be undertaken on homogenised tissue that has undergone a suitable nucleic acid-extraction procedure.

1.3.3. Cerebral Spinal Fluid

Cerebral spinal fluid (CSF) is not an ideal sample for testing for rabies, as the virus is not always shed into the fluid surrounding the brain. However, in the latter stages of infection (and later than can be detected in the blood) antibodies to the virus may be detected in the CSF using the FAVN test. This information may be of use when the vaccination history is unknown, or unavailable, because as opposed to detection of antibody in the blood (which could come from vaccination/previous exposure), antibodies present in the CSF are indicative of a rabies encephalitis.

1.3.4. Serum

As mentioned above, differentiating between antibodies derived from exposure to the virus and those obtained by vaccination by FAVN is not possible in blood samples. Therefore, although a reliable way of ensuring vaccination has been successful, antibody testing of serum cannot be used alone to determine infection.

Table 3
Relative sensitivities of various diagnostic tests from human patients in the USA between 1980 and 1996

	Detection of antigen			Isolation of virus	Detection of RNA	Detection of antibodies	
Sample	Cutaneous nerve	Corneal epithelium	Brain biopsy	Oral secretions		Serum	CSF
Number of cases with samples submitted for testing	20	9	3	15	15	22	15
Number of positive diagnosis's	15	3	3	9	15	11	3
% Positive	75	33.3	100	60	100	50	20

Adapted from Jackson, Wunner. Rabies. 2nd Edn. Original data: Noah et al. 1998

1.3.5. Corneal Impressions

This test has been shown to produce positive results in rabies patients (using FAT and PCR), but it is very difficult to obtain a suitable specimen (a clean glass microscope slide is rubbed vigorously onto the corneas) and should only be undertaken by an ophthalmologist. With other more reliable and less invasive tests available, corneal impressions should not be considered as a first-line testing method.

Table 3 is a summary of sample sites and their relative recovery rates from human patients, taken from a study of patients in the USA between 1980 and1996.

1.4. Safety

Rabies virus and all other lyssaviruses are categorised by the UK Advisory Committee on Dangerous Pathogens (ACDP) as level 3 pathogens to human health and are designated as Specified Animal Pathogens Order (SAPO) level 4 pathogens for animal health. When working with live virus or suspected cases of rabies, all work should be undertaken under class 3 biological safety conditions to avoid possible exposure. It is advisable to seek local guidance on all matters relating to these safety issues before commencing any work with ACDP3/SAPO4 pathogens.

There are a number of commercial vaccines available to protect against rabies (phylogroup I) viruses, and it is advisable that workers who undertake studies with these viruses are appropriately vaccinated. It should be noted, however, that the vaccines currently available are not efficacious against the phylogroup II lyssaviruses (i.e. Mokola virus, Lagos Bat virus). Effective PEP is also available and highly effective for the phylogroup I rabies viruses, at preventing disease. Again, however, it should be noted that treatment against phylogroup II viruses by PEP has not been established.

1.5. New Techniques

1.5.1. Neutralisation Assay Using Lentiviral Pseudotypes

Current surveillance techniques rely on conventional serological assays (FAVN/RFFIT) to measure VNAs in serum samples. However, as these tests require the addition of "live" replicating virus, it can only be undertaken in an ACDP3/SAPO4 facility. As most rabies endemic regions are in developing countries, where there are few facilities of this type, it is not possible to routinely undertake this test. Wright and colleagues (8) have circumvented this problem by creating a non-infectious, pseudotype lyssavirus for use in the detection of rabies VNAs. These virus particles are constructed in vitro and have the core of the Human Immunodeficiency Virus (HIV) and the envelope protein (the glycoprotein) of the lyssavirus. This novel virus-like particle has no replication processes, so, although it can form viral particles and infect cells (and critically – display viable lyssavirus surface antigen with which the VNAs bind), they cannot actively replicate within the host cell and so are non-infectious. This means that they can be handled within category 2 facilities. The addition of a fluorescence gene (such as "green-fluorescent protein" of firefly luciferase) into the construct of the pseudotype allows for a reporter signal to be detected without the need for an FITC conjugate. Wright and colleagues (8) have shown that their modified test acts with both high sensitivity and specificity towards sera previously tested with a traditional FAVN. Although in its early stages, this is potentially a very important step in bringing rabies surveillance to areas of the world where there are no facilities to handle live virus safely.

1.5.2. ISH for the Detection and Strain Differentiation of European Bat Lyssaviruses

This test allows the detection of specific rabies (and related rabies virus) messenger and genomic RNA within samples that have previously been formalin-fixed and mounted onto glass slides for histopathology.

After a de-waxing step, specific probes are hybridised to the sample and then detected using non-radioactive DIG-labelled riboprobes based on the nucleoprotein gene. The method allows for the visualisation of classical rabies virus, EBLV-1, and EBLV-2 virus RNA transcripts within wax-embedded fixed tissue samples.

2. Materials

2.1. Fluorescent Antibody Test

2.1.1. Principle of Test

The most widely used test and regarded as the "Gold Standard" for rabies diagnosis is the FAT, which is recommended by both the WHO and OIE. In rabies infected cells, specific inclusions consist of nucleocapsid aggregates of virus particles. Murine antibodies, conjugated to FITC, will specifically stain these inclusions by direct immunofluorescence. With correct sampling of the brain, this test can supply a quick and accurate result in less than

2 h. Composite brain samples (including the brain stem) are fixed in high-grade cold acetone and stained with a specific conjugate. This slide is then viewed under a fluorescence microscope.

2.1.2. Materials

2.1.2.1. Buffers and Reagents

1. Anti-rabies FITC conjugate (Fujirebio Diagnostics Inc. 201 Great Valley Parkway, Malvern, PA 19355).
2. Acetone.
3. Distilled H_2O.
4. 0.1 M Phosphate buffered saline (PBS) pH 7.4.

2.1.2.2. Equipment

1. Microscope slides.
2. Coplin jar.
3. Freezer capable of maintaining −70°C (±2%).
4. Humidified CO_2 incubator capable of maintaining −37°C (±2%).
5. Inverted fluorescent microscope capable of excitation at 488 nm (FITC), using an excitation filter with narrow pass-band windows in the blue (475–490 nm).

2.2. Rabies Tissue Culture Infection Test

2.2.1. Principle of Test

The RTCIT is a confirmatory test for the isolation and culture of Lyssavirus from a homogenate of suspect tissue in mouse neuroblastoma cells. The test is OIE prescribed and can be used to detect rabies virus in a range of specimen types. The inoculum is introduced into the cells and incubated for up to 4 days. After this period, the cells are stained with FITC conjugate and examined by direct immunofluorescence for nucleocapsid inclusions within the neuroblastoma cells.

2.2.2. Materials

2.2.2.1. Buffers and Reagents

1. Antibiotic trypsin versine (ATV) solution.
2. Antibiotic solution (so when added to 1 L of MEM gives the following concentrations: penicillin 100 U/ml; streptomycin 100 µg/ml; mycostatin 25 U/ml).
3. 0.1 M PBS (pH 7.4).
4. PBS plus antibiotics (at concentrations mentioned above).
5. Anti-rabies FITC conjugate (Fujirebio Diagnostics Inc. 201 Great Valley Parkway, Malvern, PA 19355).

2.2.2.2. Media

1. Tissue culture minimal Eagle's media (MEM): Glasgow MEM (containing glutamine, GIBCO 21710—25) 400 ml, penicillin/streptomycin antibiotic solution (100×) 5 ml, decomplemented foetal bovine serum 50 ml, tryptose phosphate broth 50 ml, nystatin suspension (400×) 1.25 ml.
2. Stock of confluent mouse neuroblastoma cells (N2A – ATCC) (~80–95%) in a 75 ml flask.

2.2.2.3. Equipment

1. 96-well microtitre plates with lid.
2. Pipettes capable of dispensing 50–200 µl volumes.
3. Humidified CO_2 (5%) incubator capable of maintaining 37°C.
4. Inverted fluorescence microscope capable of excitation at 488 nm (FITC), using an excitation filter with narrow pass-band windows in the blue (475–490 nm).

2.3. Mouse Inoculation Test

2.3.1. Principle of Test

The mouse inoculation test (MIT) is usually used as a confirmatory tool for rabies diagnosis. Mice (3–4 weeks old) are inoculated intra-cerebrally (i.c.), intra-dermally, or intra-muscularly usually with a brain homogenate from a suspect case. The mice are then observed over 28 days for signs of clinical rabies. This technique is currently used for all human contact cases and is also used in rabies virus production (NIH test for vaccine potency). Owing to the nature of this test procedure, a special mention should be made of the potential risk of aerosol exposure and needlestick injuries. In the UK, an appropriate Home Office licence is required to undertake this test. It is also imperative to have the appropriate husbandry expertise and equipment available before this test is undertaken.

2.3.2. Materials

In addition to laboratory mice 3–4 weeks old.

2.3.2.1. Buffers and Reagents

1. 1% Virkon solution.
2. 0.1 M PBS (pH 7.4) + antibiotics (PBSA).
3. Isofluorothane anaesthetic.
4. Oxygen supply.

2.3.2.2. Equipment

1. General mouse husbandry equipment (Mouse cages, cage racks, bedding, food, water bottles).
2. Fluovac scavenging unit with fluosorber cartridge.
3. Isofluorothane chamber.
4. 1 ml Luer-Lock syringe.
5. Disposable needles Luer-Lock, e.g., 25 G, 26¾ G.
6. Mouse isolator.

2.4. Fluorescent Antibody Neutralisation Test

2.4.1. Principle of Test

The FAVN test is used to evaluate the levels of rabies neutralising antibody levels within the serum of post-vaccinated individuals (11). A constant dose of previously titrated challenge virus is incubated with serial dilutions of patient sera, along with appropriate positive and negative controls (including low level controls and known titrations of sero-neutralising antibody samples). During incubation, any rabies VNA will bind and neutralise the added “live” virus. Any virus that is not neutralised at this stage

will then infect the BHK cells that are added to the suspension. After 2-days incubation, the BHK cells are fixed and stained to look for non-neutralised virus (fluorescent foci). The titre of the test serum is then calculated by the Spearman–Karber method and recorded in IU/ml by comparison of the value obtained with the test serum and the standard test serum.

2.4.2. Materials

2.4.2.1. Buffers and Reagents

1. BHK MEM + 10% foetal calf serum and antibiotic solution (penicillin 100 U/ml; streptomycin 100 μg/ml; mycostatin 25 U/ml concentration in BHK MEM).
2. 0.1 M PBS (pH 7.4).
3. ATV solution.
4. CVS strain ATTC VR 959, CVS 11 (not exceeding passage 8).
5. Anti-rabies fluorescein isothiocyanate (FITC) conjugate (Fujirebio Diagnostics Inc. 201 Great Valley Parkway, Malvern, PA 19355).

2.4.2.2. Equipment

1. 96-well microtitre plates.
2. Humidified CO_2 incubator capable of maintaining 37°C (±2%) with 5% CO_2.
3. Fluorescence microscope capable of excitation at 488 nm (FITC), using an excitation filter with a narrow passband in the blue (475–490 nm).
4. Refrigerator capable of maintaining temperatures between 2 and 8°C.
5. Water bath capable of maintaining temperature of 56°C.
6. Pipettes capable of dispensing between 50 μl and 5 ml.

2.5. Use of Monoclonal Antibodies for the Characterisation of Lyssaviruses

2.5.1. Principle of the Test

The development of monoclonal antibody (Mab) production described by Köhler and Milstein in 1975 involves the use of producing immortal monoclonal antibody-secreting cells known as "Hybridoma" cells. These are produced be fusing B-lymphocytes from mice with immortal myeloma cancer cells. These hybridomas have developed into becoming a vital tool in studying the immunology and epidemiology of rabies viruses. Vital to the process of producing Mabs are a number of steps that involve screening and selecting the appropriate cells. This is achieved by exploiting the ability (or lack of) of fused cells to grow under certain conditions. Myeloma cells are deficient in the enzyme hypoxanthine guanine phosphoriboseyltransferase (HGPRT) unlike hybridomas (that have this ability owing to their B-lymphocyte component); this means that growing up suspect hybridoma cells on hypoxanthine-aminopterin-thymidine (HAT) medium can select positively for cells that have undergone fusion. To avoid creating revertant myeloma

cells, cultures are treating intermittently with 8-azaguanine that kills non-HGPRT dependent cells, thus maintaining pure hybridoma cultures.

2.5.2. Materials

1. Polytetrafluoroethylene (PTFE)-coated glass slides.
2. Eagle's minimal essential media (EMEM).
3. Anti-mouse immunoglobulin G conjugated to FITC.

2.6. Use of Filter Paper (FTA®) Technology for Shipment, Storage, and Detection of Rabies Virus RNA

2.6.1. Principle of the Test

FTA® Gene Guard System is a commercially available product produced by the Whatman company (Whatman International Ltd, Springfield Mill, James, Whatman Way, Sandling Road, Maidstone, Kent. ME14 2LE UK). As rabies viruses are classed as ACDP3/SAPO4 pathogens, the transport and handling of infectious materials is tightly regulated. FTA® cards have been designed to allow the safe shipment and storage of viral RNA (12). The cards are impregnated with a chemical formula that lyses cell membranes and denatures proteins on contact. Nucleic acids are immobilised and stabilised on the cards at room temperature, which can then remain stable for many years. Crucially, these cards can be shipped through normal mail routes, with no special handling restrictions.

2.6.2. Materials

2.6.2.1. Equipment

1. Indicating FTA® classic card.
2. Tools for specimen collection.

2.7. Extraction of RNA Using Trizol™

2.7.1. Principle of the Test

Trizol™ is a monophasic solution of phenol and guanidine isothiocyanate for the isolation or total RNA from tissues and cells. Trizol™ lyses cells and disrupts cell components, whilst maintaining the integrity of the RNA within the sample. The addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. The RNA remains exclusively in the aqueous phase and is recovered by precipitation with isopropanol. The RNA can be stored at –80°C.

2.7.2. Materials

2.7.2.1. Reagents and Buffers

1. Trizol™ reagent (Invitrogen) phenol/guanidine isothiocyanate.
2. Chloroform.
3. Isopropanol.
4. 75% Ethanol.
5. RNase-free high pure liquid chromatography (HPLC)-grade water.

2.7.2.2. Equipment

1. Class III safety cabinet.
2. Pipettes capable of dispensing between 0.5 and 1,000 µl.
3. Refrigerated centrifuge.

4. 2-ml screw-cap tubes.
5. Scissors and dissection equipment.
6. Disposable pestle and mortars suitable for homogenising small volumes of tissue.

2.8. Extraction of RNA Using the Multi-Enzymatic Liquefaction Technique Total Nucleic Acid Isolation System

2.8.1. Principle of the Test

The multi-enzymatic liquefaction technique (MELT™) total nucleic acid isolation system allows for the enzymatic digestion of tissue, which is designed to be safer to use than phenol/chloroform-based systems. The MELT™ reagents also irreversibly destroy RNAses, allowing lysates to be stored at room temperature for up to 1 week. This enzymatic digestion step is coupled with the MagMax™ viral RNA isolation kit, which utilises magnetic bead-based technology, to elute RNA from the MELT™ lysate. The MagMax™ system can be used manually, with a magnetic stand processing up to six samples at a time, or automatically using the Kingfisher 96 magnetic particle processor that can process up to 96 samples at once.

2.8.2. Materials

2.8.2.1. Reagents and Buffers

1. 100% Ethanol.
2. 100% Isopropanol.
3. β-mercaptoethanol.

2.8.2.2. Equipment

1. Class III safety cabinet.
2. Pipettes capable of dispensing 50–1,000 μl volumes.
3. Dissection equipment for the removal of brain tissue.
4. 2-ml screw-cap tubes.
5. Universal tubes.
6. Vortex mixer.
7. Centrifuge.
8. MELT™ total nucleic acid isolation system (Ambion Inc.).
9. MagMax™ viral RNA isolation kit (Ambion Inc.).
10. Kingfisher 96 magnetic particle processor and consumables (Thermo Fisher Scientific Inc.).

2.9. Hemi-Nested Reverse Transcriptase Polymerase Chain Reaction

2.9.1. Principle of Test

This method allows for the high-throughput screening of samples for rabies and rabies-related viruses (13). For rabies viral RNA to be analysed by the PCR, double-stranded cDNA must first be synthesised from extracted viral RNA (see Subheading 3.7 or Subheading 3.8). This hemi-nested reverse transcriptase-PCR (hnRT-PCR) (14) procedure is designed for the high-sensitivity detection of rabies and rabies-related viral RNA. In a traditional nested PCR, subsequent rounds of PCR are undertaken, with each set of primers being from within the sequence of the previous round's products. In hemi-nested PCR, the 5′ primer (JW12)

remains the same in both rounds of PCR (hence "hemi"-nested). Only the 3′ primer changes, allowing for the amplification of small copy numbers of first-round product. This procedure allows for a highly sensitive technique for the detection of all lyssavirus genotypes.

2.9.2. Materials

2.9.2.1. Buffers and Reagents

2.9.2.1.1. First Round Primers

1. JW12 RT/PCR primer (5′ ATGTAACACCYCTACAATG 3′).
2. JW6 DPL RT/PCR primer (5′ CAATTCGCACACATTT TGTG 3′).
3. JW6 M RT/PCR primer (5′ CAGTTAGCGCACATCT TATG 3′).
4. JW6 E RT/PCR primer (5′ CAGTTGGCACACATCT TGTG 3′).

2.9.2.1.2. Second Round Primers

1. JW12 RT/PCR primer (5′ATGTAACACCYCTACAATG3′).
2. JW10 ME1 RT/PCR primer (5′ GTCATCAATGTGTGRT GTTC 3′).
3. JW10 DLE2 RT/PCR primer (5′ GTCATCAAAGTGTGRT GCTC 3′).
4. JW10 P RT/PCR primer (5′ GTCATTAGAGTATGGT GTTC 3′).

2.9.2.1.3. Others

1. HPLC grade water.
2. 28 U/μl RT buffer.
3. Amplification buffer.
4. 0.1 M Dithiothreitol (DTT).
5. 10 mM dNTPs.
6. Amplitaq Gold® DNA polymerase.
7. 2.5 mM Tetramethylammonium chloride (TMAC).
8. Dimethyl sulphoxide (DMSO).
9. Agarose.
10. TAE 1× stocks of Tris/acetic acid/EDTA buffer.
11. Ethidium bromide 10 mg/ml stock.
12. Gel loading buffer (for 10 ml: Xylene cyanol 0.025 g bromophenol blue 13. 0.025 g glycerol 4 ml distilled water 6 ml).
13. DNA marker (such as Φ0174).

2.9.2.2. Equipment

1. Pipettes capable of dispensing between 0.5 and 1,000 μl.
2. Vortex mixer.
3. Micro centrifuge.
4. Ice buckets and ice.
5. 0.2-ml PCR tubes.

6. 0.5-ml sterile screw-cap tubes (for RT step).
7. 1-ml sterile screw-cap tubes (for master mix).
8. PCR thermocycler.
9. Microwave oven/water bath (for dissolving agarose in TAE).
10. Gel mould.
11. Conical flask.
12. Fume hood.
13. Gel electrophoresis tank/power pack.
14. Molecular imaging system.
15. Measuring cylinder.

2.10. Real-Time (Quantitative) Polymerase Chain Reaction (qPCR) for the Detection of Genotypes 1, 5, and 6 Lyssaviruses

2.10.1. Principle of Test

The real-time PCR described here (15) allows for the detection of Genotype 1, 5, and 6 Lyssavirus total RNA from clinical samples using a differential real-time PCR Taqman assay. The use of four Taqman fluorogenic probes specific for the three genotypes (and an internal control transcript – β-actin mRNA) allows the real-time quantification of the amplicons, with a built-in control system. This method also includes a reverse-transcriptase step, using the primer JW12 to allow for the generation of cDNA from RNA; this allows the whole reaction to take place within a single closed tube environment, minimalising the risk of contamination.

2.10.2. Materials

2.10.2.1. Buffers and Reagents

1. 0.05 μM JW12 RT/PCR primer (5′ATGTAACACCYCTA CAATG3′).
2. 0.05 μM N165-146 PCR primer (5′GCAGGGTAYTTRTA CTCATA3′).
3. 0.05 μM Bat Rat β actin intronic 1 RT/PCR primer (5′CGATGAAGATCAAGATCATTG3′).
4. 0.05 μM Bat Rat β actin reverse PCR primer (5′AAGCA TTTGCGGTGGAC3′).
5. 0.05 μM Rab GT1 consensus Taqman® probe.
6. 0.05 μM FAM-TAMRA (5′ACAAGATTGTATTCAAAGT CAATAATCAG3′).
7. Rab GT5 Taqman® probe.
8. Yakima Yellow-Deep Dark Quencher 1 (5′AACARGGTTGT TTTYAAGGTCCATAA3′).
9. Rab GT6 Taqman® probe.
10. 3.6 nM Cy5-Black Hole Quencher 2 (5′ACARAATTGTCT TCAARGTCCATAATCAG3′).
11. 0.2 μM β Actin Taqman® probe.
12. ROX-Deep Dark Quencher 1 (5′TCCACCTTCCAGCAG ATGTGGATCAGCAAG3′).

13. 10% (v/v) Triton X100.
14. 10 mM DNTPs.
15. 5 U/µl Taqman Gold® DNA polymerase.
16. 20–40 U/µl RNAsin®.
17. 200 U/µl MMLV reverse transcriptase.
18. HPLC grade water.
19. 100× concentrated, Tris-ethylenediaminetetraacetic acid (Tris–EDTA) buffer.

2.10.2.2. Equipment

1. Real-time PCR System (e.g. MX3000P real-time PCR system with appropriate consumables. Stratagene® or equivalent).
2. Pipettes capable of dispensing volumes 0.5–1,000 µl.
3. Vortex mixer.
4. Micro centrifuge.
5. Ice buckets and ice.
6. 0.2-ml PCR tubes.
7. 0.5-ml sterile screw-cap tubes (for RT step).
8. 1-ml sterile screw-cap tubes (for master mix).

3. Methods

3.1. Fluorescent Antibody Test

3.1.1. Method

1. Make small smears of brain samples on a microscope slide. Include known positive smears as quality controls.
2. Place in a Coplin jar containing acetone stored at –70°C for 20 min to fix the smears.
3. Remove from Coplin jar allow to air-dry.
4. Add a drop of working strength conjugate (sufficient to cover smear), and incubate in a humid atmosphere at 37°C for 30 min.
5. Wash slides in 0.1 M PBS pH 7.2 twice to remove loose conjugate.
6. Rinse in distilled H_2O and allow to air-dry.
7. Read using fluorescence microscope.

3.1.2. Interpretation and Results

Rabies-virus infected cells appear as bright "apple" green fluorescent inclusions within the peri-nuclear area of the brain cells. Compare the fluorescence on the positive control slides to the test slides as a reference standard.

3.2. Rabies Tissue Culture Infection Test

3.2.1. Preparation of Cells

1. Remove media from a stock of confluent mouse neuroblastoma (N2A) cells and add 5 ml of ATV into the flask. To remove the cell sheet from the flask, rock the flask back and forth a few times before removing the ATV and incubating the flask at 37°C for 5 min.
2. After 5 min, agitate the flask to dislodge the cell monolayer and separate the cells (drawing up and expelling solution with a pipette may aid cell separation).
3. Add fresh media to the flask as to make a final concentration of ~2×10^5 cells/ml.
4. Dispense 200 µl of cell suspension into individual wells on a 96-well microtitre tray and incubate in a humid 5% CO_2 incubator for a minimum of 30 min to allow cells to attach to the microtitre well base.

3.2.2. Sample Preparation and Addition

1. Homogenise 1 g of brain material in 10 ml of phosphate-buffered saline with antibiotics in a universal tube and allow to stand so that any large particles of brain material settles at the bottom of the tube.
2. Dispense 100 µl of the supernatant into each of the wells in replicate.

3.2.3. Changing the Media

1. After 48 h of incubation, wash the cells by aspirating all of the liquid in the well and discard (aspirates may be kept and retested if limited sample is available).
2. Add 200 µl of fresh media to each well, cover, and return to incubator for required length of incubation (up to 3 days to a total of 4 days).

3.2.4. Fixing and Staining

1. After the required incubation time, remove the media from the wells by aspiration.
2. Rinse the cells briefly by immersion in 80% acetone before submerging in fresh 80% acetone for a minimum of 20 min (this step renders the wells non-infectious).
3. Remove the microtitre tray from acetone, remove any excess acetone by inversion and, allow to air-dry.
4. Add 50 µl of FITC anti-rabies monoclonal globulin to each well
   (a) Incubate at 37°C in CO_2 (5%) atmosphere for 30 min.
5. Remove excess conjugate by inversion and rinse plates by 2× immersion in 0.1 M PBS.
6. Blot plates on absorbent tissue to dry.

3.2.5. Interpretation and Results

The presence of live virus is detected by direct immunofluorescence of the cell sheet using FITC conjugated murine antibodies, which specifically stain the nucleocapsid inclusions formed by the replicating virus in the cell sheet.

Positive results can be obtained after 18 h (equal to one replication cycle within the neuroblastoma cell line). When sufficient test material is available, it is possible to set up multiple plates that can be viewed at 1–4 days, until a positive result is observed.

3.3. Mouse Inoculation Test

3.3.1. Method

1. Samples that require testing should be homogenised in PBSA as described in 3.2.2. Using appropriate safety procedures for the manipulation and handling of live mice for experimental procedures, ensure that the mice to be inoculated are fully anaesthetised prior to inoculation (i.e. not moving when touched).
2. Inoculate intra-cerebrally with between 30 and 50 µl (maximum volume) sample supernatant. Extreme care must be taken not to push the needle too far through the skull. For peripheral inoculations, 20–50 µl is used and is usually inoculated via the footpad of the mouse.
3. Ensure needle/syringes are disposed of using appropriate decontamination procedures.
4. Inoculated mice should be left to recover from the anaesthetic and are placed in a flexible film isolator for observation.

3.3.2. Interpretation and Results

1. Any mice that are moribund or die within 5 days of the inoculation being administered are considered to have become sick from the procedure (or inoculum), not from rabies infection.
2. The rest of the mice should be observed twice daily for signs of sickness. If mice are observed to be sick or dead, they should be removed from the isolator. Sick mice should be euthanised, ensuring death has occurred by approved methods (schedule 1 killing; cervical dislocation).
3. Brains are removed and a FAT is undertaken (see Subheading 2.1) to look for evidence of rabies infection.
4. After 28 days, healthy mice should be killed by sedation and approved methods for ensuring death. All mice should be disposed of according to appropriate health and safety guidelines due to the nature of the inoculum used.

3.4. Fluorescent Antibody Neutralisation Test

3.4.1. Method

1. Inactivate test sera by heating in a water bath set at 56°C for 30–45 min (maximum). Once inactivated samples should be used immediately or stored at 4°C until required.
2. Add 50 µl of serum to 100 µl BHK MEM + FCS in a microtitre well and mix.
3. Serial dilute the sample to a dilution of 1:19,683 (to allow for a titre to be determined). In addition to this test, appropriate controls should also be serially diluted, using a virus titration plate.
4. In a safety cabinet, prepare pre-dilutions of CVS to obtain a working preparation of 100 $TCID_{50}$/50 µl.

5. Add 50 µl to each test well and incubate for 60–90 min (maximum) at 37°C to allow the antibody virus reaction to take place.
6. Add 50 µl of BHK cell suspension (5×10^5 cells/ml) to all wells (excluding the media control well). Incubate at 37°C in CO_2 (5%) atmosphere for approximately 48 h.
7. After incubation, fix the cells by removing the media and immersing the plate briefly in 80:20 acetone before immersion in a clean solution of 80% acetone for a minimum of 20 min (45 min).
8. Remove and air-dry.
9. Add 50 µl of FITC anti-rabies monoclonal globulin (diluted to working strength in 0.1 M PBS pH 7.2).
10. Incubate at 37°C in CO_2 (5%) atmosphere for 30–45 min (maximum), shake out conjugate, and rinse plate twice in 0.1 M PBS.

3.4.2. Interpretation and Results

1. The plates are read under a fluorescent microscope capable of excitation at 488 nm (FITC), using an excitation filter with a narrow passband in the blue (475–490 nm).
2. View the whole well for "apple green" fluorescent foci (stained nucleocapsid aggregates of virus particles).
3. Where the serum dilution is less, it may be necessary to view with a higher magnification to confirm presence of infected cells. Ensure that the wells are examined for cytotoxicity of the cell sheet (if this occurs, samples must be re-tested).
4. For the test to be valid the negative control well for each sample (i.e. no serum added) must be positive for virus (fluorescent foci present).
5. End points dilutions for virus replication are determined by noting the last well in the series to be negative. The titre of the sample can then be determined using a defined statistical method (such as the Spearman–Karber method).
6. Quality control maybe evaluated by comparing the positive control titres to previous test runs, ensuring they fall within limits for the means from the previous runs.
7. Final serum titrations (quantitative analysis) should be provided in IU/ml.

3.5. Use of Monoclonal Antibodies for the Characterisation of Lyssaviruses

1. Material for testing against rabies monoclonal antibodies raised against the rabies nucleoprotein (Mab-RNPs) is derived from infected mouse brain material.
2. The brain is removed from the mice once clinical symptoms are apparent (i.e. observing complete paralysis) and either

3.5.1. Methods

used to make brain impressions on PTFE-coated glass slides (with enough replicates to test all Mabs) or by making a 10% brain suspension in EMEM.

3. For testing against rabies glycoprotein (Mab-G), use either 10% mouse-brain suspension containing >10,000 $MICLD_{50}$ (median lethal dose for mice inoculated intracerebrally or cell cultures of virus) where supernatant contains >10,000 infectious units per ml.
4. Typing of the virus using either Mab (RNP of G) is based upon the same premise. PTFE-coated slides are prepared with virus replicates; these slides are then incubated with Mabs raised against different viral strains (and at differing dilutions).
5. An anti-mouse immunoglobulin G conjugated to FITC is then added to bind to the Mab and the resulting fluorescence is recorded against a control homologous virus immunogen. The correct dilution of Mab is essential to allow resolution between closely related isolates whilst maintaining consistency of results.

3.5.2. Interpretation of Results

1. A "positive" result is when the intensity and distribution of fluorescence is identical to the homologous virus.
2. A "weak positive" shows much less intensity, and a "negative" reaction implies no specific fluorescence.
3. Using this method, a matrix can be drawn up of viral strain against Mab reaction.
4. Different strains of virus can then be compared against other viruses in the matrix to distinguish them from one another (see Table 4 for example, adapted from ref. (16)).

Table 4
Comparison of rabies viruses using monoclonal antibodies

Serotype virus	Mab-N reference number				
	2	5	6	7	11
Classical virus standard (CVS) 11	+	–	–	–	–
Lagos bat virus	+	+	+	+	–
Mokola virus	+	+	+	+	+
Duvenhage Africa virus	+	–	–	–	–
European bat lyssavirus type 1	+	–	–	–	–
European bat lyssavirus type 2	–	–	–	–	+

+ positive reaction, – negative reaction. In this example, Mabs were raised against Mokola virus (genotype 3)

3.6. Use of Filter Paper (FTA®) Technology for Shipment, Storage and Detection of Rabies Virus RNA

3.6.1. Method

1. Each indicating FTA® classic card is suitable for four samples. The card has four sample areas labelled A–D. The colour of the card changes from pink to white once the sample is added to the middle of each spot.
2. The samples are allowed to dry for at least 1 h at room temperature.
3. The card is then folded as directed and sealed within a sealable protective pouch.
4. The card can then be stored at room temperature until required.
5. As the FTA® card inactivates all infectious material, the cards can be posted though existing mail routes without any special restrictions.
6. Once in the laboratory, a punch can be used to remove a small section of the filter paper and this can then be used for RNA amplification and other downstream techniques.

3.7. Extraction of RNA Using Trizol™

3.7.1. Method

1. All steps must be undertaken within a Class III cabinet. Sample tubes that need to be removed from the cabinet for centrifugation must be disinfected with a suitable anti-virucidal agent such as Virkon disinfectant at 1% concentration prior to removal.
2. Homogenise 50–100 mg of tissue to be analysed per 1 ml of Trizol™ reagent.
3. Incubate at room temperature (RT) for 5 min.
4. Add 0.2 ml of chloroform and shake vigorously for 10–15 s; incubate for a further 3 min.
5. Centrifuge the sample at 12,000 × *g* at 4°C for 15 min.
6. Transfer the upper aqueous phase to a fresh 2-ml tube containing 0.5 ml isopropanol per 1 ml.
7. Incubate for 10 min at RT. Centrifuge at 10,000 rpm at 4°C for 10 min. Discard the supernatant, taking care not to disturb the pellet.
8. Wash the pellet in 20 µl 75% isopropanol per 1 ml Trizol™ and leave to air-dry (5–10 min).
9. Re-suspend in 10 µl RNase-free HPLC water. Store at –80°C.

3.8. Extraction of RNA Using the MELT™ Total Nucleic Acid Isolation System

3.8.1. Method

3.8.1.1. Tissue Digestion Using MELT™

1. Place ≤10 mg of fresh or frozen tissue into a clean 2-ml screw-cap tube.
2. Add 96 µl of MELT buffer + 4 µl MELT cocktail, and vortex for 10 min to dissolve the tissue in the MELT enzyme.
3. Centrifuge the sample to clarify, and pellet any undissolved tissue (10,000 rpm for 5 min at 4°C).
4. Store the sample at this point, prior to RNA purification at room temperature, up to 1 week.

3.8.1.2. RNA Purification Using MagMax™

1. Add 100 µl of the homogenate to a 2-ml screw-cap tube (manual) or a well in a 96-well test plate (automated).
2. Add 100 µl binding solution, 1.6 µl β-mercaptoethanol, and 100 µl 100% ethanol, and 10 µl binding beads to each sample.
3. Using the magnetic stand manual purification system, the samples are placed in the magnetic stand, and the beads within the sample that have bound to the RNA are captured onto the side of the tube. This allows for removal of the supernatant from the tube, leaving behind the RNA.
4. Remove the magnet and add the next wash solution.
5. The beads will then go back into solution.
6. Repeat the wash step three times (or four if the optional "DNAse" step is included), to purify the RNA from the tissue homogenate.
7. Once purified, the RNA is eluted into the supplied elution buffer or HPLC-grade water.
8. Store at –80°C until further processing is required.

3.9. Hemi-Nested Reverse Transcriptase Polymerase Chain Reaction

3.9.1. Method

Prior to the hnRT-PCR being carried out, the viral RNA should be extracted from samples using a suitable method (see Subheading 3.7 or Subheading 3.8). As with all PCR experiments, this procedure is highly dependent on user accuracy and the maintenance of a contamination-free environment. To ensure all enzymes are kept at the appropriate temperature, it is advisable to work on ice as much as possible.

3.9.1.1. Reverse Transcriptase Step

1. The first stage in the hnRT-PCR is to create double-stranded cDNA from the extracted RNA. Prepare the following master mix calculations *per sample* in a PCR clean room:

RT mix	Volume added per reaction (µl)	Final concentration
HPLC grade water	1.5	
5× RT buffer	2.0	(×1)
10 mM dNTPs	1.0	(200 µM each dNTPs)
0.1 M DTT	1.0	(10 mM)
28 U/µl RNAsin	0.5	(14 U)
7.5 pM/µl JW12 primer	1.0	(7.5 pM)
200 U/µl MMLV-RT	1.0	(200 U)
Total volume	8.0	

2. Transfer the tubes on ice to a suitable area away from the clean room and add 2 µl sample RNA making a total of 10 µl.

3. Incubate the tubes in a water bath set at 42°C for 60 min. This allows the generation of double-stranded cDNA from the original RNA template.
4. Remove from the water bath and place the samples on ice to stop the reaction.
5. Once cDNA has been generated, the nucleic acid is stable enough to be stored at –20°C until required.

3.9.1.2. First Round PCR Step (JW12/JW6 Primers)

1. In a PCR clean room, prepare a master mix of reagents in a clean tube on ice, using the following mix calculations *per* sample:

Reagent	Amount per sample (µl)	Final concentration
HPLC-grade water	33.075	
10× amplification buffer	5	1× amplification buffer
10 mM dNTPs	1	200 µM each dNTPs
7.5 pM/µl JW12	1	7.5 pM/50 µl
7.5 pM/µl JW6 (DPL)	1	7.5 pM
7.5 pM/µl JW6 (E)	1	7.5 pM
7.5 pM/µl JW6 (M)	1	7.5 pM
Amplitaq Gold	0.25	2.5 U
2.5 mM TMAC	1	0.05 mM
DMSO	0.675	1.35%
Total volume	45	

2. Outside of the clean room, and in a suitable area, add 5 µl cDNA to the tubes (total volume of 50 µl). Run on a thermocycler using the protocol detailed below.

Cycle parameters for first round hnRT-PCR

Denature	10 min	95°C	
Denature	1 min 30 s	95°C	×5 cycles
Anneal	1 min	45°C	
Pause	20 s	50°C	
Extend	1 min 30 s	72°C	

(continued)

(continued)

Denature	30 s	95°C	×40 cycles
Anneal	1 min	45°C	
Pause	20 s	50°C	
Extend	1 min	72°C	
Denature	30 s	95°C	×1 cycle
Anneal	1 min	45°C	
Pause	20 s	50°C	
Extend	10 min	72°C	
Chill	Pause	4°C	

3. Once the reaction is complete, the PCR product can be stored at 4°C until required.

3.9.1.3. Second Round PCR Step (JW12/JW10 Primers)

1. In a PCR clean room, prepare a master mix of reagents in a clean tube on ice, using the following mix calculations *per* sample:

Reagent	Amount per sample (µl)	Final concentration
HPLC water	38.065	
10× amplification buffer	5	1× amplification buffer
10 mM dNTPs	1	200 µM each dNTPs
7.5 pM/µl JW12	1	7.5 pM/50 µl
7.5 pM/µl JW10 (ME1)	0.65	7.5 pM
7.5 pM/µl JW10 (DLE2)	0.65	7.5 pM
7.5 pM/µl JW10 (P)	0.65	7.5 pM
Amplitaq Gold	0.25	2.5 U
2.5 mM TMAC	1	0.05 mM
DMSO	0.735	1.47%
Total volume	49	

2. Outside of the clean room, and in a suitable area, add 1 µl first round JW12/JW6 product to the tubes (total volume of 50 µl). Run on a thermocycler using the protocol detailed below.

Cycle parameters for second round hnRT-PCR

Denature	10 min	95°C	
Denature	1 min 30 s	95°C	×5 cycles
Anneal	1 min	45°C	
Pause	20 s	50°C	
Extend	1 min 30 s	72°C	
Denature	30 s	95°C	×30 cycles
Anneal	1 min	45°C	
Pause	20 s	50°C	
Extend	1 min	72°C	
Denature	30 s	95°C	×1 cycle
Anneal	1 min	45°C	
Pause	20 s	50°C	
Extend	10 min	72°C	
Chill	pause	4°C	

3. Once PCR reaction is complete, the product can be stored at 4°C until required.

3.9.1.4. Visualisation of DNA with Agarose Gel Electrophoresis

To interpret the data received from the PCR reaction, the product (or part of) is run on a 2% agarose gel run with a marker (such as Φ0174) to allow the determination of the size of the DNA product that has been amplified.

1. Mix agarose and TAE in a conical flask, at appropriate quantities to produce a 2% gel (i.e. 2 g agarose with 100 ml TAE). Mix and melt the agarose in a microwave oven.
2. Allow the molten gel to cool (whilst maintaining liquid state). Add 1–2 μl of ethidium bromide solution in a fume cupboard. Pour the gel into the mould, with both ends sealed with autoclave tape. Select a suitably sized well-forming comb and place into the mould before the gel sets. Leave the gel to solidify for at least 30 min.
3. To enable loading of a DNA (or RNA) sample, it must be mixed with a suitable volume of gel loading buffer. A DNA size marker (e.g. Φ0174) must also be mixed with loading buffer, to be run alongside the samples. Generally, a 10% volume of loading buffer is added to the sample (i.e. 1 μl loading buffer to 9 μl sample). Alternatively, commercially available loading buffers may be used at concentrations as advised by the manufacturer.
4. Remove the well-forming comb. Remove the tape from the ends of the gel and place the solidified gel in the gel tank. Add fresh TAE to the gel tank. Load the samples and DNA marker

into the wells and immediately attach the tank to the power supply and increase the voltage to 120 V. Separate the samples for approximately 1 h.

5. When the sample has separated (the loading buffer dye will give an indication of the progress of sample separation), switch the power off and disconnect from the power supply. Remove the gel and allow excess buffer to drain off. Place the gel in a tray and illuminate over a UV transilluminator.
6. A positive PCR result is observed in the form of a bright band; the size of the PCR product obtained can be confirmed by comparing it's position against the bands of the marker, which are of known size (refer to data sheet provided with the marker for size of bands). The positive controls should also produce bands, whereas the negative controls should produce no band.

3.9.2. Interpretation and Results

The size of the first-round PCR product should be ~606 bp in size, and that of the second round PCR product should be smaller at ~585 bp. For diagnostic samples, it is essential that a housekeeping gene from the tissue (such as the ubiquitous 18S gene) also be amplified (especially when rabies negative results are expected), as this will ensure that RNA extraction has been achieved successfully. If further identification of the DNA is required, it is possible to perform downstream identification (such as gene sequencing) on the PCR product from either round of PCR.

3.10. Real-Time qPCR for the Detection of Genotypes 1, 5, and 6 Lyssaviruses

3.10.1. Method

Sufficient mastermix should be prepared using the following proportions *per* sample, at least three negative controls and three positive controls corresponding to the three different genotypes should be factored into this master mix.

1. In a PCR clean room, prepare a master mix of reagents in a clean tube on ice, using the following mix calculations *per* sample:

3.10.1.1. Lyssavirus Genotype 1, 5, and 6 Master Mix

Reagent	Amount per sample (µl)
Water	22.75
10× PCR buffer	5.00
25 mM $MgCl_2$	12.00
10 mM dNTPs	1.00
20 µm JW12 primer	1.00
20 µm N165-146 primer	1.00
5 µm RabGT1 con	1.00
5 µm RabGT5	1.00
5 µm RabGT6	1.00

(continued)

(continued)

10% (v/v) Triton X100	1.00
20–40 U/μl RNAsin	0.25
200 U/μl MMLV reverse transcriptase	0.50
5 U/μl Taq polymerase	0.50
Total	48.00

3.10.1.2. β-Actin Master Mix

1. In a PCR clean room, prepare a master mix of reagents in a clean tube on ice, using the following mix calculations *per* sample:

Reagent	Amount per sample (μl)
Water	24.75
10× PCR buffer	5.00
25 mM $MgCl_2$	12.00
10 mM dNTPs	1.00
20 μM Bat Rat β actin intronic	1.00
20 μM Bat Rat β actin reverse	1.00
5 μm β actin ROX	1.00
10% (v/v) Triton X100	1.00
20–40 U/μl RNAsin	0.25
200 U/μl MMLV reverse transcriptase	0.50
5 U/μl Taq polymerase	0.50
Total	48.00

2. An aliquot of 2 μl of total RNA (~1 μg/μl), previously extracted from samples using a suitable method (i.e. phenol/chloroform) is added to each tube (one sample and one β-actin tube per sample).
3. Samples should be set up as per the specific system requirements and placed on the quantitative PCR machine running the following parameters:

3.10.1.3. Parameters for qPCR

42°C	30 min	
94°C	2 min	
94°C	30 s	45 cycles
55°C	30 s	
72°C	20 s	

3.10.2. Interpretation and Results

Qualitative PCR machines, by their nature, will output results which will need to be interpreted. The output of the machines are usually visualised as a plot of absorbance against PCR cycles. These plots are sigmoidal in shape and correspond to the increase of fluorophore activity in each well as DNA is produced and the fluorophore on each specific primer is cleaved and starts to fluoresce. There is a threshold value on the *x*-axis (known as the Cycle Threshold or C_T), that corresponds to a sample being flagged up as positive. As the system uses a number of different primers attached to different fluorophores, it is possible to detect a number of different DNA sequences in one sample. It is also possible to detect the size of the DNA amplified by running on a 2% agarose gel with suitable markers (although, this will not differentiate between genotypes).

4. Notes

1. Rabies infections are invariably fatal in the absence of the appropriate post-exposure prophylaxis. There are a number of techniques detailed here, which when used correctly can be used to detect rabies virus strains within patient's tissues, or serum. Diagnosing the presence or absence of rabies virus in any clinical picture allows patient management and treatment to be directed in appropriate manner, as well as enhancing epidemiological data. Due to the nature of the infecting virus, it is essential to have a detailed medical history, and correct sampling is important to ensure that an accurate clinical picture is observed. When undertaking diagnostic tests for any lyssavirus, proper biosafety measures are extremely important to protect both the technician undertaking the test and the environment from becoming contaminated with live virus. Used under appropriate conditions, the tests described here are extremely effective at being able to determine the presence of lyssaviruses in tissues. With the advent of PCR techniques, genetic and molecular information about any isolate can be discerned, further aiding the microbiologist in tracking trends and movements of strains of virus.
2. As lyssaviruses are classified as ADCP3 organisms, ensure that appropriate personal protective equipment (gloves, laboratory coats, goggles etc.) is worn when dealing with live virus or samples suspected of containing virus (7).
3. The virus itself is susceptible to killing by UV light, heat, or immersion in alcohols and detergents; for this reason, various steps in the FAT (Subheading 2.1) and RTCIT (Subheading 2.2) procedures maybe performed outside of class 3 conditions once the samples have been treated with acetone and are no longer classed as "live."

4. In addition to the safety aspects of handling live virus, it should also be noted that a number of the reagents and chemicals used are also potentially harmful. DMSO, TMAC, TAE buffer, and ethidium bromide are all potentially hazardous and should be treated with appropriate care and attention to health and safety. All potential hazards should have an accompanying COSHH sheet (Control of Substances Hazardous to Health) and the guidelines for handling these substances and the possible health risks are outlined in those documents (7).
5. When preparing samples for FAT slides (3.1.1), it is important that the smear is as thin as possible, ideally, so that there is a single layer of cells (which will aid in reading the slide). One way of achieving this is to invert the inoculated slide onto fibre-free blotting paper and pressing firmly to spread the sample; repeating this technique two or three times will produce a fairly uniform cell monolayer (7).
6. When performing the RTCIT (Subheading 2.2) on saliva samples (human or animal), it may be necessary to dilute the sample in PBSA (e.g. 1:50 or 1:100), as the enzymes present in neat saliva can destroy the cell monolayer, rendering the test invalid.
7. When homogenising tissue for the RTCIT (3.2.2), one technique is to use frozen aliquots of PBS + antibiotics. Remove a 10-ml aliquot from the freezer 5 min prior to adding the sample. The tissue sample is added to the partially defrosted PBSA and is shaken vigorously (the ice within the tube will act like a pestle homogenising the sample within the tube). This solution can be re-frozen and stored at −8 ± 2°C until testing required.
8. When taking a full-thickness punch biopsy from the nuchal region (Subheading 1.3), it is important that the biopsy contains a number of hair follicles. It is in the nerve cells around these follicles that the virus can usually be detected (1).
9. The Kingfisher 96 basically performs the same process as Subheading 2.8 (extraction of RNA using the MELT™ total nucleic acid isolation system), but the samples are loaded into 96-deep-well plates, and the automated robot undertakes all the washing processes. As the process is split into a digestion part and an extraction/purification part, a number of samples can be digested and stored, before being batched onto the Kingfisher machine and extracted/purified in one step. For the high throughput of samples the machine may save many man-hours in time.
10. The size of a PCR product (3.9.2) can be estimated by comparison against a band of known size; this is done by using a marker or "Ladder" run alongside the samples (such as Φ0174). A number of manufacturers now supply pre-poured gels for electrophoresis, if pouring gels in-house is a problem (14).

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Chapter 17

Simultaneous Detection and Differentiation of Respiratory Syncytial Virus and Other Respiratory Viral Pathogens

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Abstract

Rapid and accurate detection of respiratory syncytial virus (RSV) provides pathogen-specific diagnosis, allows implementation of appropriate infection control measures, and improves patient management. One diagnostic challenge is that respiratory infections, which can be caused by several viral pathogens including RSV, usually present with similar signs and symptoms that are nearly indistinguishable by clinical diagnosis. We have described in the chapter a rapid, high-throughput laboratory technique that can detect a panel of common viral pathogens in one single reaction. With the combination of target-enriched multiplexing PCR amplification and Luminex suspension array identification, 12 common respiratory viruses, including RSV A and B, influenza virus A and B, parainfluenza virus 1, 2, 3, and 4, human metapneumovirus, rhinoviruses, enteroviruses, and SARS coronavirus, are detected and differentiated simultaneously within five hours.

Key words: Respiratory syncytial virus, Multiplex PCR, Suspension array

1. Introduction

1.1. Respiratory Syncytial Virus and Related Diseases

Respiratory syncytial virus (RSV) is known to be the leading cause of bronchiolitis and pneumonia in infants and young children (1, 2). Illness begins most frequently with rhinorrhea, sneezing, and cough. Most children experience recovery from illness after 8–15 days. During their first RSV infection, 25–40% of infected infants and young children have signs or symptoms of bronchiolitis or pneumonia, and 0.5–2% require hospitalization. There are high-risk groups prone to severe RSV disease that include premature infants, children with congenital heart and chronic lung diseases, and immunosuppressed patients or those with a congenital immunodeficiency, in which 25% require hospitalization (3–5). Infections with RSV of genotype A or B may result in differing

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clinical manifestations and outcomes (6, 7). RSV causes hazardous nosocomial infection and produces outbreaks each year with widespread infections in both children and adults, including medical personnel, who may have a mild enough illness that does not cause absences from work. Furthermore, the spread of the virus is facilitated by the number of young infants admitted during an outbreak who tend to shed high titers of virus for prolonged periods (8). Other viruses that can mimic RSV infection include adenoviruses, influenza virus (INF) A and B, parainfluenza viruses (PIV), human metapneumovirus (hMPV), and rhinoviruses. Patients with acute respiratory infections can present with similar signs and symptoms regardless of the viral pathogen, and clinical differential diagnosis of infections caused by these viruses can be difficult.

1.2. Conventional Laboratory Diagnostic Methods

Early and accurate virus-specific laboratory diagnosis significantly enhances patient management. There are currently a variety of laboratory tests available to detect and differentiate respiratory viruses, which have been developed over the last two decades. The use of rapid tests in the diagnosis of RSV infection allows implementation of appropriate infection control measures, thus reducing nosocomial spread (9) and may be useful for consideration of timely treatment with aerosolized ribavirin in severe cases. Direct fluorescent antibody (DFA) tests have good sensitivity for RSV, but are not as good for other respiratory viruses (10, 11). Rapid EIA antigen tests are available for RSV and Influenza A and B; they are easy to perform, and more rapid than DFA, but have slightly lower sensitivity and specificity (12, 13). Among the respiratory viruses mentioned previously, the vast majority will be grown in traditional viral culture cell lines used in clinical virology laboratories with the exception of hMPV, which is not yet widely used in routine diagnostic laboratories (14). Furthermore, cell culture has a slow turnaround time with most viruses showing no detectable cytopathic effects until 3 to 14 days later. However, a shell vial technique incorporated with mixed fresh cells (R-Mix, Diagnostic Hybrids, Inc., Athens, OH) can be performed which speeds up the turnaround time to 2 days and has a sensitivity similar to conventional cell culture (15). The diagnosis of RSV infections using serology has no practical applications in clinical use.

1.3. Molecular Assays for Detection of RSV

As is the case with other respiratory viral pathogens, molecular assays based on in vitro nucleic acid amplification have been implemented gradually in clinical laboratories for RSV detection. These molecular assays, led by PCR, in general, have demonstrated to be superior to routine diagnostic methods in terms of their high sensitivity and specificity (16–18), which are especially useful in adult patients when viral shedding is short and low (19, 20). While cell culture remains the gold standard for RSV diagnosis,

the molecular assays have been considered the "platinum" standard to be used as the reference to validate new diagnostic kits for RSV detection (2, 12). However, these organism-specific monoplex assays are resource-intensive; when a respiratory viral infection is considered, the list of possible etiologies is necessarily long. Multiplex RT-PCR-based techniques have widely been reported for simultaneous detection of a panel of respiratory viral pathogens in a single reaction (21–25). Several products, including the Hexaplex assay (Prodesse, Waukesha, WI), the PLx-RVP assay (EraGen Biosciences, Madison, WI), the ID-Tag RVP (TM Biosciences, Toronto, Canada), and the ResPlex II (Qiagen, Valencia, CA), are commercially available (22, 26–30). This chapter describes a complete molecular diagnostic procedure covering common RNA viral pathogens causing respiratory tract infections.

1.4. Simultaneous Detection and Differentiation of 12 Respiratory Viruses Including RSV

The following procedure consists of three components (Fig. 1). The first, specimen processing and nucleic acid extraction, is done by using an easyMAG automated total nucleic acid extraction instrument (bioMerieux, Durham, NC), based on a magnetic silica particle-based technique (31). The kits are available from bioMerieux and work on a variety of clinical specimens, including

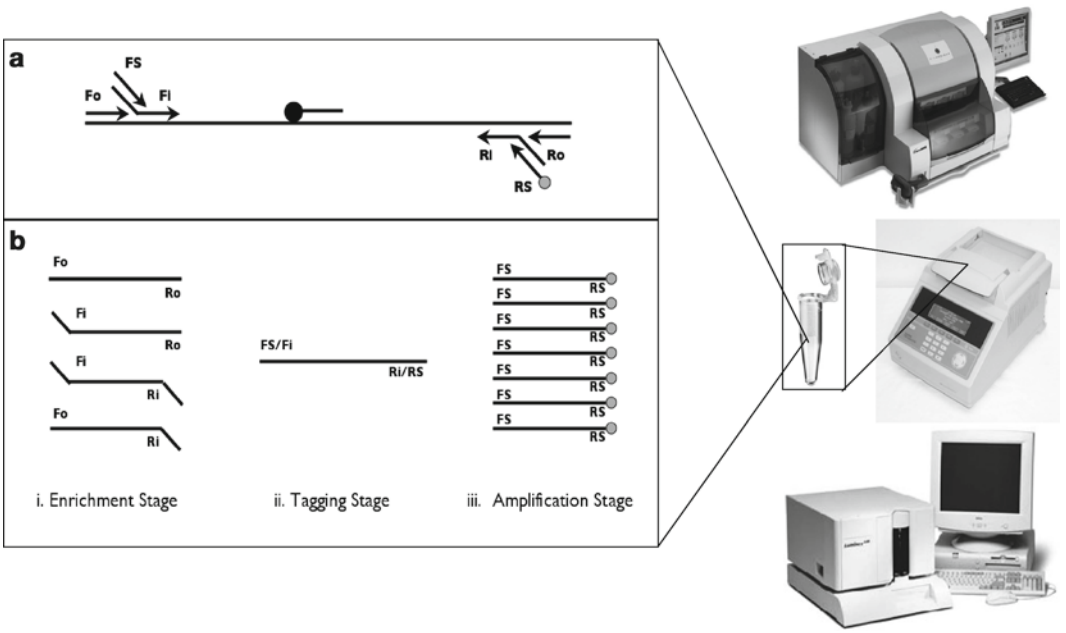

Fig. 1. A complete protocol simultaneously detects and differentiates a panel of respiratory RNA viruses. This contains nucleic acid extraction by easyMAG (bioMerieux), nucleic acid amplification by TEM-RT-PCR (Qiagen), and amplification product identification by a liquid chip system (Luminex). The *left figure* is an illustration of TEM-PCR amplification. (**a**) Nested gene-specific primers are designed to enrich the targets during initial PCR cycling. Later, universal SuperPrimers are used to amplify all targets. (**b**) Different primers are involved in the process at each of the three major stages, enrichment, tagging, and amplification. The *solid lollipop* indicates specific probes, covalently linked with luminex color-coated beads. The *gray circles* indicate biotin molecules. Modified from (33) with permission.

Table 1
Viral pathogens and their gene targets covered by the ResPlex II kit

Pathogens	Abbreviations	Targeted gene(s)
Severe acute respiratory syndrome coronavirus	SARS	Nucleoprotein, polymerase
Influenza A virus	INF-A	Nonstructural protein
Influenza B virus	INF-B	Nonstructural protein
Respiratory syncytial virus A	RSV-A	Nonstructural protein
Respiratory syncytial virus B	RSV-B	Nonstructural protein
Parainfluenza virus type 1	PIV-1	Nucleoprotein
Parainfluenza virus type 2	PIV-2	Nucleoprotein
Parainfluenza virus type 3	PIV-3	Nucleoprotein
Parainfluenza virus type 4	PIV-4	Nucleoprotein
Human metapneumovirus	hMPV	Fusion protein
Rhinoviruses	RhV	5′ Untranslated region
Enteroviruses	EnV	5′ Untranslated region

nasopharyngeal swabs and aspirates and yields high quantity and quality total nucleic acids (32). The second, nucleic acid amplification, uses a target-enriched multiplexing (TEM)-PCR (33), which contains two nested primer sets for each organism and a set of Super Primers, to simultaneously amplify INF-A, INF-B, PIV-1, PIV-2, PIV-3, PIV-4, RSV-A, RSV-B, hMPV, rhinoviruses (RhV), enteroviruses (EnV), and severe acute respiratory syndrome (SARS) coronavirus (CoV) gene in a single reaction tube (27, 29). The target gene from each of the viruses is listed in Table 1. The third, amplicon detection and identification, is reached by using a microsphere-based liquid array (Luminex, Austin, TX). A median fluorescence intensity (MFI) is reported for each target. A positivity cut-off is used to determine if the sample is positive for a particular target (34, 35). The last amplification and detection components are included in the ResPlex II kit, which is available from Qiagen.

2. Materials

2.1. Special Instruments

1. easyMAG automated extraction instrument (BioMerieux, Durham, NC).
2. GeneAmp 9700 PCR System (Applied Biosystems) using 9600 emulation mode.

3. LiquiChip 200 Workstation (Luminex, Austin, TX).
4. Vortexer.
5. Microcentrifuge with rotor for 1.5-mL microcentrifuge tubes.
6. Heat Block or thermal cycler set at 52°C.
7. Multichannel pipet.
8. Positive displacement pipets.

2.2. Nucleic Acid Extraction

1. easyMAG reagents – Buffers 1–4, Magnetic Silica (BioMerieux, Durham, NC).
2. RNase-free water.
3. 1.5-mL nuclease-free microcentrifuge tubes and racks.
4. Nuclease-free PCR tubes (thin-walled 0.2-mL PCR tubes).
5. RNase-free pipette tips with hydrophobic filters.

2.3. TEM-PCR Amplification

1. ResPlex II Kits (Qiagen).
2. Nucleic acid decontaminating solutions or 10% bleach.
3. RNase-free water.
4. 1.5-mL nuclease-free microcentrifuge tubes and racks.
5. Foil to protect reagents from light.
6. RNase-free pipette tips with hydrophobic filters.

2.4. Liquid Chip Detection

1. 96-well, flat-bottomed, microtiter plates with lids.
2. 1.5-mL nuclease-free microcentrifuge tubes and racks.
3. Foil to protect reagents from light.
4. RNase-free pipette tips with hydrophobic filters.

3. Methods

3.1. Nucleic Acid Extraction by EasyMAG

NOTE: Additional information can be obtained from the Generic Protocol provided with the easyMAG instrument.
NOTE: Samples for testing include nasopharyngeal aspirates, nasopharyngeal swabs, and other respiratory secretions and swabs.

1. Press the Daily Use Icon.
2. Begin the extraction request by entering the sample type. With each sample entry, confirm the type of sample. These should be set to "other" and "primary."
3. Define the sample volume and elution volume. For extraction on nasal collections, program a *0.2 mL sample volume with a 55 μL elution volume.*

4. Ensure the protocol is Generic.
5. Press the enter button when finished.
6. After all samples are entered, press the Organize Runs icon (opposing arrows).
7. Create a new run by pressing the New Run icon (sun).
8. Enter a run name, and then press OK.
9. Create the run layout using the arrow icons to arrange the sample list in the correct order for your run.
10. Press the Load Run (instrument with bottles) icon.
11. Place the sample tray and pipette tips in the correct positions.
12. Using the barcode scanner, scan in the position and the sample tray for each position.
13. Press the Printer Icon to print the run. Press OK on the print window when it appears.
14. Pull the sample tubes from the instrument and place them in the metal sample rack.
15. Pipette 200 µL of sample into each sample tube. Use the printout as a guide.
16. When finished, place the sample tray back into the instrument. The position and sample tray will have to be scanned into the instrument. Close the lid of the sample module.
17. Press the Add Lysis icon. The lysis buffer will be added by the instrument and incubated for approximately 10 min.
18. While the sample is lysing on the instrument, prepare the magnetic silica.
19. Prepare the magnetic silica, using the multichannel pipette, choose program 1 (Press the *Select* button until *P1* is displayed and then press *Enter*.). Place one 1-mL BioHit tip on the pipettor. The pipettor program is set up as:

    Program 1
    Fill 550 µL
    Dispense 550 µL
20. Pipette 550 µL of RNase-free water into a 1.5-mL tube.
21. Vortex the silica well and pipette 550 µL of silica into the tube containing the water. Vortex this mixture well.
22. Select an empty ELISA plate. Using the multichannel pipette, choose program 2 (Press the *Select* button until *P2* is displayed, and then press *Enter*.). Place one 1-mL BioHit tip on the pipettor. The pipettor program 2 is set up as:

    Program 2
    Fill ≈1,000 µL
    Dispense ≈100 µL back into source tube

Dispense 125 µL, ×8
Purge

23. Aspirate volume and dispense the first "shot" back into the original tube.
24. Pipette the subsequent eight dispenses into eight wells of the ELISA plate strip. Be sure to purge at the end of the pipetting step.
25. When the lysing step has been completed, an alarm will sound. Open the lid of the sample module. Using multichannel pipettor program 3, place eight 1 mL BioHit tips on the pipettor (Press the *Select* button until *P3* is displayed, then press *Enter*.). Pipettor program 3 is set up as:

    Program 3
    Fill and mix 100 µL
    Fill and mix, ×4 (800 µL, 900 µL, then three, 1,000 µL mixes)
26. Beginning with the ELISA plate, aspirate all eight wells on the ELISA plate.
27. Place the tips into the sample tube tray just about where the tubes bend; then dispense and mix in the sample tube tray.
28. When all silica has been dispensed into the sample tube trays, press the Start icon.
    NOTE: Do not open the easyMAG sample module lid during testing phases! Doing so will abort the run.
29. The run will continue until all samples are completed. "Finished" will display on the bar at the bottom of the screen. While the run is in progress, label a 1.5-mL tube for each sample and control for long-term storage.
30. Complete the run by pressing the View Results Icon (Magnifying Glass).
31. Press the Printer Icon and press OK.
32. The report will print. A window will pop up reading "The run results are being printed. Mark this run ASSESSED?" Press Yes.
33. Quickly check the report for any errors. If an error was signaled, repeat the extraction and refer to troubleshooting.

3.2. RT-PCR Amplification by TEM-PCR

1. Before using AmpCheck reagent for the first time, make a 1/50 dilution by adding 245 µL RNase-free water into the tube containing the 5 µL AmpCheck and mix.
2. Make sure the thermal cycler is preheated to 50°C before placing samples into the unit.
3. Thaw the template RNA, dNTP Mix, 5× QIAGEN OneStep RT-PCR Buffer, and RNase-free water, and place them on ice.

Table 2
Composition of reaction mix for QIAplex RT-PCR amplification reaction

Component	Volume/reaction	Final concentration
RNase-free water[a]	Variable	–
5× QIAGEN OneStep RT-PCR Buffer[a, b]	10 µL	1×
dNTP Mix (containing 10 mM of each dNTP)[a]	2 µL	400 µM of each dNTP
ResPlex II SuperPrimers	6 µL	–
QIAGEN OneStep RT-PCR Enzyme Mix[a]	2 µL	–
RNase inhibitor	Variable	5–10 units/reaction
Template RNA (added at step 4)	Variable	–
Total volume	*50 µL*	–

[a]Included in the QIAGEN OneStep RT-PCR Kit
[b]Contains 12.5 mM $MgCl_2$

4. Mix the ResPlex II SuperPrimers by vortexing briefly.

   NOTE: It is important to mix the solutions completely before use to avoid localized differences in salt concentration.

5. Prepare a master mix according to Table 2. The master mix typically contains all the components required for RT-PCR except the template RNA. Prepare a volume of master mix 10% greater than that required for the total number of reactions to be performed. A negative control (without template RNA) should be included in every experiment. An additional reaction for amplification of AmpCheck should be included as an amplification check.
6. Mix the master mix thoroughly and dispense appropriate volumes into PCR tubes.
7. Mix gently, for example, by pipetting the master mix up and down a few times.
8. Add template RNA to the individual PCR tubes. Include a negative control by substituting RNase-free water for the RNA sample volume. Include an amplification check with 5 µL AmpCheck (diluted 1/50) instead of template RNA.
9. Program the thermal cycler according to the program outlined in Table 3.
10. Start the RT-PCR program while the PCR tubes are still on ice. Wait until the thermal cycler has reached 50°C. Then, place the PCR tubes in the thermal cycler.

Table 3
Optimized cycling protocol using the GeneAmp 9700 PCR system running in 9600 emulation mode

Reverse transcription	35 min	50°C
Initial PCR activation step[a]	15 min	95°C
Enrichment cycling		
Denaturation	30 s	94°C
Annealing	1 min	52°C
Extension	1 min	72°C
Number of cycles	15	
2-step cycling		
Denaturation	15 s	94°C
Annealing/extension	1.5 min	70°C
Number of cycles	6	
3-step cycling		
Denaturation	15 s	94°C
Annealing	15 s	52°C
Extension	15 s	72°C
Number of cycles	30	
Final extension	3 min	72°C

[a]HotStarTaq DNA Polymerase is activated by this heating step. Omniscript and Sensiscript® Reverse transcriptases are inactivated and the cDNA is denatured

3.3. Amplicon Detection and Differentiation by Luminex

1. Before use, Detection Buffer and Stopping Buffer should be brought to room temperature (15–25°C).

   NOTE: These buffers can be stored at room temperature so that they are ready to use.

2. Heat a heating block or thermal cycler to 52°C.
3. Heat Stopping Buffer to 52°C, and keep it at 52°C until use.
4. Mix the ResPlex II Bead Mix by vortexing for at least 15 s.

   NOTE: Protect the ResPlex II Bead Mix and reaction mix from light. Foil should be used to cover the tubes and 96-well plate.

5. Prepare the detection mix according to Table 4.

Table 4
Composition of detection mix

Component	Volume/reaction
Detection buffer	35 µL
ResPlex II bead mix	10 µL
Total volume	*45 µL*

6. The detection mix contains all of the components needed for the reaction except for the PCR products and the diluted Streptavidin–PE. Prepare a volume of detection mix 10% greater than what is required for the total number of assays to be performed.
7. Vortex the reaction mix thoroughly. Dispense 45 µL into each well of a 96-well, flat-bottom plate.
8. For each assay, add 5 µL of the RT-PCR reaction (from the previous section) into a sample well. Mix thoroughly by pipetting up and down.
9. Incubate samples at 52°C in the dark for 10 min.

   NOTE: Keep samples at 52°C until the detection reaction is completed.
10. While the samples are incubating, proceed with step 11.
11. Make a fresh 1:1 mixture of Detection Buffer: Streptavidin–PE at room temperature. Make enough to use 10 µL of the mixture per assay. For example, add 50 µL Streptavidin–PE to 50 µL Detection Buffer. Prepare a volume 10% greater than what is required for the total number of detection assays to be performed.
12. Cover with foil or keep in a dark place to protect from light.
13. After incubation (step 9), add 10 µL Detection Buffer:Streptavidin–PE to each sample. Keep the samples at 52°C, and mix briefly by pipetting up and down.
14. Incubate at 52°C in the dark for 5 min.
15. Keep the samples at 52°C, add 120 µL Stopping Buffer (prewarmed to 52°C) to each reaction.
16. Keep the samples at 52°C and protect them from light until analysis.

3.4. Result Output and Interpretation

Samples are analyzed on the LiquiChip 200 Workstation using QIAplex MDD Software. (Alternatively, the Luminex 100 IS System or Luminex 200 System with QIAplex MDD Software can be used). A mean fluorescence intensity (MFI) is reported for each target. Table 5 provides an example of data, from a ResPlex II

Table 5
Example data results of RexPlex 2 panel

Sample	CTRL	EnV	hMPL	INFA	INFB	PIV1	PIV2	PIV3	PIV4	RhV	RSVA	RSVB
NTC	14	15	9	20	7	14	10.5	11	12	15.5	8	13
Neg	28	15	16	14	16	21	16	25	17	21.5	12	19
kit+	*1,208.5*	12.5	22.5	26	10	10	8	28	7.5	13	15	14
RSV+	*1,366*	14.5	10	17	14	8	14	9	23	6	10	*3,767.5*
INFA+	*1,288*	13.5	12	*2,845*	15	10	15	18	22	12	11	15
INFB+	*1,425*	15	25	15	*1,958*	15	19	22	16	15	15	10
PIV1+	*1,259*	18	12	18	22	*2,583*	22.5	25	28	15	12	15

run. The positivity cutoff is dependent on the instrument and software used for analysis. The cutoff value for each target is determined as the sum of the mean plus four times the standard deviations of the negative controls.

4. Notes

1. HotStar *Taq* DNA Polymerase (contained in the Qiagen OneStep RT-PCR Enzyme Mix) requires initial activation by incubation at 95°C for 15 min before amplification can take place. This incubation also partially inactivates the reverse transcriptase. The thermal cycling program includes steps for both reverse transcription and PCR. Do not activate HotStarTaq DNA Polymerase until the reverse-transcription reaction is finished.
2. It is important to maintain an RNase-free environment during extraction and RT-PCR setup steps. Set up all reaction steps in an area separate from the other areas (extraction, amplification, and detection). Dead air boxes can be used for each step/area to avoid contamination between areas.
3. Set up all RT-PCR reactions on ice. RNA is the target, which is fragile. Keeping the reaction mixes cold will reduce RNA degradation.
4. Use disposable, RNase-free tips containing hydrophobic filters to minimize cross-contamination.
5. Use of the GeneAmp 9700 PCR System (Applied Biosystems) running in the 9600 emulation mode for the amplification step is recommended. Performance of this protocol using other equipment has not been verified yet.
6. From the beginning of the hybridization procedure in the detection step, the detection reaction must be kept at 52°C until analysis on the LiquiChip 200 Workstation.
7. Protect the ResPlex II Bead Mix and reaction mix from light. Foil should be used to cover the tubes and 96-well plate.
8. In a clinical validation study on 360 archived frozen nasopharyngeal swabs and aspirates, the sensitivity and specificity for RSV detection were 73.3 and 100%, when compared to a standard combined from cell culture and monoplex real-time TaqMan PCR. Sensitivities and specificities were 72.2–90% and 99.7–100%, respectively, for other respiratory viruses. When a full panel (96 tests) was run, the hands-on time and the test turnaround time were estimated as 55 min and 6 h, respectively (27).

9. There is no validation data on PIV-2, PIV-4, enterovirus, and rhinovirus. There is some cross-reactivity between PIV-1 and PIV-3 in the ResPlex assay; of 27 samples testing positive for parainfluenza, eight samples (29.6%) had IMF values higher than the cutoff values for PIV-1 and PIV-3 (27).
10. The assay was useful to detect and recognize viral coinfections, which are usually related to more severe clinical manifestations (30).
11. Respiratory DNA viruses, such as bocavirus (36), adenovirus, and a recently reported respiratory polyomavirus (37), were not covered in the ResPlex II kits evaluated in the study. A new version of the ResPlex II kit, which covers bocavirus and adenovirus in addition to those described in the chapter, has been available from Qiagen since July 2008.
12. We thank Melinda McCormac, Shufang Meng, and Wray Estes for their excellent technical support. This project was partially supported by a research and development grant from Qiagen. The protocol contains partial information in product inserts of the easyMAG and the ResPlex II kits and the Standard Operating Manual of the Molecular Infectious Disease Laboratory, Vanderbilt University Hospital.

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Chapter 18

Rotaviruses

Jim Gray and Miren Iturriza-Gómara

Abstract

Rotaviruses can be detected easily, and methods have been developed to visualise their characteristic morphology, to detect rotavirus proteins through immunological methods or the virus genome, either directly by polyacrylamide gel electrophoresis or after reverse transcription of the viral RNA and amplification by PCR. The abundance of virus particles found in clinical samples during an acute infection makes the detection of rotavirus proteins, mainly VP6, the method of choice for virus detection. Molecular methods are generally reserved for the characterisation of a diverse population of viruses circulating in many mammalian species. Characterisation methods have been developed to determine diversity within genes encoding viral structural proteins, VP4, VP7, and VP6 and the non-structural protein and viral enterotoxin, NSP4. The combined use of the detection and characterisation methods described in this chapter allows novel rotavirus strains resulting from genetic reassortment among common strains, reassortment among animal and human strains and zoonotic strains to be identified. Also, strains in which diversity is generated through the accumulation of point mutations during virus replication are identified. The development of safe and effective rotavirus vaccines necessitates the detection and characterisation of rotaviruses of genomic and antigenic diversity circulating in both the human and animal populations.

Key words: Virus morphology, Antigen detection, Genome detection, Re-assortment, Genotyping algorithm

1. Introduction

Rotaviruses are the major cause of gastroenteritis among children of less than 5 years of age and of acute diarrhoea in the young of many mammalian species. Rotavirus infections are responsible for more than 600,000 deaths each year, mostly of infants and young children in developing countries (1).

Rotaviruses are non-enveloped viruses, 75 nm in diameter and are characterised by their wheel-like structure (Latin *rota*=wheel) (Fig. 1). Rotaviruses possess a genome of 11 segments of double-stranded (ds) RNA, encoding six structural and six non-structural proteins. Rotavirus genomes can re-assort during dual infection of a

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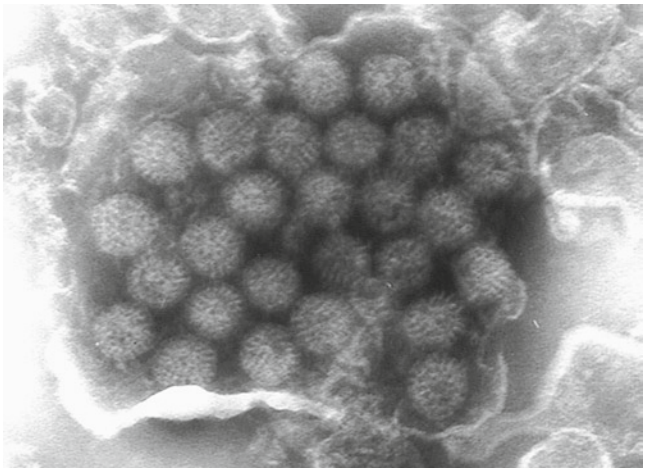

Fig. 1. Electron micrograph of negative-stained (PTA) 75 nm diameter rotavirus particles. Magnification ×50,000.

single cell, resulting in the broad diversity of rotavirus strains co-circulating in the population. The classification scheme for rotaviruses is based on the immunological reactivities or genomic sequences of three structural proteins, VP6 (the middle layer, defining group and sub-group), VP7 (the outer layer, defining G-type [glycoprotein]), and VP4 (the surface spike, defining P-type [protease-sensitive]), and the detection and characterisation of rotaviruses has concentrated on the development of methods for the detection of these proteins and the genes that encode them. Rotaviruses are excreted in faeces during acute infection and may be present at concentrations of up to 10^{12} particles per gram (mL).

Laboratory diagnosis of human rotavirus infections can be performed by electron microscopy (EM) through the identification of its characteristic morphology (Fig. 1). Although this method does not distinguish between different rotavirus groups, this can be accomplished through the use of group-specific antisera and immune EM. Rotaviruses can be cultured in foetal African green monkey kidney cells (MA104 cell line) but need to be grown in media containing trypsin, required to cleave the protease-sensitive protein, VP4, necessary for virus attachment. Primary rotavirus isolation in cell culture is performed in primary or secondary rhesus monkey kidney (RMK) cells but is relatively inefficient. Cell culture is used for the preparation of rotavirus antigens from RMK cell-adapted strains rather than for laboratory diagnosis. The detection of rotavirus VP6 protein, the middle layer and most abundant virus protein, is the target of choice for diagnostic assays. Indirect immunofluorescence, particle agglutination, in-house or commercial enzyme-linked immunosorbent assays, and, more recently, immunochromatographic near-patient test devices have all gained widespread use for the

diagnosis of rotavirus infections. The detection of the rotavirus genome by polyacrylamide gel electrophoresis (PAGE) can be used to detect and to distinguish among different groups as they possess distinct migration patterns.

Molecular amplification methods, such as RT-PCR, are regarded as too sensitive for the detection of rotaviruses in faeces due to their ability to detect asymptomatic infections, which are common in infants and young children. Molecular methods are used for the characterisation of rotavirus G and P-types (see Fig. 2 for recommended testing algorithm), for the determination of

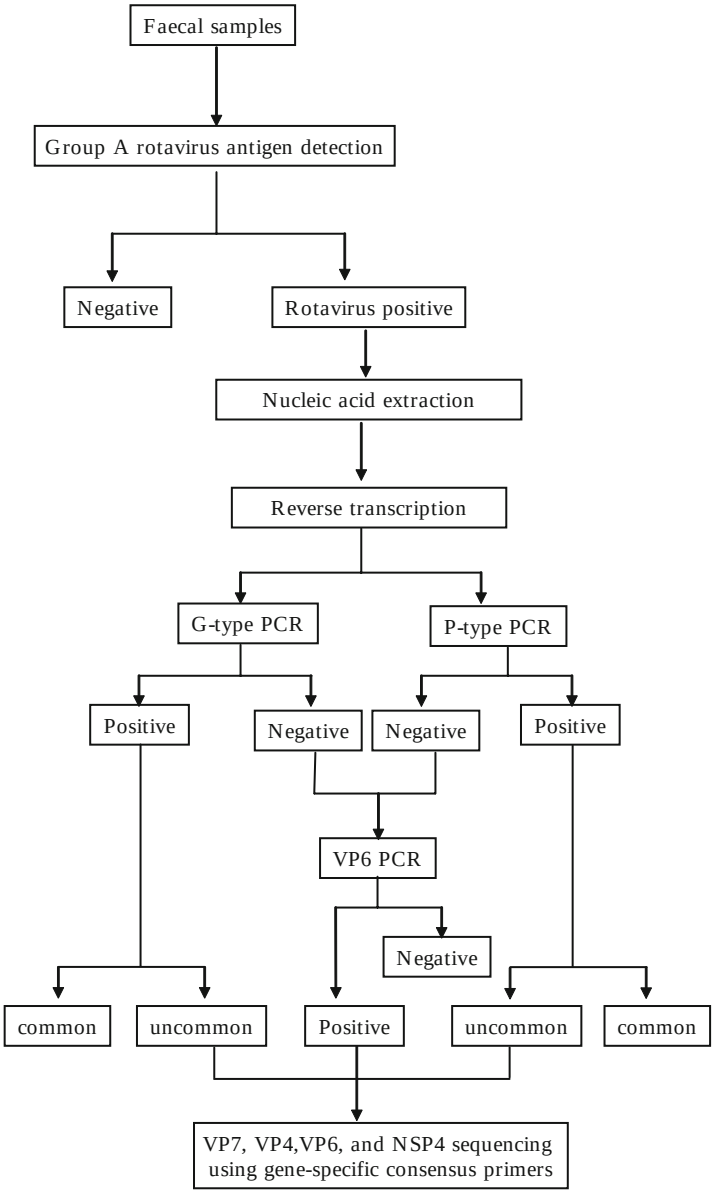

Fig. 2. Rotavirus genotyping algorithm.

zoonotic infections, and for the detection of rotaviruses infecting extra-intestinal sites, such as the central nervous system, where low viral loads are expected.

Nucleic acid sequence analysis may be required when rotavirus-positive strains fail to genotype. Failures to genotype by RT-PCR may be associated with the accumulation of point mutations at the type-specific oligonucleotide primer-binding positions (2–4), or with the emergence of a novel type for which oligonucleotide primers are not available or included in the PCR mix (5, 6). In these cases, amplicons can be obtained using the gene-specific consensus primer, which can be sequenced directly to assign a genotype and investigate whether the methods need to be updated through the modification of existing primers for the detection of common genotypes or the inclusion of a new primer for the detection of a novel or emerging genotype (4–8).

The emergence of novel strains in the human population is thought to occur through zoonotic transmission. Sequence analysis of the rotavirus genes encoding NSP4 and VP6 provides a tool for monitoring interspecies transmission, as these two genes appear to co-segregate during reassortment, and also to segregate according to the species of origin (9, 10).

2. Materials

2.1. Electron Microscopy

1. Faeces, prepared as a 10% suspension in balanced salt solution (BSS) 199 (Invitrogen, Paisley).
2. 3% Phosphotungstic acid (PTA), adjusted to pH 6.3 with potassium hydroxide.
3. Waxed slide.
4. Carbon–formvar-coated copper grids.
5. EM forceps (Aga Aids, Scientific Ltd., Stansted, UK).
6. Transmission electron microscope.

2.2. Immune Electron Microscopy

1. Faecal suspension (see Subheading 2.1).
2. Rotavirus group A, B, and C-specific antibodies.
3. 3% PTA, pH 6.3.
4. Waxed slide.
5. Carbon–formvar-coated copper grids.
6. 500 µg/mL Protein A solution (Sigma, Dorset, UK).
7. Incubator.
8. Transmission electron microscope.

2.3. Virus Culture

1. Faecal suspension (see Subheading 2.1).
2. Primary or secondary rhesus monkey kidney (RMK) cells.
3. MA104 cells.
4. M199 (balanced salt solution).
5. Penicillin (25,000 Units/mL) + streptomycin (25,000 µg/mL) stock.
6. Amphotericin B (250 µg/mL).
7. Fungizone (5 µg/mL) + ceftaxidime (20 µg/mL) + vancomycin (20 µg/mL) (FCV).
8. Foetal bovine serum (FBS).
9. Shell vials (Trac bottles; Bibby sterilin, Staffordshire, UK) or Tissue culture 8-well chamber slides (Lab-Tek™, Nunc, VWR International Ltd, Leicester, UK).
10. Sterile pipettes (10 mL).
11. Sterile Pasteur pipettes (1 mL).
12. Sterile universal containers.
13. Trypsin (10×: 25 mg/mL).
14. Versene (10×: 2.0 mg/mL NaEDTA).
15. Crystalline trypsin Factor IX (Sigma, Dorset, UK).
16. Sterile phosphate-buffered saline (PBS) solution 0.1 M, pH 7.4.

2.4. Enzyme-Linked Immunosorbent Assay

1. 96-well flat-bottomed microtitre plate (Becton Dickinson, Oxnard, CA).
2. 0.1 M sodium carbonate–bicarbonate buffer (CBB), pH 9.6.
3. 0.1 M PBS, pH 7.3 (Oxoid, Hampshire, UK), containing 0.05% Tween 20 (PBS-T).
4. 5 and 1% solutions of skimmed milk powder (SMP) (Oxoid, Hampshire, UK) in PBS.
5. Anti-group A rotavirus antibodies (goat, rabbit, or sheep; Abcam, Cambridge, UK).
6. Faecal suspension (see Subheading 2.1).
7. Anti-rotavirus mouse monoclonal antibody (Abcam, Cambridge, UK).
8. Horseradish peroxidase-labelled anti-mouse antibodies (Sigma, Dorset, UK).
9. Substrate buffer: 0.05 M phosphate citrate buffer. pH 6.0 (Sigma, Dorset, UK).
10. 0.1 mg/mL tetramethylbenzidine (TMB; Sigma) containing 0.014% hydrogen peroxide (H_2O_2) in substrate buffer (Sigma, Dorset, UK).

11. 2 M sulphuric acid.
12. Spectrophotometer with 450 nm wavelength filter.
13. Microtitre plate washer (Nunc, VWR International Ltd, Leicester, UK).
14. Incubator.

2.5. Rotavirus Molecular Detection and Typing

1. 2-mL screw-cap tubes (Sarstedt, Leicester, UK).
2. 5-mL screw-cap tubes (Sarstedt, Leicester, UK).
3. Bacteriological loop (10 μL).
4. Vortex.
5. Microfuge.
6. Heating block at 56°C.
7. Heating block at 95–100°C.
8. Water bath at 37°C.
9. Thermal cycler.
10. Real time PCR equipment: ABI PRISM 7500 SDS (Applied Biosystems, Cheshire, UK).
11. Agarose-gel casting tray and combs.
12. Electrophoresis tank.
13. Electrophoresis power pack.
14. Gel imaging equipment (UV) or UV transilluminator.
15. Balanced Salt Solution (M199, MEM or equivalent).
16. L6-buffer (Severn Biotech, Kidderminster, UK).
17. L2-bufer (Severn Biotech, Kidderminster, UK).
18. Size-fractionated silica (Severn Biotech, Kidderminster, UK).
19. 70% Ethanol.
20. Acetone.
21. Nuclease-free water.
22. RNase inhibitor (RNasin, Invitrogen, Paisley, UK).
23. Reverse transcriptase (MMLV-RT, Invitrogen, Paisley, UK).
24. Random hexamers (Invitrogen, Paisley, UK).
25. DNA polymerase (Taq polymerase, Invitrogen, Paisley, UK).
26. 10× PCR buffer (Invitrogen, Paisley, UK).
27. 50 mM $MgCl_2$ solution (Invitrogen, Paisley, UK).
28. dNTP mix (Invitrogen, Paisley, UK).
29. Taqman real-time PCR master mix (Invitrogen, Paisley, UK).
30. Oligonucleotide primers and probe.

(a) VP6 oligonucleotide primers (11, 12) and probe

VP6-F	5′ GAC GGV[a] GCR[b] ACT ACA TG GT 3′
VP6-R	5′ GTC CAA TTC ATN[c] CCT GGT G 3′
Product: 382 bp	
Probe	VP6P [FAM]5′-CCA CCR AAY[d] ATG ACR CCA GCN GTA-3′ [MGB]
Nested primers	
VP6-NF	5′ GCW[e] AGA AAT TTT GAT ACA 3′
VP6-NR	5′ GAT TCA CAA ACT GCA GA 3′
Product: 147 bp	

[a]V = A, C, or G
[b]R = A or G
[c]N = A, T, C, or G
[d]Y = C or T
[e]W = A or T

(b) NSP4 oligonucleotide primers (10)

NSP4-F	5′ GGC TTT TAA AAG TTC TGT TCC GAG 3′
NSP4-R	5′ GGT CAC ACT AAG ACC ATT CC 3′
Product size: 743 bp	

(c) G-typing oligonucleotide primers

First round: product 881 bp (4, 13)		
VP7-F	5′ ATG TAT GGT ATT GAA TAT ACC AC 3′	(nt 51–71)
VP7-R	5′ AAC TTG CCA CCA TTT TTT CC 3′	(nt 914–932)
Second-round typing primers (4, 14): All typing primers are included in the mix (14)		
Genotype G1:		
aBT1	5′ CAA GTA CTC AAA TCA ATG ATG G 3′	(nt 314–335)
Product size; 618 bp		
Genotype G2:		
aCT2	5′ CAA TGA TAT TAA CAC ATT TTC TGT G 3′	(nt 411–435)
Product size; 521 bp		
Genotype G3:		
G3	5′ ACG AAC TCA ACA CGA GAG G 3′	(nt 250–269)
Product size; 682 bp		
Genotype G4:		
aDT4	5′ CGT TTC TGG TGA GGA GTT G 3′	(nt 480–499)

(continued)

(continued)

Product Size; 452 bp		
Genotype G8:		
G8	5′ TTR TCG CAC CAT TTG TGA AAT 3′	(nt 176–198)
Product size; 756 bp		
Genotype G9:		
G9	5′ CTT GAT GTG ACT AYA AAT AC 3′	(nt 757–776)
Product size; 179 bp		
Genotype G10:		
G10	5′ ATG TCA GAC TAC ARA TAC TGG 3′	(nt 666–687)
Product size; 266 bp		
Genotype G12:		
G12	5′ GGT TAT GTA ATC CGA TGG ACG 3′	(nt 536–556)
Product size; 396 bp		

(d) VP7 nested consensus primer (for sequence typing)

VP7-RINT	5′ ANA YNG ANC CWG TYG GCC A 3′	(nt 331–344)
Product size; 293 bp		
Y = C or T		
W = A or T		

(e) P-typing oligonucleotide primers

First-round consensus primers (15)		
VP4F	5′ TAT GCT CCA GTN AAT TGG 3′	(nt 132–149)
VP4R	5′ ATT GCA TTT CTT TCC ATA ATG 3′	(nt 775–795)
Product size: 663 bp		
Second-round type-specific primers (4, 15, 16)		
VP4F	5′ TAT GCT CCA GTN AAT TGG 3′	(nt 132–149)
P(4) (previously genogroup 2)		
2T-1	5′ CTA TTG TTA GAG GTT AGA GTC 3′	(nt 474–494)
Product size; 483 bp		

(continued)

(continued)

P(6) (previously genogroup 3)		
3T-1	5′ TGT TGA TTA GTT GGA TTC AA 3′	(nt 259–278)
Product size; 267 bp		
P(8) (previously genogroup 1)		
1T-1D	5′ TCT ACT GGR TTR ACN TGC 3′	(nt 339–356)
Product size; 345 bp		
P(9) (previously genogroup 4)		
4T-1	5′ TGA GAC ATG CAA TTG GAC 3′	(nt 385–402)
Product size; 391 bp		
P(10) (previously genogroup 5)		
5T-1	5′ ATC ATA GTT AGT AGT CGG 3′	(nt 575–594)
Product size; 583 bp		
P(11)		
P(11)	5′ GTA AAC ATC CAG AAT GTG 3′	(nt 305–323)
Product size; 312 bp		

31. Molecular-grade agarose suitable for separation of low molecular weight DNA fragments (<1 kB) (Ultrapure™ Agarose-1000, Invitrogen Paisley, UK).
32. Tris-Borate Buffer (TBE).
33. Electrophoresis loading buffer.
34. Molecular weight marker (100 bp ladder; Invitrogen Paisley, UK).
35. Ethidium bromide (5 mg/L in TBE).

2.6. Polyacrylamide Gel Electrophoresis

1. Pipettors.
2. Vortex mixer.
3. Water bath (variable temperature).
4. Microfuge.
5. Freezer (–20°C or –70°C).
6. Electrophoresis apparatus (gel assembly and electrophoresis tank).
7. Power pack.
8. Side-arm flask (degassing).
9. Vacuum pump.
10. Laboratory scale, spatula, and weighing boats.

11. Plastic/glass dishes.
12. Timer.
13. Orbital rotator.
14. Gel dryer (vacuum or air).
15. Benchkote™ (Whatman).
16. Eppendorf tubes.
17. Distilled water.
18. 10% SDS stock (Add 1 g of SDS to 10 mL of distilled water. Dissolve in a 65°C water bath).
19. 1 M sodium acetate (NaAc) containing 1% SDS (Dissolve 8.2 g of sodium acetate in 60 mL of distilled water. Add 10 mL of 10% SDS stock and mix. Adjust the pH to 5.0 with glacial acetic acid, and make up to 100 mL with sterile distilled water. Heat the solution to 42°C if a precipitate is present prior to use).
20. Phenol–chloroform (1:1) (Mix equal volumes of citrate-saturated phenol, pH 4.3, and chloroform. Place in a dark or foil-covered bottle. Store at 4°C).
21. 3 M NaAc, pH 5.0 (Dissolve 4.92 g of sodium acetate in 10 mL of distilled water. Make up to 20 mL with distilled water).
22. 30% acrylamide-bis acrylamide stock solution (Dissolve 30 g of acrylamide and 0.8 g of *N,N*′methylene bis-acrylamide in 100 mL of distilled water. Filter before use. Place the solution in a dark or foil-covered bottle, and store at 4°C; pre-made solutions can be purchased from several manufacturers: Invitrogen, Paisley, UK; Sigma, Dorset, UK).
23. 1 N hydrochloric acid (HCl) (Add 8.6 mL of concentrated HCl to 91.4 mL of sterile distilled water).
24. Resolving gel buffer (1.5 M, pH 8.8) (Dissolve 18.15 g of Tris base in 40 mL of distilled water. Adjust the pH to 8.8 with 1 N HCl. Make up to 100 mL with distilled water).
25. Stacking gel buffer (0.5 M, pH 6.8) (Dissolve 5.98 g of Tris base in 50 mL of distilled water. Adjust the pH to 6.8 with 1 N HCl. Make up to 100 mL with distilled water).
26. 10% (w/v) ammonium persulphate (APS) (Dissolve 0.1 g of APS in 1 mL of distilled water just prior to use. Store at 4°C for a maximum of 3 days).
27. 5× Tris-glycine running buffer (Dissolve 15.1 g of Tris base and 94 g of glycine in distilled water, and make up to 1,000 mL with distilled water).
28. 1× Tris-glycine running buffer (Dilute 200 mL 5× Tris-glycine buffer with 800 mL of distilled water).

29. PAGE sample running dye (Dissolve 10 mg of bromophenol blue and 1 mL of glycerol in 5 mL of stacking gel buffer. Make up to 10 mL with distilled water).
30. 10% resolving gel:

	1.5-mm gel		0.75-mm gel	
Reagent	**1×**	**2×**	**1×**	**2×**
dH_2O	15.8 mL	31.6 mL	9.9 mL	19.8 mL
30% acryl stock	10.0 mL	20.0 mL	6.3 mL	12.5 mL
Resolving buffer, pH 8.8	3.75 mL	7.5 mL	2.4 mL	4.8 mL
TEMED	15 µL	30 µL	10 µL	20 µL
10% APS	450 µL	900 µL	282 µL	564 µL

31. 4% spacer gel:

	1.5-mm gel		0.75-mm gel	
Reagent	**1×**	**2×**	**1×**	**2×**
dH_2O	6.8 mL	13.6 mL	5.1 mL	10.2 mL
30% acryl stock	1.6 mL	3.2 mL	1.2 mL	2.4 mL
Stacking buffer, pH 6.8	1.25 mL	2.5 mL	0.9 mL	1.8 mL
TEMED	5 µL	10 µL	4 µL	8 µL
10% APS	150 µL	300 µL	112 µL	225 µL

32. Silver staining (For one 15 cm × 15 cm × 1.5 mm polyacrylamide gel):

Buffers	
Fixing solution 1	Add 80 mL of ethanol and 10 mL acetic acid to 110 mL of dH_2O
Fixing solution 2	Add 20 mL of ethanol and 1 mL acetic acid to 180 mL of dH_2O
Silver nitrate solution	Dissolve 0.37 g of $AgNO_3$ in 200 mL of dH_2O
Developing solution	Add 2 mL of 36% formaldehyde to 250 mL of dH_2O. Just before use, dissolve 7.5 g of NaOH in this solution
Stopping solution	Add 10 mL of acetic acid to 200 mL of dH_2O

3. Methods

3.1. Electron Microscopy

1. Place 10 µL PTA solution onto a waxed slide.
2. Emulsify a small portion of the faecal suspension into the PTA, to achieve a slightly opalescent suspension.
3. Float the EM grid onto the faecal suspension and leave for 1 min.
4. Place 10 µL of distilled H_2O onto the waxed slide adjacent to the specimen.
5. Remove the grid with sterile forceps, and remove excess liquid by touching the side of the grid to a piece of blotting paper.
6. Float the grid onto the distilled H_2O and leave for 1 min.
7. Remove the grid and blot as before.
8. Allow the grid to air-dry while held in the forceps.
9. Place the grid in a clean Petri dish, and irradiate with UV light for 1 min.
10. Examine the grid by EM at ×30,000–50,000 magnification.

3.2. Immune Electron Microscopy

1. Float three carbon–formvar-coated copper grids onto three drops (10 µL) Protein A solution.
2. Incubate in a moist chamber at room temperature for 20 min.
3. Immediately, on a second waxed card, mix 5 µL of faecal suspension with 5 µL rotavirus group-specific antibodies.
4. Place the card containing the virus–antibody mixture in a moist chamber at room temperature.
5. Remove the grids from the Protein A solution and blot by touching the edge of the grid to a piece of filter paper.
6. Float the grid onto a 10 µL drop of distilled H_2O, and blot as before.
7. Repeat the washing step once.
8. Float the grid onto the virus–antibody mixture and incubate in a moist chamber at 37°C for 1 h.
9. Repeat the wash procedure (two washes with distilled H_2O).
10. Blot to remove excess liquid and float the grid onto a 10 µL drop of PTA, and leave for 1 min.
11. Blot, and allow the grid to air-dry.
12. Place the grid under UV light for 1 min.
13. Specific virus–antibody interactions are characterised by the presence of immune complexes spread evenly across the grid when viewed by EM.

3.3. Virus Culture

3.3.1. MA104 Cell Maintenance

1. Remove the medium from confluent monolayers of MA104 cells and rinse with sterile PBS.
2. Add 5 mL of Trypsin (1:10 dilution) and 5 mL of Versene (1:10 dilution) and leave for 5 min at room temperature.
3. Remove Trypsin-Versene, and incubate the cells at 37°C for 5–10 min or until the cells begin to detach from the bottle.
4. Add 10 mL of growth medium and re-suspend the cells with a sterile pipette.
5. Split the cells into two fresh bottles and add growth medium to give a concentration of 1 to 5×10^5 cells/mL (to 10- or 25-mL final volume for 25- or 75-cm^2 flasks, respectively). Incubate at 37°C in an atmosphere of 5% CO_2.

3.3.2. Shell-Vial Preparation

1. After trypsinisation and resuspension of MA104 cells, dilute them to a concentration of 5×10^5 cells/mL in growth medium.
2. Add 500 µL of the cell suspension to each vial, and incubate at 37°C in an atmosphere of 5% CO_2 until the cells are confluent. Seed RMK cells at the same concentration, 5×10^5 cells/mL.

3.3.3. Rotavirus Activation

1. Treat 100 µL of virus suspension with 100 µL of M199-containing antibiotics + 10 µg/mL Trypsin Factor IX.
2. Incubate at 37°C for 30 min.

3.3.4. Inoculation of Shell Vials

1. 24 h before inoculation with rotavirus, remove the growth medium from the confluent shell vials, and replace with serum-free M199-containing antibiotics.
2. Remove the medium and add 200 µL of the activated rotavirus solution. Centrifuge the vials at 573 *g* for 1 h at 30°C.
3. Remove the inoculum and add 1 mL of virus culture medium. Incubate at 37°C in an atmosphere of 5% CO_2.

3.3.5. Culture of Rotaviruses from Clinical Specimens

1. Clarify the rotavirus-positive 10% faecal specimens (in BSS) by centrifugation at 6200 *g* for 15 min.
2. Treat 100 µL of the clarified supernatant with an equal volume of M199-containing 10 µg/mL Trypsin Factor IX as described in Subheading 3.3.3.
3. Inoculate the RMK cells as described Subheading 3.3.4.
4. In the first passage (P_0), add one drop of FCV antibiotic solution to each virus-inoculated vial/tube after removal of the inoculum and addition of the virus culture medium.
5. When cell lysis is evident, freeze (–70°C) and thaw the cell-culture medium and use it to inoculate a fresh vial of RMK cells as described above.

6. After two passages, inoculate MA104 cells as described above; continue passaging to achieve high viral titres.
7. Once the rotaviruses are adapted to growth in MA104 cells, the titre can be increased by inoculating the contents of three shell vials onto one 25-cm^2 flask of MA104 cells as described above, except that, after activation of the rotavirus, incubate the inoculated cells at 37°C in an atmosphere of 5% CO_2 for 1 h.
8. The presence of virus can be detected by IF using VP6-specific monoclonal antibodies.

3.3.6. Detection of Rotavirus in Cell Culture by Immunofluorescence

1. After 18-h incubation, wash the infected cells in the shell vials with PBS and fix them with methanol for 15 min. Alternatively, scrape the cells from the vial/cell culture tube and mix well, add a drop of the infected cells to a clean slide, let dry, and then fix in acetone for 15 min.
2. Cover the fixed cells with anti-VP6 monoclonal antibody at an appropriate dilution in PBS.
3. Incubate at 37°C for 30 min. Wash the slides/vials twice with PBS for 10 min each with gentle rocking.
4. Cover the infected cells with FITC-conjugated anti-mouse antibody at an appropriate dilution in PBS-containing the counter stain (0.005% Evans Blue). Incubate at 37°C for 30 min.
5. Wash twice as before. Air-dry the slides/vials. Mount slides or remove the glass cover slip from the shell vial and mount it onto a clean slide.
6. The cytoplasm of infected cells will be apple green, whereas the nuclei will be red. The specific green fluorescence will have a particulate appearance that is evenly distributed throughout the cell's cytoplasm, indicating positive rotavirus.

3.3.7. Rotavirus Titration

1. Inoculate chamber slides with 300 µL of a suspension of MA104 cells containing 5×10^5 cells/mL in growth medium. Grow at 37°C in an atmosphere of 5% CO_2 until they reach confluency.
2. Remove growth medium and replace with serum-free M199-containing antibiotics 24 h before the cells are to be used for rotavirus titration.
3. Make serial tenfold dilutions of the rotavirus suspension, and activate the virus dilutions as described in Subheading 3.3.3.
4. Inoculate each well in the slide with a different activated virus dilution and incubate at 37°C for 1 h.
5. Remove the inoculum and add 300 µL of serum-free M199-containing antibiotics without trypsin.

6. Incubate the chambers at 37°C in an atmosphere of 5% CO_2 for 18 h. Remove the medium and wash with PBS.
7. Perform IF as described in Subheading 3.3.6.
8. Count the fluorescent focus in at least two wells inoculated with different dilutions and calculate the Fluorescent Focus-Forming Units (FFU)/mL of virus suspension.

3.4. Enzyme-Linked Immunosorbent Assay

1. Coat each well of a 96-well flat-bottomed microtitre plate with 100 µL predetermined concentration (~10 µg/µL) of goat anti-group A rotavirus antibodies in carbonate–bicarbonate buffer pH 9.6 for 2 h at 37°C, or overnight at 4°C.
2. Aspirate the coating solution and wash the wells 3× with 300–400 µL PBS-T.
3. Add 200 µL PBS containing 5% SMP to each well and incubate at 37°C for 30 min, to block the uncoated site.
4. Remove the blocking agent by aspiration.
5. Add 25 µL 10% faecal suspension and 75 µL PBS-T containing 2.5% SMP to the well, and incubate at 37°C for 30 min. Include three negatives and one positive controls in each assay plate.
6. Wash the wells 5× with PBS-T, as before.
7. Add 100 µL mouse anti-rotavirus antibody (diluted in PBS-T + 2.5% SMP) to each well, and incubate at 37°C for 30 min.
8. Wash the wells 5× with PBS-T, as before.
9. Add 100 µL HRP-labelled anti-mouse antibodies to each well (diluted in PBS-T + 2.5% SMP), and incubate at 37°C for 30 min.
10. Wash the wells 5× with PBS-T, as before.
11. Add 100 µL TMB/H_2O_2 in substrate buffer, pH 6.0, to each well, incubate at room temperature for 15–30 min.
12. Add 100 µL 2 M sulphuric acid to each well to stop the reaction.
13. Measure the optical density (OD) values at a wavelength of 450 nm. Specimens with an OD value of >3 standard deviations (SD) above the mean of the negative controls are regarded as rotavirus antigen positive.

3.5. Rotavirus Molecular Detection and Typing

3.5.1. Specimen Preparation: 10% Faecal Suspensions

1 Upon receipt, the specimens should be stored at 4°C.

2 Prepare 10–20% faecal suspensions in BSS in 2-mL screw-cap tubes.

3 Add 1.5 mL of BSS to a labelled tube.

4 Add 150 µL of liquid sample or a bacteriological loop full (the size of a garden pea) from semi-solid samples.

5 Store the 10% faecal suspension at 4°C until processing.

6 Once all investigations have been carried out the 10% suspensions can be stored at −70°C.

3.5.2. RNA Extraction

This method was described by Boom et al. (17). Add 200 µL of 10% faecal suspension to 1 mL of lysis buffer-L6 buffer and 20 µL of size fractionated silica in a 1.5 mL screw-capped microcentrifuge tube. Nuclease-free water and a rotavirus positive faecal suspension are included in each run to act as negative and positive controls, respectively.

1. Vortex for 10 s and incubate at room temperature with gentle mixing for 15 min.
2. Pellet by centrifugation (microcentrifuge) for 15 s (16,200 *g*), discard the supernatant using a disposable pastette. Collect the supernatants for disposal of toxic waste.
3. Wash the pellet with 1 mL of lysis buffer L2 twice, with 1 mL 70% ethanol twice, and with 1 mL acetone once (store the wash fluids for disposal).
4. After removal of the acetone (perform carefully as pellets may become dislodged) centrifuge and place tube with lid open at 56°C in a dry heating block for 5 min.
5. Elute the nucleic acid from the silica by adding 49 µL of RNase-free distilled water and 1 µL of RNasin, vortex and incubate at 56°C for 15 min.
6. Pellet by centrifugation at 16,200 *g* for 2 min and extract the supernatant (avoid disturbing the silica. Recentrifuge if silica becomes suspended). This can be stored at 4°C for 24 h or −70°C for longer.

3.5.3. Random Priming Reverse Transcription

1. Transfer 40 µL of extracted nucleic acid to a clean PCR tube.
2. Denature the dsRNA for 5 min (between 95 and 100°C).
3. Chill the tubes on ice for 2 min.
4. Prepare RT mix for $N+2$ (N = number of tubes in test).

10× buffer II	7.0 µL
50 mM $MgCl_2$	7.0 µL
Random hexamers (6 ng/µL)	1.0 µL
dNTPs (10 mM)	2.0 µL
M-MLV (200 U/µL)	2.0 µL
RNase-free H_2O	11.0 µL
	30.0 µL

5. Add 30 µL to each tube containing the extracted RNA.
6. Incubate the tubes at 37°C for 1 h.
7. Incubate the tubes at 95°C for 5 min.
8. Chill the tubes on ice for 2 min.
9. The total volume should be 70 µL.
10. The cDNA can be used directly in the PCR amplification, and stored at −20°C for further use.

3.5.4. Rotavirus VP6-Specific PCR: Detection PCR

Block Based Protocol

1. Prepare PCR mix for $N+2$ (N = No. of tubes in test).

1× mix		
	10× buffer II (Invitrogen)	4.5 µL
	50 mM $MgCl_2$	2.0 µL
	dNTPs (10 mM)	1.0 µL
	Taq polymerase (5 U/µL) (Invitrogen)	0.2 µL
	Primer VP6-F (20 pmol/µL)	1.0 µL
	Primer VP6-R (20 pmol/µL)	1.0 µL
	RNase-free H_2O	35.3 µL
		45.0 µL

2. Add 45 µL of PCR mix to each PCR tube.
3. Add 5 µL cDNA (from the RT reaction).
4. Briefly spin in microcentrifuge (pulse for 5 s).
5. Transfer tubes to the PCR machine.
6. Add tubes to thermocycler and cycle at the following temperatures for the following times:

94°C	2 min	×1
94°C	1 min	×35
55°C	1 min	
72°C	1 min	
72°C	7 min	×1
15°C	Hold	

3.5.5. Nested VP6-PCR

1. Prepare PCR mix for $N+2$ (N = number of tubes in test).

10× PCR buffer	5.0 µL
50 mM $MgCl_2$	2.0 µL
dNTPs (10 mM)	1.0 µL
Taq polymerase (5 U/µL)	0.4 µL
Primer VP6NF (20 pmol/µL)-Forward	1.0 µL
Primer VP6NR (20 pmol/µL)-Reverse	1.0 µL
RNase-free H_2O	38.6 µL
	49.0 µL

2. Add 49 µL of PCR mix to each clean PCR tube.
3. Add 1 µL first-round product in post PCR laboratory, briefly spin in microcentrifuge (pulse for 5 s).
4. Add tubes to thermocycler and cycle at the following temperatures for the following times:

94°C	2 min	×1
94°C	30 s	
42°C	30 s	×35
72°C	30 s	
72°C	5 min	×1
15°C	Hold	

3.5.6. Detection of Rotavirus by Use of a Duplex5′-Nuclease Taqman Assay

Nucleic acids extracted from faecal suspensions and converted into cDNA are used in a PCR with primers specifically designed to amplify a 379 base-pair fragment of the gene encoding rotavirus inner capsid protein VP6 by real time PCR assay using an ABI PRISM 7500 Sequence detection system (TaqMan).

1. Dilution of cDNA

   Dilute cDNA 0.4 times (1/2.5) as follows: 10 µL of cDNA added to 15 µL of water. 10 in 25; d = 0.4.

2. Master Mix

   Prepare the Taqman mix for $N+2$ (N = number of samples) as follows:

Ingredients	[*Original*]	[*Final*]	Volume (µL)/assay
RNAse-free H_2O			5.325
TaqMan Master Mix	2×	1×	12.5
ROX (Invitrogen)			0.05
VP6-F primer	20 µM	0.8 µM	1.0
VP6-R primer	20 µM	0.8 µM	1.0
VP6-P probe	20 µM	0.1 µM	0.125

Ensure the Master Mix is thoroughly mixed. Aliquot 20 µL of master mix in each well.
Add 5 µL of 1/2.5 diluted cDNA.

Seal the plate with the optical covers. Ensure that there are no bubbles in the wells. If necessary, centrifuge the plate briefly.

3. Cycling
Follow the instruction manual to use the ABI PRISM 7500 SDS.

Use the following "STANDARD 7500 RUN"

50°C for 2

95°C for 2

and 40 cycles of 95°C for 15 s

60°C for 1 min

All the wells should be assayed using the dye and quencher of the probe.

3.5.7. Rotavirus NSP4-Specific PCR

1. Prepare PCR mix for $N+2$ (N = No. of tubes in test).

1× mix		
	10× buffer II (Invitrogen)	4.5 µL
	50 mM $MgCl_2$	2.0 µL
	dNTPs (10 mM)	1.0 µL
	Taq polymerase (5 U/µL) (Invitrogen)	0.2 µL
	Primer NSP4-F (20 pmol/µL)	1.0 µL
	Primer NSP4-R (20 pmol/µL)	1.0 µL
	RNase-free H_2O	35.3 µL
		45.0 µL

2. Add 45 µL of PCR mix to each PCR tube.
3. Add 5 µL cDNA (from the RT reaction).
4. Briefly spin in microcentrifuge (pulse for 5 s).
5. Transfer tubes to the PCR machine.
6. Add tubes to thermocycler and cycle at the following temperatures for the following times:

94°C	2 min	×1
94°C	1 min	
48°C	1 min	×35
72°C	1 min	
72°C	7 min	×1
15°C	Hold	

3.5.8. Rotavirus Genotyping PCRs

3.5.8.1. G-Typing Consensus PCR (VP7)

1. Prepare first-round PCR mix for $N+2$ (N=No. of tubes in test).

1× mix		
	10× buffer II (Invitrogen)	4.5 µL
	50 mM $MgCl_2$	2.0 µL
	dNTPs (10 mM)	1.0 µL
	Taq polymerase (5 U/µL) (Invitrogen)	0.2 µL
	Primer VP7-F (20 pmol/µL)	1.0 µL
	Primer VP7-R (20 pmol/µL)	1.0 µL
	RNase-free H_2O	35.3 µL
		45.0 µL

2. Add 45 µL of PCR mix to each PCR tube.
3. Add 5 µL cDNA (from the RT reaction).
4. Briefly spin in microcentrifuge (pulse for 5 s).
5. Transfer tubes to the PCR machine.
6. Add tubes to thermocycler and cycle at the following temperatures for the following times:

94°C	2 min	×1
94°C	1 min	
52°C	1 min	×35
72°C	1 min	
72°C	7 min	×1
15°C	Hold	

3.5.8.2. G-Typing Multiplex PCR

1. Prepare second round PCR mix for $N+2$ (N = No. of tubes in test).

1× mix		
	10× buffer II (Invitrogen)	4.8 µL
	50 mM $MgCl_2$	2.5 µL
	dNTPs (10 mM)	1.0 µL
	Taq polymerase (5 U/µL) (Invitrogen)	0.2 µL
	Primer VP7-R (20 pmol/µL)	1.0 µL
	Primer G1 (20 pmol/µL)	1.0 µL
	Primer G2 (20 pmol/µL)	1.0 µL
	Primer G3 (20 pmol/µL)	1.0 µL
	Primer G4 (20 pmol/µL)	1.0 µL
	Primer G8 (20 pmol/µL)	1.0 µL
	Primer G9 (20 pmol/µL)	1.0 µL
	Primer G10 (20 pmol/µL)	1.0 µL
	Primer G12 (20 pmol/µL)	1.0 µL
	RNase-free H_2O	30.5 µL

2. Add 48 µL round 2 mix to each PCR tube.
3. Add 2 µL of first round product (see Subheading 3.6.1).
4. Briefly spin in microcentrifuge (pulse for 5 s).
5. Transfer tubes to the PCR machine.
6. Add tubes to thermocycler and cycle using the following programme:

94°C	4 min	×1
94°C	1 min	
42°C	2 min	×30
72°C	1 min	
72°C	7 min	×1
15°C	Hold	

3.5.8.3. VP7 Semi-nested Consensus PCR (for Sequence Typing)

1. Prepare PCR mix for $N+2$ (N = No. of tubes in test).

1× mix		
	10× buffer II (Invitrogen)	4.5 µL
	50 mM $MgCl_2$	2.0 µL
	dNTPs (10 mM)	1.0 µL

(continued)

(continued)

	Taq polymerase (5 U/μL) (Invitrogen)	0.2 μL
	Primer VP7-F (20 pmol/μL)	1.0 μL
	Primer VP7-RINT (40 pmol/μL)	1.0 μL
	RNase-free H_2O	35.3 μL
		45.0 μL

2. Add 45 μL of PCR mix to each tube.
3. Add 2 μL first-round amplicon from
4. Briefly spin in microcentrifuge (pulse for 5 s).
5. Transfer tubes to the PCR machine.
6. Add tubes to thermocycler and cycle at the following temperatures for the following times:

94°C	2 min	×1
94°C	1 min	
50°C	1 min	×35
72°C	1 min	
72°C	7 min	×1
15°C	Hold	

3.5.8.4. P-Typing Consensus PCR (VP4)

1. Prepare first-round PCR mix for $N+2$ (N=No. of tubes in test).

1× mix		
	10× buffer II (Invitrogen)	4.5 μL
	50 mM $MgCl_2$	2.5 μL
	dNTPs (10 mM)	1.0 μL
	Taq polymerase (5 U/μL) (Invitrogen)	0.2 μL
	Primer VP4F (20 pmol/μL)	1.0 μL
	Primer VP4R (20 pmol/μL)	1.0 μL
	RNase-free H_2O	34.8 μL
		45.0 μL

2. Add 45 μL to each tube.
3. Add 5 μL cDNA.
4. Briefly spin in microcentrifuge (pulse for 5 s).
5. Transfer tubes to the PCR machine.
6. Add tubes to thermocycler and cycle at the following temperatures for the following times:

94°C	2 min	×1
94°C	1 min	
50°C	1 min	×35
72°C	1 min	
72°C	7 min	×1
15°C	Hold	

3.5.8.5. P-Typing Multiplex PCR

1. Prepare second-round mix for $N+2$ (N=No. of tubes in test).

1× mix		
	10× buffer II (Invitrogen)	4.8 µL
	50 mM $MgCl_2$	2.5 µL
	dNTPs (10 mM)	1.0 µL
	Taq polymerase (5 U/µL) (Invitrogen)	0.2 µL
	Primer VP4-F (20 pmol/µL)	1.0 µL
	Primer P[4] (20 pmol/µL)	1.0 µL
	Primer P[6] (20 pmol/µL)	1.0 µL
	Primer P[8] (20 pmol/µL)	1.0 µL
	Primer P[9] (20 pmol/µL)	1.0 µL
	Primer P[10] (20 pmol/µL)	1.0 µL
	Primer P[11] (20 pmol/µL)	1.0 µL
	RNase-free H_2O	32.5 µL
		48.0 µL

2. Add 48 µL second-round mix to a new 0.2 mL tube.
3. Add 2 µL of first-round product.
4. Briefly spin in microcentrifuge (5 s).
5. Transfer tubes to PCR machine room.
6. Add tubes to thermocycler and cycle at the following temperatures for the following times:

94°C	4 min	×1
94°C	1 min	
45°C	2 min	×30
72°C	1 min	
72°C	7 min	×1
15°C	Hold	

3.5.9. Agarose-Gel Electrophoresis

1. Add 2 g of Ultrapure™ agarose 1000 (or an appropriate gel for the analysis of nucleic acid <1 kB) to 100 mL 1× TBE to give a 2% gel.
2. The concentration of the gel used will be determined by the expected size of the amplicon and the electrophoresis buffer used.

500–2,500 bp	2.0% gel in TAE	1.0% gel in TBE
60–700 bp	4.0% gel in TAE	3.0% gel in TBE
20–250 bp	6.0% gel in TAE	5.0% gel in TBE

3. Melt in microwave at full power for 2 min. Any microwaved solution may become superheated and boil vigorously when moved or touched. Use extreme care in handling. Remove the boiling solution from the microwave oven, allow to stand for a few seconds at room temperature, and release the air by gentle swirling. Heat using several short 20–30 s intervals with gentle swirling between pulses to re-suspend the powder.
4. Cool to 45°C then pour into gel plate (size 14 cm [W] × 16 cm [L]) fitted with two 22 or 24 slot combs.
5. Add 10 µL of PCR product to 10 µL sample buffer in a microtitre plate.
6. Remove the comb and end pieces and add 40 µL of size markers or diluted sample from the microtitre plate to the appropriate well.
7. Place the gel plate in the gel tank and add 1× TBE level with the gel (do not flood the plate at this stage).
8. Run the products into the gel for 5 min at a constant voltage of 150 V.

3.6. Polyacrylamide Gel Electrophoresis

3.6.1. Phenol–Chloroform Extraction of RNA from Stool

1. On ice, place 450 µL of a 10% stool suspension (prepared in water) into an Eppendorf tube.
2. Add 50 µL of a pre-warmed solution of 1 M NaAcetate with 1% SDS.
3. Vortex for 10 s, and incubate at 37°C for 15 min.
4. Add an equal volume of phenol–chloroform.
5. Vortex for 1 min, and incubate for an additional 15 min at 56°C.
6. Open and immediately re-seal the tubes before further vortexing.
7. Vortex for 1 min and then centrifuge at 13,800 *g* for 3 min.
8. Transfer the upper aqueous phase to a fresh tube.
9. Add 250 µL of phenol–chloroform to the dsRNA solution.

10. Repeat steps 5–8 (phenol extraction).
11. To the dsRNA solution, add 1/10 volume (~40 µL) of 3 M sodium acetate (pH 5.0) and 700 µL of ice-cold absolute ethanol. Mix gently by inversion 4–6 times, and incubate at –20°C for 2 h and at –70°C for 30 min.
12. Centrifuge at 13,800 *g* for 15 min at 4°C. Decant the ethanol immediately, and invert the tube onto a paper towel to dry for >15 min.
13. Using the pipette, re-suspend the dsRNA pellet in 30 µL of loading buffer.

3.6.2. Polyacrylamide Gel Electrophoresis

1. Clean the glass plates with soap and water, and then wipe with 70 or 96% ethanol. Allow the ethanol to evaporate.
2. Assemble the glass plates for gel casting according to the manufacturer's instructions. Mark the top level of the resolving gel on the plate with a marker pen, remembering to leave room for the stacking gel above the resolving gel.
3. Prepare the resolving gel according to the recipe in Subheading 2.6. Pipette the acrylamide solution between the glass plates to the mark and overlay the gel with a layer of water-saturated iso-butanol (to ensure formation of an even interface and exclusion of oxygen).
4. Depending on its size, allow the gel to set for at least 45 min and up to 2 h until the interface between the gel and the overlay is visible.
5. Pour the liquid from the top of the resolving gel, wash the top of the gel three times with distilled water, and remove excess liquid by inserting a piece of filter paper between the glass plates and allowing the excess liquid to absorb into filter paper.
6. Place the gel apparatus upright, prepare the stacking gel, and load it on top of the resolving gel. Position the comb immediately.
7. Allow the gel to polymerise for at least 45 min to 1 h before loading the samples.
8. Remove the comb, and assemble the glass plates in the electrophoresis apparatus.
9. Add running buffer to the bottom reservoir, and insert the glass plates into the electrophoresis tank. Fill the wells with the electrophoresis buffer, and remove air from under the gel bottom.
10. Load each dsRNA sample in PAGE buffer into the designated gel wells. When using a large-format gel electrophoresis system (e.g. Hoefer SE600), electrophorese at 100 V or 20 mA

for 16–20 h. When using a small-format system (e.g. Bio-Rad Mini Protean 3), electrophorese at 150 V for ~2 h.

3.6.3. Silver Staining of dsRNA in Gels

1. Pour out the running buffer, and remove the gel from between the glass plates.
2. Cut the bottom right corner for gel orientation. Discard the stacking gel.
3. Add 200 mL of fixing solution 1 to each gel, and rotate at room temperature for 30 min on an orbital shaker.
4. Aspirate fixing solution 1 and replace with 200 mL of fixing solution 2. Rotate for 30 min at room temperature on the orbital shaker.
5. Make up $AgNO_3$ just before use. Work carefully as $AgNO_3$ stains hands and surfaces. Aspirate fixing solution 2, and add 200 mL of silver nitrate staining solution. Rotate for 30 min at room temperature on the orbital shaker.
6. Prepare developing solution by adding the NaOH to the previously prepared formaldehyde and water solution.
7. Aspirate the silver nitrate staining solution, and wash the gel twice with distilled water for 2 min each time.
8. Add approximately 50 mL of developing solution to the gel and agitate by hand for 30 s to remove any black precipitate.
9. Aspirate the developing solution and then add the remaining developing solution (~200 mL).
10. Rotate for ~5 min at room temperature or until RNA bands are visible.
11. Drain off the developing solution and add the stopping solution to prevent further color development.
12. Rotate for 5–10 min at room temperature before rinsing the gel in distilled water.
13. Dry the gel in a standard vacuum gel dryer (e.g. Bio-Rad Laboratories, Hercules, CA).
14. Alternatively, cover the gel with cellophane sheets and dry overnight at room temperature (Hoefer Easy Breeze) or simply seal the gel in a plastic bag. The gel can also be temporarily stored in a 20% ethanol/1% glycerol mixture or in a 5% acetic acid solution.

3.6.4. Nucleic Acid Sequence and Phylogenetic Analysis

Nucleic acid sequencing can be performed using a number of platforms, and this will dictate the method to be used. Also, increasingly, sequencing is done commercially, and there are a number of companies that can provide these services, offering a choice of platforms (see http://www.nucleics.com/DNA_sequencing_support/sequencing-service-reviews.html for a review

on customer-sequencing services available). For rotavirus genotype identification, direct amplicon sequencing is recommended. Typically, the amplicons of interest are purified and sequenced using the consensus primers in both directions. Chromatograms are analysed and a consensus sequence generated using both sequence strands.

Sequence and phylogenetic analyses can be performed using any of a number of software packages which are either freely (e.g. GCG, Phylip, Bioedit) or commercially available (Bionumerics, Applied Maths, Kortrijk, BE, DNAStar, Lasergene, WI, USA, Vector NTI, Invitrogen, Pailey, UK). In the first instance, a Basic Local Alignment Search Tool (BLAST) may be sufficient to find available sequences in GenBank which are closely related to the query sequence and assign a genotype (http://www.ncbi.nlm.nih.gov/blast/producttable.shtml). Also, an online tool maintained by the University of Leuven (http://rotac.regatools.be/) for genotype assignation of the different gene segments through alignments and comparison with sequences derived from known genotypes (18). However, it is advisable to create a local database containing sequences of known rotavirus prototype strains and of strains representative of the different rotavirus genotypes derived from humans and animals, including the most recently available strains in order to perform more detailed analyses. First, compare any new sequence to sequences of prototype strains in the database. Once the type has been determined, compare it to more recent representative strains of each of the genotypes to account for drift due to accumulation of point mutations. A genotype can be assigned when the query sequence is ≥80% homologous at the nt level.

In order to examine complementarily of the type-specific oligonucleotide primers and to investigate the possibility that mismatches may account for failure to genotype a "common" rotavirus strain, the sequence of interest should be aligned with no less than 15 sequences from strains of the same type which may be obtained from public databases, and the type-specific oligonucleotide primer sequence, using a multiple alignment tool such as Clustal W. The identification of mismatches between the query sequence and the oligonucleotide primer sequence, particularly in the vicinity of the 3′ end, is likely to result in failure to bind or reduced efficiency in the PCR reaction (3).

For the construction of phylogenetic trees, the topology should be confirmed using at least two different methods. Neighbour joining and Maximum parsimony are recommended. When the number of sequences in the comparison is not too large (<15), Maximum likelihood may be performed. When the number of sequences in the comparison is ≥15, the algorithm will require too much computer memory. Genetic lineages or clades should be confirmed with bootstrapping.

4. Notes

1. *Electron microscopy.* (1) 1% Uranyl acetate may be used as an alternative negative stain. To make 1% uranyl acetate, dissolve 100 mg of uranyl acetate in 10 mL of distilled water. Adjust the pH to 4.5, filter, and refrigerate in a dark bottle. Allow to reach room temperature before use. (2) Staining due to excess mucus in the specimen can be overcome by allowing the smear to dry on the slide and then re-suspending it in distilled water. Alternatively, a 20% suspension can be partially clarified to remove bacteria, followed by concentration by differential centrifugation.
2. *Immune electron microscopy.* A series of antibody dilutions can be used to achieve equivalence between antigen and antibody in order to maximise the formation of immune complexes.
3. *Virus culture.* (1) Always include un-inoculated cell cultures to detect cytopathic effects associated with cell degeneration. (2) If a CO_2 incubator is not available, use an ordinary incubator, making sure that the caps on the vials/flasks are tightly screwed. (3) Using shell vials for virus inoculation allows centrifugation of the sample to facilitate attachment. This has been shown to increase infectivity by >30%. Alternatively, use roller tubes that are prepared as described for shell-vials, but carry out inoculation at 37°C for 1 h without centrifugation. (4) When growing rotavirus from clinical samples, cytopathic effect and cell lysis can take several days (up to 1 week) and might be difficult to distinguish from the cell death that will also appear in the control vials/tubes. If no lysis is observed after 3 days post-inoculation, refresh the medium and incubate three more days before passaging. (5) Optimum concentrations of serologic reagents used in IF should be predetermined by titration. (6) When chambered glass slides are used, the plastic chambers may be removed from the slide after step 7 and the cells fixed with acetone. Alternatively, they can be removed just before mounting, in which case the cells must be fixed using methanol.
4. *Enzyme-linked immunosorbent assay.* (1) Concentration of serologic reagents should be predetermined through titration. (2) A second filter with a wavelength of 620 nm can be used in the spectrophotometer to minimise the background signal.
5. *Rotavirus molecular detection and typing.* (1) The nested VP6 PCR is to be used for the detection of low viral loads, e.g. in asymptomatic or extra-intestinal infections. (2) The G-typing consensus PCR provides sufficient DNA for direct sequencing if the genotype is not determined in the second round

genotype-specific multiplex reaction. (3) The VP7 semi-nested consensus PCR provides increased sensitivity and should be used in conjunction with DNA sequencing for samples which failed to G-type. (4) The VP7-RINT primer is used at a concentration of 40 pmol/μL in order to take account of its degenerate nature. (5) Figure 3 illustrates the primer locations and product sizes in the VP7 and VP4 multiplex G- and P-typing PCRs.

6. *Polyacrylamide gel electrophoresis.* Troubleshooting PAGE (1) *Gel sandwich leaks while casting.* Plates, spacers, and gasket must be completely clean; wash if necessary. Replace chipped plates, especially if near the spacers. Check the plate and spacer alignment, and realign if necessary. Tighten the clamps only as far as needed to create a seal. (2) *Sample wells are damaged or irregular.* Remove air bubbles before inserting combs; slide comb into the solution at an angle. Allow acrylamide gels to set for a minimum of 1 h. Rinse out unpolymerised gel with running buffer. Remove the comb at a slight angle and very slowly to prevent damage to the gel. (3) *Gel polymerization is incomplete.* Use only recent stocks of the highest quality reagents. If the dry APS does not crackle when added to water, replace with fresh stock. Solutions with extreme pH values (especially acidic) might not polymerise. Remove oxygen from the gel environment; de-gas the monomer solution for 5–10 min before pouring, and then overlay the gel surface with water-saturated *n*-butanol. Adjust the gel solution temperature so that it is at least 20°C, especially for low-percentage acrylamide gels. Increase the TEMED or APS concentration. (4) *Upper buffer chamber leaks.* Check that the glass plates, spacers, and clamps are aligned and fit snugly into the upper chamber gaskets. Check that the gaskets are centered and fit along the upper chamber groove. Check that the gasket is not damaged or pinched. (5) *Stained sample collects near buffer front.* Molecules are not sufficiently restricted by the resolving gel pore size; increase the percentage acrylamide concentration. (6) *Stained sample collects near the top of the gel when buffer has reached the bottom.* The gel pore size is too small; decrease the resolving (or stacking) percentage acrylamide concentration. (7) *Band resolution is poor.* Begin electrophoresis as soon as the sample is loaded to prevent low-molecular weight species from diffusing. Conduct the separation at a lower current or voltage setting. Allow the gel to polymerise fully. (8) *Sample preparation.* Desalt the sample. Adjust the sample volume or concentration. Increase the glycerol or sucrose to increase sample density. (9) *Tracking dye does not sharpen into concentrated zone in stacking gel.* Pour a taller stacking gel; for best results, allow the height of the stacking gel to be 2.5 times that of the sample in the well.

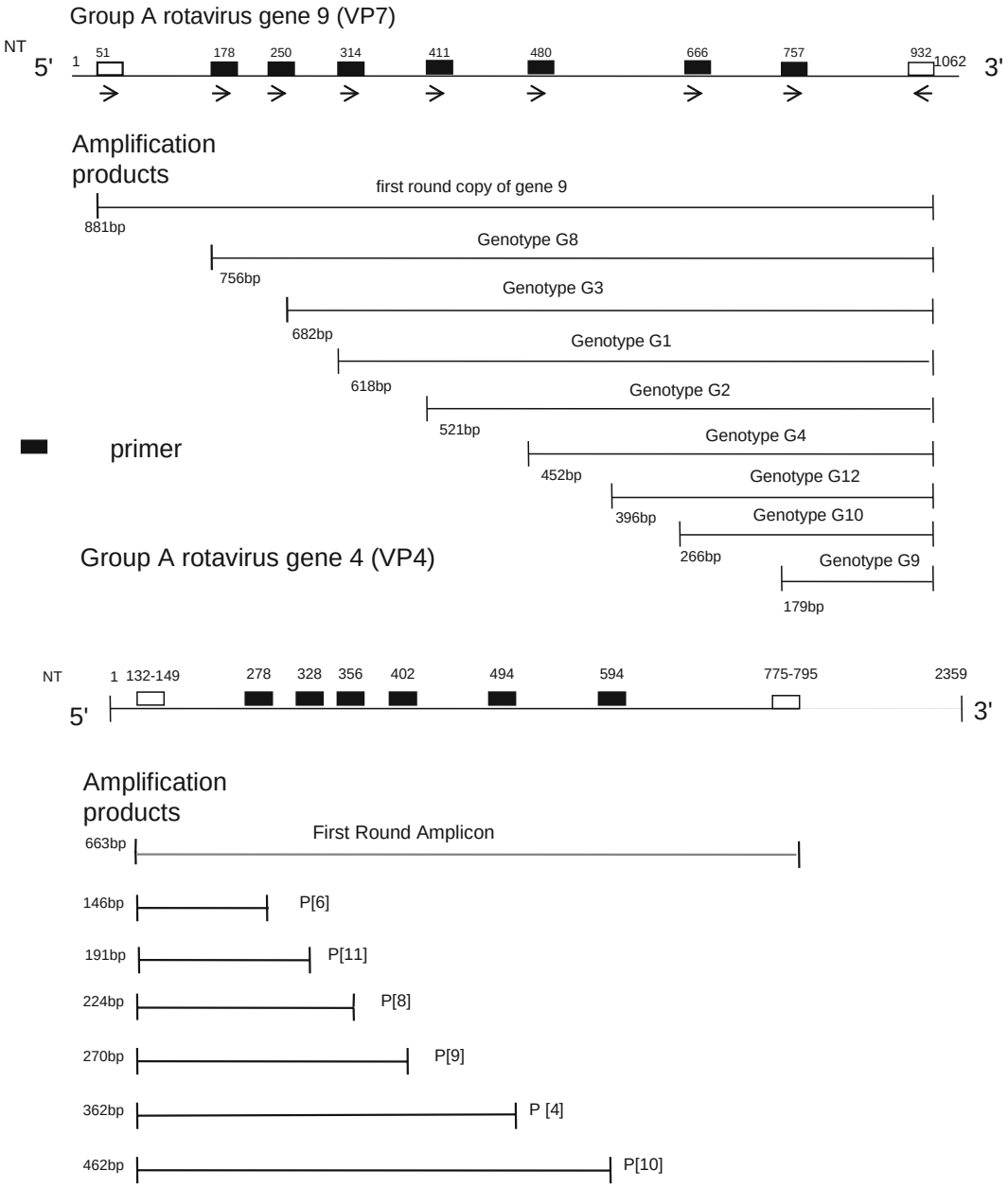

Fig. 3. Diagrammatic representation of the primer locations and product sizes in the VP7 and VP4 multiplex G- and P-typing PCRs.

Dispose of outdated acrylamide solutions, and use only the highest grade of acrylamide. When preparing samples, avoid using solutions with high-salt concentrations. (10) *Dye front curves up ("smiles") at the edges.* To reduce the running temperature, be sure the lower buffer chamber is filled to the correct level. Circulate the coolant. Pre-chill the buffer.

Decrease the current or voltage setting. Run the gel in the cold room. (11) *The run is unusually slow (or fast)*. Check for leaks; plates and spacers must be aligned and free of grease. To increase or decrease the migration rate, adjust the voltage or current setting by 25–50%. (12) *Bands are skewed or distorted*. Check gel preparation and polymerization: De-gas the stacking gel solution, and avoid trapping air bubbles under the comb teeth. Overlay the running gel with water-saturated *n*-butanol before polymerization begins to avoid forming an uneven gel surface.

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Chapter 19

Detection of Crimean–Congo Hemorrhagic Fever, Hanta, and Sandfly Fever Viruses by Real-Time RT-PCR

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Abstract

The development of sensitive and specific nucleic acid diagnostic assays for viral pathogens is essential for proper medical intervention. This chapter describes four fluorescence-based PCR assays to detect the Crimean–Congo Hemorrhagic Fever (CCHFV), Andes (ANDV), Hantaan (HANV), and Sandfly Fever Sicilian (SFSV) Viruses. These assays are based on species-specific hydrolysis probes targeting the nucleocapsid protein gene for CCHFV and SFSV and the glycoprotein gene for ANDV and HANV. All four assays were optimized for LightCycler 2.0 (Roche Diagnostics, Indianapolis, IN) or Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.; Idaho Technology Inc., Salt Lake City, UT). The assays were evaluated using the protocols described in the Subheading 3. The limits of detection were approximately 5, 2, 2, and 5 plaque-forming units (PFUs) for CCHFV, ANDV, HTNV, and SFSV assays, respectively. The sensitivity and specificity of the assays were evaluated with test panels that consisted of 20–60 known positive and 30–135 known negative samples, representing 7–34 genetically diverse bacterial and viral species. The CCHFV assay detected 59 out of the 60 positive samples and no false positives, resulting in 98.3% sensitivity at LOD of 5 PFU and 100% specificity. The ANDV and HTNV assays correctly identified all the positive samples with no false positive reactions; therefore, the sensitivity and specificity of these assays were determined to be 100% at LOD of 2 PFU. The SFSV assay missed three positive samples and cross-reacted with one of 48 negative samples, resulting in 95% sensitivity at LOD of 5 PFU and 98% specificity.

Key words: CCHF virus, Sandfly fever virus, Andes virus, Hantaan virus, Fluorescence-based real-time assay

1. Introduction

The Crimean–Congo hemorrhagic fever, hanta, and sandfly fever viruses belong to family *Bunyaviridae*, which includes five known genera, more than 60 distinct species and numerous strains, varieties, and isolates within each species. Only members of the

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Hantavirus, *Nairovirus*, *Orthobunyavirus*, and *Phlebovirus* genera can infect vertebrates while members of the *Tospovirus* genus infect plants and arthropods. All bunyaviruses are enveloped with a helical capsid symmetry. The virion ranges from approximately 90 to 120 nm in diameter and contains a three-segmented genome ranging in size from 13.5 to 21 kb. The three segments: large (L), medium (M), and small (S), encode structural and nonstructural proteins, primarily, the RNA-dependent RNA polymerase (L), the virion glycoproteins (Gn and Gc), and the nucleoprotein (N), respectively (1–3).

All bunyaviruses, except hantaviruses, are transmitted by arthropod vectors, including ticks, mosquitoes and phlebotomine flies. Hantaviruses are transmitted by contact with rodents or their contaminated tissues, secreta, or excreta. Bunyaviruses cause several diseases whose symptoms may vary from self-limiting febrile illnesses to severe hemorrhagic fever, encephalitis, renal failure, and cardiopulmonary syndromes depending on the viral species and serotype.

The Crimeran–Congo hemorrhagic fever virus (CCHFV) causes Crimean–Congo hemorrhagic fever disease. The virus has a wide geographic distribution, which coincides with its competent tick vectors (e.g., *Hyalomma*, *Dermacentor*, and *Rhipicephalus* spp.) in many parts of Africa, Central and Western Asia, Eastern Europe, and the Middle East. There is a considerable genetic variability among different CCHFV strains isolated from different geographic regions, which may account for differences in disease severity. The primary transmission cycle occurs among vertebrates, including domestic ruminant animals such as cattle, sheep, and goats. Humans are incidental hosts, and infections can occur by the bites of infected ticks or by handling infected animals or contaminated material. The CCHF disease is characterized by a sudden onset of symptoms after an incubation period that may vary from 3 to 9 days depending on the route of infection. The symptoms include a combination of severe headache, fever, dizziness, and myalgia of the neck, lower back, and joints. Hemorrhage may begin as early as 3–5 days after onset of symptoms. Recovery is slow in symptomatic cases, and mortality rates may reach 30% or higher due to uncontrolled hemorrhage (4).

Hantaviruses cause two serious and sometimes fatal human diseases: hemorrhagic fever with renal syndrome (HFRS), and hantavirus pulmonary syndrome (HPS). HFRS occurs primarily in Europe and Asia and is caused by old-world hantaviruses, e.g., Hantaan (HTNV), Dobrava–Belgrade (DOBV), Puumala (PUUV), Seoul (SEOV), and Tula (TULV) viruses. HFRS disease severity varies with the serotype, with case fatality rates up to 15%. HFRS affects approximately 200,000 people each year predominantly in Asia (5). HPS is caused by New-World hantaviruses, e.g., Andes (ANDV), Black Creek Canal (BCCV), Bayou

(BAYV), New York (NYV), and Sin Nombre (SNV) viruses. Many other New-World hantaviruses have been isolated from wild-caught rodents, but their association with HPS has not been clearly established (6–9). Thousands of cases of HPS have been reported in the U.S., Canada, and many other countries in South and Central America since 1993. According to one Pan-American Health Organization (PAHO) report, 2,296 cases were reported in North and South Americas between 1993 and 2004, with 384 deaths (10).

The Sandfly fever virus group, including the Sandfly Sicilian (SFS) and Sandfly Naples (SFN) viruses, causes a self-limiting disease known as Sandfly, phlebotomus, or paptaci fever, which is generally manifested by general malaise and mild fever with a few exceptions (11). These viruses are transmitted by phlebotomine sandflies in the Mediterranean and the Middle East regions (12–14).

Because of their great diversity and wide distribution, rapid and sensitive diagnostic methods for their detection are of critical importance. Early detection is the most efficient method for limiting the potential for widespread infections. Diagnosis of these, and many other viral infections, can be achieved by a variety of virological, immunological, and molecular methods. Virological methods, such as virus isolation, are important for assessing viability and discerning certain phenotypic characteristics of viral species or strains, but they require biosafety level 3 or 4 facilities and are not suitable for rapid detection or definitive identification of the viral species or strains. Furthermore, several of these viruses are difficult to replicate in cell cultures. Immunological methods that are frequently used for detecting antigens or antibodies, are generally simple to perform, inexpensive, and can be used under field conditions, but they are insufficiently sensitive to detect early infections or specific enough to define species or strain identity. Molecular assays, including real-time RT-PCR, are becoming increasingly available for the detection of bunyaviruses, e.g., CCHFV (14–17) and hantaviruses (18–20). These assays have the advantages of high sensitivity, specificity, and speed. Here, we describe RT-PCR assays for detecting CCHF, hanta, and Sandfly fever viruses.

2. Materials

1. LightCycler 2.0 (Roche Diagnostics, Indianapolis, IN) or Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.; Idaho Technology Inc., Salt Lake City, UT).
2. LightCycler capillaries 20 μL (Roche Diagnostics, Indianapolis, IN).

3. LightCycler carousel centrifuge 2.0 (Roche, Indianapolis, IN).
4. Bench-top refrigerated centrifuge 5417R (Eppendorf, Westbury, NY).
5. Microcentrifuge (Stratagene, La Jolla, CA).
6. Heat block for 55°C and 95°C (Eppendorf, Westbury, NY).
7. Vortex MV1 (VWR Scientific products, West Chester, PA).
8. Pipetman P-2, P-10, P-20, P-100, P-200, P-1000 (Gilson, Middleton, WI).
9. Pipette tips (sterile with aerosol barriers and general purpose (E&K SCIENTIFIC, Campbel, CA)).
10. Safe-Lock 2 mL sterile tubes (Eppendorf, Westbury, NY).
11. Powder-free latex gloves (Microflex MC Reno, NV).
12. Trizol LS reagents (Invitrogen, Carsblad, CA).
13. Water-saturated chloroform (Sigma–Aldrich, Saint Louis, MS).
14. Absolute and 75% ethanol (Sigma–Aldrich, Saint Louis, MS).
15. 2-Propanol (Sigma–Aldrich, Saint Louis, MS).
16. Glycogen (5 mg/mL) (Ambion Inc., Austin, TX).
17. Bovine serum albumin (1 ng/μL) (Sigma–Aldrich, Saint Louis, MS).
18. One-step QRT-PCR SuperScript Kit (Invitrogen, Carlsbad, CA).
19. RNAseout (40 U/μL) (Invitrogen, Carsblad, CA).
20. $MgSO_4$ (50 mM) (Invitrogen, Carsblad, CA).
21. Nuclease-free water or diethylpyrocarbonate (DEPC)-treated water (Ambion Inc., Austin, TX).
22. DNA Zap (Molecular BioProducts, Inc., San Diego, CA).
23. RNase AWAY (Ambion Inc., Austin, TX).

3. Methods

3.1. Preparation of Viral Cultures

1. Grow Vero E6 cell monolayer to 80% confluency in 175 cm^2 cell-culture flasks in cell-growth medium (Eagle's minimal essential medium (EMEM), 5% fetal bovine serum, 1% penicillin–streptomycin, 1% tricin, and 0.2% amphotericin B.) at 37°C in 95% air, 5% CO_2 environment.
2. Infect cells with a multiplicity of infection of one to ten of virus and incubate for 1 h at 37°C.
3. Remove the virus and wash the cells with prewarmed PBS (see Note 1).

4. Add 60 mL of fresh prewarmed cell-growth medium and incubate at 37°C in 95% air, 5% CO_2 environment.
5. Examine the cell monolayer daily and harvest at days 15–16 postinfection (see Note 2).
6. Pellet cells by centrifugation in 1.5-mL microcentrifuge tubes for 5 min.
7. Store crude viral stock preparations at –80°C (for long term use – see Note 3).

3.2. Purification of Viral RNA

TRIzol LS reagent from Invitrogen is a ready-to-use reagent for isolating total RNA from cells and tissues. The reagent is a monophasic solution of phenol and guanidine isothiocyanate. During sample homogenization or lyses, TRIzol LS reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with 2-propanol. The following protocol describes the procedures used to purify viral RNA from cell cultures.

1. Lyse cells in TRIzol LS reagent by repetitive pipetting (use three volumes of Trizol LS reagent per one volume of cell suspension).
2. Incubate the homogenized samples for 5 min at room temperature.
3. Transfer 1 mL of the homogenized sample into 1.5-mL microcentrifuge tube.
4. Add 250 μL of chloroform.
5. Cap the tube securely. Vortex for 15 s, then incubate on ice for 10 min.
6. Centrifuge the sample at no more than 20,000 × *g* for 20 min at 4°C.
7. Remove the upper aqueous phase (about 600 μL) to a fresh, sterile, Rnase-free tube (see Note 4).
8. Precipitate the RNA from the aqueous phase by mixing with 600 μL of isopropanol.
9. Incubate overnight at –20°C, and then centrifuge at 20,000 × *g* for 20 min at 4°C.
10. Remove supernatant carefully (see Note 5).
11. Add 1 mL of 75% ethanol. Incubate on ice for 15 min.
12. Centrifuge at 20,000 × *g* for 20 min at 4°C.
13. Remove supernatant carefully (see Note 5).
14. Air-dry the RNA pellet completely at room temperature.

15. Add 100 µL of DEPC water. Heat at 65°C for 3–5 min to dissolve RNA.
16. Store at –70°C until use.

3.3. Design of Primers and Probes (See Table 1)

The primers and probes were designed from the target sequence with the aid of PrimerSelect V 7.0.0 (DNAStar Inc., Madison, WI). The melting temperature was determined according to Breslauer et al. (21). The settings used for primer design were as follows: melting point (T_m): 58–63°C (optimum 60°C); GC content: 30–80% (optimum 50%); 3′ GC clamp: none; size: 18–27 (optimum 20); secondary structure: 0–8 with a maximum of three base pairs (bp) self-complementarities at the 3′ end. The settings used for probe design were as follows: T_m: 8–12°C higher than PCR primers (optimum T_m = 70°C); GC content: 30–80% with preferably more Cs than Gs; no runs of four or more identical nucleotides and no G at the first base (see Note 6).

3.4. Real-Time One-Step RT-PCR

1. Mix 10 µL of 2× RT-PCR mix (a buffer containing 4 mM of each dNTP and 6 mM $MgSO_4$), 0.8 µL of each upper and lower primers (10 µM), 0.2 µL of Taqman probe (10 µM), 0.1 µL of RNAseOut (40 U/µL), and 0.1 µL of BSA (1 ng/µL). Note: Use 0.4 µL of Taqman probe (10 µM) for the R.A.P.I.D. instrument.

Table 1
Primers and probes used for CCHFV, SFV, and hantaviruses real-time RT-PCR

Virus	Oligo name	Oligo sequence
Crimean–Congo hemorrhagic fever	CCHFV598U	AAACAGGGGTGGTGATGAGA
Crimean–Congo hemorrhagic fever	CCHFV650P	TGAACATGTGGAGTGGTGTAGGGAATT
Crimean–Congo hemorrhagic fever	CCHFV640P1	CATGTGGACTGGTGCAGGGAGTT
Crimean–Congo hemorrhagic fever	CCHFV732L	GAACGGCCTGACTTGTTGAT
Hantavirus Andes	ANDVM2812U	GCAACAAAAGATTCATTCC
Hantavirus Andes	ANDVM2854P	CACATCACAACAAACAAGCTTGAAT
Hantavirus Andes	ANDVM2912L	ACGTGGTCTCTTGTATTCC
Hantavirus Hantaan	HANVM299U	GATCAGTCACAGTCTAGTCA
Hantavirus Hantaan	HANVM338P	TCCACTGAAGTTGACTTGAAAGG
Hantavirus Hantaan	HANVM397L	TGATTCTTCCACCATTTTGT
Sandfly fever Sicilian	SFSS1087U	TGTCAAAAGGCTCTGCATTG
Sandfly fever Sicilian	SFSS1014P	TGCCTGAACTATGCACTCATCCAAGC
Sandfly fever Sicilian	SFSS1260L	TGAGAATGGGGGATTCACAT

2. Add 0.4 μL of RT/Platinum Taq mix (as provided), then spin briefly and return to ice.
3. Dispense 15 μL of the mix into LightCycler capillaries in a hood dedicated to dispensing reagents.
4. Move the reactions to a hood dedicated to dispensing template nucleic acids and add 5 μL of RNA template to each of the capillary tubes.
5. Add 5 μL of water to the negative control tubes.
6. Cap the capillary tubes using the tool provided by the vendor.
7. Perform one-step RT-PCR as follows: one cycle at 50°C for 30 min, followed by 45 cycles at 95°C for 5 s, and 60°C for 30 s.

3.5. Analysis of Results

The real-time RT-PCR data were analyzed using the LightCycler Data Analysis software 4.0 or the R.A.P.I.D. LightCycler Data Analysis software 3.5 at gain setting of 8. To determine the linearity and the quantitative performance of each assay, regression analysis was done by using Microsoft Excel. For analysis of the sensitivity and specificity results, all reactions that were performed included at least one positive control that contained the minimum amount of viral RNA that could be detected, i.e., 2 plaque-forming units (PFU) for ANDV and HTNV and 5 PFU for CCHFV and SFSV. The positive control for each run established the threshold cycle (C_t) value for positive samples. Samples yielding C_t values that marginally exceeded this threshold value were flagged for retesting. When C_t value was confirmed to exceed the threshold after retesting, such a sample was considered negative (i.e., the sample contains <2 PFU). Calculations of sensitivity and specificity were determined as follows: Sensitivity $\% = [(TP/(TP+FN)) \times 100]$; specificity $\% = [(TN/(TN+FP)) \times 100]$, where TP is the number of true positive samples, FN is the number of false negative samples, TN is the number of true negative samples, and FP is the number of false positive samples.

3.6. Expected Results

3.6.1. Dynamic Range and Limit of Detection

Optimum primers, probes, and $MgSO_4$ concentrations were determined from varying amounts of primers (0.1–0.5 μM), probe (0.1–0.5 μM), and $MgSO_4$ (3–6 mM) in separate experiments for each condition. The optimum conditions (0.4 μM of each primer, 0.1–0.2 μM of Taqman probe, and 5 mM $MgSO_4$) were then used to determine the dynamic range and limit of detection (LOD). Serial RNA dilutions containing from approximately 0.25 up to 500,000 PFU were tested in triplicates. The limits of detection were approximately 5, 2, 2, and 5 PFU for CCHFV, ANDV, HTNV, and SFSV assays, respectively (Figs. 1–4). When the C_t values and log PFU numbers were analyzed by regression analysis, a linear correlation was obtained over at least five orders of magnitude with R^2 values of 0.99, 0.96, 0.99, and 0.99 for CCHFV, ANDV, HTNV, and SFSV, respectively.

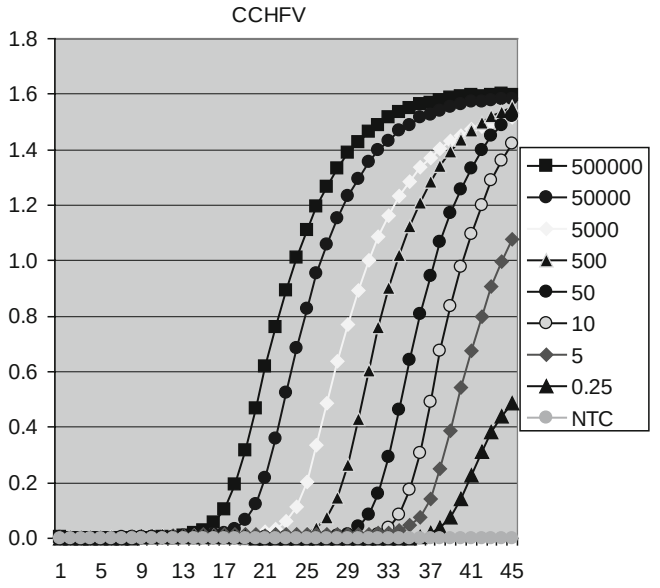

Fig. 1. Limit of detection of the Crimean–Congo hemorrhagic fever virus assay expressed in PFU. Tenfold serial dilutions of purified viral RNA (0.25–500,000 PFU) were tested. Each curve represents the mean fluorescence value of three different runs.

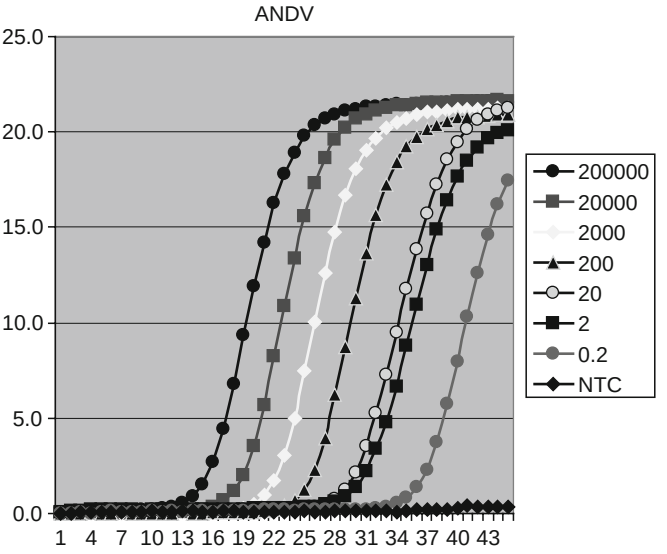

Fig. 2. Limit of detection of the Andes virus assay expressed in PFU. Tenfold serial dilutions of purified RNA (0.2–200,000 PFU) were tested. Each curve represents the mean fluorescence value of three different runs.

3.6.2. Sensitivity and Specificity (See Table 2)

The specificity of the assay was then evaluated with a test panel that consisted of 20–60 positive samples at LOD of 2–5 PFU and negative samples that contained no nucleic acids or nucleic acids from genetically diverse bacterial and viral species at concentrations

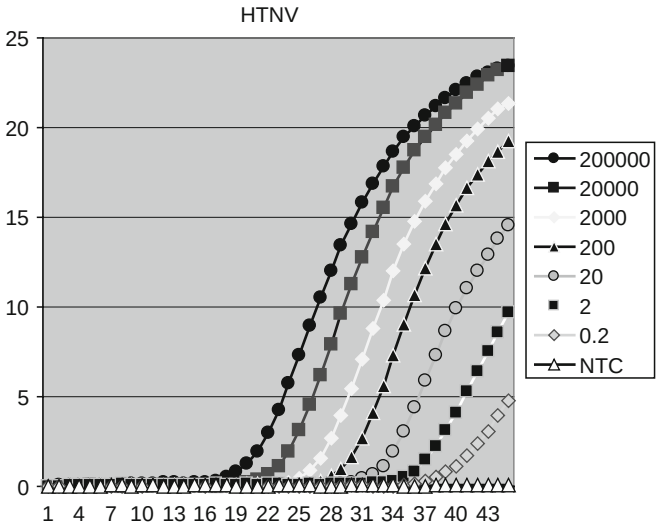

Fig. 3. Limit of detection of the Hantaan virus assay expressed in PFU. Tenfold serial dilutions of purified RNA (0.2–200,000 PFU) were tested. Each curve represents the mean fluorescence value of three different runs.

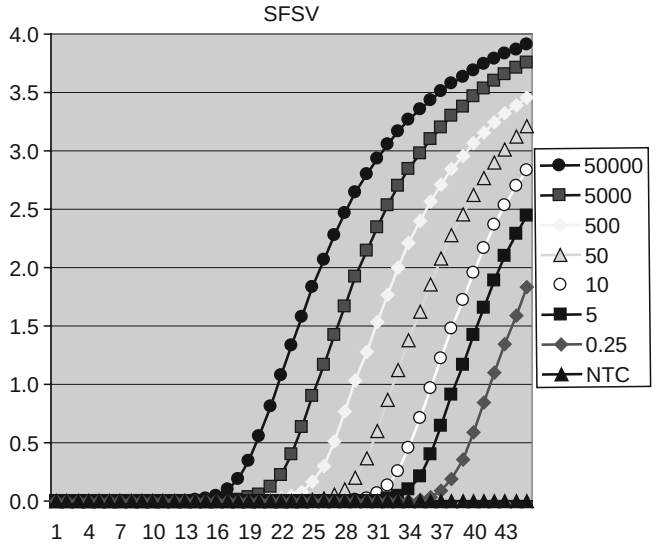

Fig. 4. Limit of detection of the Sandfly fever Sicilian (SFS) virus assay expressed in PFU. Tenfold serial dilutions of purified SFS RNA (0.25–50,000 PFU) were tested. Each curve represents the mean fluorescence value of three different runs.

ranging from 20 to 1,000 PFU. The ANDV and HTNV assays correctly identified all the positive samples with no false positive reactions; therefore, the sensitivity and specificity of these assays were determined to be 100% at LOD of 2 PFU. The CCHFV assay detected 59 of the 60 positive samples and no

Table 2
Sensitivity and specificity of CCHFV, ANDV, HTNV, and SFSV assays

	CCHFV		ANDV		HTNV		SFSV	
	Known positive	Known negative	Known positive	Known negative	Known positive	Known negative	Known positive	Known negative
Taqman positive	59	135	20	30	20	30	57	48
Taqman negative	1	0	0	0	0	0	3	1
Total	60	135	20	30	20	30	60	49
Sensitivity %	98.3		100.0		100.0		95.0	
Specificity %	100.0		100.0		100.0		98.0	

false positives, resulting in 98.3% sensitivity at LOD of 5 PFU and 100% specificity. The SFSV assay missed three positive samples and cross-reacted with one of 48 negative samples, resulting in 95% sensitivity at LOD of 5 PFU and 98% specificity.

4. Notes

1. For the preparation of viral cultures (Subheading 3.1) ensure that the buffers and growth medium used are prewarmed as those used directly from the fridge will inhibit cell growth.
2. Examine cells daily; 80% cytopathic effect should be observed at days 15–16, and the cells can then be harvested.
3. Cells can be stored at −20°C for short-term experiments, equivalent to less than 3 months.
4. Take care not to disturb the interface between the phases.
5. Ensure that the precipitate is not disturbed, this may be difficult to visualize owing to its transparent nature.
6. Ensure that the PCR is carried out in an appropriate environment, which is free from exposure to contaminating RNA or DNA.

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Chapter 20

Detection of SARS Coronavirus

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Abstract

The emergence of severe acute respiratory syndrome (SARS) and its subsequent worldwide spread challenged the global public health community to confront a novel infectious disease. The infection is caused by a coronavirus of animal origin. In this epidemic, molecular detections of SARS coronavirus RNA were shown to be useful for the early diagnosis of SARS. Although this pathogen was eradicated in humans, SARS or SARS-like viruses might reemerge from animals or from laboratory incidents. In this chapter, we describe several polymerase chain reaction (PCR) protocols for detecting SARS coronaviruses. These assays were routinely used for clinical diagnosis during the SARS outbreak.

Key words: SARS coronavirus, RT-PCR, Molecular detection, Clinical diagnosis

1. Introduction

Coronavirus is a genus of viruses in the family *Coronaviridae* under the order *Nidovirales* (1). The virus has a corona-like morphology and its genome contains a single segment of single-stranded RNA of positive polarity. Viruses under this genus have genome sizes ranging from 28 to 32 kb, which are the biggest among the RNA viruses. The genomic RNA of these viruses contains five major open reading frames (Orfs) that encode the replicase polyproteins (Orf 1a and Orf 1ab), spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins, in that order. Based on antigenic and genetic analysis, coronaviruses are traditionally classified into three groups. Coronaviruses from groups 1 and 2 have been found to infect mammals. By contrast, group 3 viruses are primarily avian viruses. Many coronaviruses are also pathogenic to their hosts (2). Animal coronaviruses can cause severe respiratory, enteric, neurological, or hepatic disease in their hosts. With the exception of severe acute respiratory syndrome (SARS) coronavirus, all human coronaviruses are often only

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associated with mild respiratory and gastrointestinal diseases. Interestingly, virus surveillance studies over the last few years have identified many novel coronaviruses from different animals. These suggest that there might be a wealth of "unknown" coronaviruses which are yet to be identified.

SARS is the first novel infectious respiratory disease in this century. The disease is caused by a coronavirus originating from animals and the clinical presentations of the disease have been extensively reviewed (3). Further studies also indicated that the virus is a distant relative of bat coronaviruses (4), suggesting bats might be natural carriers of the precursor of SARS coronavirus. However, the nature reservoir of SARS coronavirus is still not confirmed. As the majority of SARS patients seroconverted in the second week of disease onset, serological tests might not be a practical approach for early SARS diagnosis (3). Because of these reasons, the focus of early diagnosis was mainly concentrated on the development of conventional and quantitative reverse transcriptase (RT) polymerase chain reaction (PCR) assays (3). Besides, several molecular tests which employ non-PCR-based methods, such as loop mediated isothermal amplification (5), rolling circle amplification (6) and nucleic acid sequence-based amplification (7), were also developed for the detection of SARS coronavirus RNA.

Here, we share our experiences on the molecular diagnosis of SARS and other coronaviruses. The protocols described in this chapter are directly adopted from our previous publications (8, 9). The first and second assays are manual RT-PCR (Subheadings 3.2, 3.3, and 3.4) and real-time quantitative RT-PCR (Subheading 3.5) assays, respectively, for SARS coronavirus detections (see Notes 1–3). As there is a possibility that other SARS-like coronaviruses found in bats or other mammals might have zoonotic potential, we also present another PCR assay which is able to detect groups 1 and 2 viruses (Subheading 3.6). This assay might be useful to screen the SARS-like patients whom are negative in the first two assays. It should be noted that the primer set used in the third assay can cross react with a wide range of coronaviruses. Therefore, the identities of all the positive PCR products from the third assay should be formally confirmed by DNA sequencing. In our experiences, this assay is able to detect other common human coronaviruses (e.g. HKU1, NL63, OC43, and 229E).

2. Materials

2.1. RNA Extraction

1. QIAamp virus RNA mini kit (Qiagen).
2. Ethanol, 96–100%.
3. Autoclaved RNase-free water or its equivalents.

4. Clinical samples stored in 1–3 mL of viral transport medium. For 1 L of viral transport medium, dissolve 2 g of sodium bicarbonate (Merck), 5 g of bovine serum albumin (Sigma-Aldrich), 200 μg of vancomycin (Sigma-Aldrich), 18 μg of amikacin (Sigma-Aldrich), and 160 U of nystatin (Sigma-Aldrich) in 1 L of Earle's balanced salt solution (Sigma-Aldrich) and filter the solution using a 0.22 μm pore size filter (see Note 4).

2.2. Reverse Transcription

1. SuperScript II reverse transcriptase, 200 U/μL (Invitrogen).
2. 5× First strand buffer [250 mM Tris–HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$] (Invitrogen).
3. 0.1 mM dithiothreitol (Invitrogen).
4. Random hexamers, 150 ng/μL (Invitrogen).
5. RNaseOUT recombinant ribonuclease inhibitor, 40 U/μL (Invitrogen).
6. Deoxynucleotide triphosphates (dNTP) mix, 10 mM each.
7. Autoclaved RNase-free water or equivalents.
8. Heating block or equivalents.

2.3. PCR for SARS Coronavirus

1. AmpliTaq Gold DNA polymerase, 5 U/μL (Applied Biosystems).
2. 10× Gold PCR buffer (Applied Biosystems).
3. dNTP mix, 10 mM each.
4. 25 mM $MgCl_2$ solution (Applied Biosystems).
5. 10 μM PCR forward primer, 5′-TACACACCT CAGCGTTG-3′.
6. 10 μM PCR reverse primer, 5′-CACGAACGTGACGAAT-3′.
7. Themocycler (GeneAmp 9700, Applied Biosystems) (see Note 5).

2.4. Gel Electrophoresis

1. 50× TAE buffer (Bio-Rad).
2. Seakam LE agarose powder (Cambrex).
3. 6× Gel loading buffer [10 mM Tris–HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol, 60% glycerol, and 60 mM EDTA].
4. 1 kb plus DNA ladder markers (Invitrogen).
5. Ethidium bromide, 10 mg/mL.
6. Agarose gel electrophoresis apparatus.
7. Power supply (PowerPac Basic, Bio-Rad).
8. Gel documentary machine or equivalents.

2.5. Quantitative RT-PCR

1. TaqMan EZ RT-PCR Core Reagents kits (Applied Biosystems).
2. 50 μM PCR forward primer, 5′-CAGAACGCTGTAG CTTCAAAAATCT-3′.

3. 50 μM PCR reverse primer, 5′-TCAGAACCCTGTGATGA ATCAACAG-3′.
4. 10 μM probe, 5′-(FAM)TCTGCGTAGGCAATCC(NFQ)-3′ (FAM, 6-carboxyfluorescein; NFQ, nonfluorescent quencher; Applied Biosystems).
5. Quantitative PCR machine (ABI Prism 7000 Sequence Detection System, Applied Biosystems).
6. PCR reaction plates (MicroAmp optical 96-well reaction plate, Applied Biosystems).
7. Optical adhesive covers (Applied Biosystems).
8. Benchtop centrifuge (Allegra X-15R, Beckman Coulter) with microplate carriers (SX4750μ, Beckman Coulter).

2.6. PCR for Groups 1 and 2 Coronaviruses

1. AmpliTaq Gold DNA polymerase, 5 U/μL (Applied Biosystems).
2. 10× Gold PCR buffer (Applied Biosystems).
3. dNTP.
4. 25 mM $MgCl_2$ solution (Applied Biosystems).
5. 10 μM PCR forward primer, 5′-GGTTGGGACTATCCTA AGTGTGA-3′.
6. 10 μM PCR reverse primer, 5′-CCATCATCAGATAGAAT CATCAT-3′.
7. Themocycler (GeneAmp 9700, Applied Biosystems).

3. Methods

3.1. RNA Extraction

1. For a new kit, perform the following procedures before specimen processing (see Note 6):
   (a) Add 1 mL of AVL buffer to a tube of lyophilized carrier RNA (310 μg). Dissolve the carrier RNA thoroughly, then transfer to the buffer AVL bottle and mix thoroughly. Store the AVL buffer at 4°C for up to 6 months (see Note 7).
   (b) For every 19 mL of AW1 buffer, add 25 mL of ethanol (96–100%). Mix it well. Store the AW1 buffer at room temperature for up to 12 months.
   (c) For every 13 mL of AW2 buffer, add 30 mL of ethanol (96–100%). Mix it well. Store the buffer AW1 at room temperature for up to 12 months.
2. Equilibrate all reagents to room temperature before use.

3. Transfer 140 µL of the sample into a 1.5 mL microcentrifuge tube (see Note 8).
4. Add 560 µL of prepared buffered AVL with carrier RNA to the microcentrifuge tube.
5. Briefly vortex the tubes for 15 s and incubate at room temperature for 10 min.
6. Briefly centrifuge the microcentrifuge tube. Add 560 µL ethanol (96–100%) and mix by pulse-vortexing for 15 s.
7. Briefly centrifuge the microcentrifuge tube.
8. Transfer 630 µL of the solution from the tube to a QIAamp spin column placed in a provided 2 mL collection tube. Centrifuge at 6,000 × *g* (8,000 RPM) for 1 min at room temperature/4°C. Place the spin column in a clean 2 mL collection tube. Discard the tube containing the filtrate.
9. Open the spin column and repeat step 8.
10. Add 500 µL buffer AW1. Centrifuge at 6,000 × *g* (8,000 RPM) for 1 min. Place the spin column in a clean 2 mL collection tube. Discard the tube containing the filtrate.
11. Add 500 µL buffer AW2. Centrifuge at 20,000 × *g* (14,000 RPM) for 3 min. Place the spin column in a clean 2 mL collection tube and centrifuge at 20,000 × *g* for another 1 min. Place the spin column in a clean 1.5 mL microcentrifuge tube. Discard the tube containing the filtrate.
12. Apply 50 µL buffer AVE equilibrated to room temperature directly on the membrane of the column. Close the cap and incubate at room temperature for 1 min.
13. Centrifuge at 6,000 × *g* (8,000 RPM) for 1 min. Collect the filtrate for cDNA synthesis. Store the RNA at –20 or –70°C.

3.2. Reverse Transcription

1. Prepare a reverse transcription master mix sufficient for the designated number of samples in a sterile 1.5 mL microcentrifuge tube as shown below:

Reagent	Volume per reaction	Volume for *N* reactions	Final concentration
5× First strand buffer	4 µL	4 × *N*µL	1×
0.1 mM DTT	2 µL	2 × *N*µL	0.01 mM
10 mM dNTP	1 µL	*N*µL	0.5 mM
Random primers (150 ng/µL)	1 µL	*N*µL	7.5 ng/µL
Reverse transcriptase (200 U/µL)	1 µL	*N*µL	200 U/reaction
Ribonuclease inhibitor (optional)	1 µL	*N*µL	40 U/reaction
Total volume of master mix	10 µL	10 × *N*µL	–

2. Vortex and centrifuge the tube briefly. Keep the tube on ice.
3. Add 10 µL of master mix solution into separate 0.5 microcentrifuge tubes. Label the tube accordingly and keep these tubes on ice.
4. Add 10 µL of purified RNA samples into these tubes accordingly.
5. Vortex and centrifuge the tubes briefly.
6. Stand the tubes at room temperature for 10 min and then incubate at 42°C for 50 min.
7. Inactivate the transcription reaction by incubating the tubes at 95°C for 5 min and then chill the samples on ice. Store the cDNA samples at –20°C (see Note 9).

3.3. PCR for SARS Coronavirus

1. Prepare a PCR master mix sufficient for the designated number of samples in a sterile 0.5 mL microcentrifuge tube according to the following table. Include at least one positive control and one negative control (water) for each run. Add additional controls (e.g. purified RNA from the studied samples) as necessary:

Reagent	Volume per reaction	Volume for *N* reactions	Final concentration
10× PCR buffer	5 µL	5 × *N*µL	1×
$MgCl_2$, 25 mM	5 µL	5 × *N*µL	2.5 mM
dNTP, 10 mM	0.5 µL	0.5 × *N*µL	0.1 mM
Forward primers, 10 µM	1.25 µL	1.25 × *N*µL	0.25 µM
Reverse primers, 10 µM	1.25 µL	1.25 × *N*µL	0.25 µM
DNA polymerase (5 U/µL)	0.25 µL	0.25 × *N*µL	1.25 U/reaction
Water	34.75 µL	34.75 × *N*µL	–
Total	48 µL	48 × *N*µL	–

2. Vortex and centrifuge the tube briefly. Keep the tube on ice.
3. Aliquot 48 µL of the master mix into separate 0.5 mL microcentrifuge tubes and label the tube accordingly.
4. Add 2 µL of cDNA generated from the reverse transcription reactions to these tubes accordingly. For the positive control, add 2 µL of SARS coronavirus cDNA into the reaction. For the negative control, add 2 µL of autoclaved water.
5. Vortex and centrifuge the tubes briefly.
6. Run the PCR in the following condition:

Step	Temperature	Time
Heat activation	94°C	8 min
Thermal cycling (40 cycles)		
Denaturing step	95°C	30 s
Annealing step	50°C	40 s
Extension	72°C	15 s
Final extension	72°C	2 min
Soak	4°C	∞

7. After the run, analysis the PCR products by gel electrophoresis. Alternatively, the products can be kept at –20°C for short-term storage.

3.4. Agarose Gel Electrophoresis

1. Place a gel-casting tray onto a gel-casting base. Level the base.
2. Prepare 2% agarose gel by weighing out 1 g of agarose powder. Add it in a 250 mL bottle containing 50 mL 1× TAE buffer. Microwave bottle with a loosened cap until the gel starts to bubble and become transparent (see Note 10).
3. Cool the melted agarose to about 60°C and pour it into the gel-casting tray. Insert a comb to the tray.
4. Allow the gel to solidify at room temperature.
5. Remove the comb from the tray.
6. Place the tray into the electrophoresis chamber with the wells at the cathode side.
7. Fill the buffer chamber with 1× TAE buffer at a level that can cover the top of the gel.
8. Mix 0.5 μL of the DNA markers with 2 μL of 6× gel loading dye and 9.5 μL of water on a parafilm sheet by repeated pepitting.
9. Mix 10 μL of the PCR products with 2 μL of 6× gel loading dye on a parafilm sheet by pepitting up and down several times.
10. Apply the mixture to the corresponding well of the gel.
11. Close the lid of the electrophoresis apparatus and connect the electrical leads, anode to anode (red to red) and cathode to cathode (black to black).
12. Run the gel at 100 V for 30 min.
13. Turn off the power, remove the cover and retrieve the gel.
14. Soak the gel in 1× TAE with 0.5 μg/mL ethidium bromide for 15 min. Wash the gel with water briefly (see Note 11).

15. Place the gel on top of the transilluminator. Switch on the power of the gel documentary machine (see Note 12).
16. Adjust the position of the gel and record the results. The size of the expected product for the virus is 182 bp (see Note 13).

3.5. Quantitative RT-PCR

1. Turn on the quantitative RT-PCR machine. Activate the Detection Manager from the supplied software and confirm the reporter, quencher, passive reference dyes are FAM, NFQ, and ROX, respectively. Set the cycle conditions as follows:

Step	Temperature (°C)	Time
UNG treatment	50	2 min
Reverse transcription	60	40 min
Heat inactivation	95	5 min
Thermal cycling (50 cycles)		
Denaturing	95	15 s
Annealing and extension	55	1 min

2. In the reaction plate template, input the necessary information for the corresponding samples (e.g. positive standard, negative control, or name of the clinical specimen). Include at least one set of tenfold serially diluted positive controls with known copy numbers of the target sequence (e.g. 10^6 to 10 copies/reaction) and three negative controls (water) in each run. For the positive controls, key in the copy numbers of the target sequence used in the corresponding reactions.
3. Prepare a PCR master mix sufficient for the designated number of samples in a sterile 2.5 mL screw cap tube according to following table. Add additional controls (e.g. purified RNA from the studied samples) as necessary.

Reagent	Volume per reaction	Volume for *N* reactions	Final concentration
Water	6.2 µL	6.2 × *N*µL	–
5× TaqMan EZ buffer	5 µL	5 × *N*µL	1×
Manganese acetate, 25 mM	3 µL	3 × *N*µL	3.0 mM
dATP, 10 mM	0.75 µL	0.75 × *N*µL	0.3 mM
dUTP, 10 mM	1.5 µL	1.5 × *N*µL	0.6 mM
dCTP, 10 mM	0.75 µL	0.75 × *N*µL	0.3 mM

(continued)

(continued)

dGTP, 10 mM	0.75 μL	0.75 × *N*μL	0.3 mM
Forward primers, 50 μM	0.4 μL	0.4 × *N*μL	0.8 μM
Reverse primers, 50 μM	0.4 μL	0.4 × *N*μL	0.8 μM
Probe, 10 μM	1 μL	1 × *N*μL	0.4 μM
rTth DNA polymerase (2.5 U/μL)	1 μL	1 × *N*μL	2.5 U/reaction
AmpErase UNG (1 U/μL)	0.25 μL	0.25 × *N*μL	0.25 U/reaction
Total	21 μL	21 × *N*μL	–

4. Close the cup. Vortex and centrifuge the tube briefly.
5. Aliquot 21 μL of the master mix into the corresponding wells of the reaction plate.
6. Add 4 μL of the samples into the corresponding wells carefully (see Note 14).
7. Seal the reaction plate with an adhesive cover. Make sure each reaction well is sealed properly.
8. Briefly centrifuge the reaction plate.
9. Insert the plate to the quantitative PCR machine and perform the RT-PCR cycle.
10. After the reaction, examine the threshold cycles (Ct) and the amplification curves of the reactions. For a good experiment, the Ct values deduced from the standards should correlate with the $\log_{10}$ copy numbers of the target sequence used in these reactions (Fig. 1a). Positive clinical samples will generate amplification signals above the threshold (Fig. 1b). By contrast, signals from the water controls and negative samples will below the threshold line. Based on the Ct values from the reference standards, the amounts of input target in the positive reactions will be calculated by the software automatically (see Notes 15 and 16).

3.6. PCR for Groups 1 and 2 Coronaviruses

1. Prepare a PCR master mix sufficient for the designated number of samples in a sterile 0.5 mL microcentrifuge tube according to following table. Include at least one positive control and one negative control (water) for each run. Add additional controls (e.g. purified RNA from the studied samples) as necessary.

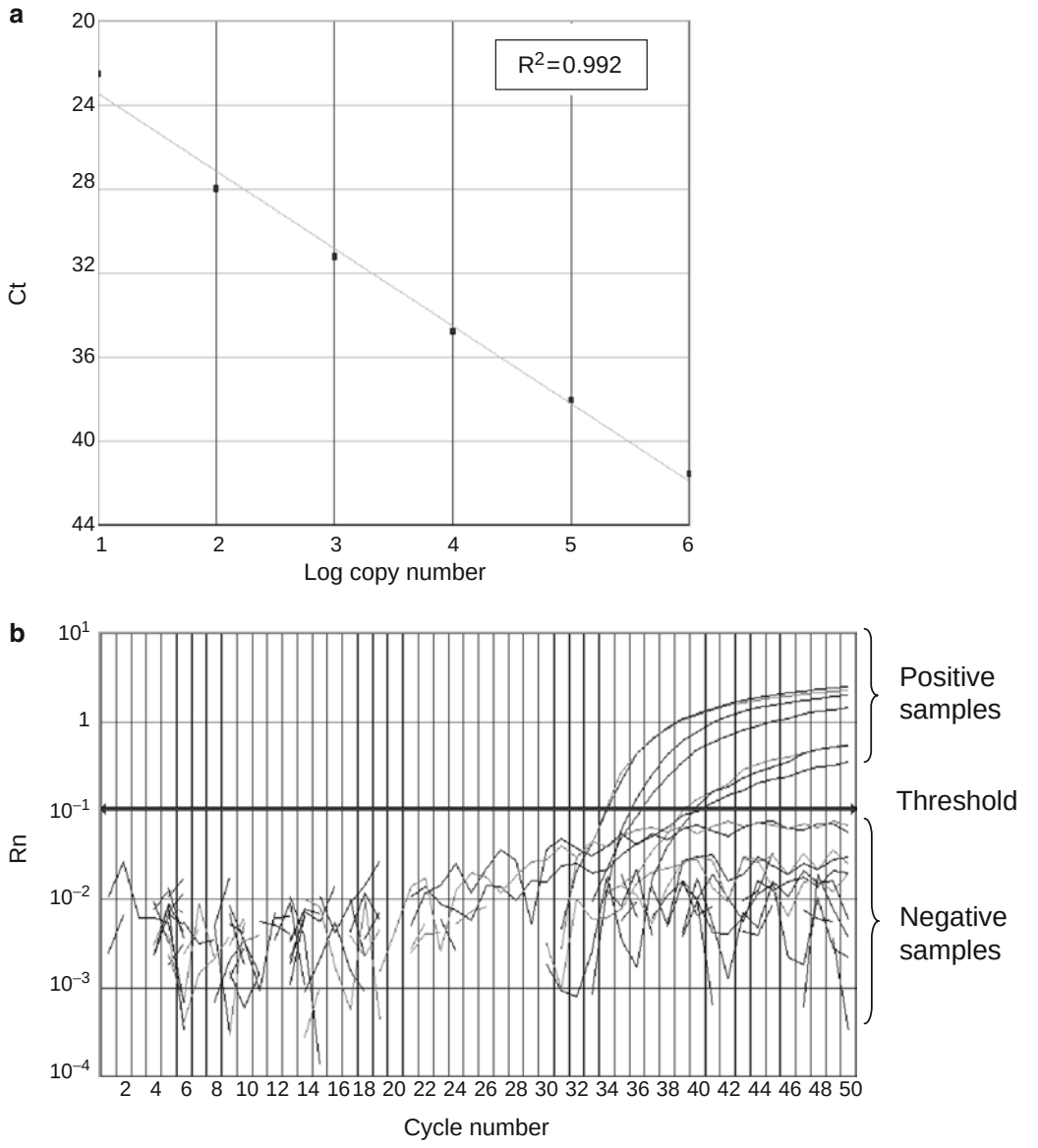

Fig. 1. Quantitative RT-PCR assay for SARS-CoV. (**a**) Standard curve for quantitative analysis of ORF 1b of SARS-CoV. The threshold cycle (Ct) is the number of PCR cycles required for the fluorescent intensity of the reaction to reach a predefined threshold. The Ct is inversely proportional to the logarithm of the starting concentration of the input target. The correlation coefficient (R^2) between these two parameters is shown. (**b**) An amplification plot of fluorescence intensity against the PCR cycle. The fluorescence signals for positive and negative samples are indicated. SARS-CoV. The *X*-axis denotes the cycle number of a quantitative PCR assay. The *Y*-axis denotes the fluorescence intensity. Figures are directly adopted from our previous work (8).

Reagent	Volume per reaction	Volume for *N* reactions	Final concentration
10× PCR buffer	5 μL	5 × *N*μL	1×
25 mM $MgCl_2$, 25 mM	5 μL	5 × *N*μL	2.5 mM
dNTP, 10 mM	1 μL	*N*μL	0.2 mM
Forward primers, 10 μM	1 μL	*N*μL	0.2 μM
Reverse primers, 10 μM	1 μL	*N*μL	0.2 μM
DNA polymerase (5 U/μl)	0.2 μL	0.2 × *N*μL	1 U/reaction
Water	34.8 μL	34.8 × *N*μL	–
Total	48 μL	48 × *N*μL	–

2. Vortex and centrifuge the tube briefly. Keep the tube on ice.
3. Aliquot 48 μL of the master mix into separate 0.5 mL micro-centrifuge tubes and label them accordingly.
4. Add 2 μL of cDNA generated from the reverse transcription reactions (Subheading 3.2) to these tubes accordingly. For the positive control, add 2 μL of coronavirus cDNA into the reaction. For the negative control, add 2 μL of autoclaved water.
5. Vortex and centrifuge the tubes briefly.
6. Run the PCR using the following conditions:

Step	Temperature (°C)	Time
Heat activation	94	10 min
Thermal cycling (45 cycles)		
Denaturing step	94	30 s
Annealing step	48	30 s
Extension	72	40 s
Final extension	72	2 min
Soak	4	∞

7. After the run, analysis the PCR products by gel electrophoresis (Subheading 3.4). The size of the expected product is 440 bp (see Note 17). Alternatively, the products can be kept at −20°C for short-term storage.

4. Notes

1. In our evaluation, the performance of the quantitative RT-PCR assay is better than the manual RT-PCR assays (3). In addition, the quantitative results generated from the real-time RT-PCR might provide additional data from prognosis (3).
2. In our patient cohort, respiratory samples (e.g. nasopharyngeal aspirate, throat swab) collected from patients within the first week of disease onset have the highest positive rates for SARS coronavirus. By contrast, fecal samples have the highest positive rate after the first week of onset. However, to increase the chance of identifying SARS patients in a nonepidemic period, we recommend testing multiple specimens available from suspected patients.
3. For respiratory samples isolated from early disease onset, the detection rates could be enhanced by increasing the initial extraction volume of the NPA sample from 140 to 560 μL (10).
4. Viral transport medium contains a high concentration of antibiotic to inhibit bacterial growth.
5. The primers and probe used in these assays are perfectly matched to those sequences deduced from SARS coronaviruses in human and civets, including those isolated in 2004.
6. Personal protection equipment should be worn by the healthcare worker taking specimens from suspect or probable SARS patients (http://www.who.int/csr/sars/infectioncontrol/en/).
7. AVL Buffer containing carrier RNA might form white precipitates when stored at 4°C. The precipitate can be dissolved in the buffer by heating the bottle in a water bath. Cool the buffer to room temperature before use.
8. For extracting RNA from suspected infectious samples, the procedure must be handled in a biosafety level (BSL) 2 containment using BSL 3 working practices. (http://www.who.int/csr/sars/biosafety2003_12_18/en/).
9. General procedures to prevent PCR cross contaminations should be strictly followed. Aerosol-resistant filtered pipette tips could minimize possible carryovers of amplicons. Separate pipettes and areas are used for sampling processing, PCR and post-PCR analysis. It is essential to include multiple positive and negative controls in the PCR reactions when a large number of samples are tested at the same time.
10. Agarose solutions can be superheated in microwave oven. Do not handle bottle immediately after microwaving. Always wear heat-resistant gloves when handling melted agarose.
11. Ethidium bromide is a known mutagen and may be carcinogenic. Handle solutions of ethidium bromide with gloves.

12. UV light can cause severe skin and eye damage. Wear safety glasses and close the photography hood before turning on the UV transilluminator.
13. The conventional RT-PCR protocol is highly specific to SARS coronavirus isolated from respiratory samples. However, we observed a small number of false-positive results from RNA isolated from stools. To overcome this problem, all of our positive fecal samples were retested by the quantitative RT-PCR as described in Subheading 3.5 or using a SYBR green-based RT-PCR assay (11) for confirmation.
14. When performing step 6 in Subheading 3.5, the RNA samples, including those positive standards, must be handled with extreme care. Cross contamination might lead to false-positive or unreliable quantitative results.
15. The amplification curves of all positive samples in the quantitative RT-PCR assays must be examined individually. We occasionally find some clinical specimens might yield high backgrounds and the analytical program might misclassify these samples as positive samples.
16. To exclude negative results due to the poor recovery of RNA, poor performance of the RT-PCR reaction, the presence of PCR inhibitors or human errors, we subsequently modified our quantitative RT-PCR assays to use a duplex assay. The revised test allows simultaneous detection of SARS coronavirus and endogenous 18 S rRNA derived from host cells (12). The primers and probe for 18 S rRNA are commercially available (TaqMan Ribosomal RNA Control Reagents, Applied Biosystems).
17. The identities of the positive products should be formally confirmed by DNA sequencing.

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Chapter 21

Detection of West Nile Virus

Elizabeth B. Kauffman, Mary A. Franke, Susan J. Wong, and Laura D. Kramer

Abstract

West Nile virus (WNV; *Flavivirus*, *Flaviviridae*) is a spherical enveloped virion containing single-stranded, positive-sense RNA, approximately 11 kb in length. The virus is the most widely distributed flavivirus in the world. Genetic analysis reveals two major lineages of virus, I and II, and several possible newly recognized lineages. Lineage I strains are most commonly associated with outbreaks of neurologic disease, although lineage II virus has led to large epidemics of fever, as in South Africa in 1974. Infection with WNV leads to a wide range of diseases from mildly febrile to severely neurologic, but asymptomatic infections occur most frequently. Approximately one in 140 infected individuals develop neurologic disease. The virus is maintained in an enzootic cycle, where it is transmitted between ornithophilic mosquitoes of the *Culex* genus and predominantly passeriform birds. Equines and humans are considered incidental hosts since they do not mount high enough viremia for mosquitoes to become infected following feeding. Laboratory diagnosis of WNV infection is predominantly serological, although caution is advised because of the high degree of cross-reactivity among flaviviruses. Field specimens, especially mosquitoes and dead birds, collected as part of surveillance programs, are tested for the presence of viral nucleic acid, viral antigen, or infectious virus. Rapid test protocols have been developed in response to the expansion of WNV in the United States. Since WNV is classified as a Biosafety Level-3 (BSL-3) agent by CDC, it is recommended that once this virus is identified in a diagnostic specimen, all infectious virus should be handled in a BSL-3 laboratory in Class II biosafety cabinets by laboratory staff who are trained to work at this level of containment. Assay protocols are described and the necessary equipment and supplies listed.

Key words: West Nile virus, Flavivirus, RT-PCR, Microsphere immunoassay, BSL-3, MAC-ELISA, Plaque reduction neutralization assay (PRNT), Nucleic acid detection

1. Introduction

West Nile virus (*Flavivirus*, *Flaviviridae*) is a spherical enveloped virion containing single-stranded, positive-sense RNA, approximately 11 kb in length, with a single open reading frame. Infection with the virus leads to a wide range of diseases from mildly febrile

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to severely neurologic, but asymptomatic infections occur most frequently. While the disease appears to be more severe in older individuals, all ages may become severely ill. The virus is the most widely distributed flavivirus in the world. Genetic analysis reveals two major lineages of virus, I and II (1), and several possible newly recognized lineages, III (2), IV (3), and V (4). Lineage 1 includes three sublineages: 1a, which is distributed in Africa, the Middle East, Europe, and the Americas; 1b, found in Australia is also known as KUN; and lineage 1c, which includes strains isolated in India. Lineage II is generally confined to sub-Saharan Africa and Madagascar. Lineage I strains are most commonly associated with outbreaks of neurologic disease, although lineage II virus has led to large epidemics of fever, as in South Africa in 1974 when 10,000 cases were recognized (5). There have been approximately 29,000 cases of WN disease in the US between 1999, when the virus was introduced into the New York City area, and December, 2009.

The virus is maintained in an enzootic cycle where it is transmitted between ornithophilic mosquitoes of the *Culex* genus and predominantly passeriform birds. Equines and humans are considered incidental hosts since they do not mount high enough viremia for mosquitoes to become infected following feeding. There is mounting evidence that certain small mammals also may become involved in the enzootic cycle, but to a significantly lesser extent than birds.

1.1. Clinical

Laboratory diagnosis of WNV infection is predominantly serological (6), although caution is advised because of the high degree of cross-reactivity among flaviviruses (7, 8). Paired acute and convalescent sera are recommended for confident determination of etiology of disease, where a fourfold rise between the two is necessary for confirmation (9), or a fourfold difference between related flaviviruses. Diagnosis by serological methods is particularly difficult in areas where Japanese encephalitis and/or dengue are cocirculating, e.g., India and tropical Americas, respectively. Secondary infections present additional problems in interpretation. Serologic assays include the standard IgM and IgG enzyme-linked immunosorbent assay (ELISA), immunofluorescent antibody assay (IFA), microsphere immunoassay (MIA), and confirmatory plaque reduction neutralization test (PRNT). Many horses are now vaccinated against WNV and therefore the diagnostician must be certain to distinguish naturally occurring antibody from that resulting from vaccination. Laboratory protocols for the standard IgM ELISA (MAC-ELISA) and the MIA, as well as the PRNT assays, are described below. Procedures for RT-PCR and virus isolation assays are seldom used for clinical samples because of transient and low viremia.

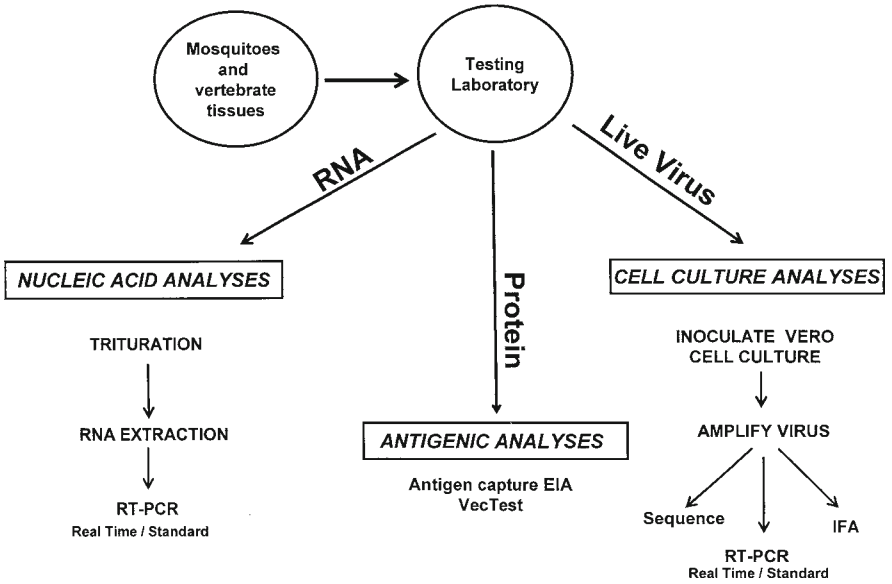

Fig. 1. Flow chart for surveillance of field specimens for WNV. Mosquitoes and birds, after collection in the field, are sent to the Arbovirus Lab where they are tested for WNV by methods that detect viral nucleic acid, viral antigen, or live virus.

1.2. Surveillance

Field specimens, especially mosquitoes and dead birds, collected as part of surveillance programs, are tested for the presence of viral nucleic acid, viral antigen, or infectious virus. Rapid test protocols have been developed in response to the expansion of WNV in the US (10–12). In some locations, sentinel birds are tested for antibody, using protocols similar to those used in equine and human diagnostics. A flow chart for testing field specimens is presented in Fig. 1. Protocols commonly used for avian and mosquito surveillance are RT-PCR (standard and real-time), isolation of infectious virus, antigen-capture dipstick assay (VecTest), and an indirect ELISA for detection in WNV Ab in bird serum. However, with appropriate modification, all of the methods described here can be used for clinical diagnosis.

1.3. Safety

It should be noted that WNV is classified as a BSL-3 agent by CDC (13). It is recommended that once WNV is identified in a diagnostic specimen, all infectious virus should be handled in a Biosafety Level-3 laboratory in biosafety cabinets (BSC) by laboratory staff who are trained to work at this level of containment. The staff must wear appropriate personal protective gear such as lab coats, goggles, and gloves to protect themselves from infection. It is best practice for all submitted specimens to be handled as infectious material.

2. Materials

2.1. General Lab Equipment and Supplies

1. Vortex Mixer.
2. Microfuge, Eppendorf Model 5415D, rotor with aerosol-tight lid.
3. Microfuge, refrigerated, Eppendorf Model 5415R, rotor with aerosol-tight lid.
4. Biological Safety Cabinet (BSC), Class II Type A/B3.
5. Phosphate buffered saline solution (PBS).
6. Microcentrifuge tubes, 1.5 and 0.6 ml, and 2.0 ml safe-lock (Eppendorf cat. no. 38-2236335-2).
7. Water, RNase/DNase-free.
8. Ethanol, 70% and 95–100%.
9. β-Mercaptoethanol (β-ME), 14.3 M.
10. Single and multichannel pipettes and pipette tips.
11. Aluminum foil.

2.2. Preparation of Homogenized Vertebrate and Mosquito Samples

1. Retsch Mixer Mill, MM 301 (Retsch Inc., Newtown, PA).
2. TissueLyser adapter set (hold 2×24 microfuge tubes) for Mixer Mill 301, (Qiagen Inc., Valencia, CA).
3. Refrigerated microfuge, rotor with aerosol-tight lid.
4. Daisy 4.5 mm steel Airgun shot (BBs), zinc plated.
5. Mosquito diluent: PBS supplemented with 20% heat-inactivated fetal bovine serum (FBS), 100 units/ml of penicillin, 100 μg/ml of streptomycin, 10 μg/ml gentamicin, 1 μg/ml Fungizone (Amphotericin B).
6. BA-1 diluent: Hanks M-199 salts, 1% bovine serum albumin, 350 mg/l of sodium bicarbonate, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 10 μg/ml gentamicin, 1 μg/ml of Fungizone in 0.05 M Tris buffer, pH 7.4. Store at 2–8°C, expiration 3 weeks.
7. ABI lysis solution, 2×, nucleic acid purification solution (Applied Biosystems cat. no. 430 5895). Dilute to 1× with PBS.
8. Qiagen RLT Buffer supplemented with β-mercaptoethanol (see Qiagen RNeasy Kit instructions).

2.3. RNA Purification by RNeasy Mini Kits

1. RNeasy® Mini Kit (250 reactions), Qiagen cat. no. 74106.
2. Collection tubes, 2 ml, Qiagen cat. no. 19201.

2.4. RNA Purification by ABI 6100 Nucleic Acid PrepStation

1. ABI Prism 6100 Nucleic Acid PrepStation, Applied Biosystems.
2. Lysis solution, 2×, nucleic acid purification (Applied Biosystems cat. no. 430 5895).
3. Elution solution, RNA purification (Applied Biosystems cat. no. 430 5895).
4. Wash solution I, RNA purification (Applied Biosystems cat. no. 431 5891).
5. Wash solution II, RNA purification (Applied Biosystems cat. no. 432 5890).
6. Purification tray, total RNA (Applied Biosystems cat. no. 430 5673).
7. Plates, TC II reaction plate, 96 well (Applied Biosystems cat. no. 430 6737).
8. Reagent reservoirs, 120 ml (Applied Biosystems cat. no. 430 4831).
9. Splash Guards (Applied Biosystems cat. no. 431 1758).
10. Plates, deep well (Applied Biosystems cat. no. 430 8641).
11. Optical adhesive covers (Applied Biosystems cat. no. 431 1971).
12. Tissue pre-filter tray (Applied Biosystems cat. no. 432 8129).
13. 96-Well flat-bottom plate (Costar cat. no. 3895).
14. Wide bore 1,000 µl micropipette tips.

2.5. Standard RT-PCR

1. Thermocycler, Applied Biosystems, Model 2720.
2. PCR enclosure, Labconco Purifier.
3. Primers (Table 1, sets 4 and 5). Stocks (25 µM) are prepared in RNase/DNase-free water, and stored in aliquots at –20°C in a manual defrost freezer.
4. Owl EasyCast horizontal B2 mini gel system (Owl Separating Systems Inc., Portsmouth, NH).
5. Gel electrophoresis combs (variety of sizes).
6. Ultra-pure agarose (Invitrogen cat. no. 15510-027).
7. Ethidium bromide, 10 mg/ml (Invitrogen cat. no. 15585-011).
8. TAE buffer (Tris, acetic acid, EDTA), 50×, (Invitrogen cat. no. 24710-030).
9. 10× Blue juice gel loading buffer (Invitrogen cat. no. 10816-015).
10. 1 KB ladder (1 µg/ml) (Invitrogen cat. no. 10787-026). Working solution: 10 µl 1 KB ladder, 10 µl Blue juice and 80 µl H_2O.
11. Power supply, VWR Model 300.

Table 1
Primers and probes for detection of WNV by standard or real-time RT-PCR

Set	Target	Description	Sequence (5′–3′)	Size (bp)	Ref
1	WNV env	1160 Forward 1229 Reverse 1186 Probe	TCA-GCG-ATC-TCT-CCA-CCA-AAG GGG-TCA-GCA-CGT-TTG-TCA-TTG 6FAM-TGC-CCG-ACC-ATG-GGA-GAA-GCT-C-TAMRA	70	(11)
2	WNV NS5	NS5 Forward NS5 Reverse NS5 Probe	GCT-CCG-CTG-TCC-CTG-TGA CAC-TCT-CCT-CCT-GCA-TGG-ATG 6FAM-TGG-GTC-CCT-ACC-GGA-AGA-ACC-ACG-T-TAMRA	168	(31)
3	WNV NS1	3111 Forward 3239 Reverse 3136 Probe	GGC-AGT-TCT-GGG-TGA-AGT-CAA CTC-CGA-TTG-TGA-TTG-CTT-CGT 6FAM-TGT-ACG-TGG-CTG-AGA-CGC-ATA-CCT-TGT-TAMRA	149	(10)
4	WNV capsid/PrM	212 Forward 619 Reverse	TTG-TGT-TGG-CTC-TCT-TGG-CGT-TCT-T CAG-CCG-ACA-GCA-CTG-GAC-ATT-CAT-A	432	(11)
5	WNV NS5	WN 9483 WN 9794	CAC-CTA-CGC-CCT-AAA-CAC-TTT-CAC-C GGA-ACC-TGC-TGC-CAA-TCA-TAC-CAT-C	326	–

12. UV transilluminator.
13. Polaroid camera with UV-light filter (use with Polaroid film 667).
14. Qiagen One-step RT-PCR Kit, Qiagen cat. no. 210212.

2.6. Real-Time RT-PCR

1. Thermocycler: ABI Prism 7500 Real-time PCR System (Applied Biosystems).
2. Eppendorf centrifuge, model 5810R with A-2-DWP rotor, or similar centrifuge that will handle 96-well plates.
3. TaqMan One-Step RT-PCR Kit, Applied Biosystems cat. no. 4309169. The kit has two components. Vial 1: DNA polymerase mix (2×) contains AmpliTaq Gold DNA polymerase, dNTPs with dUTP, Passive Reference 1, and optimized buffer components. Vial 2: RT enzyme mix (40×) contains MultiScribe reverse transcriptase and RNase inhibitor.
4. 96-Well optical reaction plates, ABI Prism cat. no. 4306757.
5. Optical adhesive covers (Applied Biosystems cat. no. 4311971).
6. Primers and probes (Table 1, sets 1–3). Stocks of primers (100 μM) and probes (25 μM) are prepared in RNase/DNase-free water, and stored in aliquots at −20°C in a manual defrost freezer.
7. WNV standards: RNA, purified from a WNV viral stock with known titer, is diluted to 1×10^6 PFU/ml, and then serial tenfold dilutions equivalent to 1,000, 100, 10, 1, and 0.1 PFU/10 μl are made and stored in 100 μl aliquots at −70°C.
8. Negative extraction controls: extraction buffer without sample is included in each RNA extraction procedure and tested by RT-PCR assay.
9. Positive extraction control: tissue from a WNV-positive crow is homogenized in 1× ABI lysis buffer (Subheading 3.1.2), and stored at −70°C in 100 μl aliquots. For each RNA extraction, an aliquot is diluted 1:10 with lysis buffer and included in the RNA purification procedure and tested by RT-PCR.

2.7. Isolation of Virus in Cell Culture

1. Incubator, 37°C, 5% CO_2.
2. Inverted light microscope with phase contrast optics.
3. Confluent cell monolayers: cell lines commonly used for infection of arboviruses are listed in Table 2. Cells are diluted to 2×10^5 cells/ml and seeded into six-well plates at 3 ml/well or T25 or T75 flasks at 5 or 15 ml/flask, respectively. The monolayer should be ready for infection 3–5 days later. Before infecting, examine cell monolayers for density and vitality under the microscope. Monolayers should be at least 90% confluent, but not overgrown. Maintenance medium (cell line-specific growth medium with FBS reduced to 2%) is used after the cells reach confluency and during virus infection.

Table 2
Cell lines used for arbovirus isolation

Cell line	ATCC	Derivation	Culture medium
Vero	CCL-81	African green monkey kidney	Eagle's MEM + 10% FBS
C6/36	CRL-1660	*Aedes albopictus* (Asian tiger mosquito)	Eagle's MEM + 10% FBS
BHK	CRL-10314	Baby hamster kidney (Syrian golden hamster)	DMEM + 10% FBS

4. Test samples: homogenized vertebrate or mosquito tissue (Subheading 3.1), infected cell cultures, oral swabs, serum, or CSF.
5. PBS-2%. Calcium/magnesium-free PBS supplemented with 2% FBS.
6. BA-1 diluent (see Subheading 2.2, item 6).
7. Mosquito diluent (Subheading 2.2, item 5).

2.8. VecTest Antigen Capture Assay

1. VecTest kit (Medical Analysis Systems, Camarillo, CA). Kits contain antigen assay dipsticks, grinding solution, copper-coated BBs, tubes, and racks.
2. Swabs (polyester fiber tipped applicators, Fisher cat. no. 14-959-90).

2.9. WNV IgM Capture ELISA (MAC-ELISA)

1. Coating buffer: carbonate/bicarbonate buffer, pH 9.6. Dissolve 1.59 g sodium carbonate and 2.93 g sodium bicarbonate in 500 ml water; adjust pH to 9.6 and bring to 1l with water. Store at 4°C.
2. Wash buffer: PBS with 0.05% Tween 20, pH 7.2.
3. Blocking buffer (BB): PBS, 5% milk, 0.5% Tween 20. Prepare fresh.
4. Stop solution: 1 N H_2SO_4.
5. Coating antibody: AffiniPure Goat Anti-Human IgM, $Fc_{5\mu}$ Fragment Specific, 2.0 mg/vial (Jackson Immunoresearch Laboratories, West Grove, PA, cat. no. 109-005-043).
6. WNV antigen: WNV COS-1 tissue culture antigen, P120-4 (Hennessy Research, Shawnee, KS). The dilution factor for each lot of viral and control antigen is determined by preliminary assay.
7. Normal antigen: normal control COS-1 uninfected tissue culture antigen, N-130-4 (Hennessy Research).
8. Positive control serum: PRNT-confirmed WNV-positive patient specimen.

9. Negative control serum: normal negative human control serum.
10. Detecting antibody conjugate: horseradish peroxidase (HRP) conjugated monoclonal antibody (Mab), broadly cross-reactive for the appropriate viral antigenic group (e.g., SLE, Strain MSI-7, IgG fraction of mouse monoclonal antibody 6B6C-1, DC153-100, Hennessy Research).
11. K-Blue substrate (stabilized chromogenic substrate for HRP-based ELISA tests, containing both 3,3′, 5,5′ tetramethyl-benzidine (TMB) and hydrogen peroxide (H_2O_2) in a one bottle format), Neogen Corporation, Lexington, KY, cat. no. 300175. Store at 4°C; avoid prolonged exposure to light.
12. Immulon 2 HB flat-bottomed 96-well plates.
13. Test tubes, 16×100 mm.
14. Microplate washer (SKATRON Washer 300/Model 12010).
15. Microplate reader (Molecular Devices Vmax Plate Reader).
16. Reagent reservoirs.

2.10. WNV Microsphere Immunoassay

1. Wash buffer: PBS, 0.05% Tween, pH 7.4 (Sigma, cat. no. P-3813).
2. Blocking/storage buffer (PBN): PBS, 1% BSA, 0.05% sodium azide, pH 7.4 (Sigma, cat. no. P-3688 and S-8032).
3. Luminex 100 system (Luminex Corporation, Austin, TX), consists of Luminex 100 instrument, XY plate handling platform, sheath fluid delivery system, software, and computer.
4. Calibration microspheres (Beads) (Luminex L100-CAL1 & L100-CAL2). Store in the dark at 4°C.
5. Luminex 100 Multi-analyte microspheres w/carboxylated surface (Luminex L100-C…-01) coupled to WNV truncated envelope protein (E) (L2 Diagnostics, LLC, New Haven, CT) as described in refs. 14, 15. Store the coupled beads at 4°C in the dark.
6. Luminex 100 sheath fluid (Luminex 40-5000).
7. WNV-positive control serum: PRNT-confirmed WNV-positive patient specimens, pooled. Store in 15 µl aliquots at –70°C or at 4°C for 1 month.
8. Negative serum control: previously tested human sera with no detectable WNV antibodies in IgM capture and IgG ELISAs, pooled. Store in 15 µl aliquots at –70°C or at 4°C for 1 month.
9. Detecting antibody conjugate: Goat F(ab′)2 anti-human IgG conjugated to R-Phycoerythrin, (Biosource International, cat. no. AH1707). Store in the dark at 4°C.
10. Multiscreen vacuum manifold (Millipore Corporation, cat. no. MAVM0960R).

11. Multiscreen filter plates with 1.2 μm pore size (Millipore Corporation, cat. no. MSBVN1250).
12. Titer plate shaker, Lab-Line Instrument, Inc., Model 4625.
13. Sonicator Bath, Cole-Parmer, Model B3-R.
14. Flat-bottom, medium binding, polystyrene, breakaway 96-well plates.
15. Reagent reservoirs.

2.11. Plaque Reduction Neutralization Test

1. Confluent Vero cell monolayers in six-well plates (Subheading 2.7, item 3).
2. BA-1 diluent (Subheading 2.2, item 6).
3. 2× MEM + 10%FBS: 2× minimal essential medium with 10% fetal calf serum, 200 units per milliliter penicillin, and 200 μg/ml streptomycin. Store at 4°C, expiration 3 weeks.
4. 1.2% Oxoid agar: add 1.2 g of Oxoid agar to 100 ml of H_2O in a 250 ml Wheaton bottle. Autoclave on liquid cycle, store at room temp, and microwave immediately before use.
5. 0.33% Neutral Red Solution (Sigma cat. no. N2889).
6. 96-Well cell culture plate, U-bottom (Falcon cat. no. 3077).
7. Test tubes, 12 × 75 mm snap-cap (Falcon).
8. Virus stocks, with known Vero cell titer in PFU (plaque-forming units)/ml. The viruses used in each assay vary and are based on the patient's history.
9. Undiluted positive control serum. PRNT-confirmed positive patient specimen specific for each virus employed in the assay.

2.12. Indirect ELISA for Bird Surveillance

2.12.1. Preparation of Crude Antigen

1. Sonicator, Branson Digital Cell Disrupter, Model S 250D, equipped with large capacity cuphorn.
2. Vero cell monolayers in T75 flasks (Subheading 2.7, item 3).
3. WNV stock virus with a known Vero cell titer.
4. 0.2 M glycine, pH 9.5.
5. 0.5% Triton X-100 in PBS.

2.12.2. Indirect ELISA

1. PBS with 0.05% Tween 20 (PBS-T).
2. Coating buffer (Subheading 2.9, item 1).
3. PBS with 0.05% Tween 20 and 0.5% bovine albumin (PBS-T + BA). Use as diluent for test serum and conjugated secondary antibody.
4. Binding buffer: 1× PBS with 0.05% Tween 20 and 2.0% Casein.
5. HCL 1:20 (300 ml dH_2O + 15 ml hydrochloric acid).
6. Immulon 1B flat-bottom 96-well plates.

7. ELISA AutoPlate Washer, ELx 405.
8. ELISA ultra microplate reader, ELx 808, Bio-Tek Instruments, Inc.
9. Humidity chamber (e.g., plastic container with moist paper towels).
10. WNV-positive control avian serum.
11. WNV-negative control avian serum.
12. Goat anti-Bird IgG-heavy and light-chain antibody, conjugated with HRP (Bethyl Labs, Montgomery, TX, Cat. no. A140–110P).
13. TMB 2-component microwell peroxidase substrate kit (KPL, Inc., Gaithersburg, MD, cat. no. 50-76-00). Bring to room temperature before use.

3. Methods

3.1. Preparation of Homogenized Vertebrate and Mosquito Samples

3.1.1. Mosquitoes

Since mosquito samples are commonly used for both virus isolation and RT-PCR, the samples are first homogenized in mosquito diluent, clarified by centrifugation, and then aliquots are taken for cell culture and/or RNA purification by RNeasy (see Note 1). RNA also may be extracted from the pellet using the ABI 6100 system.

1. Mosquitoes are collected in the field, sorted by species, and pools of 10–50 are placed in 2.0-ml safe-lock microfuge tubes, each containing a single BB. Samples are stored at –70°C until homogenized.
2. Remove mosquito samples from the freezer and place on ice. Add 0.75–1.0 ml of mosquito diluent to each tube of mosquitoes.
3. Place the tubes in the 24-well Mixer Mill adapter racks that have been pre-chilled at 4°C, and then secure in the Mixer Mill. Homogenize for 30 s at 24 cycles/s, and then place on ice for 5 min. It is important to keep the samples as cold as possible to minimize the activity of RNases released from the tissue. Microfuge at 4°C for 5 min at 8,000 × *g*. The supernate is ready for virus isolation or purification of RNA by RNeasy mini kits. Transfer to a 1.5-ml microfuge tube and store at –80°C. Save the pellet if RNA is to be purified by ABI 6100.
4. Treatment of pellet for RNA purification: Add 0.75–1 ml of 1× ABI lysis solution to the pellet and homogenize for 30 s at 24 cycles/s. Cool the material on ice for at least 10 min to

reduce the bubbles. *Do not centrifuge* after homogenization in ABI lysis buffer to avoid precipitation of RNA that has been incorporated into micelle structures formed from lysis buffer detergent and cellular lipids. Homogenized samples can be stored at 4°C for 24 h, –20°C for 1 week, and –70°C for a month. If samples are to be kept longer, they should be transferred to a new tube without the BB. Frozen samples may need to be heated to 37°C for 5 min to dissolve crystals in the lysis buffer.

3.1.2. Vertebrates

1. Vertebrate tissue received by the laboratory for testing is stored in specimen jars at –80°C. For detection of WNV, brain tissue is tested most often. All mammalian samples should initially be confirmed as rabies negative.
2. Place the frozen tissue in its jar on a bed of ice in a BSC. Thaw the tissue slightly and cut three sections of less than 1 mm^3 from different areas since the virus may not be evenly distributed throughout the tissue. A 1-mm cube of tissue weighs approximately 10 mg. Place the sections together in a 2.0 ml safe-lock tube containing one BB and 1 ml of buffer (1× ABI lysis solution for Applied Biosystems 6100 extraction, RLT for Qiagen RNeasy extraction, or BA-1 diluent for virus isolation).
3. Place the tubes in the Mixer Mill 24-well adapter racks. Homogenize for 4 min at 24 cycles/s, and then place on ice for 5 min. To achieve uniform homogenization, stop the mixer mill after 2 min and rotate the tubes in the sample adapters.
4. For RNA purification by ABI 6100, homogenize tissue in ABI Lysis Buffer, and place on ice for 30 min to reduce bubbles. *Do not centrifuge.* It is ready for RNA purification.
5. For RNA purification by RNeasy, homogenize tissue in RLT lysis buffer, and clarify by centrifugation for 3 min at 10,000 ×*g*. Purify RNA from the supernate by Qiagen RNeasy.
6. For virus isolation in cell culture, homogenize tissue in BA-1 diluent, centrifuge for 3 min at 10,000 ×*g*, and transfer the supernate to a new microfuge tube. It is ready to be plated on cells for virus isolation, or store at –80°C for later use.

3.2. Nucleic Acid Detection

3.2.1. RNA Purification by RNeasy Mini Kit (See Note 2)

All RNeasy procedures should be carried out at room temperature (15–25°C). Check buffer RLT for precipitate and if necessary incubate at 80°C until dissolved. Add β-ME to RLT buffer (10 µl/ml RLT; use within 1 month). Prepare working solution of buffer RPE by adding four volumes of 96–100% ethanol. Refer to the RNeasy Mini Handbook for further details (16).

1. Vertebrate and mosquito homogenates are prepared as described in Subheadings 3.1.2 and 3.1.1, respectively.
2. Ethanol promotes selective binding of RNA to the silica-based membrane in the column. For samples that are homogenized in RLT (vertebrates), place 350 µl of clarified homogenate in a microfuge tube, add 350 µl of 70% ethanol, and mix well by pipetting up and down. For samples that are homogenized without RLT lysis buffer (mosquitoes), add 100 µl of clarified homogenate to 350 µl of RLT, and then add 250 µl of 96–100% ethanol and mix well. *Do not centrifuge after the addition of ethanol.*
3. Bind RNA to the silica-based membrane by transferring the 700 µl sample with ethanol to an RNeasy mini spin column positioned in a 2-ml collection tube. Microfuge for 15 s at 8,000 × *g* (10,000 rpm). Carefully transfer the spin column to a clean 2 ml collection tube.
4. Wash the membrane with bound RNA three times. For the first wash, add 700 µl of *RW1 Buffer* to the column, microfuge for 15–30 s at 8,000 × *g*, and then carefully transfer the spin column to a clean 2-ml collection tube.
5. For the second wash, add 500 µl of *RPE Buffer* to the column, microfuge for 15–30 s at 8,000 × *g*, and then carefully transfer the spin column to a clean 2-ml collection tube.
6. For the third wash, add 500 µl of *RPE Buffer* to the column, microfuge for 2 min at 8,000 × *g*, and then carefully remove the column and place in a 1.5 ml collection microfuge tube. It is very important that all ethanol be removed from the sample to prevent degradation of RNA; use care in removing the column after the last spin so that the column does not contact the flowthrough. If carryover is observed or suspected, transfer the spin column to a clean 2-ml collection tube and repeat the centrifugation.
7. To elute RNA from the column, add 30–50 µl of RNase-free water onto the center of the membrane without touching, and allow it to adsorb for 1–10 min. Centrifuge for 1 min at 8,000 × *g*. If the expected yield is greater than 30 µg, repeat the elution with another 30–50 µl of water, reusing the collection tube.
8. The purified RNA is ready for use in standard or real-time RT-PCR assays. Store at –20 or –70°C.

3.2.2. RNA Purification by 6100 Nucleic Acid PrepStation (See Note 2)

3.2.2.1. Prefiltration

Samples that have large amounts of collagenous, fibrous, viscous, or particulate material can block the purification membrane and potentially contaminate other samples in the tray due to overflow during washes. Mosquito samples are always prefiltered because of the difficulty of attaining uniform, particulate-free homogenates. Vertebrate tissue lysates usually do not require prefiltration.

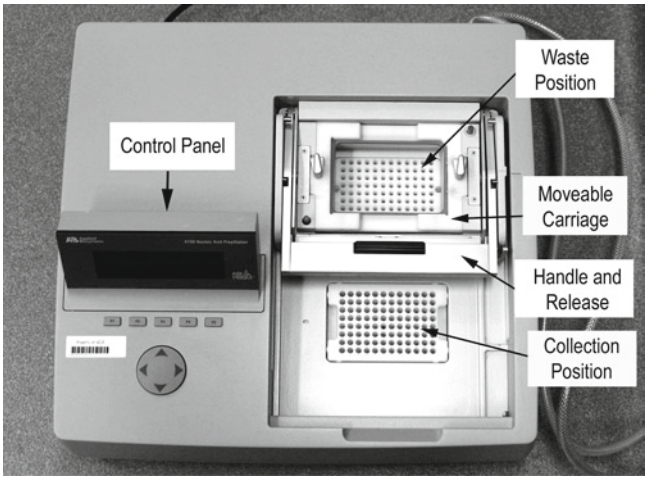

Fig. 2. ABI Prism 6100 Nucleic Acid PrepStation. A movable carriage holds a 96-well plate (tissue prefiltration or total RNA purification tray). The carriage has two locations (collection and waste) and three height settings (sealed, touch off, and release). The waste position is used for washing the samples; fluids are vacuumed out of the wells and discarded into a waste container. Disposable splash guards in the waste position prevent cross-contamination of samples. The collection position is used for collecting filtered lysate (using the prefiltration tray) or eluting purified RNA (using the RNA purification tray). The control panel, which consists of a display screen, function keys F1–F5, and arrow keys, controls the timing and pressure of the vacuum. Stored methods can be recalled with the use of a user name and pin number.

1. Load disposables on the 6100 PrepStation as described in the manufacturer's User Guide (17) and Fig. 2. Place a deep-well plate in the collection compartment and a splash guard in the waste compartment. Place a tissue prefilter tray in the purification tray carriage and secure it in place by turning the two knobs. Move the carriage to the collection position and push the carriage handle down until the carriage locks into position (seals). Cover unused wells by taping over them, or else the vacuum may not attain the correct level.
2. Prewet all wells with 40 µl of wash I, and then load samples by pipetting 100–600 µl of homogenized tissue lysate into each well of the prefilter tray, using a 1,000 µl micropipette with wide bore tips.
3. Perform prefiltration with the 6100 Quick Run program, entering "collection" for position, "60 s" for time, and "80%" for vacuum.
4. After the vacuum is complete, check that all the material has passed through the filter. If liquid remains, repeat the run. Care should be taken not to greatly exceed the recommended prefiltration time to avoid excessive foaming in the deep-well plate.

5. Touch off: set the carriage handle to the touch off position and carefully release the handle on the vacuum station so that the tips are still in the wells (this move requires practice to avoid jerky action). Gently move the vacuum station forward and back so that the tips touch the sides of the wells and any drops that may be attached are released.
6. Using the release lever, carefully (requires practice to avoid jerky action) release the purification tray handle, and move the carriage to the waste position. Remove prefiltration and deep-well plates from the instrument.
7. If not used immediately for RNA purification, store the deep-well plate at 4°C for up to 12 h, or at −20 or −80°C for long term.

3.2.2.2. RNA Purification of Vertebrate Tissue Lysates and Prefiltered Mosquitoes

1. Program the 6100 Nucleic Acid PrepStation with the steps described in Table 3. Load disposables on the 6100 PrepStation (Fig. 2). Place a Costar 96-well plate in the collection compartment and a splash guard in the waste compartment. Place a total RNA purification tray in the purification tray carriage and secure it in place by turning the two knobs. Move the carriage to the waste position and push the carriage handle down until the carriage locks into position (seals).
2. Prewet all wells with 40 µl of wash I.

Table 3
Purification of RNA on the ABI 6100 Nucleic Acid PrepStation

Step	Description	Volume (µl)	Position[a]	Vacuum (s)	Vacuum (%)
–	Prewet all wells with wash I	40	Waste	None	N/a
1	*Load samples* Prefiltered mosquitoes Vert homogenates	 50 100	Waste	180	80
2	Add wash solution 1	500	Waste	120	100
3	Add wash solution 2	400	Waste	120	100
4	Add wash solution 2	400	Waste	120	100
5	Add wash solution 2	400	Waste	120	100
6	Preelution vacuum	–	Waste	300	100
7	Touch off at waste	–	Touch off	–	–
8	Add elution solution	150	Collection	120	40
–	Touch off at collection	–	Touch off	–	–

[a]Position of the vacuum station (Fig. 2)

3. Load prefiltered *mosquito* lysates from the deep-well collection plate (Subheading 3.2.2.1) by placing 50 µl of each sample into the corresponding well of the Purification tray. Dilute *vertebrate* homogenates (Subheading 3.1.2) 1:1 with 2× ABI lysis buffer and load 100 µl (Table 3, step 1).
4. After loading, incubate samples for 3 min and then vacuum for 120 s by pressing the Start button (F1) (Table 3, step 1).
5. Add 500 µl of wash I to each well, using a multichannel pipette and being careful not to splash. The tips should be changed for each row to prevent cross-contamination. Vacuum for 120 s at 100% vacuum (Table 3, step 2). For all wash steps, add buffer to all wells to maintain vacuum efficiency.
6. Add 400 µl of wash II as in step 2. Vacuum for 120 s at 100% vacuum (Table 3, steps 3–5).
7. Perform a preelution vacuum to ensure that no wash buffer containing ethanol remains. Vacuum for 300 s (5 min) at 100% (Table 3, step 6).
8. Touch off (Subheading 3.2.2.1, item 5). Elution: move vacuum station to the collection position. Add 150 µl of elution buffer to each well. Incubate for 3 min. Vacuum for 2 min at 80% (Table 3, step 10).
9. Touch off (Subheading 3.2.2.1, item 5) and move the carriage to the waste position. Remove the collection plate. The samples are ready for RT-PCR. Store the deep-well plate at 4°C for up to 12 h, or at –20 or –80°C for long term.
10. Move the vacuum station to the *waste* position, flush the purification tray with 70% alcohol, and vacuum at 100%.

3.2.3. Standard RT-PCR (See Note 3)

1. Prepare Master Mix (MM) in a PCR enclosure that is located in a room separate from the thermocycler and gel electrophoresis procedures. Keep all reagents on ice or use cold blocks. Use the reagents supplied in the Qiagen One-Step RT-PCR kit. The WNV primer sets used for standard RT-PCR are listed in Table 1 (sets 4 and 5). Each reaction requires 40 µl of MM and 10 µl of sample. Determine total volume of MM needed for the entire set of reactions and prepare enough for an extra one or two reactions (Table 4).
2. Set up the reaction tubes by pipetting 40 µl MM into each RT-PCR tube, and then add 10 µl of the RNA sample. Secure caps on the tubes and place in the thermocycler.
3. Thermocycler conditions: for both WNV primer sets (Table 1, sets 4 and 5), the thermal cycling consists of 50°C for 30 min to synthesize the first-strand cDNA; 95°C for 15 min to inactivate the reverse transcriptase and to activate DNA *Taq* polymerase;

Table 4
Master Mix for standard RT-PCR

Component	Microliters per reaction
Water, DNase/RNase-free	14
5× Buffer	10
dNTP	2
Forward primer (25 μM)	1
Reverse primer (25 μM)	1
Q-solution	10
Enzyme	2
Total	40

35 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 1 min for PCR amplification; and a final elongation at 72°C for 10 min.

4. Analyze 20 μl of each RT-PCR product mixed with blue juice (four parts sample one part blue juice), on a 1.5% agarose gel with TAE running buffer and 0.5 μg/ml ethidium bromide. Place the gel on a transilluminator, and with safety shield in place, turn on UV-light to visualize bands. Photograph the gel for a permanent record.

3.2.4. Real-Time RT-PCR

3.2.4.1. Master Mix

1. Prepare MM in a PCR enclosure that is located in a room separate from the thermocycler and any downstream processing. Keep all reagents on ice or use cold blocks. Use the reagents supplied in the ABI TaqMan One-Step RT-PCR Kit. The WNV primer sets used for standard RT-PCR are listed in Table 1 (sets 1–3).
2. Each reaction consists of 40 μl of MM and 10 μl of sample. Determine total volume of MM needed for the entire set of reactions and prepare enough for an extra one or two reactions (Table 5).

3.2.4.2. TaqMan Plate Setup

1. Place 96-well optical reaction plate in a cold block.
2. Add 40 μl of MM to each well (may be done with multichannel pipette).
3. Add 10 μl of RNA template, serial dilutions of WNV standards, or DNase/RNase-free water (no template control) to each well.

Table 5
Master Mix for real-time RT-PCR

Component	Microliters per reaction
Water, DNase/RNase-free	12.25
2× Universal PCR Master Mix	25.00
40× Multiscribe + RNase inhibitor	1.25
Forward primer (100 μM)	0.5
Reverse primer (100 μM)	0.5
25 μM TaqMan Probe	0.5
Total	40.00

4. Cover the reaction plate with an optical adhesive cover, seal well.
5. Centrifuge the reaction plate so that all liquid is in the bottom of the wells (1,000 × *g* for 3 min).

3.2.4.3. Real-Time RT-PCR

1. Place the plate in the ABI 7500 instrument and turn it on. Open the software and choose File>New. Refer to the ABI Absolute Quantification Getting Started Guide (pp. 40–45) to set up the run (18).
2. Enter sample information and concentrations of the standards.
3. The thermocycler conditions for the WNV primers listed in Table 1 are: 30 min at 48°C and 10 min at 95°C (rep 1); 15 s at 95°C and 1 min at 60°C (rep 40). Save the file as a *.sds file. You can also save as a *.sdt (template) file for future use.

3.2.4.4. Analyze Data

1. When the run is complete, go to Results/Amplification Plot; select the entire plate (upper left corner) and click Analyze. Using the Results Tab, you can view the results, change parameters, omit samples, and manually set baseline and threshold. If any parameters are changed, the data must be reanalyzed. Refer to ABI Absolute Quantification Getting Started Guide, pp. 40–45 (18).
2. The default baseline is automatically set from 6 to 15 cycles. Reset the cycles if the amplification begins before a C_T of 15.
3. The threshold is automatically set by the software and should be located in the geometric phase of the amplification curve. For the WNV primer sets, the threshold is usually maintained at 0.2.

4. View the Standard Curve, which displays the values for the samples designated as standards.
5. The C_T value is the amplification cycle at which fluorescence increases above the threshold. The R_n value is relative change in fluorescence at the end of the amplification. These two values are used to analyze the data. Export these values for each test and standard sample into an Excel file. Express results as C_T values or relative PFU calculated by linear regression from the standard curve. A sample is determined to be positive if the C_T value is equal to or less than the threshold C_T value, and the R_n value is two or more times the average of eight negative wells.

3.3. Isolation of Virus in Cell Culture (See Note 4)

1. Decant media from six-well plate (or T-25 flask) containing a confluent monolayer of cells. Vero cells are used most often (Subheading 2.7, item 3).
2. Inoculate cells with 100 µl of mosquito or vertebrate homogenate (Subheading 3.1) (use 200 µl for T-25 flask).
3. Mock-infect at least one well or flask for each assay, by inoculating with 100 µl (or 200 µl) of BA-1 diluent.
4. Incubate at 37°C, 5% CO_2, for 60 min, rocking plates every 15–20 min.
5. At the end of the infection period, add 3 ml (5 ml for T-25 flask) of cell maintenance medium to each well. *Do not remove the inoculum*.
6. Incubate at 37°C, 5% CO_2, for up to 7 days.
7. Examine plates each day under the phase microscope, using the uninfected control for comparison. The presence of virus usually gives rise to morphological changes in the host cell known as cytopathic effect (CPE). CPE may consist of cell rounding, fusion, swelling or shrinking, death, or detachment from the surface and is rated on a scale of 1+, which means that less than 25% of the cells are affected, to 4+ where all of the cells in the monolayer are involved. Early examination of inoculated cells may reveal damage caused by toxicity from a component of the inoculum. The distinction between CPE and cytotoxicity often can be resolved during subsequent daily examinations, since CPE due to viral infection is progressive, spreading through the monolayer with time.
8. When no or questionable CPE is observed, the samples may be passed for two additional rounds as follows. Decant media from a fresh uninfected cell monolayer (six-well plate or T-25 flask). Transfer medium from the first passage to the new cells (0.5 ml for six-well plates and 1.5 ml for T-25 flask). Adsorb at 37°C, 5% CO_2, for 60 min, rocking every 15–20 min. Add maintenance medium (3 ml per well of six-well plate; 6 ml per

T25). Incubate at 37°C, 5% CO_2 for 4–5 days and examine for CPE, using the uninfected control for comparison. If no CPE is observed after 1 week, repeat the procedure for a third passage.

9. The virus responsible for CPE is identified by IFA of infected cells and/or RT-PCR with RNA extracted from the supernatants (12). Harvest of cells is usually performed at the time when 50% of the monolayer affected.
10. To harvest virus, add 800 ml of culture supernate to a cryotube containing 200 µl of FBS. Mix well, a using micropipette, and transfer 500 µl to a second tube to give two 500 µl aliquots. Store at −80°C.
11. For analysis of the viral culture by PCR, purify RNA from 100 µl of culture fluid by Qiagen RNeasy kit or by Qiagen QIAamp kit and perform standard RT-PCR (Subheading 3.2.3) or real-time RT-PCR (Subheading 3.2.4).
12. To prepare slides for IFA (12), thoroughly scrape the infected cell monolayer in the well or flask. Break up clumps of cells by trituration, using a 1,000 µl micropipette. Transfer material to a 15 ml centrifuge tube and vortex to further disrupt clumps of cells. Dispense 20 µl to each well of two ten-well slides. Dry slides by placing on a warming tray, and then fix by immersing in −20°C acetone for 15 min. Allow to air-dry and store at −80°C.

3.4. VecTest Antigen Capture Assay (See Note 5)

1. *Avian oral swabs (corvids only)*: In a BSC, open the bird's beak carefully as the bill can be sharp and insert a swab into the oral cavity. Move it vigorously around the entire oropharyngeal cavity and the proximal esophagus. Remove the swab and swirl it for 3–5 s in 1.0 ml of VecTest buffer (Grinding Solution provided with the VecTest kit) in a 10-ml plastic tube, and then press the tip against the side of the tube to release remaining fluid and discard. If the solution is full of debris, centrifuge for 5 min at 8,000 ×*g*.
2. *Mosquito pools*: Homogenize up to 50 mosquitoes in 1 ml of Grinding Solution (provided in VecTest kit) in Mixer Mill for 4 min at 25 cycles/s (Subheading 3.1.1). Clarify the homogenate at 8,000 ×*g* for 4 min.
3. The VecTest should be performed in a BSC. Transfer 250 µl of the swab solution or clarified mosquito homogenate into a conical microcentrifuge tube (supplied with the kit), and then insert the VecTest strip with arrows pointing down (Fig. 3). After 15 min, remove the strip and check for the presence of reddish-purple lines in both the test and control zones.
4. If lines have developed in both zones the sample is considered positive, while development of a line only in the control zone

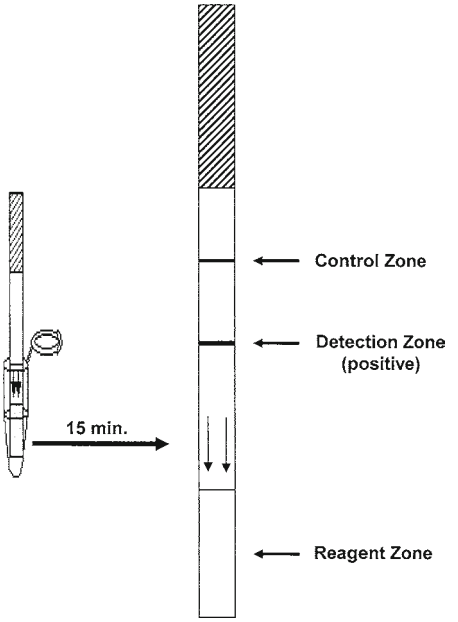

Fig. 3. VecTest. Diagram of the VecTest assay. The reagent zone of the test strip is coated with WNV monoclonal antibody (mAb) conjugated to colloidal gold. These antibodies will migrate up the test strip with the test material. The detection zone contains immobilized unlabeled WNV mAb that will capture WNV antigen as it migrates up the strip. The control zone contains immobilized reagents that will capture any gold-conjugated mAbs that do not bind to the detection zone to demonstrate that the sample migrated through the test zone. The bottom of the dipstick, indicated by *arrows*, is placed in a tube containing the test material, which will be wicked onto the strip and migrate through the three zones. After 15 min, the strip is removed and read. A positive sample will develop a reddish-purple color in both the control and the detection zones. Negative samples develop color only in the control zone. If no color develops, then the test is invalid.

is negative (Fig. 3). If no lines develop, discard the strip and repeat the assay with a fresh VecTest strip. Sometimes it is difficult to determine if samples are positive or negative. It is helpful to have several people independently interpret results. Treat borderline results as negative.

5. Samples not tested on the day of collection may be refrigerated overnight at 4°C. If a delay of >24 h occurs before testing, store the solution frozen at −20°C.

3.5. Serologic Assays

3.5.1. MAC-ELISA

This protocol is based on a procedure developed at CDC (8, 19). For single specimens, at least 10 µl of serum or 200 µl of cerebrospinal fluid (CSF) are needed. Paired specimens consist of acute (0–45 days after onset) and convalescent (>45 days after onset or at least 3–7 weeks after the acute) samples Store all diagnostic specimens at 4°C prior to testing. Store at −70°C after all anticipated testing has been completed. Avoid repeated freeze–thaw cycles.

3.5.1.1. Day 1

1. Coat the inner 60 wells of 96-well Immulon 2 plates with 75 μl/well of goat anti-human IgM diluted 1:500 in coating buffer. Incubate at 4°C overnight. Coated plates can be kept at 4°C in a humidified chamber for up to 1 week.
2. Plates should be kept in an enclosed, humidified environment during all incubation times with the exception of the blocking and substrate steps.

3.5.1.2. Day 2

1. Using an automated plate washer, aspirate the coating antibody and blot plates on paper towels. Add 200 μl per well of BB and incubate at room temperature for 30 min.
2. Prepare 1:400 dilutions of patient serum samples and negative control serum in wash buffer, enough for six replicate wells of 50 μl each. Note: CSF samples are not diluted. Dilute positive control serum to the concentration determined in a preliminary assay. Working dilutions of test and control sera can be prepared and refrigerated up to 7 days before use.
3. Wash wells containing BB 5× with wash buffer, using the automated plate washer. Wells should be filled to the top for each wash cycle.
4. Add serum samples to the plate, 50 μl per well. Each serum sample (patient samples, positive control, and negative control) is added to a block of six wells. Each CSF sample is added to a block of four to six wells, depending on amount available.
5. Incubate plates for 1 h at 37°C in a humidified chamber.
6. During incubation, prepare WNV antigen (WNV-infected cell lysate) and normal antigen (uninfected cell lysate) dilutions in wash buffer. Wash wells 5× with wash buffer using the automated plate washer.
7. Add 50 μl of WNV antigen to three wells of each serum block and normal antigen to the other three wells.
8. Incubate plates overnight at 4°C in a humidified chamber.

3.5.1.3. Day 3

1. Prepare 1:2,000 dilution of HRP-conjugated mAb in BB. Wash wells 5× with wash buffer and add 50 μl of conjugate to all wells.
2. Incubate plates 1 h at 37°C in a humidified chamber. At this time, remove K-Blue (TMB) substrate from the refrigerator, and turn on the microplate reader.
3. Wash plates 10×, turning the plates 180° in the washer after the first five washes to promote consistent results.
4. Add 75 μl of K-Blue (TMB) substrate to each test well and to two unused blank wells. Immediately cover plates to block

out light. Incubate at room temperature for 10 min. A blue color will develop in antibody-positive wells.

5. Add 50 μl of stop solution to all test wells and the two blank wells. Allow the plate to sit at room temperature for 1 min. Reactive wells will turn yellow.
6. Using a 450 nm filter, set the plate reader to zero on the blank wells, and then read the entire plate.

3.5.1.4. Calculations and Interpretation of Results

1. Calculate the P/N ratio for the positive control using the plate reader software. The P/N ratio must be >7.00 for a valid test.

$$\frac{\text{Mean OD of the positive control serum reacted on WNV antigen (P)}}{\text{Mean OD of the negative control serum reacted on WNV antigen (N)}}$$

2. Calculate the P/N ratio of each clinical specimen to determine if it contains IgM to the viral antigen, which would indicate recent infections with that virus. A ratio of >7.00 is considered IgM reactive. If the ratio is 3.0 or less the result is nonreactive. If the ratio falls between 3.01 and 7.00 the result is indeterminate.

$$\frac{\text{Mean OD of the test specimen reacted on WNV antigen (P)}}{\text{Mean OD of the negative control serum reacted on WNV antigen (N)}}$$

3. When the result is reactive or indeterminate, nonspecific reactivity is ruled out by calculating the ratio of the specimen's mean WNV antigen OD to the specimen's mean normal antigen OD. The ratio must be ≥2.0 to rule out nonspecific reactivity.

$$\frac{\text{Mean OD of the test specimen reacted on viral antigen (P)}}{\text{Mean OD of the test specimen reacted on normal antigen (N)}}$$

4. Interpretation of MAC-ELISA results is made in the context of collection date, onset date, and corresponding IgG results from a convalescent specimen. In most cases, IgM is detectable 8 days postonset of symptoms. WNV IgM is able to persist for at least 45 days, and sometimes for as long as 1 year (20–22).
5. Reactive specimens are confirmed by PRNT, which must be performed with both the original specimen and convalescent serum.
6. If an early acute serum (collected less than 8 days after onset) is nonreactive (P/N ≤ 3.00), a convalescent serum specimen is requested and tested before that patient can be clinically considered to have no serological evidence of recent viral infection. Likewise, nonreactive results for spinal fluid collected less than 8 days after onset are inconclusive.

7. If the test result is indeterminate (P/N 3.01–7.00), a convalescent serum specimen is requested, and then the paired specimens are tested by PRNT.
8. The IgM MAC-ELISA is restandardized whenever a new reagent lot is introduced. Standardization is performed by titration, comparing the optical densities of the reagents reacted on viral and normal antigen. All materials are tested in their appropriate dilution buffers. The mean optical density of the positive control serum reacted on the viral antigen should be set to approximately 3.3–3.7 (this varies from lot to lot). The normal control serum reacted on the viral antigen should have an OD of approximately 0.3.

3.5.2. WNV Microsphere Immunoassay

1. Bring reagents and specimens to room temperature. Prepare 1:100 dilutions of patient serum samples, and positive and negative serum controls. Lightly vortex the beads coupled to the WNV envelope, sonicate for 1–2 min, and then dilute in PBN to a concentration of 50,000 beads/ml. Prepare enough diluted beads to dispense 50 µl containing 2,500 beads to each test well. Keep beads in the dark until use.
2. The assay is performed in a Multiscreen filter plate. Block the test wells by adding 100 µl of PBN, and incubating at room temperature for 2 min. Wash wells with 190 µl of wash buffer, and remove the wash using the Multiscreen vacuum manifold. Add 20 µl of PBN to all wells to keep them wet.
3. Add 50 µl of each test sample, and positive and negative control to the plate.
4. Lightly vortex and sonicate diluted beads for 30 sec, and then add 50 µl (containing 2,500 beads) to each test well. Cover the plate with aluminum foil, and incubate at 37°C for 30 min while shaking gently (speed 3) on a titer plate shaker. Wash plate 3× with 190 µl of wash buffer. Use care to avoid cross-contamination of wells while pipetting.
5. While plate is incubating, dilute the detection conjugate (goat antihuman R-phycoerythrin) in PBN. The dilution will have been predetermined from block titration with current reagents.
6. Add 50 µl of diluted detection conjugate to each test well. Cover with foil and incubate for 30 min at 37°C, shaking gently on the shaker. Wash wells 2× with 190 µl of wash buffer.
7. Resuspend the beads by adding 125 µl of PBN to each test well. Mix with a multichannel pipette to reconstitute beads bound to the filter; avoid creating air bubbles. Transfer 75 µl of the reconstituted beads from each well to a 96-well plate.
8. Read samples on the Luminex 100, analyzing a minimum of 100 beads per well for both bead designation and

R-phycoerythrin fluorescence. The Luminex 100 will calculate the median fluorescent intensity (MFI) of collected events for each microsphere set.

9. Calculate the P/N ratio for the positive control. The P/N ratio must be >4.00 for a valid test.

$$\frac{\text{MFI of the positive control serum reacted on rWNV – E coupled beads (P)}}{\text{MFI of the negative control serum reacted on rWNV – E coupled beads (N)}}$$

10. Calculate the P/N ratio of each clinical specimen to determine if it contains antibody to the WNV E antigen, which would indicate infection, past or recent. The P/N ratio must be >4.00 to be considered reactive. If the ratio is <4.00, then the result is nonreactive.

$$\frac{\text{MFI of the test specimen reacted on rWNV – E coupled microspheres}}{\text{MFI of the negative control reacted on rWNV – E coupled microspheres}}$$

11. Interpretation of MIA results is made in the context of the collection and onset dates, and the MIA and/or MAC ELISA results from convalescent specimens. If the MIA result is reactive (P/N > 4.00), the specimens are submitted for confirmatory testing by PRNT. If the MIA result is nonreactive (P/N < 4.0), and it is an early acute serum sample (collected less than 8 days after onset) a convalescent serum specimen is requested and tested before that patient can be clinically considered as having no serological evidence of recent viral infection. The WNV-E MIA can be performed as a multiplex assay with WNV NS-5 (see Note 6).

3.5.3. Plaque Reduction Neutralization Test

The PRNT is considered the gold standard procedure for the identification of arboviruses, and the protocols are well established (23–25). The test can help distinguish false-positive results from the MAC-ELISA and other serologic assays. A fourfold rise in titer between acute and convalescent sera is indicative of a current infection. The PRNT also is useful for epidemiological studies that address antibody seroprevalence in a population. Since the test is costly, time-consuming, and requires a BSL-3 facility, it usually is not used as a screening tool.

1. Dilute test serum and positive control serum 1:5 in BA-1 diluent and heat-inactivate at 56°C for 30 min to remove nonspecific neutralizing substances. Note: with low serum volumes, higher serum dilutions may be used. Aliquot 100 µl of BA-1 diluent in columns 2–12 of a 96-well plate, and then add 100 µl of diluted and heat-inactivated serum samples to columns 1 and 2, using one row for each test or control serum. Make twofold serial dilutions of test sera as follows. Mix contents of column 2 with a multichannel micropipette,

and transfer 100 µl to column 3. Repeat the dilution procedure in columns 3–12 and discard the last 100 µl. Prepare an identical plate for each virus used in the assay.

2. The viruses chosen for neutralization in each PRNT assay are determined by patient history, with information on travel to areas endemic for arboviruses such as DEN, JEV, and YFV of high importance. Thaw each virus stock and dilute to 200 PFU/100 µl in fresh BA-1 diluent. Each virus will be "back-titered" to verify its concentration; take an aliquot of the 200 PFU/100 µl dilution and prepare 100, 10, and 1 PFU/100 µl dilutions in BA-1 diluent.
3. For the neutralization step, add 100 µl of the 200 PFU/100 µl virus stock to each dilution of test serum and positive control serum. Each well now will contain 100 PFU/100 µl of virus, and the starting dilution of each test serum sample (in column A) will be 1:10. Add the virus control "back-titration" dilutions to three vacant wells of the same 96 well plate. Incubate the plate at 4°C overnight or 37°C for 1 h.
4. At the end of the neutralization incubation, Vero cell monolayers will be infected with the neutralized virus. Remove the medium from six-well plates containing confluent cell monolayers (Subheading 2.7, item 3). Inoculate 100 µl of each virus/serum mixture into a separate well. For back-titration of virus, inoculate duplicate wells with 100 µl of each dilution. Rock the plates gently to evenly distribute the inoculum over the monolayer, and incubate at 37°C for a 1 h infection period to allow adsorption of virus to the cells.
5. During the infection period, prepare a nutrient agar overlay by combining equal parts of 45°C 2× MEM + 10% NCS and 1.2% agarose that has been melted by microwave and cooled to 45°C; hold the mixture at 45°C until use. At the end of the1 h infection period, add 3 ml of agar overlay to each well, allow it to solidify at room temperature and incubate at 37°C until plaques develop (approximately 2 days for EEEV, 3 days for WNV, 5 days for SLEV, and 4–5 days for JEV).
6. When plaques start to appear stain the cells by adding to each well a second agar overlay, similar to the first but with the addition of 1.5% neutral red. After an overnight incubation the plaques should be visible and ready to count.
7. Using a light box, count plaques and record numbers daily until no further significant increase in plaque number is observed. Neutralization titers are determined as the highest dilution of test serum or positive control serum that inhibits formation of at least 90% of the plaques as compared with the virus control back titration. The back titration of the

100 PFU/100 µl dilution should exhibit 30–100 plaques per well. If <20 plaques or >100 plaques develop, the assay should be repeated.

8. Interpretation of results. A fourfold difference in PRNT titers between related flaviviruses as well as a fourfold rise in titer between paired acute and convalescent sera are required for confident determination of etiology of disease. If paired acute and convalescent sera are not included in the PRNT, it is impossible to determine if neutralizing antibody detected by the assay is due to a recent or past infection. When secondary infections with a different flavivirus occur, results can be confusing and easily misinterpreted.

3.5.4. Indirect ELISA for Detection of WNV Antibody in Avian Serum

This screening assay has been used to study the prevalence of WNV infection within wild bird populations (25, 26). A similar assay has been used for detection of IgG in clinical samples.

3.5.4.1. Preparation of Crude Antigen

1. Infection. Seed two T-75 flasks with Vero cells and allow to grow to 90–95% confluency (Subheading 2.7, item 3). Infect one flask with WNV at an MOI of 1.0 PFU/cell. The other, uninfected, flask will be processed as negative control antigen. Incubate at 37°C until CPE reaches 3+ (most cells rounded, usually at 48 hpi). Freeze the flasks with their contents at −70°C overnight.
2. Thaw and add 1.5 ml of 0.2 M glycine, pH 9.5, to the contents of each flask, and then transfer to 50-ml centrifuge tubes.
3. Sonicate indirectly in an ice water bath at 100 mV for three bursts of 20 s. Allow 1 min of cooling between bursts.
4. Place tubes in a 37°C water bath for 4.5 h, vortexing every 45–60 min.
5. Inactivate antigen: Add an equal volume of 0.5% Triton X in PBS (3 ml/T-75 PREP) and leave at 4°C for 2 h with occasional vortexing.
6. Centrifuge at 10,000 × *g* for 10 min at 4°C. Store the supernate in aliquots at −70°C.
7. To ensure inactivation of virus in the antigen preparation, inoculate Vero cell monolayers and monitor for CPE. Note: Triton X-100 will produce cell toxicity; therefore, pass at least one time to eliminate possibility that apparent toxicity is not the result of viral infection.
8. Titrate antigen at a dilution range of 1:50–1:1,000 to determine the optimal concentration for use in ELISAs.

3.5.4.2. Indirect ELISA

1. Coat 96-well ELISA Plates. Dilute crude antigen (negative and positive) in coating buffer to a concentration previously determined by checkerboard titration. Apply 50 µl per well, placing negative antigen in columns 1, 4, 7, and 10 and positive antigen in columns 2, 3, 5, 6, 8, 9, 11, and 12. Incubate in a moist chamber at 4°C overnight (37°C for 1 h).
2. Wash plates with PBS-T, using a plate washer. Blot plates dry by inverting over a stack of paper towels and slapping several times.
3. Block plates by adding 100 µl of binding buffer to each well. Place in humidity chamber and incubate at 37°C for 1 h. Remove BB by inverting plates over paper towels and slapping several times. *Do not rinse.*
4. Dilute each test serum 1:200 in PBS-T + BA and add 50 µl to three wells (one coated with negative antigen and two with positive antigen). Include blank (PBS-T + BA) and diluted positive and negative control serum samples in each assay.
5. Incubate the plates in a humidity chamber at 37°C for 1 h. Wash with PBS-T using the plate washer, and blot dry.
6. Dilute wild bird conjugate (HRP conjugated goat anti-bird antibody) 1:1,000 or 1:2,000 in PBS-T + BA, and add 50 µl to each well. Incubate in a humidity chamber at 37°C for 30 min. Wash with PBS-T using the plate washer and blot dry.
7. Prepare the substrate by mixing equal volumes of room temperature TMB peroxidase substrate and peroxidase substrate solution B in a clean polypropylene or glass container immediately before use. Add 50 µl to each well, cover plates, and incubate at room temperature for 8 min. A deep blue color will develop. Stop the reaction by adding 50 µl of 1:20 HCl to each well. The color will turn yellow.
8. Wipe the bottom of the plate and read within 30 min in the plate reader at a wavelength of 450 nm.
9. Compute the positive/negative (P/N) value of each sample by dividing the mean OD of positive antigen-containing wells by the OD of the negative antigen-containing wells. Samples with a P/N values ≥2 are considered positive and usually are tested further by PRNT for confirmation.

4. Notes

1. Lysis buffers, RLT (RNeasy Kit) or ABI lysis solution, contain guanidine thiocyanate, which inactivates RNases that are released during homogenization of the tissues. The RNA from mosquitoes homogenized in these lysis buffers is well

preserved, but the integrity of live virus is compromised and the samples cannot be used for virus isolation. When mosquito samples are homogenized initially in mosquito diluent, the quantity and quality of RNA obtained from mosquito samples will be somewhat compromised. It is recommended that the samples be kept chilled during all steps of the procedure to minimize RNA degradation.

2. Two methods for viral RNA purification have been presented. The RNeasy method provides a good yield and is useful if a small number of samples is being processed. When mosquito samples need to be retested by RT-PCR, aliquots may be taken directly from the material homogenized in mosquito diluent. The ABI 6100 semiautomated procedure is faster than RNeasy because of the 96-well format, but the ABI chemistry relies on RNA extraction in the presence of cells, which prevents its use with medium from virus-infected cells or tissue homogenates. Furthermore, the material homogenized in the presence of ABI lysis buffer cannot be clarified by centrifugation after RNA extraction, increasing the potential for contaminating material that can interfere with RT-PCR.
3. Real-time RT-PCR assays are specific and high-throughput ones. For bird and mosquito surveillance, specimens are confirmed as WNV when positive results are obtained with two different primer/probe sets. If results are equivocal, RNA often is reextracted from the tissue homogenate by RNeasy, and a standard RT-PCR assay is performed.
4. Since RT-PCR is accurate, efficient, and fast, WNV isolation in cell culture is performed only if expanded amounts of the virus isolate are needed for further testing. However, virus isolation procedures commonly are performed for mosquito pools other than *Culex pipiens* or *C. restuans* to detect other arboviruses (flaviviruses, alphaviruses, and bunyaviruses). Vero cells are used most of the time since most arboviruses will replicate in these cells. Other cell lines such as BHK and C6/36 also have been used (Table 2). If CPE is observed, the virus is identified by IFA, RT-PCR, and/or sequencing (Fig. 1).
5. Two dipstick immunochromatographic assays for WNV are in popular use, the VecTest (Medical Analysis Systems, Camarillo, CA) and RAMP test (Rapid Analyte Measurement Platform, Response Biomedical Corporation, Vancouver, BC). Both assays are rapid and easy to perform, require minimal training, and can be used for mosquito and bird surveillance. Although the specificity of the assays is very high (98–100%), the sensitivity is significantly less than cell culture or RT-PCR assays (27, 28). Sensitivity Thresholds in $\log_{10}$ PFU/ml have been reported to be 1.0 for viral plaque assay, 0.1 for RT-PCR,

5.8 for VecTest, and 3.3 for RAMP (28). Comparison of the tests with avian oropharyngeal swabs similarly has shown that RAMP is more sensitive than VecTest and both tests are less sensitive than RT-PCR (29, 30). VecTest and RAMP are most reliable for testing corvid species of birds and possibly house sparrows because sensitivity of the assays drops well below 50% for other species. Despite the ease of use and superior sensitivity of the RAMP test, its cost (approximately $350 for the reader and $13–15 per test) may have deterred its widespread use. Care should be taken in handling material during the RAMP assay because unlike VecTest, the virus is not inactivated in the RAMP lysis buffer. It is recommended that both assays be performed in a BSC.

6. An MIA assay using recombinant WNV NS5 also has been developed (15). It would be feasible to perform a multiplex MIA that tests for WNV-E and NS-5 in one tube, as long as the NS-5 and envelope proteins are conjugated to microspheres with different fluorescent signals. Recombinant WNV NS-5 is available from Pei-Yong Shi (Wadsworth Center, NYSDOH, Albany, NY).

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Chapter 22

Point of Care Testing: Diagnosis Outside the Virology Laboratory

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Abstract

Numerous point-of-care tests (POCTs) are available to diagnose viral infections in both hospital and community settings. The ideal POCT is rapid, sensitive, specific, and simple to perform. This chapter will describe the benefits of POCTs, factors that can influence the accuracy of POCTs and highlight some limitations of POCT strategies. The sensitivity, specificity, and turn-around time of available POCTs are included for common conditions including respiratory viral infections (e.g. influenza, RSV) and blood-borne viral infections (e.g. HIV).

Key words: Point-of-care test, Influenza, Respiratory Syncytial Virus, HIV

1. Introduction

A point-of-care test (POCT) is defined as an analytical or diagnostic test undertaken in a setting distinct from a normal hospital laboratory (1). It may be performed by a health care professional or non-medical person. In the past two decades, there has been a rapid expansion in the use and number of diagnostic tests available in a point-of-care format. In the literature, there are many descriptive names given including bedside testing, near-patient testing, physician's office testing, extra-laboratory testing, decentralised testing and offsite, ancillary, or alternative site testing (2). To distinguish POCTs from other rapid diagnostic methods, the test is able to be performed by non-laboratory trained staff and without the use of complicated and poorly transportable equipment (e.g. microscopes, centrifuges). It can be also used by laboratories as a rapid alternative to other "usual" laboratory tests, or where testing facilities are limited.

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POCTs have been developed for many areas of clinical medicine. These include biochemical assays (e.g. glucose, sodium, cardiac enzymes, and cholesterol), haematological assays (e.g. haemoglobin, prothrombin/INR, ESR, and HbA1C), hormonal assays (e.g. βHCG, LH, and FSH) and drug assays (e.g. alcohol, amphetamines, and cannabinoids). In infectious diseases, POCTs can be used to detect antigens (e.g. of *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Legionella pneumophila*, influenza viruses, and respiratory syncytial virus) or pathogen-specific antibodies (e.g. HIV antibodies).

To establish a valuable role in clinical practice, a POCT needs to fulfil a number of key features (Table 1) (3). To provide advantages over traditional testing or at least comparability, a POCT needs to be rapid, sensitive, and specific. Furthermore, the provision of rapid results should improve patient or hospital outcomes and be cost-effective. When evaluating rapid tests such as POCTs, clinicians need to understand the difference between the time taken to perform the test, often referred to as turn-around time (TAT), and clinical turn-around time (e.g. incorporating patient assessment, test ordering, sample collection, transport if required, test performance, and clinical action). As POCTs are often designed to be performed by clinical staff without laboratory training, they need to be simple to perform and interpret and should require no significant infrastructure. Clinicians need to be aware of the impact that disease prevalence can have on positive and negative predictive values when utilising POCTs in clinical practice.

This chapter will describe the most common POCT design used in the detection of viral agents, describe a number of clinical

Table 1
Important features of POCT

Important features of a POCT
• Is highly sensitive and specific
• Gives a result that improves treatment (and reduces cost) by reducing inappropriate treatment and hospitalisation
• Can be done rapidly (15–30 min)
• Is simple to perform and interpret by non-laboratory personnel
• Contains internal controls to help assure the validity of results
• Does not require expensive or elaborate equipment
• Has temperature stable components that allow easy and prolonged storage
• Is relatively inexpensive

illnesses with examples of the available POCTs, and highlight special situations where a POCT may be most beneficial. As few POCTs have been directly compared and often the sensitivity and specificity calculated on different "gold standards", caution is advised when comparing different POCTs. Examples of POCTs in the text and tables may not reflect the number of different tests available worldwide and should not be interpreted as a recommendation of one assay over another.

2. Test Format

The diagnosis of infection may be achieved through the detection of either specific antigens from the site of infection (e.g. species-specific influenza antigens in respiratory secretions) or specific antibodies from blood and other bodily fluids (e.g. HIV antibodies in whole blood). There are a variety of formats utilised for these assays. The two most frequently used formats are the lateral-flow immunoassay and the dot blot immunoassay (4).

The lateral-flow immunoassay (also referred to as immunochromatographic test or ICT) uses a chromatographic pad with three zones: a sample application area, a conjugate pad, and a capture line (Fig. 1) (4). The clinical sample, which may have undergone lysis treatment, is applied to the sample pad and flows laterally by capillary action. Upon reaching the conjugate pad, if the target antigen or antibody is present in the sample, it binds to the conjugate forming an immune complex. The complex then continues to flow laterally along the strip and is captured by the line, coated with an appropriate antibody/antigen. The presence of a coloured line is a positive reaction. Most assays also have a positive control line included.

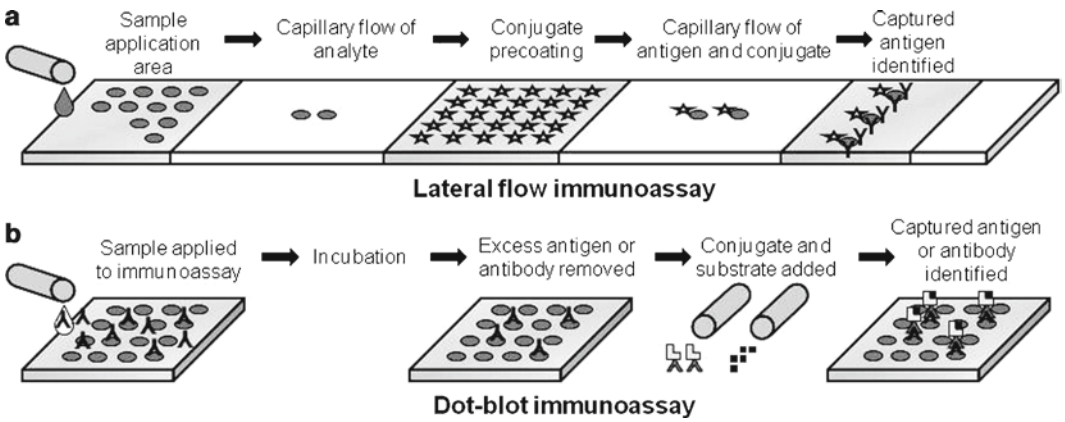

Fig. 1. Test format. (**a**) Antigen detection by lateral flow immune assay (**b**) antibody detection by dot-blot immunoassay (modified from Carpenter (4)).

The dot-blot immunoassay (also referred to as flow-through immunoassay) has bound antigen or antibody coupled to a membrane (Fig. 1). The patient sample is incubated with the membrane forming an immune complex. Excess antigen or antibody is then removed prior to the addition of conjugate and substrate. The presence of a coloured product indicates the presence of the specific antigen or antibody. Controls are usually included.

3. Quality Assurance

A number of pre-analytical, analytical, and post-analytical factors influence the quality of pathology tests, and it is important that clinicians using or interpreting POCT results understand these principles. Essential components include specimen collection, kit storage (conditions and duration), the performance of the test, test interpretation, use of internal and external controls, and reporting of results.

Most POCTs have been designed to be performed by non-laboratory staff. In the USA, laboratory testing including point-of-care testing is regulated by the Clinical Laboratory Improvement Amendments of 1988 (CLIA) (5). Tests are classified according to their complexity. To receive a CLIA waiver, tests must use direct, unprocessed specimens and be easy to perform with negligible chance of error. Waived tests can be performed by persons without formal laboratory training and outside traditional laboratories. Many of the tests discussed in this chapter have received CLIA waiver. Although simplification of the test will enable non-laboratory trained practitioners to perform it, this does not negate the need for training, particularly in areas such as specimen collection. This is particularly relevant in the setting of respiratory viruses, where upper respiratory tract samples are required.

Most POCTs contain internal controls and recommend the use of external controls. Internal controls are contained in the test format and indicate that a sample has been correctly processed. Unlike quantification of nucleic acid in molecular tests and fluorescent microscopy for respiratory viruses, internal controls in POCTs do not give any indication of sample quality. Most manufacturers recommend that external controls (usually supplied by the manufacturer) are run: (a) by each new operator prior to performing the test on patients, (b) when a new lot/batch of test kits is used, (c) upon receipt of a new shipment of test kits, (d) when the temperature of the storage or test area falls outside the recommended range, and (e) at periodic intervals determined by the testing facility (usually based on their volume of testing).

The recording of POCT results will depend on the environment in which it is introduced. Care must be taken to record

results in clinical notes or the laboratory information system. External controls should also be documented and records retained. Following introduction of a POCT, formal investigation to verify that the testing device performs to the required analytical sensitivity and specificity must be undertaken and the findings recorded and retained.

Given the importance of quality assurance when delivering POCT services, a number of national, medical, or hospital bodies have released statements on the use of POCTs (1, 6, 7). These highlight the need for clinical governance, training, and review, following the introduction of a point-of-care testing programme.

4. Financial Considerations

The financial impact of POCTs needs to be considered before delivering POCT services. Cost effectiveness has been established for a number of devices especially when used in high prevalence or outbreak situations and when investigations, admission, or repeat appointments could be avoided (8–10). Care needs to be taken when using these data in lower prevalence situations and sometimes in outbreak situations (11). Conditions such as community acquired pneumonia (CAP) are particularly challenging given the number of potential pathogens responsible (i.e. a number of POCTs may be required to establish a diagnosis, thus increasing costs). Despite the best diagnostics, an aetiology may be established in only 46% of CAP (12). The costs of consumables (e.g. swabs), ordering and storage, quality assurance, and reporting are often not factored into analyses.

Who bears the cost? This varies between different countries and regions. Costs may be incurred by the patient, the hospital, the laboratory, or the doctor, and these factors need consideration when designing POCT services.

5. Point of Care Testing for Respiratory Viruses

One of the major applications of POCTs has been in the rapid detection of respiratory pathogen antigens in community and hospital respiratory infections. The diagnosis of respiratory viruses is important to enable early infection control procedures and both timely and appropriate treatment wherever available. Methods to identify respiratory viruses include: a) viral culture with virus confirmation with monoclonal antibodies, b) antigen detection by staining of clinical specimens with monoclonal fluorescent

antibodies, c) rapid antigen detection by POCT, d) direct viral genome detection by nucleic acid testing (NAT), and e) acute and convalescent serology (most frequently complement fixation or haemagglutinin inhibition tests) demonstrating a fourfold rise in virus-specific antibodies. POCTs for respiratory viruses are available for three respiratory viruses: influenza, respiratory syncytial virus (RSV), and adenovirus (which is covered separately in Point-of-Care Testing for Other Viruses).

5.1. Influenza Virus

Numerous influenza POCTs are available employing a variety of formats (Table 2) (13, 14). For influenza POCTs, the target is usually the conserved internal influenza viral protein (e.g. nucleoprotein). Many POCTs now distinguish both influenza A and B in the same test. Although type-specific influenza POCTs exist, subtyping is not possible. This is required to distinguish seasonal from pandemic/outbreak strains. Furthermore, given the increasing proportion of oseltamivir-resistant H1N1 influenza A (15), subtyping is required to determine optimal treatment strategies.

POCTs do not perform equally for all influenza viruses, as demonstrated by their poor performance in the diagnosis of human influenza A H5N1 infection (16). In vitro studies suggest that the analytical sensitivity is comparable between different influenza viruses (including influenza A H5N1) when grown in culture (17). As a result, the reduced sensitivity reported is likely to be related to patient and disease factors rather than the analytical sensitivity of the test. Variation in test sensitivity is influenced by epidemiology and disease prevalence (e.g. during seasonal outbreaks), specimen type, and treatment. Higher sensitivity is observed in children compared with adults (18), when nasopharyngeal aspirates are compared with nasal or throat swabs (19) and when specimens are collected early in the illness (when viral shedding is highest) and prior to antiviral therapy (20).

The rapid identification of influenza viruses has a number of advantages. Antiviral agents are beneficial in reducing duration and severity of symptoms, secondary complications, and fatality rates. They enable earlier return to work, but need to be commenced within 48 h of symptom onset (21, 22). Timely diagnosis is therefore required for effective therapy.

Diagnosis of influenza may be associated with reduced investigations and antibiotic use, shorter admission time, and less health care costs (8). As influenza is often managed in general or family practice or in hospital emergency departments, a point-of-care format offers a number of advantages over “traditional” tests with longer turn-around times (TAT): e.g. antigen detection by staining of clinical specimens with indirect fluorescent antibodies (laboratory TAT are approximately 3–4 h) and specific influenza nucleic acid detection (laboratory TAT approximately 12–24 h).

Table 2
Frequently used influenza POCTs which identify influenza A and B (13, 14)

Test	Manufacturer	Specimen	Sensitivity (%)	Specificity (%)	Time (min)
BinaxNOW® influenza A & B[a]	Binax, Scarborough, ME, USA	NPA, NPS, NW	58–82	92–100	15
Directigen flu A + B	Becton-Dickinson, Cockeysville, MD, USA	BAL, NPA, NPS, NW, TS	81–86	91–99	15
Directigen flu A + B/EZ	Becton-Dickinson, Cockeysville, MD, USA	NPA, NW, TS	69–86	86–100	15
Flu optical immunoassay A/B	Thermo Electron, Waltham, MA, USA	NPA, NPS, TS, sputum	62–88	52–80	15
QuickVue® Influenza A + B[a]	Quidel Corporation, San Diego, CA, USA	NPA, NW, nasal swab	72–82	96–100	10
Xpect fluA and B	Remel Inc, Lenexa, KS, USA	NPS, NW, TS	83–100	100	15
ZstatFlu-II™[a]	ZymeTx Inc, Oklahoma City, OK, USA	NPA, TS	50–88	83–100	30

BAL Bronchoalveolar lavage, *NPA* nasopharyngeal aspirate, *NPS* nasopharyngeal swab, *NW* nasal wash, *TS* throat swab
[a]CLIA-waived

The portability of POCTs can assist in the diagnosis of institutional or community influenza outbreaks. The rapid identification of influenza enables early public health intervention and therapy and may influence the nature of an influenza outbreak (23).

Practitioners need to be wary of the reduced sensitivity compared with other methods when interpreting results. Reliance on POCTs will miss a significant proportion of those infected with influenza. This is particularly important in the high-risk patients, but less so when multiple patients are tested during an outbreak. There is evidence accruing that a POCT apparatus can be stored and the sample tested at a later date using nucleic acid analysis in circumstances where false positive or false negative outcomes are a possibility.

5.2. RSV

A number of RSV POCTs, targeting conserved structural proteins, are available (Table 3) (13, 24). The quoted sensitivity of RSV POCTs has been established in children during the RSV season. However, various studies have documented a lower sensitivity in adults with RSV infections, perhaps due to the lower and shorter period of RSV shedding (25, 26). Care needs to be taken outside of the RSV season as the proportion of false positive tests increases.

The rapid identification of RSV infections has a number of advantages. Given the burden of disease in paediatric hospitals during the RSV season, rapid testing may assist with infection control, patient isolation, and cohorting, thus reducing nosocomial transmission. Rapid diagnosis in paediatric patients has been associated with reductions in antibiotic use, hospital costs, and length of stay (9). Following the introduction of POCTs, Mackie et al. reported a decrease in nosocomial RSV transmission (27). A rapid diagnosis may also enable early institution of antiviral therapies (e.g. ribavirin) in high-risk patients (27).

6. Point-of-Care Testing for Blood-Borne Viruses

The global burden of blood borne viruses is a significant problem, with many infected populations living in resource poor countries with limited access to diagnostic laboratories. Diagnosis is essential to stop the ongoing spread and allow appropriate therapy. Traditional serological or molecular testing requires the patient to return for results. Recent estimates suggest that up to 31–40% of those tested for HIV may not return for test results (28, 29).

Table 3
Frequently used RSV POCTs (13, 24)

Test	Manufacturer	Specimen	Sensitivity (%)	Specificity (%)	Time (min)
BinaxNOW® RSV	Binax, Scarborough, ME, USA	NPA, NPS, NW	70–93	89–100	15
Clearview® RSV[a]	Inverness Medical Innovations, Bedford, UK	NPA, NPS, NW	93	97	15
Directigen RSV	Becton-Dickinson, Cockeysville, MD, USA	NPA, NPS, NW, tracheal aspirates	93–97	90–97	15
Directigen EZ RSV	Becton-Dickinson, Cockeysville, MD, USA	NPA, NPS, NW	89	93	15
Respi-Strip RSV	Coris Bio-Concept, Gembloux, Belgium	NPA, NPS	86–92	93–98	10
SAS™ RSV Alert[a]	SA Scientific, San Antonio, TX, USA	NPA, nasal swab	83	91	10
Sure-Vue RSV[a]	Thermo Fisher Scientific, Waltham, MA, USA	NPA, NPS, NW	96	94	15
Xpect RSV[a]	Remel Inc, Lenexa, KS, USA	NPA, NPS	96	94	15

BAL Bronchoalveolar lavage, *NPA* nasopharyngeal aspirate, *NPS* nasopharyngeal swab, *NW* nasal wash, *TS* throat swab
[a]CLIA-waived

6.1. Human Immunodeficiency Virus

Numerous POCTs for HIV antibodies are available (Table 4) (30–33). Whole blood (via fingerstick or venipuncture) or oral fluid is suitable for point-of-care testing. Envelope proteins gp41, gp120, gp160 in HIV-1, gp36 in HIV-2, and core antigen (p24) are used as target antigens (34). Some tests target both HIV-1 and 2 (e.g. OraQuick®, Clearview HIV) whilst others are specific for HIV-1 (e.g. Uni-Gold Recombigen® HIV test). POCTs need to be distinguished from home HIV tests, where samples are self-collected at home but tested in a routine laboratory (e.g. Home Access® HIV-1 Test System; Home Access Health Corp., IL, USA).

HIV POCTs using whole blood have a very similar sensitivity and specificity to currently available enzyme immunoassays used in the diagnostic laboratory. POCTs using saliva have variable results (30–32). Any positive HIV POCT should be confirmed by EIA and/or Western blot, or HIV proviral DNA, if available. The ideal testing algorithm is influenced by the disease prevalence, logistic, and economic challenges and so will vary between countries. The excellent specificity of POCTs may negate the need for confirmatory testing in high prevalence areas, especially in resource poor environments (33). The limitations of routine HIV antibody detection methods such as the "window period" in seroconversion, or waning antibody levels with advanced disease are relevant to POCTs.

The performance of POCTs varies depending on the strains and subtypes of HIV. Systematic evaluations of rapid HIV tests with non-B subtypes of HIV-1 group M, group O, and HIV-2 have established that most tests adequately detect all subtypes of group M. Performance is more variable with group O and HIV-2 strains (35–38) and thus, caution is required in geographical areas where these subtypes are frequent.

When evaluated, use of HIV POCTs is associated with an increase in the number of patients learning their serostatus when compared with traditional testing (10, 39). Patient satisfaction studies suggest that this approach is well received (10, 40). Economic analyses demonstrate lower costs as only one visit is required (10). As a result, expansion of voluntary counselling and testing services has been possible in countries with limited access to laboratories. The availability of rapid results does not negate the need for appropriate pre and post-test counselling.

POCTs have an important role in delivery of prevention of mother-to-child transmission (MTCT) strategies. In both developed and developing countries, a sizeable proportion of pregnant women seek medical care only during the time of labour and delivery. Lack of antenatal care implies that these women have no opportunities for HIV counselling and testing prior to delivery. Furthermore, HIV prevalence is often substantially higher among women who do not receive prenatal care (41). Anti-retroviral

Table 4
Frequently used HIV POCT (30–33)

Test	Manufacturer	Specimen	Sensitivity (%)	Specificity (%)	Time (min)
ADVANCED QUALITY™ Rapid HIV Test	InTec Products Inc, Xiamen, China	Whole blood, serum, or plasma	98.8	100	1
CAPILLUS™ HIV-1/HIV-2	Trinity Biotech, PLC, Bray, Ireland	Whole blood, serum, or plasma	100	98.8	3
Clearview HIV 1/2 STAT-PAK[a]	Inverness Medical Innovations, Bedford, UK	Whole blood Serum or plasma	99.7 99.7	99.9 99.9	15
Clearivew COMPLETE HIV 1/2[a]	Inverness Medical Innovations Bedford, UK	Whole blood Serum or plasma	99.7 99.7	99.9 99.9	15
Determine™ HIV-1/2	Abbott Laboratories, IL, USA	Whole blood, serum, or plasma	100	99.4	17
Efoora HIV Rapid	Efoora Inc, Buffalo Grove, IL, USA	Whole blood, serum, or plasma	96.2	98.0	21
First Response™ HIV-1/HIV-2 WB	PMC Medical Pty Ltd. Daman, India	Whole blood, serum, or plasma	100	98.8	1
GENEDIA® HIV 1/2 Rapid 3.0	Green Cross Life Science Corp, Kyunggi-do, Korea	Whole blood, serum, or plasma	100	99.3	11
HIV 1/2 STAT-PAK	Chembio Diagnostic System Inc. Medford, NY, USA	Whole blood, serum, or plasma	97.5	100	11
HIV (1+2) Antibody (Colloidal Gold)	KHB Shanghai Kehua Bio-engineering Co Ltd, Shanghai, China	Whole blood, serum, or plasma	100	100	4–30
Hema•Strip® HIV 1/2	Chembio Diagnostic System Inc. Medford, NY, USA	Whole blood, serum, or plasma	98.1	100	16
InstantScreen™ Rapid HIV-1/2 Generation 2	GAIFAR GmbH, Potsdam, Germany	Whole blood, serum, or plasma	100	100	3

(continued)

Table 4 (continued)

Test	Manufacturer	Specimen	Sensitivity (%)	Specificity (%)	Time (min)
InstantCHEK™ HIV 1 + 2	EY Laboratories Ltd, San Mateo, CA, USA	Whole blood, serum, or plasma	99.4	97.6	2
MedMira Rapid HIV test	MedMira Laboratories Inc, Toronto, Canada	Whole blood, serum, or plasma	100	97.6	5
OraQuick® Advanced Rapid HIV-1/2 Antibody test[a]	Orasure Technologies, Bethlehem PA, USA	Oral fluid Whole blood Plasma	98.1–99.8 98.1–99.9 98.1–99.8	99.8–100 99.7–100 99.8–100	21
OraScreen®	Beacon Diagnostics Inc, CA, USA	Saliva	56	98.6	20
Salivax™ HIV	ImmunoScience Inc, CA, USA	Saliva	79.4	96.0	15–45
SD Bioline HIV1/2 3.0	Standard Diagnostics Inc, Kyonggi-do, Korea	Whole blood, serum, or plasma	100	99.3	6–21
SMLX Technologies Diagnostic test	SMLX Technologies, FL, USA	Saliva	62.7	74.8	12–17
Uni-Gold Recombigen® HIV test[a]	Trinity Biotech, PLC, Bray, Ireland	Whole blood Serum or plasma	100 100	99.7–100 99.8–100	11

[a]CLIA-waived

drugs administered intra-partum or immediately post-partum, even in the absence of antepartum care, significantly reduce the risk of MTCT (42). When evaluated in pregnant women, blood-based tests have a high sensitivity (86.4–100%) and specificity (99.5–100%) (43). Although the sensitivity with oral fluid tests is only moderate (75–100%), specificity is maintained (99.9–100%) (43). When offered to pregnant women, the uptake of rapid tests varied in studies from 83 to 97%. These data highlight the benefits of POCTs for detecting HIV antibodies in this unique situation.

6.2. Hepatitis B

Hepatitis B virus (HBV) infections are a major public health problem worldwide, being responsible for considerable morbidity and mortality from chronic liver disease. Immunisation with the hepatitis B surface antigen and the provision of immunoglobulin to those exposed is now the standard of care in many countries (44, 45). This strategy is of greatest benefit to neonates born to HBV-infected women in whom rates of infection are high. As such, rapid diagnostics to confirm infection are desirable. A POCT to detect HBV surface and e antigens has been produced in an ICT format and validated using fingerstick blood samples (ICT hepatitis B sAg/eAg, Binax, Scarborough, ME, USA). The published sensitivity and specificity are 94.5–96.0% and 100% for HBsAg and 80% and 94.7–100% for HBeAg (46).

7. Point of Care Testing for Enteric Pathogens

Stool antigen detection to diagnose rotavirus and adenovirus infections has been the diagnostic method of choice of many laboratories. As such, conversion to a POCT using a lateral flow immunoassay format is readily achievable with comparable sensitivity and specificity (47). There are two important disincentives to using POCTs for diagnosis of gastrointestinal tract infections. First, most common gastrointestinal infections resolve without treatment and as such there is less clinical advantage in making a rapid diagnosis. Secondly, there are significant hazards associated with handling faecal samples without adequate safety procedures. Samples may contain dangerous and highly transmissible pathogens such as *Salmonella typhi* or enterotoxigenic *Escherichia coli*.

Hepatitis A and E infections are major diseases in developing countries, with outbreaks usually occurring due to contaminated water sources. Laboratory support in endemic regions is often insufficient for routine diagnostics, and so POCTs to detect virus-specific antibodies have been developed. A hepatitis A-specific IgM POCT has been developed, but is not commercially available (48). A POCT for Hepatitis E-specific IgM has also been

developed and evaluated using the Open Reading Frame 2.1 antigen in an ICT format. The reported sensitivity and specificity in patients in developing countries was 93.0–96.7% and 96.9–99.7% respectively (49, 50). Care should be taken when assessing POCTs for hepatitis A and E in other countries, where the incidence is significantly lower.

8. Point of Care Testing for Other Viruses

8.1. Adenovirus

Adenoviral infections are associated with numerous clinical manifestations including respiratory tract infections, conjunctivitis, and gastroenteritis. Adenoviral infections, particularly concerning conjunctivitis, are often difficult to distinguish from bacterial and other viral pathogens. POCTs have been developed and assessed in the setting of respiratory tract infections, conjunctivitis, and gastroenteritis. Antigenic targets are group reactive hexon antigens common to known human adenovirus serotypes.

Two tests are commonly available. SAS™ Adeno Test (SA Scientific Inc. San Antonio, TX, USA) is recommended for respiratory, conjunctival, and stool samples. The published sensitivity for nasopharyngeal aspirates and swabs is 72.6–95.0% with 100% specificity (51–56). The sensitivity of the SAS Adeno Test for respiratory disease is decreased later in the disease: <5 days; 80.4%, ≥5 days; 61.5% (53). When conjunctival specimens are compared, the sensitivity of the SAS™ Adeno Test appears lower (sensitivity: 54–72%, specificity: 97–100%). RPS Adeno detector (Rapid Pathogen Screening Inc., South Williamsport, PA, USA) is another POCT for adenoviral conjunctivitis, with a sensitivity of 89% and specificity of 94% for conjunctival swabs. Modelling data suggests that the use of RPS Adeno detector is cost-effective given the antibiotic use avoided following adenovirus diagnosis (57). Both kits are CLIA-waived.

8.2. Herpes Simplex Type 2 Virus

Herpes simplex type 2 (HSV-2) infections are common and frequently remain undiagnosed. As the infection is often subclinical or unrecognised, serological testing is often necessary to make the diagnosis (58). After a diagnosis is made, infected patients can be counselled that symptom awareness, condom use, and antiviral suppressive treatment may all decrease the risk of transmission to uninfected partners. Assays use whole blood (from fingerstick or venipuncture) and target HSV-2 antibodies. The performance of HerpeSelect® Express (Focus diagnostics, Cypress, California, USA) in screening those attending a sexual health clinic in San Francisco demonstrated a sensitivity of 97% and specificity of 98%

(assay time 15 min, not CLIA waived) when compared with HSV-2 Western blot (58).

8.3. Epstein–Barr Virus

Serological testing is the method of choice for the diagnosis of primary Epstein–Barr virus (EBV) infections. Traditional methods of diagnosis included demonstration of Paul-Bunnell heterophile antibodies. Paul-Bunnell heterophile antibodies are a heterogeneous group of mostly IgM-class immunoglobulins generated in response to acute EBV infection. Immunologic studies suggest that the Paul-Bunnell "antigen" is actually a complex glycoprotein structure on the surface of EBV infected cells (59).

Older methods for measuring heterophile antibodies have largely been replaced by rapid qualitative agglutination or immunochromatographic assays. A number of these are intended as POCTs and are CLIA-waived (Clearview Mono, Inverness Medical Innovations, Bedford, UK; One step+ mono test, Henri Schein Inc., Melville, NY, USA; Poly stat® mono test, Polymedco, Cortlandt Manor, NY, USA; Signify Mono cassette, Abbott Laboratories, Abbott Park, IL, USA; Clarity mononucleosis rapid, Diagnostic test group, Boca Raton, FL, USA; Accutest value+ mononucleosis rapid test, Jant Pharmacal, Encino, CA, USA) (5). Previous evaluations demonstrate that rapid solid phase immunoassays have equivalent sensitivity and specificity to in-laboratory methods (60–63). When compared with EBV-specific antibodies, the sensitivity and specificity of heterophile antibodies are 63–84% and 84–100% respectively. The sensitivity of this method is reduced in children compared with adults (63). The specificity is reduced in certain populations, e.g. false positive heterophile antibodies have been reported in the setting of non-EBV infections and autoimmune conditions (64, 65). Clinicians using Paul-Bunnell heterophile antibodies to diagnose acute EBV infection, either in a POCT format or as a routine laboratory test, need to consider the limitations of the assay.

9. Conclusions

There has been a significant increase in the use of rapid diagnostic assays such as point-of-care tests. They can provide useful diagnostic information to clinicians (Table 5), but practitioners need to be aware of their limitations (Table 6). They should not replace routine diagnostic virological investigations. When considering the implementation of a POCT programme, quality assurance, training, and economic considerations are paramount.

Table 5
POCTs may be useful in a number of clinical situations

POCTs may be useful in a number of clinical situations
• For individual patient management (accepting the limitations of the assay and in conjunction with other laboratory tests, as required)
• In non-laboratory environments where trained staff are available to perform the assays
• During peak seasonal activity: e.g. influenza, RSV
• For diagnosis of outbreaks where the reduced sensitivity of the test may be overcome by testing multiple samples
• During periods when laboratory facilities are stretched: e.g. peak of seasonal influenza and during large outbreaks
• In laboratories with limited diagnostic facilities, although quality assurance, training and cost need to be considered in resource poor settings (33)
• Where early treatment is required: e.g. antivirals in influenza
• As a surveillance tool

Table 6
Limitations of POCT

Limitations of POCT
• POCTs often have reduced sensitivity compared to standard laboratory methods including nucleic acid testing (e.g. respiratory viruses)
• Subtyping of viruses may not be available with POCTs (e.g. influenza)
• No isolate is available following POCTs for resistance testing or molecular epidemiology
• A second swab may be required for other tests (e.g. culture, PCR)
• Expense (especially if sequential testing)
• Samples are often collected by less experienced operators

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Chapter 23

Modelling Emerging Viral Epidemics for Public Health Protection

Steve Leach and Ian Hall

Abstract

Mathematical models when applied to infectious disease data can provide extremely useful insights into the possible future impacts of potential emerging epidemics and how they might be best controlled or mitigated. Modelling, which is like any other hypothesis-driven approach, aims to develop a better understanding of biological phenomena. However, diseases processes generally, and particularly those related to transmission, will in many cases be imperfectly understood or too complex to systematically describe, so models will necessarily be simplifications of the overall system. It is essential, therefore, that models are designed carefully and used appropriately. Key to this is identifying what specific questions a model might be expected to answer and what data is available to inform the model. A particular type of model might be fine for one particular situation but highly inappropriate for another. It is also important to appreciate and communicate what simplifications and assumptions have had to be made and how this might affect the robustness of the modelling results. It is also particularly important to understand that models frequently make what can be hidden assumptions about underlying processes because of the way they have been constructed and these assumptions also need to be carefully considered and made explicit, particularly for non-expert audiences. This chapter, therefore, provides a brief introduction to some of these aspects of epidemic modelling for those that might be less familiar with them.

Key words: Epidemic modelling, Emerging infectious diseases, Epidemiology, Transmission, Pandemics, Deliberate release, Policy and planning, Preparedness, Prevention and control, Public health interventions, Isolation/quarantine, Vaccination, Real-time modelling

1. Background to Epidemic Modelling

The application of mathematics within the sciences has a long and interesting history and has been used most extensively in the modelling of physical and chemical systems to better understand their underlying processes and test hypotheses. Biological systems have probably seen comparatively less use of mathematical

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modelling, due mainly to the systems under consideration being less subject to well-defined "governing laws" (such as often apply at some level in physics and chemistry) and the difficulties of observing biological systems in sufficiently well-controlled and comprehensively understood contexts. There is, nevertheless, a considerable literature associated with the use of mathematical modelling in the context of public health and infectious diseases, with early investigations exemplified by Bernoulli (1) (in relation to smallpox inoculation), Ross (2) (in relation to malaria transmission), and Kermack and McKendrick (3) (in relation to epidemics more generally). In historically more familiar areas, mathematical investigations have often been related to childhood vaccination programmes and combating sexually transmitted infections (4, 5). For the sake of brevity, the extensive background literature related to this subject will not be covered in depth here; but there is a selection of excellent reviews provided in the bibliography (4–10). Similarly, other modelling applications that have also received increasing attention recently include those that aim to provide ongoing advice in the face of such outbreaks; these often being referred to as "real-time" epidemic modelling, "now-casting," or "forecasting," depending on context (11–14). Other more recent applications of note, however, have related to providing contingency planning advice ahead of time for potentially high impact outbreaks of emerging infectious diseases (for which we have little current or certain knowledge) arising from acts of bioterrorism or from more natural pandemics, e.g. smallpox, pandemic influenza, and SARS (15–24). Consequently, it is on some of these latter areas of application to contingency planning for emerging infections that this chapter will mainly focus. This chapter is also not intended to be a comprehensive review of the literature nor a technical treatise on how to set-up or use mathematical models. Rather, it is intended to provide a short introduction and pragmatic overview to assist the familiarisation of non-specialist audiences engaged in public health protection. It will thus avoid too much technical description.

2. The Benefits of Using Models

First, what is a mathematical model? Models are generally considered as a representation of a system based on our knowledge of what we understand to be its constituent processes and the relationships that operate between them. Ideally, such representations should be formulated in such a way that it is possible to generalise about the system's behaviour in every (or most) instance where it is observed. A mathematical model "simply" achieves this representation through one or more mathematical equations.

Necessarily, models, including mathematical ones, will be simplifications of the system under consideration. However, this situation is no different to that which pertains with the types of conceptual models that more generally underlie the development, testing, and potential predictive power of scientific hypotheses. Mathematical models in this context are neither intrinsically no better nor no worse than any other used in science, as long as they are equally supported by data; though they do have some very particular and useful properties. Mathematical models do, nevertheless, often have a vocabulary and notation that is not necessarily easily accessible; and may also come with an air of precision that some might find difficult to accept. It is key then that the (simplifying) assumptions that mathematical models make are made explicit and understandable to more general audiences. This is often best achieved in a collaborative and multidisciplinary environment, including not only the mathematics, but also, for example, the epidemiology, disease and public health expertise. It is also often beneficial to engage with all the constituencies that might come to be dependent on the modelling, including those involved in risk management and risk communication. Indeed, this is well recognised in the broader sphere of risk assessment, and probably most well developed and articulated in the infectious diseases field for food-borne infection risk assessments, as per relevant guidelines of the World Health Organisation (WHO), Food and Agriculture Organisation of the United Nations (FAO), the World Organisation for Animal Health (OIE), and the Codex Alimentarius (25, 26).

3. What Makes a Good Mathematical Model?

Mathematical models do have huge potential for gaining a better understanding of the complex biological and epidemiological systems that underlie emerging infectious disease threats and thus enabling better prospects for their control. Before examining such models in detail, however, it is worth considering what makes a good mathematical model, both in relation to its use and its limitations. Keeling and Rohani (5) identify accuracy, transparency, and flexibility as important aspects that require careful consideration, which can frequently be at odds with each other. Accuracy suggests an ability to quantitatively reproduce observed epidemic data in a consistent fashion (suggesting that a model has predictive power), whereas transparency suggests that a model is well understood in terms of how its various constituent parts interact to generate the resulting epidemic dynamics. The tension here is that more complex (and often less transparent) models which seek to capture the increasingly detailed biology of the

underlying system(s) are generally more likely to better capture their quantitative dynamics (and thus seem more accurate). Put simply, the more parameters/factors that are included in a model, the more likely it is that there will be sufficient parameters that can be (independently) tuned that it is ultimately bound to more accurately reproduce at least one observed epidemic, whether for entirely the right reasons or not. They therefore become increasingly more difficult to understand in terms of how the interactions of the various constituent parts impact on the dynamics and the degree to which the model is able to be generalised. Complex models can also pose other challenges in terms of our ability to parameterise them satisfactorily and in the computational power required to operate them. Contemporary computational capabilities, however, now often make the latter less limiting than does our dearth of knowledge and data concerning diseases and their natural history and transmission, and thus their ability to be satisfactorily parameterised.

Finally, flexibility refers to the relative ease with which models can be adapted to new public health problems. Simpler models can be inherently more flexible than more complex ones, since the latter are more likely to have features that are specific to a particular disease and set of circumstances, several of which may not be relevant to the new problem to be addressed. Possibly, more importantly, they may also lack key features that become crucial. One possibility would be to have one enormously complex model that contains all possibilities in terms of disease natural history (i.e. all the factors that impact on the progression of the disease both within individuals and populations, such as incubation period, viral titres in body fluids, infectivity, etc.), public health interventions, and so forth, with the potential to turn features on and off as required. The problems with this approach would be: first, the unnecessary computational overhead that such a model would always be carrying for any problem that required investigation; second, it is unlikely that every eventuality will have been foreseen at the outset, ultimately requiring the model to be rewritten anyway; third, the potential for over-reliance on "black-box"-type approaches where the underlying model is implicitly trusted but not transparent, and the tendency to be tempted to use a model that is far more complex than is warranted given what little might be known about a particular emerging infectious disease problem. A more reasonable approach is to have a toolbox of models of differing complexity that can be used for the question(s) at hand and a cadre of modelling experts to operate them. Thus, in the spirit of Ockham's razor (27), a good model is the simplest one (or set) that is suitable to the purposes to which it is to be put, having the right balance of accuracy, transparency, and flexibility, and one that has been constructed with due reference to what is known and preferably measurable.

4. Value of Models

Mathematical representations (models) of systems can take a variety of forms and degrees of complexity, from simply descriptive or explanatory ones concerning a single or a few variables to ones descriptive of more complex multivariable systems. The latter, when suitably validated, have potentially important predictive capabilities and frequently provide insights that are not directly observable or immediately intuitive (23). They also make it possible to undertake experiments (in silico) such as the optimisation of public health interventions that would otherwise be impossible for reasons of, for example, expense, practicality (e.g. no contemporary outbreak/data/evidence) or ethics (e.g. one cannot generally deliberately infect individuals or communities).

5. Introduction to Simple Models and Their Uses in Epidemiology

At one end of the scale of complexity, popular statistical approaches to data analysis and interpretation could be considered as modelling. They provide a more systematic and useful description of a series of observations than simply scanning of a long list of numbers that demonstrates that a parameter in question (e.g. height of mature individuals) varies. If the statistical approach is a parametric one, then there will also be some underlying assumption about the form the data takes. For example, the fitting of a normal, lognormal, or Gamma probability distribution (i.e. ones that have defined parameters such as mean, variance, etc.; hence parametric), might imply that there are underlying processes that produce such a distributional form. With care, for many biological processes, it is often a reasonable assumption that there is an expected distribution to which the data could conceivably conform. In the case of the normal distribution the assumption is that whatever is being measured is subject to multi-factorial (but additive) influences; for example, many genetic loci and environmental influences acting in concert, each with their own "distribution" in relation to their action on what is being measured. Together these interactions produce a joint distribution that is closely approximated by a normal distribution by virtue of the central limit theorem. However, phenomena in nature often produce distributions that are right skewed and with zero probability of values of zero or less. Such systems are generally more closely represented by log normal distributions, where the underlying assumption is that the many factors contribute multiplicatively rather than additively. Further detail on probability distributions

and their applications are outside of the scope of this chapter and can be found elsewhere (28, 29). However, the more complex models that will be discussed later are often built around multiple components that are parameterised as probability distributions. For example, the curves in Fig. 5 and the solid line in Fig. 6 illustrate the types of skewed distribution that are often used to better describe the observed lengths of, for example, the incubation or symptomatic periods of infectious diseases, particularly when these have been sampled from a large enough number of infected individuals in a population to arrive at a sufficiently robust result. Thus, the length of any one individual's incubation period will differ from those of others with some durations (those closer to the average) more frequently observed than others. Clearly, incubation periods of less than zero would be meaningless and use of the lognormal avoids these. Further, the lognormal, and other potentially skewed distributions such as the Gamma, capture well the distribution of the duration of the positive values, probably because incubation periods depend on a wealth of multi-factorial influences (not always well understood) acting multiplicatively, such as the differing pre-existing genetic backgrounds and immunological susceptibilities of individuals to particular viruses, their age and underlying fitness in terms of any pre-existing co-morbidities, and their nutritional and socio-economic conditions; and any variations in the pathogenicity of the particular virus or microorganism, the dose of virus received by an individual and the route of infection. Incubation periods can vary enormously between different infectious agents both in terms of average incubation period and the degree to which this varies between individuals; for example, the mean (and variances) for influenza, SARS coronavirus (SARS CoV), and smallpox have been estimated to be about 1.3 days (0.5 days^2), 4.6 days (15.9 days^2), and 11.6 days (3.34 days^2), respectively. Appropriately parameterising such aspects can be hugely important to modelling the dynamics (e.g. the rate and extent of spread) of particular infections and the potential impact that different public health control options might have. The extent to which they can be accurately reflected in more complex models can be extremely important and will be discussed later.

If the observed data is a series of measurements that varies systematically over time (i.e. a time series) or varies systematically with some other factor that has also been measured (e.g. infectiousness with respect to virus levels in body fluids), then we can also use regression analysis to statistically interpret such relationships. With regression, the model that is quite often imposed is a linear one ($Y = aX + b$), where Y is the observed data and X the (independent) variable; with a and b the slope and intercept parameters, respectively. A simple example concerns crude analysis of the smallpox outbreaks that resulted from importations into

Europe from abroad between 1959 and 1973, when the disease had otherwise been eradicated from that continent. With these importations, and the outbreaks that quite frequently resulted, the greater the delay in their initial notification to the health services, the greater the likely number of cases at the end of an outbreak (30). Whilst this is entirely logical and to be expected, the clear mathematical relationship that was observed between these two variables was striking. Although for illustration here a linear regression model (Fig. 1, solid line) has been fitted, it is worth pointing out that the data on the *y*-axis has actually been expressed as $\log_{10}$ of the observations. This is because the relationship between the untransformed variables is in fact an exponential one, with the log transform simply linearising this for ease of exploratory data analysis. The reason for this relationship is that the longer an outbreak is allowed to progress unimpeded (with there being delays in public health response) the more time it will have had to grow exponentially. Indeed, epidemics often show early exponential growth (see later). Because this relationship was so striking it was possible to derive a reasonable estimate of the transmissibility of smallpox based on an equivalent regression model (31) (Fig. 1).

There are also analytically tractable response forms other than linear and exponential that might be appropriate (e.g. quadratic, logistic); right through to full non-linear regression. The fitting process that underpins regression relies on assumptions about the probability distribution of the measurement error (e.g. that the

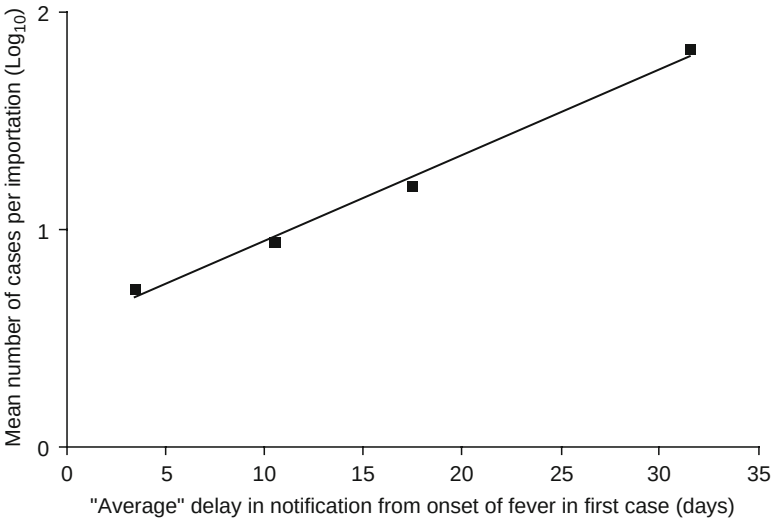

Fig. 1. Fitting a linear regression model to infectious disease data showing the strong relationship between the extent to which smallpox outbreaks expanded as delays in the public health response increased. Regression model: $y = 0.0397x + 0.5519$, where y is the average size of outbreaks and x is the delay.

deviation of the observed data from the modelled prediction is explained by the normal distribution introduced above). Fuller explanation of regression models is outside the scope of this chapter and more detail can be found elsewhere (32). However, another familiar example of a relationship (model) that is highly non-linear which often has great utility in virology and bacteriology is that between the dose of infectious agent received by an individual (often an experimental animal) and their subsequent probability of infection or death. Usually the probability of becoming infected increases as the dose increases, with the non-linear trend to some extent linearised by expressing the probability of infection or death as a probit transformation (33) plotted against the log of the dose. On the basis of such analyses two key parameters are determined that can be extremely useful when developing more complex models. The first is the infectious dose (ID_{50}) or lethal dose (LD_{50}) that gives a 50% probability that an individual might get infected or die, respectively. The second is the extent to which this changes on the probit scale with every log increase in the dose of organisms administered (i.e. the slope). The infectious dose (50%) varies considerably for different micro-organisms and can be important to parameterise appropriately when considering epidemic models. That for *Bacillus anthracis* spores, for example, might be of the order of 10^4 spores, whilst that for *Francisella tularensis* or smallpox virus has been estimated to be of the order of 10 cells/virions or so.

Moving on to more complex infectious disease models, there is an extensive ecological and epidemiological modelling literature that underpins this subject (see reviews in bibliography and references therein). The simplest models of practical utility for emerging infectious diseases are possibly best immediately understood through their schematic descriptions; as the so-called SIR (Fig. 2a), or SIS models (4, 5).

To better understand these, first imagine simply a disease that infects a person for 1 day only and in that time they infected two other people. This means that if we start on day 1 with one case, on day 2 we will have two more; and on day 3 four, day 4 eight, etc., until on day 31 we have more than one billion cases. This process can be represented mathematically by Eq. 1.

$$I_{n+1} = I_n + \beta I_n - \gamma I_n = [1 + \beta - \gamma]^{n+1} I_0 \qquad (1)$$

Note that in the simple example given above (γ) *gamma* (the reciprocal of the number of days between infection of a case and their recovery [assuming they are equally infectious throughout] which may be inferred from the generation time or the serial interval data [defined later]) = 1 and (β) *beta* (number of people infected by each case per day) = 2; and I is the number infected, and n denotes the day of the outbreak. Whilst clearly this approach provides only

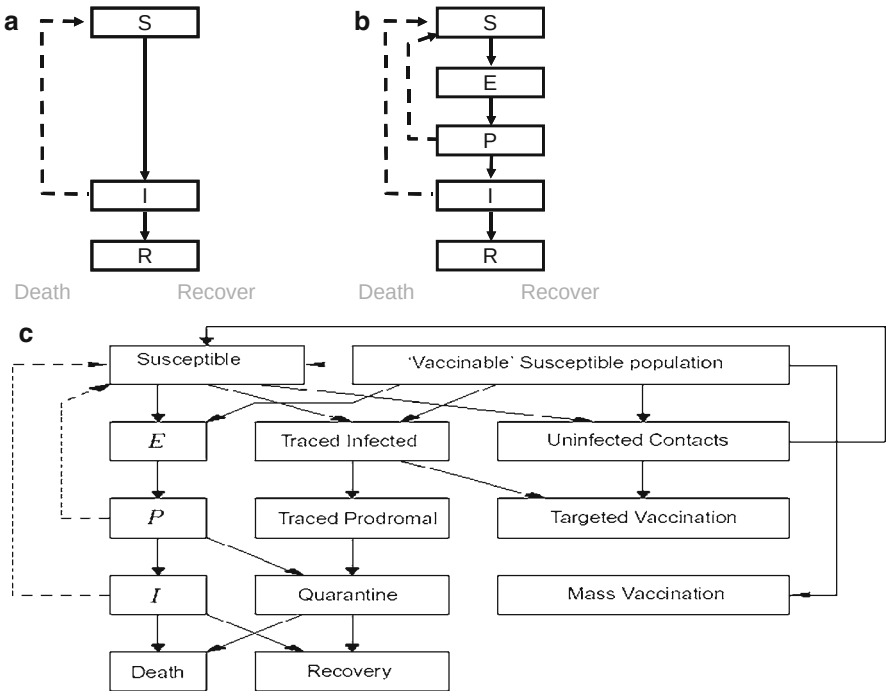

Fig. 2. Three different schematic representations of disease transmission models, from more a simplistic one (**a**) to a more complex one involving more disease states (**b**) to one involving a range of public health interventions, in this case for smallpox (**c**), (see text).

a toy model, this process of exponential growth is often observed (to a first approximation) in real-world epidemic data at the start of outbreaks, as in the smallpox example above. However, common sense tells us that epidemics will not spread that quickly and unchecked through the entire world's population. One reason for this is human contact behaviour. In the scenario above, for example, one of the four cases occurring on day 3 would have had a chance of meeting the same person as one of the other cases (i.e. shared contacts in social groups). Further, in a population of limited size the steadily increasing number of infectious cases would dictate that quite soon some will tend to meet other infected cases (or previously infected and immune individuals) rather than susceptible individuals. Consequently, the number of new contacts that result in new infections at each generation must depend both on a contact rate and a probability that the contacts will be with individuals still susceptible to infection.

This phenomenon can be captured by simply dividing a closed population into two compartments; *S*, meaning that proportion of the population that is (s)usceptible to infection and *I*, that part that is (i)nfected and (i)nfectious (note that no distinction is made between these two). This process may be represented by the differential Eq. 2 (note in this instance cases are assumed to become

susceptible again after infection wanes) and solved explicitly as in Eq. 3, taking a logistic form, where t is time and I_0 the number infected at time 0, and $I(t)$ the number infected at time t.

$$\frac{\mathrm{d}}{\mathrm{d}t}I(t) = \beta[1 - I(t)]I(t) - \gamma I(t) \tag{2}$$

$$I(t) = \frac{(\beta - \gamma)I_0}{\beta I_0 + [\beta - \gamma - \beta I_0]\exp\left[-(\beta - \gamma)t\right]} \tag{3}$$

Whilst mathematically tractable such a model is still a fairly limited representation for most diseases, particularly for those diseases where cases become immune to further infection, at least for a time (or die), for example, influenza, smallpox, and SARS CoV. To account for this the schema can be extended by dividing the population into three compartments or classes: with S, and I, as defined above and R, that part that is (r)emoved (i.e. immune or dead). The distinction between immunity and death is generally of no importance to the dynamics of the model unless considered over a time period much longer than the timescale of the disease or a single epidemic. If longer timescales are important to the problem under investigation (as with assessing vaccination programmes for vaccine-preventable childhood diseases such as measles and mumps) then disease mortality has to be factored into the schema, along with deaths from all causes and also new births, the latter providing new susceptibles through the relevant birth rate (4). Factoring such things as mortality and hospitalisation into models can clearly also be important from other perspectives, for example, when estimating the impacts on society and health-care systems. Clearly, the severity of diseases such as smallpox with a case fatality ratio estimated to be in the range of 30% would result in rather different set of consequences compared to a disease such as pandemic influenza with its usually much lower estimates for the case fatality ratio (only up to about 2.4% in the main wave of the 1918/9 pandemic and much lower in the 1957 and 1968 ones).

Such simple SIR models have been used to great effect and can be expressed most simply as a series of differential Eq. 4 that describe the time-dependent transition of proportions of the population through these stages (e.g. from I to R at rate *gamma* in Eq. 4, where *beta* and *gamma* have same definitions as in Eqs. 1 and 2).

$$\begin{aligned}
\frac{\mathrm{d}}{\mathrm{d}t}S(t) &= -\beta S(t)I(t) \\
\frac{\mathrm{d}}{\mathrm{d}t}I(t) &= \beta S(t)I(t) - \gamma I(t) \\
\frac{\mathrm{d}}{\mathrm{d}t}R(t) &= \gamma I(t)
\end{aligned} \tag{4}$$

Such models are also dependent on that part of the population that is infectious being able to transmit infection (dotted line, Fig. 2a) to that part that is susceptible at some probability or rate (e.g. between *I* and *S* with transmission rate *beta* in Eq. 4, *beta* being a composite of the number of contacts made per day and the probability that transmission occurs given that a contact is susceptible). As noted above, this rate will change over time as it depends both on the number of infected individuals and the number of susceptible individuals, as well as the more "intrinsic" transmissibility of the infection. The latter is often described by a "fundamental" parameter of many epidemic models, usually referred to as the basic reproductive number or ratio, designated R_0, defined in Eq. 5,

$$R_0 = \frac{\beta}{\gamma} 1 - I_0 \tag{5}$$

The simplest understanding of this parameter is the average number of secondary cases caused by each primary case within an entirely susceptible population (and in the absence of public health interventions). The effective reproductive ratio, R_E (Eq. 6), on the other hand, has a similar description except that the level of susceptibility to infection within the population and the effects of public health intervention are taken fully into account. Thus, R_E changes over time as the relative proportions of the infectious and susceptible population change over the course of the epidemic (Fig. 3),

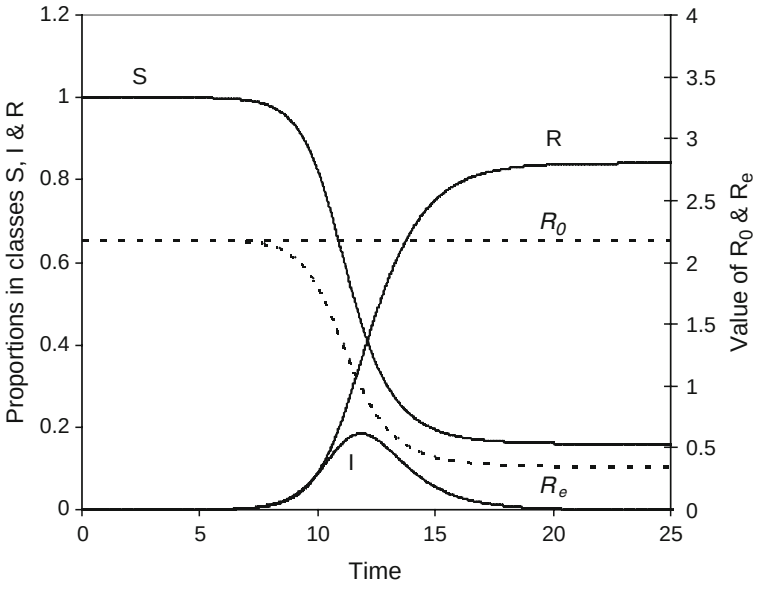

Fig. 3. Illustrative dynamics of a simple SIR model showing the change over time in the number of individuals that remain susceptible and then become infected and ultimately recover.

$$R_E = \frac{\beta S}{\gamma} = SR_0 \tag{6}$$

Even though the SIR Eq. 4 is not amenable to explicit temporal resolution, they are amenable to approximation in certain phases of the epidemic (e.g. the early exponential growth discussed above) and numerical solution. Some of the further mathematical analysis that is possible on this Eq. 4 can provide fundamental insights into aspects of the expected severity and prospects for control of epidemics. The key parameter here is R_0 (Eq. 5), which can be shown to be the parameter that defines the stability of the system (i.e. whether the disease is likely to become a major public health problem or not); since if it is greater than 1, the introduction of cases of disease into a population will likely cause an epidemic, whereas if it is less than one, the introductions will fade-out. Thus, from this parameter one can estimate the proportion of a population (or the population number if this proportion is converted by reference to the population size) that might need to be immunised to control an infection (V, i.e. that proportion that is required to bring R_E below 1 – Eq. 7, Fig. 4), and also, one may derive (4), the likely final size of an uncontrolled epidemic in a closed population (Eq. 8, Fig. 4), where R_∞ is the final attack size.

$$V = \frac{R_0 - 1}{R_0} \tag{7}$$

$$1 - R_\infty = S_0 \exp - R_0 R_\infty . \tag{8}$$

All of this assumes that the various approximations to real life that the model employs (some of which will be covered later) still allow meaningful interpretation of the model output in relation to the real setting in question. Sufficiently often these approximations do not completely compromise the results and allow useful observations to be made. A simple corollary of the relationship in Fig. 4 regarding final attack size is that in the range of R_0 between 1 and 2, one can relatively robustly infer R_0 from final attack size, and vice versa. This range of R_0 between 1 and 2 is relevant to the case of past pandemics of influenza, such that useful comparisons can sometimes be made between these two measures. For R_0 greater than 2, however, the discrimination between final attack sizes for different R_0 becomes much less and often within the bounds of the error in the data that might be available to independently determine final attack size or R_0. Therefore, for diseases such as smallpox (R_0 in the range 3–6), SARS CoV (R_0 about 3–4) and measles (with one of the highest estimated R_0's for an infectious disease, variously reported to be in the range 12 to in excess of 20), the inference of R_0 from final attack size would likely be much less clear. It is probably worth mentioning in passing that the estimates of R_0 given above vary, even for a single

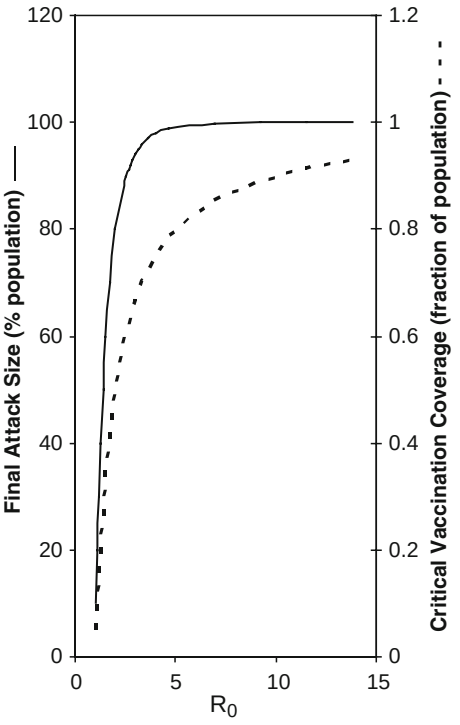

Fig. 4. The simplistic dependence of two basic epidemic disease features on the fundamental disease parameter R_0.

disease, because they will depend on time, place, and context of the study where R_0 was inferred. R_0 will be influenced, for example, depending as it does on contact rates (see above) by factors such as overcrowding and socio-economic conditions. Even for diseases with lower R_0 such as pandemic influenza, assessments are actually made more complicated by the fact that a good proportion of cases won't seek medical attention and others may well become infected and to some degree infectious whilst remaining asymptomatic. These individuals do not therefore get counted among the clinical cases (and so the final attack size may not actually be directly observed), though they might be observed through changes in their immune status if this was to be measured by serological surveys; or, more fundamentally, as a consequence of careful interpretation of the underlying epidemic dynamics (34). It is thought, for example, that maybe only about 60% of individuals infected with influenza actually develop reportable clinical symptoms, whilst by comparison cases of smallpox and SARS CoV are rarely, if ever considered, to remain completely asymptomatic following infection, which makes the epidemiology somewhat easier to interpret in these latter cases.

For the many problems that are not amenable to such explicit treatment one must turn to the numerical solution of these equations. As before, one must define the initial conditions (i.e. what

proportion of individuals are infected, immune or susceptible at the point of introduction of the infectious agent) and we also require a so-called equation of state, $S+I+R=1$. Typically, for a new or emerging infectious disease initial conditions are given such that no people are immune (R at time $0=0$) and only a small proportion are infected; though other situations can be readily investigated by adjusting the proportions for S, I, and R accordingly. Numerical schemes essentially make the continuous differential equations (like Eqs. 2 and 4) discrete with respect to time so that they become difference equations, the precise form dependent on the accuracy of the solution demanded. Difference equations introduce a time step h and the accuracy of the numerical solution to the exact one is inversely dependent on this time step. The choice of numerical scheme is dependent on the form of the equations being approximated and the available computational resources. Essentially, however, the initial conditions will be substituted into the equations (at time 0) and the results calculated for the first time step. These results are then fed back into the equations as the starting conditions for the next time step and so on until the results for sufficient time steps have been calculated to describe the required course of the epidemic. Further discussion of such methods (e.g. Euler, Runge-Kutta, etc.) can be found elsewhere (35).

6. Some Problems with Simple Models

The mathematical formulation of the SIR schema, based on the series of differential equations described above, makes a number of implicit assumptions that need to be appreciated. The first is the often criticised one of homogeneous mixing such that an infected individual has an equal probability of infecting any one susceptible individual in the population as any other. As will be discussed later, this simplification can be addressed by introducing population heterogeneities, such as the probabilities of different age classes mixing with one another or the different geographical limits that might reasonably apply to population mixing over longer distances.

Differential equation sets are also deterministic ("clockwork"), that is, each time the model is run from the same starting conditions and with the same parameters it will produce exactly the same results across each and every time step for the entire "epidemic." They are also continuous which means that they will allow fractional people to be counted among the cases. Real epidemics, however, are prone to stochasticity, based on individual events that occur probabilistically, which if neglected can present a major issue, particularly at the start and end of epidemics. For example, although the average number of secondary cases caused by each primary case (i.e. R_0) might be observed to be around 2,

say, for pandemic influenza, for any one individual case this might vary from 0 to some rather larger number than 2 depending on circumstances (e.g. the number of contacts the particular case might make with others, the concentration of virus a specific individual sheds and for how long). Thus for diseases with a R_0 closer to 1, say between 1 and 3 for diseases such as pandemic influenza, pneumonic plague or SARS CoV, a one off introduction of a single case of disease into a population would have a much greater chance of causing no further cases and not starting/contributing to an epidemic than for diseases where the R_0 is much larger, such as measles, where there would be a much greater probability of one imported case causing at least one further infection. Diseases with low R_0 therefore have a greater chance of experiencing what is termed stochastic fade-out and this can be extremely important to capture appropriately in models depending on what is being investigated. Similarly, as will be discussed in greater detail later, whilst there might be some concept of an average infectious period (i.e. length of time in I), the duration of such disease states will also vary between cases. Individual-level variability in features such as infectious and/or incubation period has already been discussed previously and where relevant can often be best captured by modelling them with lognormal or Gamma distributions. Differential equation-based models can be formulated within stochastic frameworks to take such individual-level variability into account, along with allowing for the concept of whole, discrete individuals rather than fractional ones to be enabled. The ways in which such formulations can be achieved (5) are beyond the scope of this chapter, but it is worth pointing out that the resulting models will usually have to be run large numbers of times (often 100–1000 s depending on the number and range of uncertainty on parameters that has to be stochastically varied) to generate a whole family of epidemics in order to ensure that a representative selection is collected. These then need to be statistically analysed to better understand the problem being investigated. All of this often increases the computational cost of such models.

7. Introducing Better Descriptions of Disease Natural History: Non-uniform Infectiousness

It may be sufficient for the purposes of a model to simply employ the concept of an average generation time or serial interval; the latter being the observed time between onset of specific symptoms in one case and the onset of the same symptoms in the subsequent cases caused by that case; whilst the former is the time between the infection of a primary case and the infection of each of its secondary cases (36, 37). In the models discussed so far observational data that are related to these intervals are often used as a surrogate for the period of time spent in the I class, whilst at the same time

naively assuming people are equally infectious throughout this period. This relatively unrealistic assumption of uniform infectiousness over time can, however, be solved reasonably well by introducing more infectious compartments into the model schema, each of which can be attributed different infectiousness, I_1, I_2, I_3, and so forth. Thus for influenza it might be appropriate to have an I_1 class to cover the first 24 h following symptom onset that is more infectious with individuals then passing into subsequent I_2, I_3, etc. classes of defined duration that are progressively less infectious. Influenza infectiousness is thought to peak very abruptly and then decline somewhat more slowly (19, 38).

8. Introducing Better Descriptions of Disease Stages

The simple SIR model structure (Fig. 2a), whether deterministic or stochastic, can be made more realistic in other ways. For example, as discussed previously, individuals do not necessarily progress from being susceptible to being infectious (and symptomatic) without some intervening latent period. Accepting this potentially alters the observed dynamics of epidemic models in ways that may or may not be important to the specific questions being asked (5, 36, 39). For diseases such as smallpox, for example (Fig. 2b), it might be important to consider five separate disease classes and extend the differential equation set accordingly (34); though there are many ways in which the following aspects of the disease natural history might be reasonably represented with fewer or even greater numbers of classes; for example, to better capture the time varying infectiousness discussed above (5). Thus, there could be a period between infection and the first non-specific symptoms (often referred to as the (E)xposed class), then the period with non-specific symptoms (the (P)rodromal period), followed by the (I)nfectious and (R)ecovered classes. It can be important to capture the natural history in this way for diseases such as smallpox, which have both prodromal and infectious periods, since in the case of smallpox both are infectious (signified by the two dotted lines in the Fig. 2b) but with the latter much more so than the former, according to our knowledge and analysis of previous smallpox outbreaks (30, 40).

9. Some Problems with Data and the Parameterisation of Disease Natural History

The specific timings of events at an individual level can be highly critical, especially the relative infectiousness through the infectious period, which is rarely, if ever, uniform. This can be

important; for example, in relation to modelling public health interventions, which if applied early and before the peak infectiousness of each case will clearly have more impact on the control of the onward spread of an infection than if applied later. In which case it is important to better understand and appropriately capture the disease natural history in models (Figs. 5 and 6). Thus, when modelling pandemic influenza, it is thought that if antiviral drugs (such as the neuraminidase inhibitors) are to have much impact on the onward transmission of disease (through minimising viral replication and viral loads in the secretions, as opposed to simply ameliorating the course of infection and reducing the probability of hospitalisation and death (41) the drugs probably need to be administered within (a challenging) 12–24 h of symptom onset because of the extremely short infectious period of influenza and the rapid rise and fall of viral titres and infectiousness (19, 22). Whilst serial interval times are often more easily

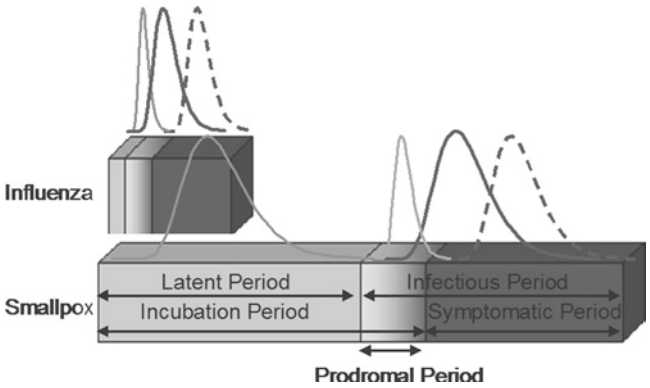

Fig. 5. Timelines of infection for two different viral diseases, given roughly to scale.

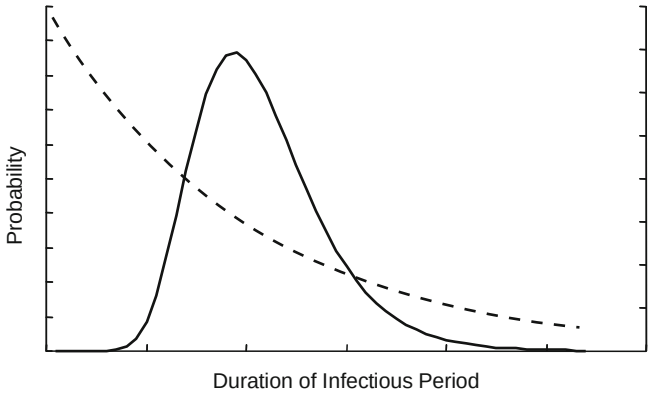

Fig. 6. Two different distributions with the same mean that models sometimes use for representing the duration of the infectious period, one exponential (*dashed line*) the other lognormal (*solid line*).

observed than some other intervals in the disease process, they are in themselves convolutions of the other intervals (periods), or parts of them. For example, as discussed previously, there is usually a period between the initial acquiring of infection by a case to the onset of symptoms (incubation period) and to the onset of infectiousness (latent period), which may or may not be coterminous periods and will vary between persons and the disease (Fig. 5). This distinction between incubation and latent period can be extremely important since diseases that become infectious before the onset of symptoms can make them much harder, or impossible, to control through the traditional means of isolating cases and quarantining contacts. Thus, diseases that can be asymptomatic at points during infection or relatively mild overall, or chronic or recurring such as influenza, HIV, or tuberculosis can theoretically be much harder to control by these means than diseases such as SARS CoV or smallpox (18). In this respect, the eradication of SARS CoV from the human population was highly dependent on (and blessed by), amongst other things, the fact that cases were generally not significantly infectious before showing symptoms and had a reasonably long incubation and infectious periods. Incubation/latent periods can often be followed by other defined periods that are relevant to the disease natural history, including: a symptomatic period, during some part of which cases are usually their most infectious (infectious period) – though, infectiousness is likely to vary over time, often rising rapidly to a peak and tailing off more gradually. For some infections there may also be a prodromal period (involving non-specific disease symptoms) between the incubation and symptomatic periods that might also be infectious (Fig. 5).

At its simplest, the parts of these periods that contribute to, for example, the serial interval are the incubation period of a secondary case and some part of the infectious period of the primary case. The latter being the time until a relevant contact has been made between persons such as to permit the transmission of infection, which will also depend on other factors, such as variation in contact rates with the rest of the population. Deconvoluting generation time distributions into their constituent distributions (or attempting the inference of the other distributions) can prove problematic to achieve in a statistically rigorous sense. Details of this are outside of the scope of this overview but such problems and their implications for epidemic models are important given what has been discussed already concerning modelling the prospects of ameliorating or controlling (or not) outbreaks of diseases such pandemic influenza, SARS CoV, and smallpox. Technical reflections on this subject have been discussed in depth elsewhere (36, 39).

Thus, although it might seem trivial to try to obtain the distributions of the different periods by direct observation, actual

measurement of some of these processes and their distributions can be problematic. For example, quite often the point in time when infection occurs (necessary for the estimation of generation interval and incubation period) is usually not observed in population-based studies for perfectly understandable and pragmatic reasons, except in those rarer situations where one person can reliably be known to have had only one contact with a single case and at a single point in time. Even then the precision of such observations is often limited to being differentiated to the nearest whole day. This will obviously matter more for diseases that have shorter generation/serial interval times: for example, a day can be a relatively long time in the course of influenza compared to smallpox (Fig. 5). Depending on the natural history of the disease, and possibly the prevalence of the disease in the rest of the community, such observations can be more or less feasible. For diseases such as smallpox or SARS CoV that possibly have a better marked clinical course in relation to infectiousness it is easier to define the timing of contacts with cases in relation to disease symptoms, as long as the disease is not so prevalent in the rest of the community that it is difficult to identify infected contacts uniquely with respect to the case that was responsible for them. Smallpox was also a disease for which contact tracing and quarantine was an important part of controlling outbreaks and so observations of the timings of contacts were more routinely made and, happily, sometimes recorded for posterity (30). Useful observational data can be more difficult to obtain for other diseases where the course might be either more rapid (influenza) and/or less well defined (influenza, measles, rubella) with respect to the infectiousness of cases in relation to their symptoms, and especially when the number of infections more widely in the community might also be quite high (such as for influenza) so as to potentially "mask" unique infection events. This problem can sometimes be overcome to some extent; for example, in the case of influenza this has been achieved by rigorous statistical analysis of studies undertaken in defined contexts, such as households where the time between subsequent infections can be more easily inferred (11), or volunteer challenge studies (38) where the time of infection is known.

10. Further Improvement of Descriptions of Disease Natural History: Non-exponential Disease Periods

The differential equation sets described above also hold an implicit assumption that the residence times in the I class, although having the correct mean duration, are exponentially distributed (they exhibit so-called Markovian dynamics because the result at a given point in time depends only on the state at the previous time as no

other history is encapsulated in the model) as opposed to something more realistic (e.g. lognormal or Gamma). As can be seen from Fig. 6, this means that although the average residence time in I is correct from both distributions (i.e. same mean for exponential and lognormal), an appreciable proportion of the residence times for the exponential will be unrealistically short (left hand end of distribution – dashed), and another proportion will be unrealistically long (right hand tail of distribution – dashed).

The consequences of adopting such simplifying assumptions may matter to a greater or lesser extent but is essentially a mathematical convenience to improve the tractability and computational ease of the problem. This assumption can, however, at a computational cost, be revised in a number of ways that allows the utilisation of more reasonable distributions. The different means of achieving this are largely beyond the scope of this chapter (5, 42). However, one simple approach is to adjust the schema described earlier and to arbitrarily break the I class down into more than one compartment (and therefore introducing another equation and term for each class). The use of several sequential equations and classes rather than one, with each class having an implicit exponential distribution for residence time, will overall combine to approximate a Gamma distribution (more like that in Fig. 6, solid line) that will also be closer in form to the distribution observed in the data.

11. Introducing Better Descriptions of Transmission

The transmission process can also itself be implemented mathematically in more than one way, for example, as a probability or rate determined by the mean estimate of the quantity R_0 or, as with the residence times in each of the disease classes mentioned above, as a more realistic distribution based on prior observations. This is sometimes represented as an "offspring distribution" (10), and often given as a distribution that closely follows observed transmission events. This approach usually better represents the variability that is observed in the transmission process. This is because, depending on the mathematical implementation, simple usage of the concept of average transmission, R_0, and, for example, implementing this as the mean of a Poisson process, can underestimate the potential impacts of low probability but high transmission events ("super-spreaders" or "super-spreading events"), and also the high probability but low transmission events. That is, a more reasonable distribution to use would probably have more dispersion than the Poisson that frequently gets used in mathematical formulations. This is explored in Fig. 7, which relates to observational data on pneumonic plague (31), which is transmissible person to person at relatively low average

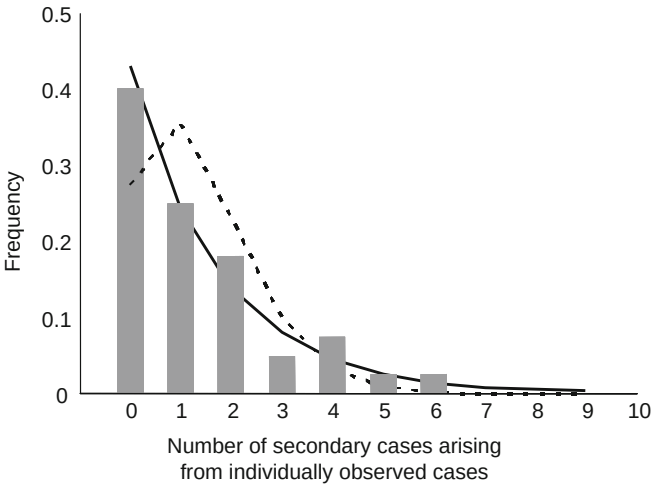

Fig. 7. Offspring distribution for pneumonic plague. Data are the vertical bars; the geometric distribution (*solid line*) is a better fit to the data than the Poisson (*dashed line*).

probability (R_0 = c. 1.3). However the data (vertical bars), which are the frequencies (y-axis) with which infected individuals have been observed to infect one, two, three, and so on susceptible individuals (x-axis) can be seen to be better reproduced by a more dispersed geometric distribution given by $f(x) = p(1-p)^x$, where x is the number of secondary cases per primary case, $f(x)$ is the frequency, and $p = 0.43$ (solid line) than a Poisson with an equivalent mean (dotted line). It can be seen from Fig. 7 that there is a greater probability than would be predicted from the Poisson of no transmission occurring from an infected case, and a greater probability of 4 or more secondary cases occurring from a primary case.

The former observation means that if a Poisson was used in a model then there would be a somewhat smaller probability of an epidemic dying out if there were only a very few initial cases, and the latter observation would mean that there would be a smaller probability in the model of generating larger outbreaks purely by chance (31).

12. Introducing More Realistic Descriptions of Public Health Controls

The model structures discussed so far are useful in deriving a better understanding of some aspects of "free-fall" epidemic dynamics, but have not really been discussed so far in relation to assessing the potential impacts of public health interventions, except in other than fairly simple ways. For example, as described already, in relation to calculating the proportion of the whole population that might need to be vaccinated in order to stop transmission

and eradicate a disease. As can be seen simplistically from Fig. 4, the higher the estimate of R_0 for a particular infectious disease the higher the proportion of the population that needs to be vaccinated to create sufficient "herd immunity" to prevent transmission; that is to bring R_E below one. For a pandemic of influenza with an R_0 value of 1.6, for example, this could be as little as around 37% of the population (43), but for smallpox with an R_0 value in the range of 3–6 this might need to be 67–80% or more, respectively (15). As stated previously R_0 can of course depend on local conditions and can vary geographically; often being higher if transmission is promoted by overcrowding and lower socioeconomic conditions. A similar approach could also be simplistically extended to the concept of isolation or quarantine, to estimate what proportion of infected cases and contacts of cases have to be found and completely isolated before they themselves become infectious. This number is similar to the critical vaccination coverage if a simplistic view of case finding, contact tracing and efficient quarantine is assumed (5). Model realism, and therefore complexity, can, however, be extended to investigate public health interventions more directly by allowing other states within the model schema and equations. For example, Fig. 2c shows an extension of the earlier SEPIR structure for smallpox to allow for a whole range of public health interventions, such as mass vaccination of some proportion of the population, case finding/reporting, contact tracing, targeted vaccination, and so forth. The equations exemplified earlier would of course have to be extended to cover these other compartments. All of these processes of course have to be understood and parameterised accordingly; one example being what proportion of the contacts of cases might reasonably be expected to be found and at what point in the course of their disease. This might be based on previously recorded experiences with the disease or with a disease of similar natural history. For diseases such as smallpox, pneumonic plague, SARS CoV, and bacterial meningitis, for example, the finding of potentially infected contacts can be relatively efficient and can often be in excess of 80%, reflecting the relative ease of finding the majority of those who have had sufficient contact with a case to facilitate disease transmission. Those predominantly getting infected tending to be those in (or visiting between) households and those in health-care facilities (see later for a discussion of transmission of pneumonic plague in different settings and contexts). Alternatively, or more likely additionally, robust analysis to parameter uncertainty (sensitivity analysis) would be undertaken. Hence, the parameters related to public health controls, as well as those related to disease natural history, would be systematically varied within ranges considered to be plausible, ensuring appropriate sensitivity and/or scenario analysis was performed [e.g. as in the case of the smallpox studies referred to earlier (20, 23)].

13. Introducing Better Descriptions of Population Mixing vs. Homogeneous Mixing

Returning to the assumption of homogeneous mixing referred to earlier; this is clearly not an entirely reasonable one, and for some purposes may considerably invalidate the use of a model depending on its application. If sufficient is known concerning the contact patterns of groups of individuals, or at least the contexts in which transmission occurs relative to one another, then such features can usefully be incorporated. This can be achieved either through splitting the population into a number of specific groups that share particular defined features (metapopulation models) or indeed into the more computationally demanding concept of individuals, each of which will have some generalised set of (measurable) features that are to some extent different to other individuals, but in combination with all of the other individuals in the model together reflect the characteristics of the population as a whole. These latter models can be implemented as either what are sometimes known as individual-based microsimulation models or network models (5, 19, 20, 22). Metapopulation models introduce heterogeneity into the mixing patterns of the population by identifying specific groups of individuals, within which there is still homogeneous mixing, but where between them there is not. Network and individual-based microsimulation models, necessarily generalise about individuals in some rational way, attributing features to each individual appropriately, and then allowing for heterogeneity of mixing at an individual-based level in respect of those features (5).

Metapopulation approaches are often much more computationally tractable, the extent to which this is true being dependent on the size of the population being simulated and the number of patches into which the population is subdivided. Such models may also have fewer parameters and therefore be easier to more reliably parameterise and understand. Different types of metapopulation approach have been used to good effect. Metapopulations can, for example, be developed on the basis of breaking the population down into different age classes, different economic, social, or functional contexts and/or by geography.

14. Age-Structured Models

Age-structured models at their simplest essentially take the SIR (or more complex compartmental)-type approaches but have a series of parallel schema (and sets of equations) running for each age group in the model (Fig. 8, for a simplistic two age class model), with transmission terms (dashed lines) not just operating

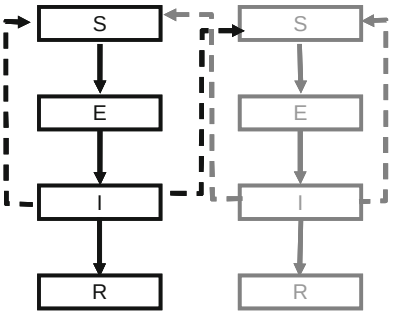

Fig. 8. Simplistic schematic for an age-structured model with two age classes (one *grey*, the other *black*).

within a particular age group, but also between different age groups at potentially different rates.

Parameterising such age-structured models requires some knowledge of the facility with which individuals in each age group infect others in that age group and in each of the others and this will depend to an extent on aspects of the natural history of the disease (e.g. the mode of transmission and the degree of intimacy of contact required; the severity of illness and the degree to which infectious individuals continue to be able to mix). With caveats, such transmission matrices can be estimated by attempting to fit models to infectious disease data where this has been stratified by age group or by reference to data that has been recorded on the relative extent to which different age classes mix with one another and with themselves, and ideally taking into account the intimacy of the contact (e.g. face-to-face conversation of some duration or some level of physical contact). These matrices have been used for some time in relation to developing a better understanding of the dynamics of, for example, childhood vaccine-preventable diseases and informing on optimal vaccination strategies. The derivation and use of such WAIFW (who acquires infection from whom) and similar matrices is beyond the scope of this broad overview but a good introduction can be found in Anderson and May (4), along with more contemporary analyses based on more recent multi-centre European studies (37, 44–46). Essentially mixing among age groups is highly assortative, that is those closer in age tending to mix more frequently with one another than with those in other age groups (but with children also mixing with parental and sometimes grandparental age classes), but with the frequency of contact between children generally being higher than mixing within any other individual age group. This potential disproportionate mixing and potential transmission of disease can have important consequences which are often important to capture in a model. For example, the initial rise in cases during an emerging uncontrolled epidemic might be seen first as a rise in the number of cases in children ahead of the rise in other age groups. Further,

such assortative mixing can have important consequences in relation to potential public health controls such as which age groups to prioritise for vaccination or the value of closing schools in order to try to limit the disproportionate contribution of children to overall disease transmission. For pandemic influenza it is thought such effects are likely to be important, since, for seasonal influenza at least, children do seem to be particularly implicated in transmission (47–49). For smallpox and SARS CoV on the other hand this is much less certain. In the case of smallpox it would seem from the limited historical observations available on populations that had not experienced smallpox for some considerable time (and so no older members of the community were already immune) that the age distribution of cases matched that of the population itself (21, 30). Even when age-dependent assortative mixing is relevant it is important to remember that public health measures such as school closure, for example, would not necessarily reduce the contact rate of that particular age group to zero unless draconian measures were also introduced to prevent them mixing in other contexts out of school. They would be likely to continue to mix to some extent in other contexts such as playing together outside and in other households, and also mix more frequently than before with other age groups such as their parents, household members, and relatives. Such effects are much more difficult to parameterise reliably or to compensate for, but are likely to be important and should not be neglected. Such effects and parameter assignments can in some instances be clarified to an extent by referring back to data from natural experiments and fitting age-stratified rates of influenza-like illness over time during an ongoing epidemic, such as happens, for example, around school holiday periods (47).

15. Socially Structured Models

Depending on what is known about the disease and the purpose of a model, the extent or facility with which disease transmission might occur might also be usefully characterised and subdivided by some social or functional context (e.g. household, workplace, school, hospital). Such contexts have and can be incorporated into a variety of types of model of differing degrees of complexity, including metapopulation and individual-based microsimulation ones (19, 20, 22). For ease, however, they will be considered here more simply in terms of metapopulation models. Taking pneumonic plague again as an example it is clear from historical data that not all contexts and inter-personal relationships were equal in terms of the extent to which transmission was observed to occur (Fig. 9).

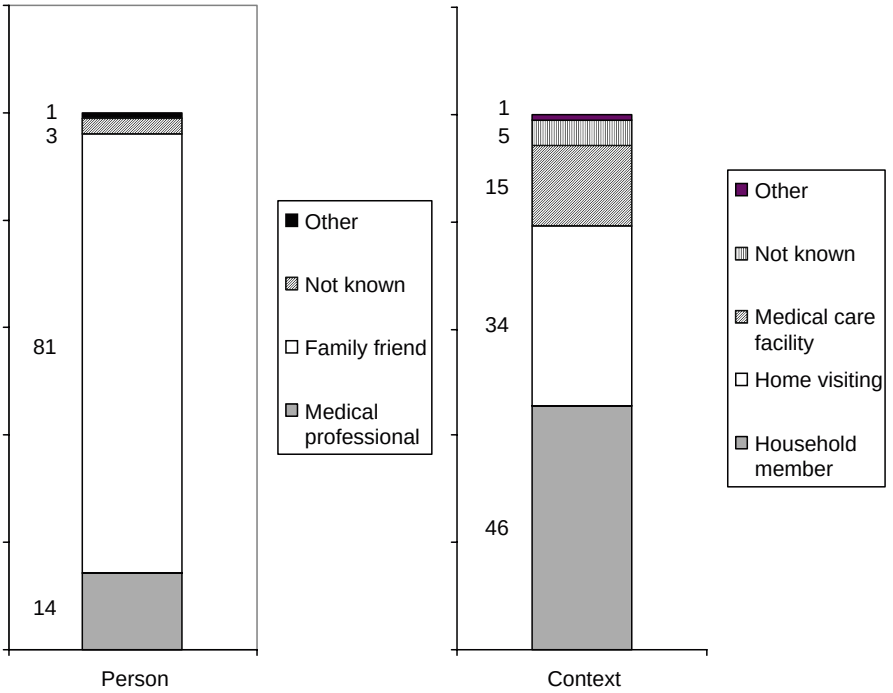

Fig. 9. The contexts in which the transmission of pneumonic plague was observed to occur.

The most frequent context for transmission was within a household (either with another member of, or visitor to, an infected household) or within a medical care facility. Hence, by far the most frequently infected individuals in this case (31) were family relatives and friends followed by health-care workers (together accounting for about 95% of transmission events). This observation in itself probably accounts for the fact that outbreaks of pneumonic plague (with a low overall reproductive potential) were readily brought under control since the infectious contacts of cases were relatively easily identified and quarantined such that R_E was rapidly reduced to below 1. Further, for smallpox, in the latter parts of the eradication era in Europe and other more developed parts of the world, transmission within the hospital context accounted for a significant proportion of all transmission (about 50%) before the disease was correctly identified and subjected to appropriate local infection controls (15, 30). Such contexts as described above clearly have parameters that relate directly to observations and data, and as such can be specifically incorporated into models with each context being represented by a separate metapopulation within the overall model structure, in much the same way as has been described already for age-structured models. In this way the relative frequencies of transmission seen in the data are then replicated by the model in the correct contexts. The contexts of home, workplace, school, etc. have also

been employed in a more sophisticated way within individual-based microsimulation models, where individuals in the model have attributed to them particular home, work, and school locations/interactions (20, 22).

16. Geographically Structured Models

Another degree of complexity that can be introduced into metapopulation models is the concept of separating the overall population into different geographical (or spatially determined) units. This can be done on the basis of relevant administrative areas such as those utilised during the collection of census information. The resulting metapopulation model in principle is not unlike that shown diagrammatically for simpler age-structured models in Fig. 8. In this case, however, each geographically distinct entity might have its own SIR (or more complex) structure, but with the connections (dotted lines in the figure) and probabilities of infection between geographic units parameterised by the extent to which proportions of the populations move between them. These connections may be viewed as largely analogous to the WAIFW matrices described earlier in relation to age-structured models, though with a typically much larger matrix that links each geographic unit with all of the others, and may have dependence on the time of day (to allow for commuting behaviour). Models that reasonably capture space in this way can be crucial when it comes to investigating interventions that have to be given a spatial context, such as vaccinating all the individuals in some geographic region based on there being cases of disease in that region (23). The concept of transmission between geographic entities can also be usefully implemented in other ways. In individual-based microsimulation models, for example, the matrix mentioned above can be converted into some more generalised movement kernel that describes the probability of any one individual moving (and/or causing infection) some distance from their home location by virtue of applying a probability based on such a kernel (20, 22). This probability typically drops off very non-linearly with increasing distance from home.

17. Closing Remarks

It is clear from the discussion above that models of varying degrees of complexity can be constructed to tackle problems related to (re-)emerging infectious disease problems and their control. Before embarking on model development for such issues it is

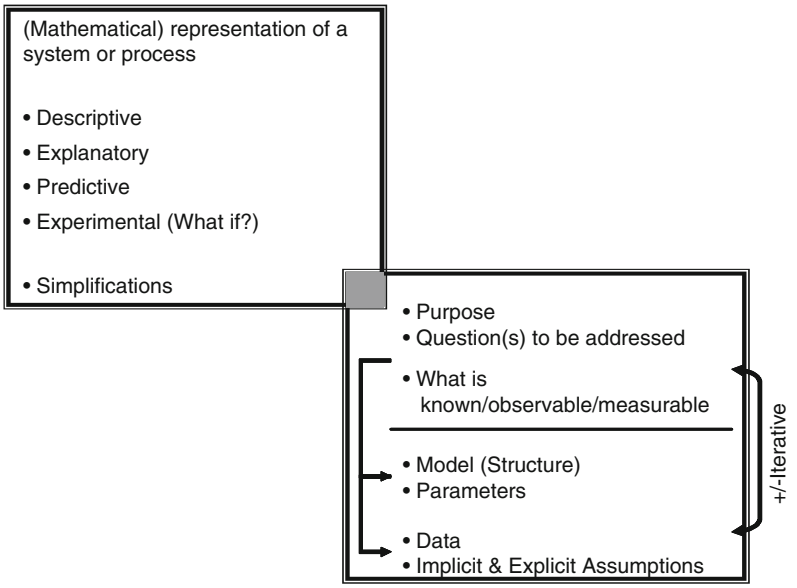

Fig. 10. Inter-relationships between policy and planning and the design of models.

generally useful for those engaged in public health protection to consider some basic practicalities. The first is that there is probably an important initial step before identifying a suitable model structure, either an existing one or one that is to be developed de novo, and that is to carefully consider the question or questions that are to be addressed in the light of what might be knowable, observable, or preferably quantifiable features of the disease; that is, the measurable features (parameters and relationships) of the underlying processes that are involved and what it is that the model is intended to determine (Fig. 10).

The former assertion in particular might seem rather facile, but less so when it is realised that in relation to issues of policy, planning, and responses, those requiring the answers to the questions are often not the ones that will be doing the modelling. Questions that might seem at first well specified by one, or even all, parties may fail to take into consideration some contingent factor that was not initially quite so obvious. So to take a very simple example, a question regarding what proportion of a population would need some particular intervention to achieve successful control of an outbreak may at first fail to take into account that it might be advisable to target a particular subset of the population on the basis of, for example, its geographic or demographic features. If the model has not been suitably constructed from the outset then the real question of how to optimise control policies will probably not be able to be addressed without reformulating the model. Similarly, with regards model parameters, it is entirely feasible to develop a model that turns out to require

data on a feature that has never been (reliably) measured or indeed can never be measured (e.g. time of infection for some diseases). Reasonable assumptions about such parameters can sometimes be made, but often a safer recourse is to reformulate (simplify) the model, if possible from the outset, in terms of other parameters that are measurable and for which there are more reliable data. This aspect needs careful consideration and communication among the various stakeholders in the modelling, particularly in relation to the question(s) that need to be addressed. Generally, as long as fit for purpose, the more parsimonious a model, the more readily it will be parameterised and executed, and produce results that are transparent and better able to be understood. As suggested earlier it is probably best to engage iteratively with as comprehensive a stakeholder group as possible that includes all of the disciplines that are relevant from the outset (Fig. 10). It is also important to make clear what are the assumptions and limitations of the models, and employ appropriate sensitivity and scenario analyses to mitigate such problems.

Finally, it is also equally important to set in place real-time data collection and analysis systems so as to be able to recalibrate and rerun models based on real-time data as it arises during an outbreak. Only in this way can an outbreak of an emerging infectious disease really be better understood at the time when the aim is to bring it under control (16).

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