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Avian Influenza Virus

Edited by

Erica Spackman

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Preface

The avian influenza (AI) virus, which is the type A influenza virus adapted to an avian host, has been an important pathogen for the poultry industry worldwide for many years. Although the cause was unknown at the time, one of the first recognized outbreaks of highly pathogenic AI (HPAI) virus occurred in Europe in the 1870s. Since 1955, when the AI virus was first identified, there have been about 24 outbreaks of the HPAI virus in domestic poultry worldwide.

Of those, perhaps the most consequential HPAI virus strain to emerge has been the Asian H5N1 HPAI virus, which was first seen in Southeast Asia around 1997. Until sometime in 2003, this strain had been mainly involved in sporadic outbreaks and was relatively contained. Then in 2004, reports of infection in poultry increased throughout Asia at about the same time as fatal cases of human infections in several Southeast Asian countries were confirmed. By 2006, the virus had spread further throughout Asia, into Africa and Europe, and as far west as the United Kingdom.

In addition to the rapid and distant spread of the Asian H5N1 HPAI virus, and the low number of human infections with a high fatality rate (approximately 50–60%), this virus's lineage has developed unusual pathogenic properties in birds in that it can cause disease and even mortality in some species of ducks. These factors have contributed to an increasing focus on the AI virus as a basic research area, and surveillance for the virus in domestic poultry and wild bird species has also increased considerably in the past few years.

This volume focuses on both the essential virological methods that are foundational for AI virus research and diagnostics as well as some of the newest molecular procedures used for basic and applied research. Since the AI virus has had such a long history as a veterinary pathogen, many of the classical virological procedures for the basic manipulation and characterization of the virus have been in use for many years. Therefore, they are well established and well optimized. These tests will form the foundation for either research or diagnostics. The recently developed molecular methods focus on elucidating the virus pathogenesis (reverse genetics), and some methods focus on the avian host response to the AI virus. Although some of these methods are applicable to type A influenza in general, the focus is the AI virus and the avian host system.

The aim of this book is to create a resource that includes both basic methods that are currently used and well established as well as some of the most exciting new methods for studying the virus itself, and to include methods that focus on work with avian hosts, an area that has been greatly lacking. In that context, this volume will be of interest to both diagnosticians and researchers, but it does assume a basic knowledge of virology and molecular biology.

I would like to thank all the authors for their contributions. I am also thankful to John M. Walker, the series editor, for his help in editing and working with me through this project.

Erica Spackman
Athens, GA, September 2007

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Chapter 1
A Brief Introduction to the Avian Influenza Virus

Erica Spackman

Summary The avian influenza (AI) virus is type A influenza isolated from and adapted to an avian host. Type A influenza belongs to the *orthomyxovirdae* virus family, is enveloped, and is pleiomorphic with a size ranging from 80–120 nm (reviewed in [1]). Type A influenza strains are classified by the serological subtypes of the primary viral surface proteins, the hemagglutinin (HA) and neuraminidase (NA). The HA has 16 subtypes (H1–H16) and contains neutralizing epitopes. Antibodies against the NA are not neutralizing, and there are nine neuraminidase or "N" subtypes. The "H" and N subtypes seem to be able to assort into any combination, and many of the 144 possible combinations have been found in natural reservoir species, although some combinations are more common than others.

All 16 subtypes have been found in ducks, gulls, or shorebirds, the natural reservoir host species of the virus. However, in these species certain subtypes are more common than others; for example, H3, H4, and H6 are most common in ducks in North America [2, 3] and although there is no clear association between host range or host restriction based on HA subtype, some subtypes are more common in some species than others, i.e., H1 and H3 in swine, H3 in horses, and H5 and H7 in chickens.

Keywords avian influenza virus; type A influenza; orthomyxovirus; host range; pathogenesis; molecular characteristics.

1. Molecular Biology

The genome of type A influenza is single-stranded, negative-sense RNA and contains eight genome segments that encode 10 or 11 proteins depending on the isolate (reviewed in [1]). The virus replication cycle, which takes place in the nucleus, has been well described (reviewed in [1]), although details on the functions of some proteins remain unclear. Additionally, relatively little is known about the molecular determinants of pathogenicity for type A influenza in hosts other than gallinaceous birds, although there is increasing evidence for the importance of the NS1 protein [4–7]. In gallinaceous birds (i.e., chickens, turkeys, and quail), the HA protein is the primary mediator of pathogenicity,

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where the proteolytic cleavage site sequence determines whether infection will be systemic (highly pathogenic) or restricted to the respiratory and enteric tracts (low pathogenic), based on which proteases recognize the sequence that is present [8].

Typical of RNA viruses, type A influenza is able to generate a high degree of genetic diversity by mutation within genes. In addition to a high mutation rate, reassortment, which is the mixing of gene segments between different strains and lineages during concomitant infection, is an additional mechanism for generating variability in the influenza genome. Certainly, the high genetic variability of the type A influenza virus contributes to the high adaptability of the virus and its ability to evade the immune system by antigenic drift (mutation) and antigenic shift (reassortment of the hemagglutinin).

In addition to the HA and NA genes, one other gene, the nonstructural (NS) gene, can be classified into separate genetic subtypes, A and B [9]. Between subtypes A and B, there is 63–68% nucleotide identity, 66–70% amino acid identity in the NS1 proteins, and 80–85% amino acid identity in the NS2 proteins. The NS subtype A is much more common and is the only subtype found in mammalian adapted isolates. However, subtype B is not host-restrictive since viruses with NS subtype B have been isolated from mammals [10], and reassortant viruses with the NS subtype B can replicate in experimentally exposed squirrel monkeys [11]. The remaining five gene segments are not divided into subtypes.

The genetic variation in the AI virus has also resulted in sufficient genetic variation among many genes to establish geographic lineages: North American or "New World" and Eurasian or "Old World." These geographic lineages have been defined for most genes, with the exceptions being the rare HA and NA subtypes for which there are too few isolates to evaluate. Due to the paucity of AI virus isolates from South America, that region has not been included but may be divergent enough to constitute a third lineage [12, 13].

2. Host Range

Although the natural host reservoir species for the AI virus is waterfowl [14], particularly ducks [3, 15], on rare occasions the virus has been transmitted to a nonnatural host species including other avian species and numerous mammalian species. On even rarer occasions a virus lineage may become adapted to, and established in, the new host species (i.e., swine H1N1, equine H3N8). It is very important to recognize that isolation of a virus from a particular species does not indicate that the host is a natural host or reservoir species. Many avian species are not natural hosts, including chickens and turkeys. However, in cases where an AI virus becomes adapted to these species, they may serve as a reservoir.

In gallinaceous birds (i.e., chickens and turkeys), AI viruses are classified as being highly pathogenic AI (HPAI) virus or low pathogenic AI (LPAI) virus. Although molecular criteria have been established by the World Organization for

Animal Health (formerly the OIE) for the identification of the HPAI virus based on the protein sequence of the HA proteolytic cleavage site [16], *in vivo* testing is more reliable for clinical classification. An AI virus isolate is classified as being HPAI if it kills at least 75% of susceptible 4- to 6-week-old chickens within 10 days postinoculation by the intravenous route [16]. Some isolates will cause 100% mortality by 36–48 hours postinoculation (reviewed in [17]). All other isolates are considered to be LPAI viruses. Biologically, the difference between HPAI and LPAI is that HPAI is a systemic infection and LPAI remains localized to the respiratory and intestinal tracts. For unknown reasons, all HPAI viruses have been either H5 or H7 HA subtypes [18, 19].

The generation of HPAI viruses appears to be a phenomenon associated with adaptation of LPAI viruses to chickens or turkeys. AI viruses from waterfowl are LPAI for gallinaceous birds, and some waterfowl adapted isolates will not even replicate in chickens [20]. However, once an H5 or H7 LPAI virus has been transmitted to chickens or turkeys, it has the potential to become an HPAI virus, although most strains remain low pathogenic. In some cases, the mutation from LPAI to HPAI has occurred within weeks of virus introduction into poultry (i.e., Chile 2002 [13] and British Columbia 2004 [21]) and in other cases has taken from months to years (i.e., Pennsylvania 1983 [22] and Mexico 1994 [23]). Although the alteration in pathotype is primarily mediated by the HA proteolytic cleavage site, the specific molecular changes vary and other genes are involved in overall virulence.

Type A influenza can replicate in numerous avian and mammalian species based on natural and experimental infections [19, 24]. For cross-species transmission of the influenza virus to occur, the host–virus interaction is critical, where extensive contact is necessary. In addition, cross-species transmission is virus strain-dependent. Although the zoonotic aspects of the AI virus are beyond the scope of this volume, one should always handle the virus with standard biosafety measures for pathogens.

3. Clinical Disease in Avian Species

In chickens and turkeys, LPAI virus infection may be subclinical; however, in the field, mild to moderate respiratory disease is the primary presentation. Lethargy and a drop in egg production are also frequently observed. Although the LPAI virus may cause mortality in the field, mortality is generally low and may be due to, or exacerbated by, secondary causes.

The primary characteristic of the HPAI virus in chickens and turkeys is rapid, high mortality, which can reach 100% within 36–48 hours postinfection (reviewed in [17]). The mean death time can vary by virus strain and is affected by numerous host factors, but it is not uncommon for chickens and turkeys to die soon enough postinfection that gross lesions are absent and clinical signs are observable for only a very short period before death. Clinical signs consist of severe depression and/or neurological signs (reviewed in [17]).

The AI virus, including most HPAI viruses, does not normally cause disease in natural host species, such as ducks. However, some isolates of the Asian H5N1 HPAI virus that emerged around 2001 and later can cause severe disease and even death in ducks and other waterfowl [25]. Only one other AI virus isolate has been documented to cause disease in a natural host species, A/Tern/SouthAfrica/61, which was also highly pathogenic for chickens. Currently, it is unclear whether the Asian H5N1 HPAI viruses identified in wild birds originate from contact with infected poultry and domestic birds or whether the virus has become endemic in some wild bird populations.

4. AI Virus Detection and Characterization Methods

The rapid detection and diagnosis of AI virus in poultry, wild birds, and other species are critical to controlling the virus. Subsequent characterization provides vital information about the ecology and biology of the virus.

Numerous tests are currently being used for AI virus detection, including real-time RT-PCR, virus isolation, and commercial antigen immunoassays. Because of the clinical importance of the presence of a viable virus isolate, and the necessity of having a virus isolate to characterize, virus isolation is probably the most critical of these methods. In the field, antibody detection is also widely used for evaluating the prior exposure of birds to the AI virus. Current antibody detection tests include commercial ELISA, agar gel immunodiffusion assay, and hemagglutination inhibition (HI) assay. Each has its advantages and disadvantages; therefore, the most important considerations are fitness for purpose and validation.

Characterization of isolates is frequently done by both classical and molecular methods. Classical methods such as HI assay, neuraminidase inhibition assay, and *in vivo* pathotyping are still the reference standards required by the World Organization for Animal Health. Some molecular methods such as sequencing have been used for many years to characterize AI virus isolates; however, more technically advanced methods like reverse genetics are relatively new tools for AI virus research. In general, techniques for avian host systems are somewhat behind those of mammalian systems due to the inherent physiological differences and a lack of reagents for avian species.

5. AI Virus Research Directions

As a highly contagious cause of disease in numerous economically important species, the AI virus will remain an important subject of research. Numerous questions remain to be answered on everything from the basic biology of the virus to the intricate details of virus replication and interaction with the host cell. Control of infection and disease will be critical areas, where vaccine development and antiviral drug development will be the major foci of research.

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Chapter 2
Avian Influenza Virus Sample Types, Collection, and Handling

Mary Lea Killian

Summary Successful detection of the avian influenza (AI) virus, viral antigen, nucleic acid, or antibody is dependent upon the collection of the appropriate sample type, the quality of the sample, and the proper storage and handling of the sample. The diagnostic tests to be performed should be considered prior to sample collection. Sera are acceptable samples for ELISA or agar gel precipitin tests, but not for real-time RT-PCR. Likewise, swabs and/or tissues are acceptable for real-time RT-PCR and virus isolation. The sample type will also depend on the type of birds that are being tested; oropharyngeal swabs should be collected from poultry, and cloacal swabs should be collected from waterfowl. This chapter will outline the collection of different specimen types and procedures for proper specimen handling.

Keywords diagnostics; virus replication; virus detection; specimen collection; sample processing.

1. Introduction

Sample selection and handling are important in the detection of the avian influenza (AI) virus. The type and quality of samples collected will determine the success of diagnosis. In addition, storage conditions from the time of collection until the specimen is processed in the laboratory are very important for the diagnosis of avian influenza.

Low pathogenic avian influenza (LPAI) is primarily a respiratory disease in poultry and in general replicates mainly in the respiratory tract in gallinaceous birds as well as in the gastrointestinal (GI) tracts of waterfowl (see Chapter 1). In contrast, highly pathogenic avian influenza (HPAI) is a systemic disease in poultry and can be isolated from most organ systems [1]. Most HPAI viruses replicate poorly in waterfowl and produce few clinical signs. Specimens collected within three days of the onset of clinical symptoms in poultry will yield the highest viral load for virus isolation or nucleic acid detection [2]. The collection of specimens from dead animals should be done as soon as possible after death to preserve the virus for testing.

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The National Veterinary Services Laboratories (NVSL) uses brain-heart infusion broth without antibiotics for the collection of tracheal and cloacal swabs. Other viral transport media containing protein buffer may also be used (tryptose, nutrient, or peptone broth) [3]. The presence of protein in the viral transport media will help prevent the degradation of live virus during handling and transport to the laboratory. Antibiotics may be added to the viral transport media to prevent microbial growth during transportation provided the specimens will not be processed for bacterial identification.

AI viruses are unstable in the environment and are easily inactivated by extreme heat or drying [1]. Transporting dry swabs to the laboratory decreases the likelihood of isolating the AI virus. The swabs should be placed into one of the previously mentioned viral transport media immediately following collection. Collection of specimens in a protein-containing solution followed by refrigeration increases the stability of the virus. The specimen should be maintained at refrigeration (2–8 °C) and transported to the laboratory as soon as possible. The virus titer will decrease if the specimen is frozen and thawed multiple times. This may result in a failure to detect virus that is present in low levels [4]. In order to avoid freezing and thawing, do not store specimens at 0 °C.

Polyester swabs with plastic handles should be used for the collection of swab specimens rather than cotton swabs with wooden handles. The wooden handles are frequently treated with substances that can inactivate live virus and inhibit the isolation of viral nucleic acid. In addition, swabs treated with calcium alginate have been shown to inactivate virus and can decrease the viral titer up to 1000-fold in 48 hours [4].

2. Materials

1. Sterile glass tubes, screw cap
2. Brain-heart infusion broth or other viral transport media
3. Sterile polyester or Dacron swabs with plastic handle

3. Methods

3.1. Collection of Swab Specimens

AI viruses replicate primarily in the respiratory and gastrointestinal tracts of poultry and waterfowl, respectively. Swab specimens can be used for virus isolation, nucleic acid-based assays, and antigen immunoassays. Swabs collected for flock surveillance should be taken from clinically ill or dead animals [5]. The virus is present in the highest quantity at this time [1, 5]. Oropharyngeal swabs are the preferred specimen from poultry for rRT-PCR testing; however, waterfowl shed the AI virus primarily from the GI tract. For this reason, cloacal swabs should be used for

detection from these species (see Chapter 11 for more detail). However, there are substances in these samples that can inhibit the PCR reaction (see Chapter 4). Therefore, virus isolation (see Chapter 6) is the best test method to accurately identify the presence of the AI virus from cloacal swab samples. Collecting cloacal swabs from small living birds may be harmful to the animal; therefore, in this case fresh feces may be used for testing [6].

Swab specimens from a single flock or species may be pooled for testing. Up to five oropharyngeal swabs or five cloacal swabs from a single poultry flock may be pooled in the same viral transport media. Oropharyngeal and cloacal swabs should not be pooled in the same viral transport media if the specimens will be tested by rRT-PCR. Combining oropharyngeal and cloacal swabs is acceptable if the specimens will be tested only by virus isolation. Swab specimens from wild birds or waterfowl should not be pooled in the same viral transport media. These birds may carry multiple types of the AI virus, and pooling the swabs in a single tube may inhibit the detection of one or more virus subtypes. Therefore, cloacal swabs from wild birds should be collected in individual tubes. If the samples are to be tested by rRT-PCR, a small amount of the swab material from up to five swabs from a single flock or species may be pooled into a separate tube for RNA extraction and tested as a single sample. If the rRT-PCR results are positive, the specimens should be tested individually by virus isolation to reduce the chance of mixing virus subtypes in a single sample.

1. Insert the swab into the cloaca or oropharyngeal area, swabbing the area thoroughly. Be sure to swab the tracheal opening and draw the swab through the choanal cleft on the upper palette when collecting swabs from the respiratory tract. The trachea may also be swabbed; however, the oropharyngeal area is easier to sample and can yield a higher titer. If collecting fecal swabs, insert the swab into freshly deposited wet feces in order to saturate the swab.
2. Place the swab into 3–4 mL of viral transport media and swirl vigorously to dispel the contents of the swab into the media.
3. Lift the swab out of the media; press the swab firmly against the side of the tube to remove any remaining liquid from the swab.
4. Discard the swab into a disinfectant solution.
5. Label each tube with a unique sample identifier.

3.2. CAvian Influenza Virusion of Tissue Specimens

Tissues are appropriate specimens for virus isolation, nucleic acid-based assays, and immunohistochemistry. Isolation of the AI virus from internal organs (other than respiratory or gastrointestinal tissues) may indicate systemic disease, which is often associated with highly pathogenic AI virus [1]. Detection of virus in tissues other than lung and trachea from animals infected with low pathogenic AI virus is very rare and may occur from contamination with secretions from the respiratory or digestive tract.

Tissues should be collected and placed into sterile plastic bags or tubes labeled with a unique identifier. Tissues to collect may include trachea, lung, air sac, intestine, spleen, kidney, brain, liver, and heart. Tissues may be processed individually or pooled; however, tissues from one system (respiratory, digestive, cardiovascular) should not be mixed with tissues or organs from other systems. Recommended tissue pools include respiratory tissues (lung, air sac, trachea), digestive tissues (liver, pancreas, proventriculus), intestinal tissues (large and small intestine, cecum), lymphoreticular tissues (spleen and Bursa of Fabricius), lung and spleen, liver and kidney, heart, and spleen/bursa. Brain and other nervous system tissues should always be processed individually. Tissues from more than one bird should never be pooled together, as individual birds within a flock may be at various stages of infection and if antibody is present in tissues from one bird, it may neutralize the virus in tissues from a second bird.

3.3. Specimen Handling and Storage

Swab specimens should be chilled at 4 °C immediately following collection for up to 48 hours [1–3]. If testing cannot be initiated within 48 hours, samples should be frozen (−70 °C or lower). Fresh tissue specimens may be frozen immediately without viral transport media and homogenized at the time of processing. Alternatively, fresh tissues may be placed in a viral transport media, frozen and thawed up to three times to disrupt the cells, and processed without homogenization.

In order to avoid freezing and thawing multiple times, specimens should never be maintained at 0 °C. Swab specimens can be maintained for weeks to months at −20 °C or below, and for up to a week at 4 °C. Tissue specimens should be stored at either 4 °C for up to 48 hours or at −70 °C or below for long-term.

3.4. Collection of Serum Samples

A presumptive diagnosis of AI virus infection may be made by detecting antibodies in sera. Without the isolation of a virus, the detection of antibody assumes a recent infection with the AI virus. Antibodies are primarily an indication of prior exposure and may be detected long after an active infection is over. Numerous tests are available for AI virus antibody detection. Sera can be tested by enzyme-linked immunosorbant assay (ELISA), agar gel immunodiffusion (AGID) assay (Chapter 6), and hemagglutination inhibition (HI) assay (Chapter 8). ELISA and AGID assays are not type-specific because they detect antibody to either the matrix or nucleocapsid proteins; the HI assay detects antibody to specific hemagglutinin (HA) subtypes.

If possible, serum should be collected no later than 7 days after the onset of clinical signs, and convalescent phase serum should be collected 2–4 weeks later

[2]. Three to four mL of blood should be collected from a wing vein into a 5-mL syringe. Following collection, pull the plunger all the way out on the syringe to allow air into the barrel and to maximize the surface area, as this will allow for the most serum to be collected. Lay the syringe on its side so the blood pools to one side of the syringe (the surface area is maximized). Allow the syringe to sit at room temperature for 24 hours to allow the serum to separate from the clot. Alternatively, the syringe can be placed at 37 °C for 4 hours and stored at 4 °C overnight. After separation, the serum can be poured off of the clot into a clean tube. Serum should always be collected within 24 hours of blood collection. The serum should be centrifuged at a low speed to remove excess erythrocytes before testing.

3.5. Transportation of Specimens to the Laboratory

Samples should be transported to the laboratory as soon as possible following collection. The specimens should be shipped on ice pack or wet ice and delivered for testing. To prevent degradation of the virus, the specimens should not be held at refrigeration temperatures for longer than 48 hours. If the specimens must be stored before transportation, the samples should be frozen (preferably at −70 °C). The specimens should be packed for shipping with sufficient ice packs to ensure the specimens stay frozen until they are received in the laboratory for testing. Samples should not be shipped on dry ice unless they are in sealed containers or are sealed, taped, and double plastic-bagged [2], because the CO_2 gas released during dry-ice sublimation will inactivate the AI virus.

In the United States, the transportation of diagnostic specimens via public roadways or airspace must follow the regulations outlined by the International Air Transport Association (IATA) and the Department of Transportation (DOT). Complete regulations can be found at www.iata.org or in the Code of Federal Regulations (49 CFR Parts 171, 172, 173, and 178).

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Chapter 3
Avian Influenza Virus RNA Extraction from Tissue and Swab Material

Erica Spackman and David L. Suarez

Summary The efficient extraction and purification of viral RNA are critical for downstream molecular applications, whether it is the sensitive and specific detection of virus in clinical samples, virus gene cloning and expression, or quantification of the avian influenza (AI) virus by molecular methods from experimentally infected birds. Samples can generally be divided into two types: enriched (e.g., virus stocks) and clinical. Clinical type samples, which may be tissues or swab material, are the most difficult to process due to the complex sample composition and possibly low virus titers. In this chapter, two well-established procedures for the isolation of AI virus RNA from common clinical specimen types and enriched virus stocks for further molecular applications will be presented.

Keywords RNA; RNA extraction; molecular; nucleic acid; specimen processing; tissue; swab material.

1. Introduction

AI virus RNA can be isolated from numerous *in vitro* and *in vivo* virus sources; however, cell lysates, cell supernatants, or chicken embryo chorioallantoic fluid are the easiest to work with because the virus load is normally high and the sample composition is defined. When the RNA will be used for gene sequencing, cloning for characterization, or expression, the use of RNA from enriched stocks is recommended. Most general RNA extraction methods or commercial kits for total RNA or viral RNA extraction may be used with enriched samples.

In contrast, clinical diagnostic samples or samples from experimentally infected animals are complex mixtures of host cells, bacteria, and other material that can interfere with the detection of the AI virus with molecular diagnostic tests. The efficient extraction and purification of the viral RNA from this material are critical for sensitive and specific detection of the virus. Specific processing methods will depend on the sample type. For animal testing, the optimal sample type varies by species and virus strain (see Table 1), as, for example, the virus tissue tropism can differ based on the host and the virus may therefore be present in different amounts in different tissue

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types. Specimen species of origin can also affect the processing procedure for cloacal swabs or fecal samples since the host's diet may introduce RT-PCR inhibitors, which are not removed during RNA extraction. The diet of domestic poultry is generally uniform, allowing for more consistent processing, but samples from wild birds can vary greatly because of the variety of foods they can consume.

Clinical specimens and animal-origin specimens (swab material and tissue) may also contain substances that can degrade RNA once it is released from the virion. Specimens containing fecal material, such as cloacal swabs, are some of the most difficult to work with and require processing methods that inactivate RNases thoroughly and that are efficient at removing PCR inhibitors or other contaminants. Some general recommendations for the selection of RNA extraction method by sample type are provided in Table 1. See Chapter 2 for additional guidance on sample collection and handling.

Numerous commercial kits for total RNA extraction are available, and standard organic extraction methods may also be optimized for AI virus RNA. Here the procedures for two methods, a silicon binding column-based method that has been widely used with AI virus [1, 2] and a method using organic solvents for nucleic

Table 1 Processing Methods and Notes by Specimen Type

Specimen Type	Processing Notes
Oropharyngeal or tracheal swabs	Any method may be used. LPAI virus tends to replicate to higher titers in the respiratory tract of gallinaceous birds.
Cloacal swabs	Organic extraction preferred. Use of an internal positive control is highly recommended for RT-PCR and real-time RT-PCR (see Chapter 4). Low pathogenic AI virus tends to replicate to the highest titers in the intestinal tracts of waterfowl.
Tissue (i.e., lung, heart, etc.)	Prepare a 10% W/V homogenate and follow with an organic extraction. Highly pathogenic AI viruses can replicate to high levels in numerous tissues; low pathogenic AI viruses are optimally detected in the respiratory tracts of gallinaceous birds and the enteric tracts of water fowl.
Environmental samples (i.e., drag swabs)	Organic extraction preferred. Use of an internal positive control is highly recommended for RT-PCR. RT-PCR–based methods are not recommended if differentiation of viable from nonviable virus is necessary, in which case virus isolation is preferred [5].
Cell culture fluid	Any.
Amnioallantoic fluid (AAF)(egg fluid)	Any. Avoid using egg fluid with excessive yolk. AAF should appear clear to yellow and may be cloudy. Brown to red or solid yellow AAF, which may indicate yolk, bacterial, or blood contamination, should be avoided.

acid isolation, will be discussed. Although specific kits are mentioned, equivalent kits and suppliers may be used with optimization.

2. Materials

Mention of trade names or commercial products in this procedure is solely for the purpose of providing specific information and does not imply recommendation or endorsement.

2.1. Method 1. Silicon Nucleic Acid Binding Column

1. Nuclease-free water.
2. Nuclease-free plastic ware (i.e., microcentrifuge tubes) (*see* **Note 1**).
3. Qiagen RNeasy Mini Kit (Qiagen, Inc.) or equivalent (*see* **Note 2**).
4. 100% Ethanol.
5. 70% Ethanol (prepared with nuclease-free water).
6. 2-Mercaptoethanol (βME).
7. Sample material (*see* **Note 3**): oropharygeal or tracheal swab material, amnioallantoic fluid (AAF), cell culture material.

2.2. Method 2. Organic Extraction

1. Nuclease-free water.
2. Nuclease-free plastic ware (i.e., microcentrifuge tubes) (*see* **Note 1**).
3. Trizol LS reagent (Invitrogen, Inc.).
4. Chloroform.
5. Isopropanol.
6. Glycogen 5 mg/mL.
7. 70% Ethanol (prepared with nuclease-free water).
8. Sample material (*see* **Note 3**): oropharygeal, tracheal, or cloacal swab material, AAF, cell culture material, tissue.

3. Methods

3.1. Method 1. Silicon Nucleic Acid Binding Column

This method is best for AAF, cell culture material, tracheal swabs, or oropharyngeal swabs. This method is not recommended for tissue, cloacal swabs, or samples that may contain fecal material.

1. Prepare all buffers and reagents in accordance with the RNA extraction kit protocol (Qiagen RNeasy Mini Kit).
2. Vortex the sample (tracheal swabs in BHI or other media) for 3–5 s, and then centrifuge the sample for 5 min at ~5 Kg to pellet any large debris. Withdraw 500 µL of supernatant and place in a 1.5 mL microcentrifuge tube. Amnioallantoic fluid (AAF) and cell culture material may be added directly to the RLT buffer.
3. Add 500 µL of kit-supplied RLT buffer. Close the tube and vortex the sample for 5 s.
4. Add 500 µL of RNA-grade 70% ethanol to the tube and mix. Pulse-centrifuge to minimize aerosol generation.
5. Add 750 µL of the supernatant from step 3 to the RNeasy column and centrifuge for 15 s at ~12 Kg, empty the flow-through from the collection tube, and repeat (all of the sample/RLT/70% ethanol mix should be applied to the column). This step may be done with a vacuum manifold instead of a centrifuge.
6. Add 700 µL of RW1 buffer to the RNeasy column, centrifuge for 15 s at ~12Kg, and place the column in a clean collection tube (the collection tube with RW1 flow-through may be discarded and replaced with a fresh collection tube). This step may be done with a vacuum manifold instead of a centrifuge.
7. Add 500 µL of RPE buffer to the RNeasy column, centrifuge for 15 s at ~12 Kg, and empty the flow-through from the collection tube. This step may be done with a vacuum manifold instead of a centrifuge.
8. Repeat step 6 for a total of two washes with RPE buffer.
9. Centrifuge the empty RNeasy column an extra 2 min at ~14 Kg and discard the collection tube.
10. Place the RNeasy column in a 1.5 mL microfuge tube and add 50 µL of nuclease-free water to the column. Incubate at room temperature for 1 min. Elute RNA by centrifuging for 1 min at ~14 Kg. Discard the RNeasy column (*see* **Notes 4** and **5**).
11. The RNA sample may be stored at 4 °C for <1 week; storage for longer than 1 week should be at −70 °C or colder. Repeated freeze-thaw cycles should be avoided.

3.2. Method 2. Organic Extraction with Trizol LS Reagent

This method can be used with AAF, oral swabs, tracheal swabs, cloacal swabs, cell culture material, and tissues.

3.2.1. Sample Preparation

1. Cloacal swabs: Vortex vigorously for 7–10 s. Centrifuge for 5 min at 12,000 g. Extract RNA from the supernatant (see Section 3.2.2).
2. Tissues: Make a 10% homogenate of tissue in PBS. Centrifuge for 10 min at 12,000 g. Extract RNA from the supernatant (see Section 3.2.2).

3. AAF and cell culture material: Use 250 µL of the AAF or cell culture supernatant or lysate directly with 750 µL of Trizol LS (see Section 3.2.2).

3.2.2. RNA Extraction

1. Add 250 µL of the supernatant from the sample prepared as described in step 1 of Section 3.2.1, to 750 µL of Trizol LS reagent or equivalent. Vortex. Pulse-spin to remove liquid from the tube lid.
2. Add 200 µL of 100% chloroform to the sample/Trizol homogenate. Vortex for 15 s. Incubate at room temperature for 7 min.
3. Centrifuge at 12,000 g for 15 min at room temperature.
4. Transfer 400–450 µL of the upper aqueous layer to a separate microcentrifuge tube marked with the sample number. Because the transfer of organic phase material with the aqueous layer will reduce RNA yield and inhibit the RT-PCR reaction, take care to avoid pipetting any of the organic phase or interface material if present.
5. Add 500 µL of 100% isopropanol. Add 1 µL of 5 mg/mL stock glycogen to the isopropanol to aid RNA precipitation. Invert tube several times to mix, and incubate at room temperature for 10 min.
6. Centrifuge at 10,000 g for 10 min at 4 °C.
7. Decant liquid. Care should be taken to ensure that the RNA pellet is not disturbed. Add 1.0 mL of 70% or 80% ethanol. Mix gently.
8. Centrifuge at 10,000 g for 5 min at 4 °C.
9. Decant ethanol without dislodging the pellet. Invert the tube on a clean tissue wipe and allow to air-dry for 10 min or until all visible signs of moisture are gone. It is important not to let the RNA pellet overdry, as this will decrease its solubility.
10. Reconstitute the pellet in 50 µL of RNase-free water and incubate at 4 °C for 1 hr to overnight before using in downstream applications (*see* **Notes 4** and **5**).
11. The RNA sample may be stored at 4 °C for <1 week; storage for longer than 1 week should be at −70 °C or colder. Repeated freeze-thaw cycles should be avoided.

4. Notes

1. Use only RNA-grade (RNase-free) reagents and supplies for all reagents, buffers, and anything that may come in contact with the sample (e.g., mix any buffers to be used with RNA in RNase-free vessels). When possible, it is recommended that disposable RNAse-free labware is used since complete decontamination of glassware is difficult and time-consuming. Change gloves often to avoid cross-contamination and to minimize RNase contamination.

2. All kit-supplied buffers and reagents should be prepared in accordance with the kit instructions.
3. Samples collected for AI virus detection are often processed for more than one test. Therefore, when collecting samples for molecular methods, the samples are often handled to maintain viable virus. Since the AI virus is enveloped, maintenance of the cold chain while preventing freeze-thaw cycles and protein-rich pH-balanced buffers are critical. Brain-heart infusion (BHI) broth has been used extensively as the AI virus transport media based on work that compared numerous media and showed that BHI broth was superior in preserving AI virus [3].
4. Failure of the RNA extraction procedure may result in false negatives and can be difficult to identify as the cause. Use of an internal positive control for real-time RT-PCR is one way to ensure the quality of RNA extraction [4]. If RNA extraction fails:

   a. Confirm that all reagents have been properly made.
   b. Confirm that virus is present in the sample at adequate titers.
   c. Confirm that all materials were RNase-free.
   d. Repeat the extraction using the same or an alternative method.

5. For critical applications, some quality assurance may be helpful and can be done prior to the downstream application. Although UV spectroscopy may be used, it will give data for any and all RNA in a sample.

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Chapter 4
Type A Influenza Virus Detection and Quantitation by Real-Time RT-PCR

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Summary Real-time RT-PCR (rRT-PCR) is a relatively new technology that has been used for avian influenza (AI) virus detection since the early 2000s for routine surveillance, during outbreaks, and for research. Some of the advantages of rRT-PCR are high sensitivity, high specificity, rapid time-to-result, scalability, cost, and quantitative nature. Furthermore, rRT-PCR can be used with numerous sample types, is less expensive than virus isolation in chicken embryos, and since infectious virus is inactivated early during processing, biosafety and biosecurity are also easier to maintain. This chapter will provide an overview of the USDA-validated rRT-PCR procedure for the detection of type A influenza.

Keywords real-time RT-PCR; hydrolysis probe; RNA; avian influenza virus detection; virus quantitation.

1. Introduction

Real-time reverse-transcription PCR (rRT-PCR) was initially introduced for AI virus detection in clinical samples in 2001, when it was used for testing poultry in live bird markets [1, 2]. It was then used to help control an outbreak of low pathogenic avian influenza (LPAI) virus in commercial poultry in 2002 [3], where it was field-validated for use in chickens and turkeys. Since then, a standard protocol has been developed in the United States, where numerous veterinary diagnostic labs have implemented the test. Subsequently, rRT-PCR has been employed as a diagnostic test worldwide during numerous outbreaks for the detection of AI virus infection in numerous species [1, 2, 4]. Real-time RT-PCR has also been used extensively for wild bird monitoring and in research as an alternative to virus isolation, particularly for quantification [5].

The primary and most reliable application of rRT-PCR is the detection of any type A influenza virus from any species. Tests for pan-type A influenza detection are often targeted to the conserved matrix (M) or nucleoprotein genes, for which substantial sequence data are available for primer and probe design. This chapter will describe a protocol that targets the M gene [1, 2]. The sensitivity of this test has been shown to be as low as 10^{-1} 50% egg infectious doses.

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The general principle of amplification of viral RNA to detectable levels of PCR DNA is the same for real-time PCR as it is for standard PCR. The difference between the two methods is in how the PCR product is detected. In real-time PCR, the PCR product is visualized with a fluorogenic probe or nucleic acid binding dye in real time (i.e., during each PCR cycle). Numerous chemistries are available for real-time PCR. The type A influenza test reported here uses hydrolysis probes, also called dual-labeled probes or Taqman probes.

In the hydrolysis/dual-labeled probe system, in addition to the target-specific PCR primers, a DNA probe with a sequence complementary to the amplification target is labeled with a fluorogenic reporter dye on one end (usually the 5′ end) and a quencher dye on the other end (usually the 3′ end). The reporter dye is excited at a specific wavelength of light (by the real-time PCR instrument), which causes it to fluoresce at a different wavelength, which is then detected by the real-time PCR instrument. If the quencher dye is in close proximity to the reporter dye, it absorbs any light produced by the reporter dye, which prevents detection by the real-time PCR instrument (this is registered as the background fluorescence level). If the probe anneals to the PCR amplicon during the extension step of the PCR reaction [6, 7], the reporter dye is cleaved from the 5′ end of the probe by *taq* polymerase (due to 5′ exonuclease activity), physically separating it from the quencher dye. When enough of the reporter dye is cleaved, a fluorescent signal will be produced that is directly correlated to the amplicon concentration in the log phase of amplification. Since the fluorescence signal is monitored every cycle, increases in the PCR product are recorded and can be reported in real time as amplification occurs. By its nature, real-time PCR is also inherently quantitative (*see* **Note 1**).

Importantly, with these methods, since a sequence-specific probe is used, the specificity of the test is high because the product is not visualized unless it can bind a sequence-specific probe; therefore, even if the primers react nonspecifically with a template in the reaction, this side reaction will not be recorded (in contrast to conventional PCR methods, where all reaction products are detected with electrophoresis).

The AI virus matrix gene assay reported here was optimized using the Qiagen OneStep RT-PCR kit; however, other kits may be used. For maximum performance, however, other kits will require separate optimization of the enzyme amount, magnesium concentration, and cycling conditions. Not all RT-PCR kits will provide acceptable performance, and they must include a polymerase with 5′ exonuclease activity. Using two-step RT-PCR procedures, where the reverse transcription and PCR reactions are each run separately, can potentially result in increased sensitivity because each reaction can be optimized separately. However, two-step RT-PCR is not recommended due to the increased potential for cross-contamination and the increased labor because of the additional pipetting steps required for this modification. Finally, it is highly recommended that the PCR primers and probe be highly purified (i.e., HPLC-purified). Mention of trade names or commercial products in this procedure is solely for the purpose of providing specific information and does not imply recommendation or endorsement.

Real-time RT-PCR can be used with RNA from any sample type; however, the extraction process is critical and must be appropriate for the specimen. Details on sample processing can be found in Chapters 2 and 3.

2. Materials

1. Real-time PCR instrument (the conditions for the Cepheid Smart Cycler are provided in this volume [2]; the conditions for the Roche Light Cycler and Idaho Technologies RAPID have been reported [1]). Many other thermocyclers use the Peltier system of heating and cooling, which typically requires increased cycle times due to the slower ramp times.
2. Reagent-grade H_2O (nuclease-free).
3. TE buffer (for probe reconstitution).
4. Qiagen OneStep RT-PCR Kit (reverse transcriptase, *taq* polymerase, dNTPs, reaction buffer) or equivalent reagents (*see* **Note 2**).
5. Dual-labeled probes (*see* **Note 3**) (Table 1).
6. Primers (*see* **Note 4**) (Table 1).
7. $MgCl_2$ (25 mM stock).
8. RNase inhibitor (13 units/µL stock).
9. Positive control RNA.
10. 25-µL Smart Cycler tubes or appropriate reaction vessel for the real-time PCR instrument being used.

3. Methods

3.1. Primer and Probe Handling and Dilution

1. Lyophilized primers and probes must be centrifuged briefly to ensure that the DNA pellet is at the bottom of the tube before they are opened and reconstituted. Nuclease-free water or TE buffer (the correct pH of the TE buffer is

Table 1 Influenza Real-Time RT-PCR Probe and Primer Sequences

Specificity		Sequence (5′-3′)
Type A influenza-matrix gene	M+25 forward primer	AGA TGA GTC TTC TAA CCG AGG TCG
	M+64 probe[a]	[FAM]-TCA GGC CCC TCC AAA GCC GA-[BHQ-1]
	M−124 reverse primer	TGC AAA AAC ATC TTC AAG TCT CTG

[a]FAM = carboxyfluorescein and BHQ−1 = BlackHoleQuencher-1.

dye-dependent) should be used for the initial reconstitution of lyophilized primers and probes (*see* **Note 5**). Concentrated stock solutions should be stored at –70 °C. Primer stock solutions should be 200 µM (200 pmol/µL) (*see* **Note 6**); probe stock solutions should be 120 µM (120 pmol/µL). The quantitation information will be supplied for each oligo by the manufacturer.
2. Mix gently by tapping the tube, and allow the oligo to reconstitute for about 10 min before dilution of the working stocks.
3. Prepare the working stocks by diluting the primer master stock 1:10 (20 pmol/µL or 20 µM final concentration) and dilute the probe master stock probe 1:20 (6 pmol/µL or 6 µM final concentration) in nuclease-free H_2O. Do not use TE buffer for the working stocks. Working stocks should be stored at 4 °C. The probes are stable at this concentration at 4 °C for approximately 1 month (*see* **Note 7**).

3.2. rRT-PCR Reaction

1. Set up the real-time PCR instrument with the following reaction conditions: The reverse transcription step is one cycle of 30 min at 50 °C and 15 min at 94 °C. This reverse-transcription step is specific for the Qiagen OneStep RT-PCR Kit, which uses a hot-start *taq* polymerase that requires a 15–min 94 °C activation stage. The PCR phase conditions for the influenza primers and probes with the Smart Cycler are 45 cycles of 94 °C for 1 s and 60 °C for 20 s. The fluorescence data are collected during the 60 °C cycle (see Table 2). Cycle times for the PCR phase may vary slightly among different real-time PCR instruments. Information on setting up and programming a specific real-time PCR instrument can be found in the user's manual.
2. Place the reaction tubes in a cooling block, taking care not to damage optical surfaces on reaction tubes.
3. Prepare the reaction master mix (everything but the template) in a clean, 1.5-mL nuclease-free microcentrifuge tube. Use aerosol-resistant pipette tips throughout the procedure. Add the components (volume per reaction) in this order: 6.95 µL of H_2O, 5 µL of kit-supplied 5X reaction buffer (or equivalent), 0.8 µL of dNTP

Table 2 Real-Time RT-PCR Thermocycling Conditions for Type A Influenza (M Gene) Probe and Primer Sets

Phase	Step	Number of Cycles	Time	Temp
Reverse transcription[a]	Reverse transcription	1	30 min	50 °C
	Heat activation of polymerase		15 min	94 °C
PCR	Denaturation	45	1 s	94 °C
	Annealing/extension[b]		20 s	60 °C

[a]The reverse-transcription conditions provided are specific for the Qiagen OneStep RT-PCR Kit.
[b]The fluorescent signal is acquired at the annealing step.

Table 3 Real-Time RT-PCR Reaction Mix Volumes and Conditions for Type A Influenza (M Gene) with the Qiagen OneStep RT-PCR Kit

	Volume (µL) per Reaction	Final Concentration
H_2O	6.95	
5X	5	1X
25 mM $MgCl_2$	1.25	3.75 mM[a]
Enzyme mix	1	
Forward primer	0.5	10 pmol
Reverse primer	0.5	10 pmol
dNTPs	0.8	320 µM ea. dNTP
Probe	0.5	0.12 µM
Rnase inhibitor	0.5	13 units
MM per rxn	17	
Template	8	
Total	25 µL	

[a]The Qiagen OneStep RT-PCR Kit buffer contains 2.5 mM of $MgCl_2$.

mix (supplied in the Qiagen kit), 1.25 µL of 25 mM $MgCl_2$, 0.5 µL of forward primer, 0.5 µL of reverse primer, 0.5 µL of RNase inhibitor, 1 µL of kit-supplied enzyme (or equivalent) (see Table 3). Add the probe last (0.5 µL per reaction). Once the probe has been added, minimize exposure of the reaction mix to light. If an internal positive control is being used, it should be added at this step (*see* **Note 8**).

4. Mix by vortexing for 3–5 s, and centrifuge briefly to remove any liquid from the lid of the tube.
5. Add 17 µL of the reaction mix to each reaction tube.
6. Add the template to the reaction tubes (8 µL per reaction) in order as follows: Add the negative-control template (H_2O) to the appropriate tube, but do NOT seal the tube yet; then add each sample template, sealing the tubes as you add the template (if applicable); then add the positive-control template to the appropriate tube and seal the tube; finally, seal the negative-control tube (*see* **Note 9**).
7. Either whole-virus RNA or *in vitro* transcribed RNA may be used for the positive-control template (*see* **Note 10**). Nuclease-free water is used as the negative-control template.
8. Centrifuge the reaction tubes briefly, and run the real-time RT-PCR with the conditions described in Table 2.

3.3. Analysis of Results

1. Examine the positive and negative controls to ensure that the reaction conditions were correct (*see* **Note 11**). Evaluate the raw data from each sample individually on the fluorogram to ensure there are no irregularities or software artifacts, as

most instruments have correction and normalization functions that can occasionally distort the results.
2. The result is recorded as a cycle threshold (C_t) value, which is the PCR cycle where the fluorescence level increases to the point where it crosses a positive-negative cutoff value. The maximum possible value is 45. A negative sample has a C_t value of 0.00. On the Smart Cycler, the default value for linear increase in fluorescence signal (30 units) is used as the positive-negative cutoff value for the type A influenza test. In a diagnostic situation, any samples with an increase in fluorescence between 20 and 40 (such samples usually have a C_t value over 35) should be considered suspect and should be retested. In general, in a diagnostic situation, any questionable samples should be retested. If the results of the second test are unsatisfactory, additional sampling from the animal or flock should be considered if possible.
3. Specific criteria for the analysis of results may vary slightly by real-time PCR instrument. When specifics are not known, use the instrument defaults or consult with the manufacturer.

4. Notes

1. Real-time RT-PCR is inherently quantitative; therefore, absolute quantitation of specimens can be done fairly easily by the addition of a standard curve (RNA from serial dilutions of a virus of known titer) during a sample run. Because the correlation of virus titer to C_t value varies by virus isolate and even virus preparation due to factors such as defective particles, it is critical to use RNA from the same virus isolate, and optimally from the same stock that the virus in the sample is derived from, for the standard curve. For this reason, quantitation is most accurate when the target virus is known. It is also important to recognize that the quantitative axis is the PCR cycle axis (x-axis), not the fluorescence level (y-axis), which can actually be lower with reactions with more starting material since the reaction components will be used up and will plateau faster. All real-time PCR instruments have settings for quantitation and setting up a standard curve. Consult your user's manual or the manufacturer for details.
2. Because of the risk of cross-contamination with a two-step RT-PCR reaction, it is highly recommended only to use a one-step RT-PCR reaction procedure. Optimization of the enzyme amount, magnesium concentration, and cycling conditions may be necessary. Not all RT-PCR kits will provide acceptable performance, and they must include a polymerase with 5′ exonuclease activity.
3. FAM (6-carboxyfluorescein) and BHQ-1 (BlackHoleQuencher-1) are the recommended dyes for the Smart Cycler and are compatible with most instruments. If a different real-time PCR instrument is available, check if alternative dyes need to be used.

4. Primers for real-time RT-PCR need to be more highly purified than primers for sequencing or conventional PCR. Therefore, it is recommended to have primers purified by HPLC.
5. Probes are light-sensitive; store them in amber tubes if available or wrap in foil, and minimize their exposure to light.
6. Example of calculation for oligo reconstitution: You have 17,786 pmol of oligo (will be on oligo information sheet from manufacturer) and a concentration of 200 pmol/μL is needed for the stock. Divide the pmol of oligo by the pmol/μL needed, or 17,786 pmol/200 pmol per μL = 88.9 μL. Therefore, for 200 pmol/μL, reconstitute the pellet in 89 μL of TE buffer or nuclease-free H_2O. The calculation for the probe is the same, except divide the number of probe pmol by 120 pmol/μL.
7. To avoid repeated freeze-thaw cycles of primers and probes, freeze the master stocks in multiple small quantities (equivalent to the total amount for 1 month of working stock volume).
8. Internal positive control: The development of an internal positive control (IPC) for this test has recently been reported [8]. Use of an IPC is recommended, especially for samples that may contain inhibitors, such as fecal material.
9. Suggestions to help prevent cross-contamination: (1) Only use aerosol-resistant pipette tips; (2) centrifuge all reagents prior to use, especially freeze-dried materials; (3) handle and prepare samples in a biological safety cabinet throughout the procedure; (4) use separate areas for each stage of the procedure (e.g. separate biological safety cabinets for RNA extraction and RT-PCR reaction preparation); (5) minimize sample handling and manipulation; (6) change gloves frequently.
10. Positive-control RNA may be whole-virus RNA that has been diluted to have a specific cycle threshold value or target gene RNA can be *in vitro* transcribed from a plasmid with the target gene inserted. *In vitro* transcribed RNA provides a consistent and abundant source of RNA, which reduces the risk of cross-contamination with molecular tests that target other genes, because only RNA from a single gene is present. Positive-control RNA is often calibrated to a cycle threshold value of 25, with a valid range of 21 to 29, although it is uncommon to see more than 1 or 2 cycles in variation.
11. Troubleshooting:

    a. The positive controls are negative: (1) The control template has degraded; (2) the probe may be old and no longer properly fluoresces; (3) the enzymes may be inactivated; (4) an incorrect thermal-cycling program or fluorescence acquisition was used (incorrect setting or incorrect channel being viewed).
    b. The negative controls are positive: (1) There may be contamination in the samples or reagents; (2) there may be nonspecific probe degradation; repeat with fresh probe and primers.
    c. Background levels are too high or too low (consult your instrument user's manual or manufacturer for the correct levels for the dye being used):

(1) The probe concentration may be wrong; (2) the probe may be degraded or too old if the background level is too low.

d. Error message or an instrument error (calibration or failure occurs: consult the manufacturer of your machine.
e. Irregular fluorescence profile occurs: (1) There is an air bubble in the reaction tube; (2) the optical surface of the tube may be damaged.
f. "Railing," a sharp decrease in fluorescence after a steady increase may be caused by a probe concentration that is too high (may cause a warning message in sample status on results screen on the Smart Cycler).

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Chapter 5
Detection and Identification of the H5 Hemagglutinin Subtype by Real-Time RT-PCR

Erica Spackman and David L. Suarez

Summary Serological methods, gene sequencing, and RT-PCR-based methods have all been used for the identification of influenza virus hemagglutinin (HA) subtypes. Compared to serological methods and gene sequencing, RT-PCR is fast, sensitive, and relatively inexpensive. However, since RT-PCR generally lacks the specificity of sequencing or serology, the most practical application of RT-PCR methods for subtype identification is either to target a few of the most important subtypes such as H5 and H7 or to use it in situations where a specific strain is being targeted, such as during an outbreak or with experimental samples.

Since identification of viruses from the Asian-origin H5N1 highly pathogenic avian influenza virus lineage is a high priority worldwide, the procedure for real-time RT-PCR (rRT-PCR) identification of the H5 H subtype is presented here. This assay can identify the H5 hemagglutinin of any genetic lineage (North American or Asian) and either pathotype (highly pathogenic or low pathogenic), but does not differentiate between subtypes or pathotypes.

Keywords H5 HA subtype; real-time RT-PCR; subtype identification; H5N1 HPAI virus.

1. Introduction

Influenza viruses are known for their extreme antigenic variation of their surface glycoproteins: the hemagglutinin (HA) and neuraminidase (NA). The influenza virus has 16 distinct antigenic subtypes of the HA subtype and 9 subtypes of the NA subtype (see Chapter 1). By definition, antibody raised against one subtype cannot neutralize the activity of another subtype. Of the 16 recognized HA subtypes, the H5 and H7 subtypes are the most important for poultry because of the potential for viruses of these subtypes to be, or to become, highly pathogenic (HP). In addition, although the other 14 HA subtypes may infect poultry and cause serious disease outbreaks, only the H5 and H7 viruses, regardless of whether they are low pathogenic or highly pathogenic, are reportable to the World Organization of Animal Health.

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Recently, it has become critical to immediately identify the HA and NA subtypes (e.g., H5N1) of influenza viruses once they are detected by type-specific tests such as virus isolation (see Chapter 6), antigen immunoassays, or RT-PCR methods (see Chapter 4). The use of serological methods, the hemagglutination inhibition (HI) (see Chapter 8) or neuraminidase inhibition (NI), (see Chapter 9) assays is the current "gold standard" reference method used to determine a virus subtype. However, serological methods do have some inherent error because of antigenic variation within a subtype and, in some cases, cross-reactivity between subtypes. In addition, the HI and NI assays are expensive and time-consuming, partly because they require a panel of sera that includes every subtype and, in some cases, more than one reference sera for a subtype in order to produce accurate results. The requirement of an extensive reagent panel restricts blind subtype identification of all 16 H and 9 NA subtypes by serological methods to mostly reference laboratories.

An alternative method for subtype identification is direct sequencing of the HA and NA genes, which is often accomplished by amplifying the target genes by RT-PCR with primers directed to the ends of the gene segments, which are conserved among all isolates and gene segments [1]. This method allows for the nonsubtype-specific amplification of HA and NA genes, but requires a gene cloning step. Gene sequencing is probably the most accurate method; however, it is slow, relatively expensive, and more technically difficult than either classical methods or RT-PCR. Therefore, gene sequencing is primarily used as a research tool and not as a primary diagnostic method.

Because of the high speed and relatively low cost, there is considerable interest in using RT-PCR-based methods directly targeted to the different HA and NA subtypes as primary diagnostic tests or as follow-up tests for type A influenza-directed tests. Numerous RT-PCR-based tests have been described for some of the most common and economically important HA and NA subtypes [2–6]. Although RT-PCR can be a valuable tool for subtype identification, particularly during outbreaks or other situations where the sequence of the target virus is known, it has similar shortcomings to serological methods due to the high sequence variation within subtypes. Therefore, false negatives are a concern. In rare cases, false positives may occur when the targeted region of a gene is conserved across subtypes. Finally, just as with serological methods, blind subtype identification of all 16 HA and 9 NA subtypes would be expensive and inefficient, as a large panel of reagents, in this case at least 25 sets of primers, would be needed to directly identify all HA and NA subtypes by RT-PCR.

Despite the previously mentioned problems with RT-PCR-based methods for blind subtype identification, because of the speed and cost, RT-PCR methods have been employed in cases where the identification of one or two subtypes is particularly critical; for example, the H5 and H7 HA subtypes because of their potential to be highly pathogenic in poultry.

Due to the current importance of the H5 HA subtype (i.e., the Asian-origin H5N1 highly pathogenic strains), a protocol will be presented. An additional advantage of this protocol is that it is known to detect all H5 subtype viruses regardless of genetic

lineage. Finally, it is critical to understand that this test can detect both low pathogenic and highly pathogenic AI viruses and can detect viruses of either the North American or Eurasian H5 H lineages [5] but does not differentiate among them.

In order to detect all H5 subtype viruses with optimal sensitivity, two forward primers—one with preferential specificity for North American lineage viruses and one with preferential specificity for Eurasian lineage viruses—are used. Each primer will amplify genes from viruses of the heterologous lineage; however, the sensitivity is decreased approximately 10- to 50-fold. When the specificity of the target is known, the primer of the other specificity may be left out and the volume can be replaced with water.

Although the product size is small (229 bp in length), this procedure may be adapted for conventional RT-PCR with minor modification (*see* **Note 1**).

As with the type A rRT-PCR test, the H5 HA subtype test can be used with RNA from any sample type. However, the extraction process is critical and must be appropriate for the specimen. Details on sample processing can be found in Chapters 2 and 3. In samples containing fecal material, the use of an internal positive control is highly recommended (*see* **Note 2**).

2. Materials

Reagent and rRT-PCR details including primer and probe handling are covered in Chapter 4. Mention of trade names or commercial products in this procedure is solely for the purpose of providing specific information and does not imply recommendation or endorsement.

1. Real-time PCR instrument. The conditions for the Cepheid Smart Cycler are provided in this volume. Conditions for air thermal cyclers will be similar. Conditions for Peltier block-type instruments can be optimized by extending cycle times.
2. Reagent-grade H_2O (nuclease-free).
3. Pipettors and aerosol-resistant tips for volumes between 1 µL and 1 mL.
4. 1.5-mL microcentrifuge tubes (nuclease-free).
5. Microcentrifuge.
6. Qiagen OneStep RT-PCR Kit (reverse transcriptase, *taq* polymerase, dNTPs, reaction buffer) or equivalent reagents (*see* **Note 3**).
7. Dual-labeled probes (see Table 1).
8. Primers (see Table 1).
9. $MgCl_2$ (25 mM stock).
10. RNase inhibitor (13 units/µL stock).
11. Positive-control RNA (*see* **Note 4**).
12. 25-µL Smart Cycler tubes or appropriate reaction vessel for the real-time PCR instrument used.

Table 1 Influenza Real-Time RT-PCR Probe and Primer Sequences; Protocols for the H5 Subtype

Specificity		Sequence (5′–3′)
H5 H subtype-all	H5+1637 Probe	[FAM]-TCA ACA GTG GCG AGT TCC CTA GCA-[BHQ-1]
	H5−1685 Reverse primer	AGA CCA GCT ATC ATG ATT GC
H5 subtype-North American	H5+1456 NA forward primer	ACG TAT GAC TAT CCA CAA TAC TCA
H5 subtype-Eurasian	H5+1456 EA forward primer	ACG TAT GAC TAC CCG CAG TAT TCA

3. Methods

3.1. Instrumentation

Information on setting up and programming specific real-time PCR instruments can be found in the user's manual. The conditions for the H5 HA subtype primers and probes on the Smart Cycler are shown in Table 2. Note that in this protocol there is a 15-minute incubation step at 94 °C after the reverse-transcription step that is necessary to activate the hot start *taq* polymerase included in the Qiagen OneStep RT-PCR Kit enzyme blend. Therefore, optimal conditions for amplification may be different with enzymes from other sources. Cycle times for the PCR phase may vary among different real-time PCR instruments; for example, the length of each step needs to be increased for instruments with a slower ramp time.

3.2. Reaction Setup

1. Reactions should be set up with the tubes in a cooling block, taking care not to damage optical surfaces. Use aerosol-resistant pipette tips throughout the procedure.
2. Prepare the reaction mix (everything but the template) by pipetting the H_2O, kit-supplied 5X reaction buffer (or equivalent), dNTPs (supplied in the Qiagen kit), and $MgCl_2$ into a clean, nuclease-free microcentrifuge tube using the volumes per reaction for each component specified in Table 3 (*see* **Note 5**). Next add the RNase inhibitor and enzyme. Add the probe last. Once the probe has been added, minimize exposure of the reaction mix to light.
3. Mix by vortexing for 3–5 s and centrifuge briefly.
4. Add 17 µL of the reaction mix to each reaction tube.
5. Add the template to the reaction tubes (8 µL per reaction). Nuclease-free water is used as the negative-control template. Add the positive control last.
6. Centrifuge the reaction tubes briefly, visually confirm that the reaction mix is the correct volume, and run the real-time RT-PCR with the temperature cycling conditions described in Table 2.

Table 2 Thermocycling Conditions for Gene-Specific Probe and Primer Sets

Phase	Step	Number of Cycles	Time	Temp.
Reverse transcription[a]	Reverse transcription	1	30 min.	50 °C
	Heat activation of polymerase		15 min.	95 °C
PCR	Denaturation	40	10 sec.	94 °C
	Annealing[b]		30 sec.	54 °C
	Extension		10 sec.	72 °C

[a]The reverse-transcription conditions provided are specific for the Qiagen OneStep RT-PCR Kit.

[b]The fluorescent signal is acquired at the annealing step.

Table 3 Real-Time RT-PCR Reaction Mix Volumes and Conditions for the H5 H Subtype

	Volume (µL) per Reaction	Final Concentration
H_2O	6.45 µL	
5X	5	1X
25 mM $MgCl_2$	1.25	3.75 mM[a]
Enzyme mix	1	
H5 + 1456 EA forward primer	0.5	10 pmol
H5 + 1456 NA forward primer	0.5	10 pmol
Reverse primer	0.5	10 pmol
dNTPs	0.8	320 µM ea. dNTP
Probe	0.5	0.12 µM
RNase inhibitor	0.5	13 units
MM per rxn	17	
Template	8	
Total	25 µL	

[a]The Qiagen OneStep RT-PCR Kit buffer contains 2.5 mM of $MgCl_2$.

3.3. Analysis of Results

Specific criteria for the analysis of results vary by real-time PCR instrument. When specifics are not known, use the instrument defaults or consult with the manufacturer. Always examine the raw data from each sample individually on the fluorogram to ensure there are no irregularities or software artifacts, as most instruments have correction and normalization functions that can sometimes distort the results (*see* **Note 6**).

On the Smart Cycler the linear increase in the fluorescence signal is used as the positive-negative cutoff threshold. The default value of 30 units is used for the H5 subtype rRT-PCR test. In general, in a diagnostic situation, any questionable samples should be retested. Due to the high level of sequence variation within an HA

subtype, a negative rRT-PCR result does not completely exclude the possibility that an H5 subtype virus is present. The results should be verified with sequencing or serological tests on an isolate.

4. Notes

1. This test can be adapted to standard RT-PCR by excluding the probe from the master mix and using the following cycle conditions for the PCR phase: 35 cycles of 94 °C for 1 min, 54 °C for 30 s, and 72 °C for 1 min. The product is 229 bp in length and can be visualized on a 1.5-2% agarose gel.
2. The development of an internal positive control (IPC) for this test has been reported [6]. Use of an IPC is recommended, especially for samples that may contain inhibitors, such as cloacal swabs.
3. Because of the risk of cross-contamination with a two-step RT-PCR reaction, it is highly recommended only to use a one-step RT-PCR reaction procedure. Optimization of the enzyme amount, magnesium concentration, and cycling conditions may be necessary. Not all RT-PCR kits will provide acceptable performance, and they must include a polymerase with 5' exonuclease activity.
4. Positive-control RNA may be whole-virus RNA that has been diluted to have a specific cycle threshold value, or target gene RNA can be *in vitro* transcribed from a plasmid with the target gene inserted. *In vitro* transcribed RNA provides a consistent and abundant source of RNA, which reduces the risk of cross-contamination with molecular tests that target other genes because only RNA from a single gene is present. Positive-control RNA is often calibrated to a cycle threshold value of 25, with a valid range of 21 to 29, although it is uncommon to see more than 1 or 2 cycles in variation.
5. Two forward primers are used in this protocol. However, if sensitivity for only one lineage is desired, the test can be run with one forward primer, by adding water to compensate for the volume of the second primer.
6. For troubleshooting tips, see Chapter 4.

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Chapter 6
Avian Influenza Virus Isolation and Propagation in Chicken Eggs

Peter R. Woolcock

Summary The avian influenza (AI) virus is usually isolated and propagated by inoculating either swab or tissue samples from infected birds into the chorioallantoic sac of embryonating chicken eggs. This is the accepted method, but occasionally an isolation may only be successful when inoculated either into the yolk sac or onto the chorioallantoic membrane of embryonating chicken eggs. Chorioallantoic fluid is harvested from eggs with dead or dying embryos and is tested for the presence of hemagglutinating antigen. If hemagglutination-positive, this indicates that the isolate may be the AI virus. The presence of the AI virus may be confirmed by either an agar gel immunodiffusion (AGID) assay, RT-PCR specific for AI virus, or a commercially available immunoassay kit specific for type A influenza. Instructions for AI virus primary isolation and propagation, preparing antigen for an AGID test, setting up an AGID test, and interpreting results are given.

Keywords avian influenza; virus isolation; agar gel immunodiffusion (AGID)

1. Introduction

Avian influenza (AI) viruses, which are type A influenza, have been isolated from a wide range of avian species, including poultry, waterfowl, ratites, passerines, and psittacines [1–4]. Despite the diverse avian host range, the AI virus can usually be isolated in embryonating chicken eggs when inoculated into the chorioallantoic sac (CAS). In the absence of bacterial contamination, amnioallantoic fluid (AAF) that hemagglutinates chicken erythrocytes indicates the probability of an influenza A virus being present, although the presence of an avian paramyxovirus (APMV) needs to be ruled out (*see* **Note 1**). The presence of the AI virus can be confirmed by an agar gel immunodiffusion (AGID) assay by demonstrating the presence of the nucleocapsid or matrix antigens, both of which are common to all influenza A viruses. Various immunoassays are now also available as commercial kits; they detect nucleocapsid or viral neuraminidase of type A influenza virus using

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monoclonal antibodies [3, 5]. Alternatively, real-time RT-PCR for the AI virus may be performed to identify the isolate.

Primary isolation of AI viruses by inoculation of the suspect sample into embryonating chicken eggs by the CAS route is the standard recommended method, but while isolating the AI virus from diagnostic specimens, Woolcock et al. [3] found that in some cases isolation was not successful unless the yolk sac (YS) or chorioallantoic membrane (CAM) routes of inoculation were used. This was the case with some AI viruses isolated from nonchicken species. If isolation is successful by the CAS route, the CAM and YS methods need not be pursued, but these latter methods should be attempted if the AI virus is suspected and an isolation is proving difficult.

Although virus isolation in chicken embryos is expensive and can take a few weeks, it is probably the most sensitive method for detecting viable virus. Because numerous agents will replicate in chicken embryos, virus isolation is not the most specific method, and all primary isolations need to be confirmed as previously mentioned.

Embryonated chicken eggs are also a standard way to propagate AI viruses because the virus can be grown rapidly and to high titers, although continuous passage in embryos will cause some egg adaptation mutation to occur. Virus titration is also normally performed in embryonated chicken eggs.

2. Materials

2.1. Virus Isolation and Propagation in Chicken Embryos

2.1.1. General Lab Equipment

1. Class II Biosafety cabinet; Class III if Asian HPAI H5N1 “bird flu” or human infectious virus is suspected or known to be present (*see* **Note 2**).
2. Egg incubator.
3. Egg candling light.
4. Centrifuge, preferably refrigerated.
5. Refrigerator.
6. −70 °C freezer or liquid nitrogen freezer.
7. 1–, 5–, 10–mL pipettes (sterile).
8. Pipette aid or a suction bulb.
9. Latex gloves or equivalent.
10. Laboratory coat or gown.
11. Biohazard bags.
12. Autoclave.
13. Egg punch; an 18 gauge syringe needle punched through a rubber stopper suffices.

2.1.2. Tissue Processing

1. Preferred specimens: trachea, lung, air sac, intestine, sinus exudates.
2. Forceps and scissors (sterile).
3. Mortars and pestles or Ten Broeck tissue grinders (sterile) or Stomacher Lab Blender and bags.
4. Silica (silicon dioxide, fine granular) (sterile).
5. Cryovials (2 mL) (sterile).
6. 15- and 50-mL polypropylene centrifuge tubes (sterile).
7. 5.0-, 0.8-, 0.45-, and 0.2-µm syringe filters (or combined filters) or antibiotic cocktail. The antibiotic cocktail should be prepared in tris-buffered tryptose broth. Recommended final concentrations/mL are: penicillin G 10,000 IU/mL, streptomycin sulfate 2,000 IU/mL, kanamycin sulfate 650 µg/mL, gentamicin sulfate 1,000 µg/mL, mycostatin 20 IU/mL.
8. 3-cc and 12-cc syringes (sterile).
9. 1.5-in. × 20 gauge syringe needles (sterile).
10. Virus transport medium (VTM). Several VTM may be used: brain-heart infusion broth or a buffered balanced-salt solution with or without the addition of serum; both should contain antibiotics.

2.1.3. Swab Processing

1. Preferred specimens: tracheal, oropharyngeal, and cloacal swabs (*see* Chapter 2).
2. Virus transport medium (VTM). Several VTM may be used: brain-heart infusion broth or a buffered balanced-salt solution with or without the addition of serum; both should contain antibiotics.
3. Vortex mixer.
4. 15- and 50-mL polypropylene centrifuge tubes (sterile).
5. 5.0-, 0.8-, 0.45-, and 0.2-µm syringe filters (or combined filters) or antibiotic cocktail. The antibiotic cocktail should be prepared in tris-buffered tryptose broth. Recommended final concentrations/mL are: penicillin G 10,000 IU/mL, streptomycin sulfate 2,000 IU/mL, kanamycin sulfate 650 µg/mL, gentamicin sulfate 1,000 µg/mL, mycostatin 20 IU/mL.
6. 3-cc and 12-cc syringes (sterile).
7. 1.5-in. × 20 gauge syringe needles (sterile).

2.1.4. Egg Inoculation

1. 9–11-day-old embryonating chicken eggs, preferably from a specific pathogen-free source (CAS and CAM methods).
2. 5–7-day-old embryonating chicken eggs, preferably from a specific pathogen-free source (YS method).
3. 70% Ethanol.

4. Egg punch.
5. 1-cc tuberculin syringes (sterile).
6. 5/8-in. × 25 gauge syringe needles (sterile).
7. Elmer's Glue-all or equivalent to seal inoculation hole in egg shell.
8. Rubber pipette bulb for CAM inoculation.
9. 3-cc or 5-cc syringe with 18 gauge needle.

2.2. AGID

1. Glass flask.
2. Vacuum pump.
3. Vacuum flask with Pasteur pipette or 1-cc syringe fitted to end of tubing.
4. Humidified chamber.
5. Agarose.
6. NaCl.
7. HCl.
8. Glycin/sarcosyl buffer: 1% (w/v) sodium lauroyl sarcosinate buffered to pH 9.0 with 0.5 M glycine.
9. AI virus reference serum and antigen (National Veterinary Services Laboratories, USDA-APHIS, Ames, IA).
10. AGID template for cutting agarose (Veterinary Diagnostic Technology, Inc., Wheat Ridge, CO).

3. Methods

3.1. Virus Isolation and Propagation in Chicken Embryos

3.1.1. Tissue Processing

1. Mince tissue(s) with sterile scissors, then triturate with a mortar, pestle, and silica or a Ten Broeck grinder or a Stomacher, using VTM at a concentration of 1:10 w/v. Transfer supernatant to a sterile centrifuge tube.
2. Clarify by centrifugation at 1780Xg for 15 min at 4 °C.
3. Save some of the supernatant for storage at −80 °C (for future reference).
4. Treat the supernatant for bacterial contamination by one of the following methods:
   a. Filter the remaining supernatant through 5.0-, 0.8-, and 0.45-µm filters with a final 0.2-µm syringe filter to provide enough material for a pair of 2-mL cryovials: one for egg inoculation and one for storage at −80 °C.
   b. Treat the supernatant with an equal volume of antibiotic cocktail (*see* **Note 3**) for a minimum of 1 hr at room temperature prior to egg inoculation.

3.1.2. Swab Processing

1. Mix the swabs in up to 5 mL of VTM (in the swab tubes they were collected in) by briefly vortexing. Even if the swab is not present, mix the material.
2. Treat the supernatant for bacterial contamination by one of the following methods:
   a. Filter the swab material through a final 0.2-µm syringe filter into a pair of 2-mL cryovials: one for egg inoculation and one for storage at −80 °C.
   b. Treat the swab material with an equal volume of antibiotic cocktail (*see* **Note 3**) for a minimum of 1 hr at room temperature prior to egg inoculation.

3.1.3. Virus Propagation and Titration

1. Prepare 10-fold serial dilutions of AAF containing infectious virus in VTM (or cell culture material) to 10^{-4} for virus propagation or to 10^{-9} for titration.
2. Inoculate 5 eggs with 100 µL of material by the CAS route with each dilution for propagation or titration as follows: For propagation, inoculate from 10^{-2} to 10^{-4} (15 eggs total). Dilution reduces the MOI, which generally yields higher titers upon propagation. For titration, inoculate the dilutions from 10^{-4} to 10^{-9} (30 eggs total). This range will normally bracket the 50% infectious endpoint.
3. Mark the eggs with a pencil or china marker (do not use a felt-tip marker), mist with 70% ethanol, and allow to dry. Carefully make a hole in the egg shell with the egg punch as shown in Figure 1 (*see* **Note 6**).
   a. For CAS inoculation, use a 1-cc syringe with tuberculin needle. Insert the entire needle vertically through the hole and inject the desired amount of inoculum (100 or 200 µL) into the allantoic sac. Avoid moving the syringer sideways once the needle is inserted to prevent tearing of the CAM, which could cause bleeding and embryo death.
4. The eggs are candled daily as with primary isolation (see Section 3.1.4); however, at 4–5 days' postinoculation, the eggs can be harvested (or chilled up to 24 hr) (*see* **Note 4**). Each embryo is tested for virus replication by hemagglutination assay (see Chapter 7).
5. When propagating virus, AAF from hemagglutination-positive eggs is pooled and aliquoted for long-term storage at −70 °C or lower. For titration, the number of hemagglutinating eggs per dilution is used to calculate the 50% infectious dose per 100 µL [3].

3.1.4. Primary Isolation

1. For primary isolation, inoculate five 9–11-day-old embryonating SPF chicken eggs per specimen by the chorioallantoic sac (CAS) route with 0.2 mL of inoculum per egg. (If necessary, because initial isolation attempts fail: Also inoculate five 9–11-day-old embryonating SPF chicken eggs by the CAM route and three 5–7-day-old embryonating eggs by the YS route.)

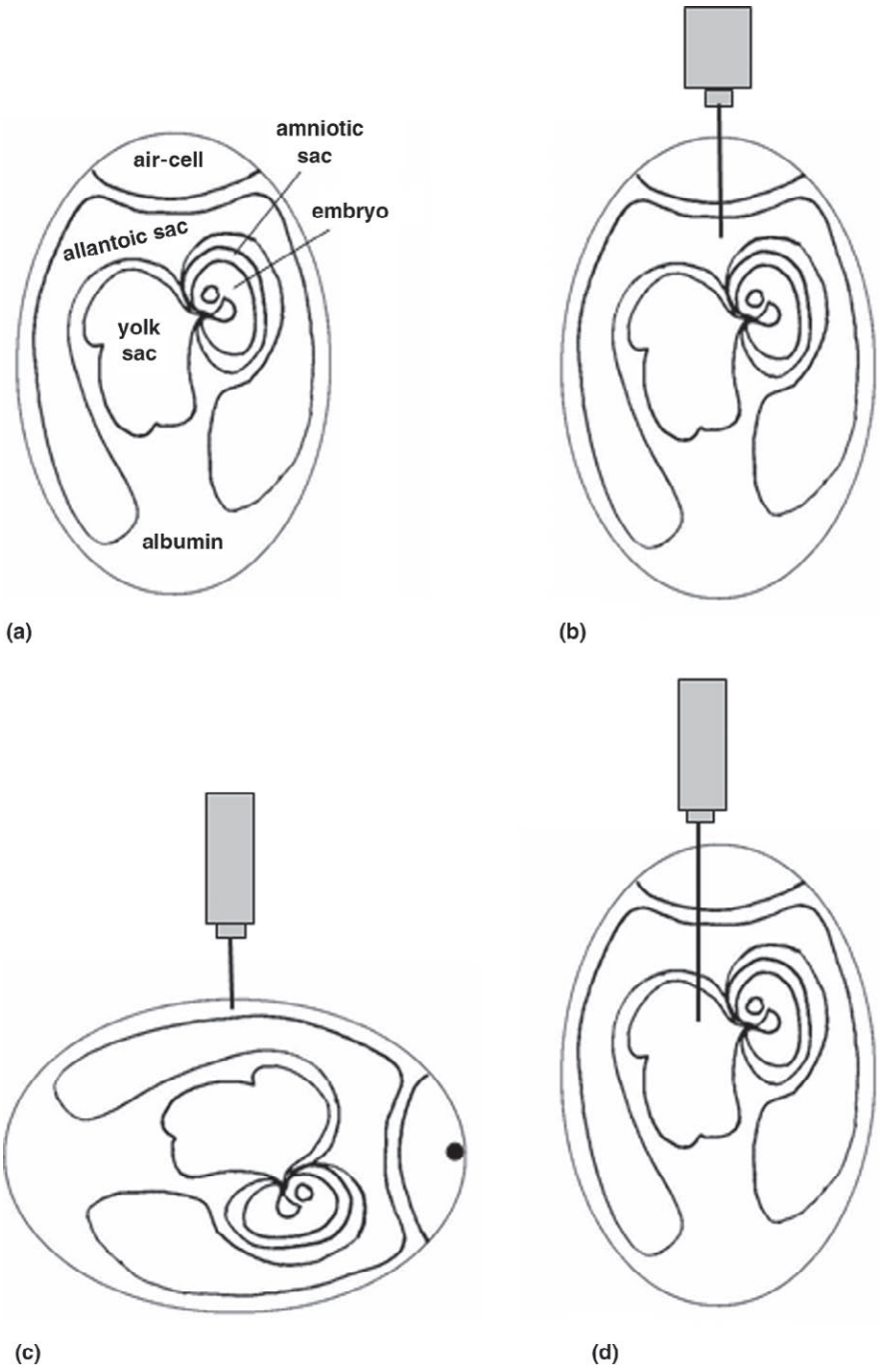

Figure 1 Approximate structure of the avian embryo (a) relative to the inoculation sites for (b) CAS, (c) CAM, and (d) YS inoculation methods. Approximate placement of the syringe is shown. Extra hole in the air cell for CAM inoculation is shown with a black dot

2. As a negative control, inoculate three eggs, for each route, with sterile VTM.
3. Mark the eggs with a pencil or china marker (do not use a felt-tip marker), mist with 70% ethanol, and allow to dry. Carefully make a hole in the egg shell with the egg punch as shown in Figure 1 (*see* **Note 6**).
   a. For CAS inoculation, use a 1-cc syringe with tuberculin needle, insert the entire needle into the lower hole (not the air-cell hole), and inoculate 200 µL of material per egg.
   b. For CAM inoculation, use a 1-cc syringe with a tuberculin needle; insert the needle into the hole on the side of the egg (not the air-cell hole) so the tip is just inside the egg shell, taking care not to puncture the egg membrane; and deposit 100 µL of material onto the CAM.
   c. For YS inoculation, use a 1-cc syringe with a 1-in. (2.5-cm) 21 gauge needle, insert the entire needle straight down through the hole, and inoculate 200 µL of material.
4. Seal eggs with glue and incubate at 37 °C with passive humidity.
5. Candle eggs daily for a period of 4 to 5 days (CAS route) or 7 days (CAM and YS routes). Remove eggs with dead embryos and chill at 4 °C. Eggs with dead embryos within 24 hours' postinoculation are discarded as nonspecific; however, highly pathogenic avian influenza (HPAI) virus can kill embryos within 24 hours (*see* **Note 3**).
6. After chilling, place the eggs in a Class II Biosafety hood and mist the egg shells with 70% alcohol. Remove the portion of the shell above the air cell with sterile forceps and harvest the amnioallantoic fluid (AAF) with a syringe and a 20 gauge 1.5-in. needle into two 2-mL cryovials for hemagglutination testing (see Chapter 7) and serial passage (if necessary). Save the remainder in a sterile centrifuge tube. Using sterile forceps, scissors, and a Petri dish, examine the embryos for lesions if necessary. The embryo body after removing the head, legs, wings, and skin can be triturated for serial passage (if necessary) (*see* **Note 7**). Alternatively, the AAF can be harvested by inserting an 18 gauge needle on a 3-cc or 5-cc syringe into the inoculation hole and withdrawing the fluid (one may have to move the needle around to avoid the embryo, membrane, and yolk sac). The AAF should be clear, slightly yellow, or occasionally milky. Red, brown, thick yellow, or dark cloudy AAF are signs of bacterial contamination and/or excessive embryo deterioration.
7. At 4–7 days' postinoculation, remaining eggs with live embryos are chilled to 4 °C for 2 to 24 hr. From all eggs, harvest AAF, CAMs, or YSs depending on the route of inoculation, and examine embryos for lesions.
8. All AAF is tested individually for each egg for the presence of hemagglutinating agents (see Chapter 7).
9. A minimum of two passages should be performed before determining that a specimen is negative for the AI virus.

3.1.5. Confirmation of the Presence of the AI Virus in a Primary Isolation

1. Once the AAF has been determined to be hemagglutination-positive, the possibility of the presence of other hemagglutinating viruses needs to be eliminated;

these include the avian paramyxoviruses (APMVs), usually types 1, 2, and 3, and are checked by the hemagglutination-inhibition (HI) test (see Chapter 8).
2. Confirmation for the AI virus can be determined in three ways:

   a. Agar gel immunodiffusion assay
   b. Commercial immunoassay kits
   c. Real-time RT-PCR

3.2. AGID

3.2.1. Preparation of AGID Antigen from AAF

The antigens may be prepared by concentrating virus from infective AAF by acid precipitation or by extraction from infected chorioallantoic membranes (*see* **Note 5**).

1. Acid precipitation: Add 1.0 M HCl to infective allantoic fluid until it is approximately pH 4.0. Place mixture in an ice bath for 1 hr, and then clarify by centrifugation at 1000 Xg at 4 °C. The supernatant fluid is discarded. The virus concentrate is resuspended in glycin/sarcosyl buffer. The concentrate contains both nucleocapsid, which is the target for the AGID assay, and matrix polypeptides.
2. Preparation of antigen from CAMs: Remove CAMs from infected eggs that have AAF with hemagglutinating activity. Rinse CAMs with PBS and then drain fluid from membranes. Homogenize the harvested membranes by trituration or with a Ten Broek grinder (prepare at least 1 mL), freeze-thaw three times, and then centrifuge at 1000Xg for 10 min. Collect the supernatant. Discard pellet. Add formalin to 0.1% final concentration.

3.2.2. Preparation of AGID Agar and Plates

1. Weigh 8.0 g of NaCl and 0.9 g of agarose and add PBS to 100 mL in a glass flask.
2. Mix well to dissolve NaCl.
3. Autoclave for 10 min or microwave for 5 min at 100% power. Mix solution well while hot.
4. Let solution cool, but not harden (agarose may be kept liquid by keeping the flask in a 56 °C bath during use), then dispense 17-mL aliquots into tubes with caps. Store in a refrigerator up to several months.
5. Reheat tube at low power in a microwave to melt agarose (or place in a boiling water bath) when you are ready to use it.
6. Plate preparation: Pour 17 mL into a 15 × 100-mm Petri dish, place on a level surface, and allow to cool.

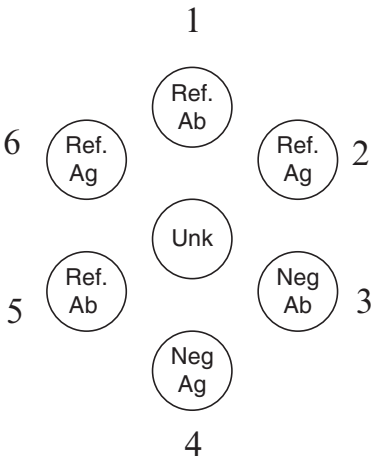

Figure 2 Layout of the AGID assay. Wells 1 and 5 contain reference (positive-control) antibody; wells 2 and 6 contain reference (positive-control) virus antigen; well 3 contains the negative-control antibody; well 4 contains the negative-control antigen (AI virus-free AAF without); the center well contains the unknown serum or antigen

7. Immediately before use, prepare wells in the agarose by pressing the pattern template (see Figure 2) into the hardened agarose. Remove agar plugs with a syringe or Pasteur pipette attached to a vacuum flask.

3.2.3. AGID Test Procedure

1. On the bottom of the Petri dish, label the outer wells of each pattern 1 through 6 clockwise (see Figure 2).
2. Using a micropipette, add 50 µL of the unknown antigen to be tested for identity to the center well. AGID may also be used to assay for antibody by using unknown or test serum in the center well instead of AAF.
3. Pipet 50 µL of AI virus reference antiserum into wells 1 and 5.
4. Pipet 50 µL of negative chicken serum as a control into well 3.
5. Pipet 50 µL of a heterologous (negative-control) antigen into well 4 (e.g., AGID antigens prepared from normal CAM antigen).
6. Pipet 50 µL of AI virus reference antigen into wells 2 and 6. Place a lid on the Petri dish and place in a humidified chamber for 24 hr at room temperature.
7. As a positive control, set up another pattern as described in steps 3 through 6 with the exception that AI virus reference antigen is placed in the center well instead of the unidentified antigen (see Figure 3).
8. Examine the plates at 24 hr for the presence of precipitin lines by holding the plate over a light source. Record observations. If negative, incubate for an additional 48 hr, since some weak positives may take this long to be visible.

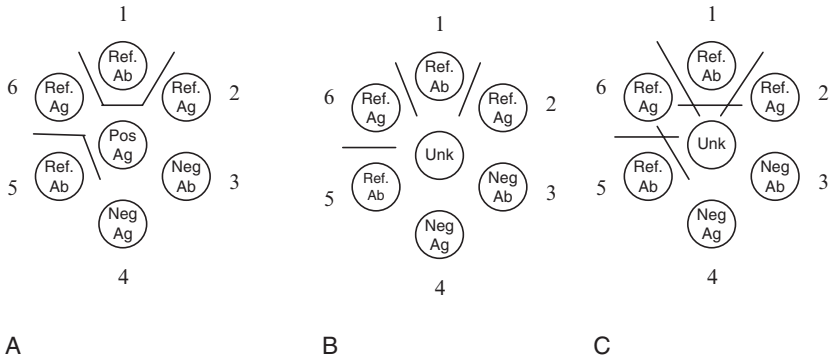

Figure 3 Examples of the expected precipitin lines in a (a) positive-control plate where a known positive antigen is in the center well. In a positive-control serum plate, the lines of identity will be between the center well and wells 2 and 6 instead of wells 1 and 5. (b) Negative plate with no precipitin lines from the unknown. (c) Negative plate with precipitin lines of non-identity. Lines of non-identity (due to nonspecific reaction) are distinguished from lines of identity by the "spurs" or they cross each other. In some cases (partial identity), only one of the lines will cross; this is still a negative result

3.2.4. Interpretation of the AGID Test

1. Positive-control plate: For a valid test, precipitin lines of identity must be observed in the positive-control plate between the wells containing reference antiserum (wells 1 and 5) and AI virus reference antigen (center well and wells 2 and 6). Precipitin lines of identity do not have spurs where the precipitin lines make an angle. If spurs are seen at points of angle, the antigen and antiserum are not homologous. Also, for a valid test, no precipitin lines should form between antigen wells and wells that contain SPF chicken serum nor between the wells that contain heterologous (negative) antigen and wells that contain either AI virus antiserum or negative SPF chicken serum (see Figure 3).
2. Test plate: In each test plate precipitin lines must form between wells containing AI virus positive-control antiserum and AI virus reference antigen. No precipitin lines should form between wells containing negative SPF chicken serum and wells containing AI virus reference antigen. No precipitin lines should form between wells containing AI virus antiserum and wells containing heterologous (negative) antigen. The sample is positive if there is a line of identity between the test sample (center well) and the positive control serum (see Figure 3).

4. Notes

1. The presence or absence of APMV can be determined by HI testing with specific APMV antisera. Tests for APMV 1, 2, and 3 are the most commonly performed.

2. A minimum of Class II Biosafety cabinet should be used for all procedures when working with suspect AI virus cases. If highly pathogenic AI (HPAI) Asian H5N1 "bird flu" virus, which is associated with human deaths, or any AI virus known to infect humans is suspected or known to be present, Class III safety conditions should be followed. Consult your occupational health professional for details on personal protective equipment.
3. If more than one embryo is dead at 24 hours, it is advisable to repeat the passage unless HPAI virus is suspected. When repeating the passage, it may be necessary to treat the material again for bacteria using a different method than that used for the first passage. Filtering is generally considered more efficient for removing bacteria but can remove infectious virus, too. Alternatively, one can test for the presence of bacterial contamination by inoculation of AAF from the dead embryo into thyoglycolate media, which is then incubated overnight at 37 °C and evaluated for bacterial growth.
4. For virus propagation, negative-control eggs are not necessary. As with primary isolations, eggs dying within 24 hours' postinoculation are discarded, unless highly pathogenic AI virus is being propagated where mortality within 24 hours is expected. Candle eggs daily and harvest AAF immediately after the eggs have died. Keep AAF from dead eggs separate from AAF from surviving eggs and test for hemagglutination separately. Eggs for propagation may be incubated for 4 to 5 days' postinoculation and then can be stored at 4 °C for up to 24 hours; the AAF from surviving eggs should be harvested within 24 hours of the eggs' being chilled.
5. Acid precipitation is preferred, unless a large volume of AAF is available for ultracentrifugation.
6. For the CAS and YS methods, the holes should be punched all the way through the membrane. To prepare an egg for CAM inoculation, lay the egg on its side, punch the hole in the air-cell piercing the membrane, and then carefully punch a hole in the side of the egg (see Figure 1) without piercing the membrane. Once the holes are punched, use a pipette bulb to drop the CAM by using the bulb to suck a small amount of air out of the egg through the hole in the air cell; the CAM will pull away from the eggshell. Only drop about 1 cm of CAM (more will fall as the egg incubates). Illuminate the egg with an egg candle to see the membrane during the procedure.
7. CAMs from HA-positive eggs may be saved for preparation of test antigen for the AI virus agar gel immunodiffusion (AGID) assay.

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Chapter 7
Hemagglutination Assay for the Avian Influenza Virus

Mary Lea Killian

Summary The hemagglutination (HA) assay is a tool used to screen cell culture or amnioallantoic fluid harvested from embryonating chicken eggs for hemagglutinating agents, such as type A influenza. The HA assay is not an identification assay, as other agents also have hemagglutinating properties. Live and inactivated viruses are detected by the HA test. Amplification by virus isolation in embryonating chicken eggs or cell culture is typically required before HA activity can be detected from a clinical sample. The test is, to some extent, quantitative [1 hemagglutinating unit (HAU) is equal to approximately 5–6 logs of virus]. It is inexpensive and relatively simple to conduct. Several factors (quality of chicken erythrocytes, laboratory temperature, laboratory equipment, technical expertise of the user) may contribute to slight differences in the interpretation of the test each time it is run. This chapter will describe the methods validated and used by the National Veterinary Services Laboratories (NVSL) for screening and identification of hemagglutinating viruses.

Keywords hemagglutination; amnioallantoic fluid; erythrocyte.

1. Introduction

The hemagglutinin protein on the surface of influenza virus particles is capable of binding to *N*-acetylneuraminic acid-containing proteins on avian and mammalian erythrocytes [1, 2]. When combined, if the influenza virus is present in a high enough concentration, there is an agglutination reaction and the erythrocytes link together to form a diffuse lattice. The hemagglutination (HA) assay is a classic diagnostic test used to screen cell culture supernatant or amnioallantoic fluid (AAF) harvested from embryonating chicken eggs.

The HA assay is not an identification assay. Other types of viruses (e.g., paramyxoviruses and adenovirus) and certain bacteria also have hemagglutinating properties [3–5]. The HA assay should be followed by a hemagglutination-inhibition assay (see Chapter 8) to determine the type and/or subtype of virus

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present. The HA assay does not necessarily indicate the presence of a viable virus [1]. The assay is also capable of detecting viral particles that have been degraded or inactivated and are no longer infectious.

Generally, enough virus is present following a single virus isolation passage for detection by HA assay [4, 5], and repeated virus passages increase the chance for cross-contamination in the laboratory. On average, a 10^5–10^6 50% egg infectious dose (EID_{50})/mL is required for detection by HA assay [5]. At the time of collection, AAF that contains erythrocytes should be discarded, as there may be some adsorption of the virus by the erythrocytes, decreasing the overall titer of the virus in the fluid or inhibiting the ability to detect the virus by HA assay.

The following test procedure is used at the NVSL. The OIE's *Manual of Standards for Diagnostic Tests and Vaccines* [6] outlines a slightly different procedure, and the results are equivalent. The OIE procedure utilizes different volumes of reagents and a different concentration of chicken erythrocytes, and the steps are performed in a slightly different order. For this reason, steps or reagents from the NVSL procedure and the OIE procedure should not be combined in the same test.

2. Materials

1. Sterile bottle with lid (sufficient size to hold blood and anticoagulant).
2. Anticoagulant: Alsever's solution or acid citrate dextrose.
   a. Alsever's solution: Weigh out reagents into a conical flask: 0.55 g of citric acid, 0.8 g of sodium citrate, 2.05 g of D-glucose, and 0.42 g of sodium chloride. Dissolve in distilled water and make up volume to 100 mL. Dispense into sterile 10-mL bottles and sterilize by autoclaving at 116 °C for 10 min. Use slow exhaust. Allow to cool, then tighten the lids and label the bottles. Store at 4 °C.
   b. Acid citrate dextrose: Weigh out reagents into a conical flask: 4 g of citric acid, 11.3 g of sodium citrate, and 11 g of D-glucose. Dissolve in 300 mL of distilled water. Make up to 500 mL with distilled water. Dispense into 100-mL bottles and put on lids. Do not tighten. Sterilize by autoclaving at 116 °C for 10 min. Use a slow exhaust. Allow to cool, then tighten the lids and label the bottles. Store at 4 °C.
3. 50-mL conical tubes.
4. U-bottom or V-bottom microtiter plate with lid.
5. Single and multichannel pipettes and pipette tips to deliver 50-µL volumes.
6. Liquid reagent reservoirs.
7. 0.1 M phosphate buffered saline (PBS). To prepare PBS, combine the following ingredients: 8.5 g of sodium chloride, 1.33 g of sodium phosphate dibasic, and 0.22 g of sodium phosphate monobasic. Dissolve in distilled water and make volume up to 1 L. Mix thoroughly and check pH. The pH should be 7.2 ± 0.1.

8. Chicken erythrocytes, 0.5% in PBS (see Chapter 8, Section 3.1).
9. Positive-control antigen.
10. Mylar microtiter plate sealers (Thermo) or equivalent.
11. AAF harvested from inoculated eggs. Sufficient quantity of AAF should be harvested to provide enough material to conduct the HA assay and subsequent characterization assays.

3. Methods

3.1. Collection and Preparation of Rooster Red Blood Cells

1. Rooster erythrocytes collected from specific pathogen-free (SPF) chickens are preferred for use in the HA assay. Erythrocytes collected from hens may contain hormones that interfere with hemagglutination. Chicken erythrocytes are typically used because the settling time is quicker and the settling patterns are typically clearer than with cells from other species [2]. Certain AI viruses may not hemagglutinate chicken erythrocytes before adaptation to growth in embryonating chicken eggs; these viruses may be more sensitive to hemagglutination with turkey erythrocytes or guinea pig erythrocytes.
2. Fresh erythrocytes should be prepared regularly. Erythrocytes will begin to hemolyze after 5–7 days, causing inaccuracies in the HA assay [7]. The erythrocyte suspension should be mixed gently before use to ensure a uniform distribution of cells in each of the test wells.
3. Prepare a sterile bottle with a lid containing an anticoagulant, either acid citrate dextrose (ACD) or Alsever's solution. Allow for 1 volume ACD to 3 volumes blood, or 1 volume of Alsever's solution to 1 volume of blood.
4. Before collecting blood, draw a small amount of anticoagulant into the syringe and expel again. This will keep the blood from coagulating in the syringe before addition to the anticoagulant in the bottle.
5. Collect 3–5 mL of blood from the wing vein or by cardiac puncture from an SPF chicken in accordance with the appropriate animal care and use procedures. Animals should not be vaccinated for Newcastle disease virus or other pathogens, as this may interfere with some serological hemagglutination-inhibition tests. Add the blood to anticoagulant. Rotate the bottle gently to mix thoroughly. Always treat the red blood cells gently to avoid hemolysis.
6. Add the suspension to a 50-mL conical tube and add a sufficient quantity of PBS to total 50 mL. Rotate gently to mix.
7. Centrifuge at approximately 800 Xg for 10 min to pellet erythrocytes.
8. Aspirate the supernatant and surface layer of white cells (Buffy coat) from the tube without disturbing the pellet of erythrocytes.
9. Wash the erythrocytes a total of three times in PBS by repeating steps 6–8.

10. Add 1 mL of packed red blood cells to 199 mL of PBS for a final erythrocyte concentration of 0.5%.

3.2. Hemagglutination Assay

1. Orient a microtiter plate (U-bottom or V-bottom) so that samples will be diluted either 8 wells or 12 wells across. Number the rows on each plate so that the contents of each row are uniquely identified.
2. Add 50 µL of PBS to every well on the plate.
3. Add 50 µL of AAF (*see* **Note 2**) or cell culture fluid to the first well in each row to be tested. Note that this will result in a 1:2 dilution of test material. A positive-control antigen and a cell control (i.e., PBS and erythrocyte-only wells) must be included each time the test is performed. If multiple plates are being tested at the same time, one positive-control antigen and one cell control should be included on a minimum of every fifth plate. PBS may be used in the cell control well.
4. Dilute the test material: Mix the contents of the first well by pipetting up and down. Pipet 50 µL from the first well and place it in the second well. Continue to make two-fold dilutions of the virus suspension across the entire row. Discard the excess 50 µL after the last row. All wells should have a final volume of 50 µL after this step.
5. Add 50 µL of 0.5% erythrocyte suspension to every well. Tap the plate gently to mix.
6. Apply adhesive plate sealer to each plate. Once the sealer has been applied, the plates may be removed from the biological safety cabinet.
7. Allow 20–30 min for the erythrocytes to settle (*see* **Note 3**).

3.3. Interpretation of Results

1. The HA assay plate should be read when the erythrocytes in the cell control wells have settled to form a solid button in the bottom of the well (hemagglutination-negative) (*see* **Note 1**). When the plate is tilted at approximately 45 degrees, the erythrocytes will stream in a "tear-drop" fashion [2, 6]. Test fluids that are hemagglutination-negative will also form solid buttons in all wells of the corresponding row. These buttons should tear-drop at the same rate as the cell control. Because 10^5–10^6 EID_{50}/mL of virus are required for hemagglutination to occur, an additional passage in chicken eggs is optional to confirm that isolations are not missed because of low levels of virus in the sample.
2. Samples showing complete hemagglutination in one or more test wells should be considered positive for a hemagglutinating agent. Hemagglutination-positive samples

should be further characterized by testing in the hemagglutination-inhibition assay (see Chapter 8) using monospecific antibodies.

3. Incomplete hemagglutination may be observed as buttons that do not tear-drop, have fuzzy margins, or form a doughnut-shaped ring in the bottom of the well (incomplete hemagglutination may not be observed if using V-bottom plates). Incomplete hemagglutination usually indicates an unbalanced proportion of erythrocytes and virus particles, allowing partial settling of the erythrocytes.
4. The endpoint of the virus titration is the highest dilution causing complete hemagglutination (initial dilution is 1:2). The endpoint dilution is considered 1 hemagglutination unit (HAU), and the number of HAUs/50 μL is the reciprocal of the highest dilution. Example: For 6 wells of complete hemagglutination with an endpoint dilution of 1:64, there are 64 HAU/50 μL.

4. Notes

1. Troubleshooting:
   a. The erythrocytes do not settle in cell control wells after 20–30 min: (1) poor quality or hemolyzed erythrocyte suspension; (2) poor quality or incorrect pH PBS; or (3) contamination of wells with viral antigen has occurred.
   b. Positive control is negative: (1) The positive-control antigen was not added to the control well, or (2) the erythrocytes have been allowed to settle for longer than 30 min.
   c. The test sample is negative for hemagglutination in first wells and is positive in last wells (lower concentration wells). This may be caused by the prozone effect. The prozone phenomenon is caused by high dose effect where reactions may be weak or negative in the first wells and stronger in the higher dilutions. Reactions may be interpreted as false negatives if the dilution series is not carried out far enough. This occurs when there is proportionately more virus in the first well(s) than available receptors on the erythrocytes and may appear as either complete buttons or a partial button with fuzzy margins in the bottom of the well.
2. All steps with infectious or potentially infectious material must be performed in a Class II biological safety cabinet. Aerosol-resistant tips and aseptic technique should be used any time an aliquot is taken from the original tube containing virus. Material carried on the surface of a pipette is a common source of laboratory contamination.
3. Assay plates should not be left too long before reading results [7]. The neuraminidase protein present on influenza viruses acts to break virus-cell bonds and may eventually begin to break apart the lattice formed by the virus and erythrocytes. Some virus strains have very high neuraminidase activity and may not allow proper hemagglutination. When these viruses are encountered, the assay should be performed at 4 °C to decrease neuraminidase activity, and the incubation period should be increased to 45–60 min before reading the plate.

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Chapter 8
Hemagglutination-Inhibition Test for Avian Influenza Virus Subtype Identification and the Detection and Quantitation of Serum Antibodies to the Avian Influenza Virus

Janice C. Pedersen

Summary The hemagglutination-inhibition (HI) assay is a classical laboratory procedure for the classification or subtyping of hemagglutinating viruses. For the avian influenza (AI) virus, the HI assay is used to identify the hemagglutinin (H) subtype of an unknown AI virus isolate or the HA subtype specificity of antibodies to AI virus. Since the HI assay is quantitative, it is frequently applied to evaluate the antigenic relationships between different AI virus isolates of the same subtype. The basis of the HI test is inhibition of hemagglutination with subtype-specific antibodies. The HI assay is a relatively inexpensive procedure utilizing standard laboratory equipment, is less technical than molecular tests, and is easily completed within several hours. However, when working with uncharacterized viruses or antibody subtypes, the library of reference reagents required for identifying antigentically distinct AI viruses and/or antibody specificities from multiple lineages of a single hemagglutinin subtype requires extensive laboratory support for the production and optimization of reagents.

Keywords hemagglutination; hemagglutination-inhibition; erythrocyte.

1. Introduction

Avian influenza (AI) viruses agglutinate erythrocytes through the interaction of the virus surface glycoprotein, the hemagglutinin (HA), with receptors on the surface of the erythrocyte. If viral particles are in sufficient quantity, the interaction of the HA protein with erythrocytes will form a complete network of linked erythrocytes and will prevent the erythrocytes from settling out or precipitating as a small pellet in the bottom of a tube or U-bottom microtiter plate. Agglutination of erythrocytes is the basis of the hemagglutination assay (see Chapter 7), and inhibition of the agglutination reaction by HA subtype-specific antisera is the basis of the hemagglutination-inhibition (HI) assay [1–3].

A panel of serum prepared against the 16 distinct HA subtypes is used in the HI assay to confirm the HA subtype identity of AI viruses and may be used to evaluate antigenic relatedness [4]. Typing is facilitated by using antisera raised against AI viruses

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with neuraminidase (NA) subtypes that are heterologous with the test virus. This helps to avoid false-positive reactions from steric inhibition (inhibition caused by the interaction of homologous neuraminidase antigen and antibodies) [5]. Importantly, in diagnostic and surveillance specimens, an AI virus with a novel HA subtype would not be detected or would produce a false-negative result in tests using antisera to the known HA subtypes [6]. Therefore, it is essential to confirm that a hemagglutinating agent that is negative by HI assay is not influenza by another test such as commercial antigen immunoassay, rRT-PCR, or agar gel immunodiffusion assay.

Furthermore, when the NA subtype is unknown, perform the HI test with several sera with different NA subtype specificities. If the NA subtype is known, use sera produced against viruses with heterologous NA subtypes. This will help to eliminate false-positive results due to steric inhibition from the NA antibodies. Reference laboratories maintain a library of antigens and antisera of each HA subtype for isolate identification and testing antigenic relatedness. Production of antibodies to the HA protein only with DNA vaccines has been reported [7] and offers an alternative method for the production of sera without NA interference.

In addition to virus subtype identification, the HI assay may be used to detect and quantitate HA subtype-specific antibodies in serum, plasma, or yolk following infection or vaccination. The presence of AI virus-specific antibodies may be detected as early as 7 days after infection by HI assay [4]. Positive serum will inhibit the HA activity of the antigen, and negative serum will have no effect. Inhibition is evidenced by the formation of a free-flowing button of cells in the bottom of the tube or microtiter plate, which will flow and form a "tear-drop" when the plate or tube is tilted.

The titer of serum is calculated as the reciprocal of the highest dilution that produces complete HI [1]. The sera of many species contain nonspecific inhibitors that may interfere with the specificity of the HI test. However, sera from the majority of avian species do not contain nonspecific inhibitors and do not need to be treated prior to testing [1]. Sera that are, or that may be problematic should be pretreated to reduce or destroy such activity, although it should be recognized that some treatments may lower specific antibody levels. The two most commonly used treatments for these inhibitors have been receptor-destroying enzyme (RDE) (*see* **Note 7**) or treatment with potassium periodate [1]. In addition to nonspecific inhibition of hemagglutination from some species—for example, turkeys, chickens and geese—may cause agglutination (autoagglutination) of the chicken erythrocytes used in the HI test. Autoagglutination will mask low levels of HI activity. Such hemagglutinating activity can be removed by pretreatment of the serum with erythrocytes of the same species as the serum being tested [1].

The HI titer of either a virus or antibodies is somewhat dependent upon the antigenic relatedness of the isolate that is the source of the antigen and the specific serum being used. This is because the titer relies on the relationship of antibodies to a virus antigen that can be highly variable, even within a subtype [6]. Therefore, the level of antibody in a serum specimen may be underestimated when using a distantly related virus of the same subtype as the antigen source. Because of this, the interpretation of HI assay results can be challenging. Therefore, using homologous reagents will give

the most accurate quantitative results, and acceptable qualitative results can usually be obtained by using reagents of the same HA subtype. Additional complications may arise when one animal (particularly in wild bird species) has antibodies to multiple subtypes or may even have concomitant active infections with different subtypes of the AI virus. Finally, steric inhibition from the antibodies to the neuraminidase protein can affect the interaction of the HA protein and the antibodies directed to it. Molecular techniques such as real-time RT-PCR (rRT-PCR) [8] and nucleotide sequence analysis of the hemagglutinin gene are being used by some reference laboratories as an alternative to HI assay for subtype identification for new isolates. However, this does not give information on the antigenic relationship of different viruses that can be used for vaccine selection.

2. Materials

2.1. Collection of Erythrocytes

1. Alsever's solution
2. Avian influenza antibody-Free and infection-free chickens (*see* **Note 1**)
3. Syringes and needles appropriate for the size of the animal and the amount of blood to be collected

2.2. Preparation of Reference Antigens

1. Nine- to 11-day-old embryonating specific pathogen-free chicken eggs.
2. 1-cc syringe with 26 gauge needle.
3. Egg punch.
4. 70% Ethanol or iodine (to disinfect the egg surface).
5. Glue.
6. Antigen source: the appropriate AI virus strain.
7. Egg incubator (37 °C with passive humidity).
8. Beta-propriolactone (BPL).
9. Cryo-vials.
10. Centrifuge tubes and centrifuge.
11. Litmus paper for the pH 4–8 range.
12. Sodium bicarbonate.
13. Calibrated pipettes and aerosol-resistant tips.
14. Phosphate-buffered saline (PBS), 0.1 M, pH 7.2.
15. Egg candle.
16. Class II Biological safety cabinet (BSC).
17. U-bottom 96-well microtiter plates, plate covers, and plate sealing tape.

18. 0.5% Suspension of chicken erythrocytes (see Section 3.1).
19. HA subtype-specific reference antigen (see Section 3.2) and antisera (*see* Section 3.3).
20. 0.4% BSA-PBS-SA.
21. Multiple- and single-channel pipettes calibrated to dispense 0.025–0.05 mL.
22. Aerosol-resistant and standard pipette tips.
23. PBS.
24. Biological safety cabinet of the appropriate level for the viruses being used (BSC).
25. 0.05 M of sodium citrate buffer solution: 1.92 g of citric acid in 200 mL of distilled water. Adjust pH to 4.5 with NaOH and filter prior to use.
26. Receptor-Destroying Enzyme (BioWhittaker, Walkersville, MD), 100 units/mL, prepared according to manufacturer's instructions.

2.3. Preparation of Reference Antisera

1. Washed packed chicken erythrocytes.
2. Centrifuge.
3. Phosphate-buffered saline (PBS), 0.1 M, pH 7.2, or 0.4% bovine serum albumin-phosphate buffered saline-sodium azide (0.4% BSA-PBS-SA). Add 4 mL of 4% BSA and 1.0 mL of sodium azide 10% solution to 96 mL of PBS (*see* **Note 3**).

2.4. HI Assay for the Identification of AI Virus Isolate HA Subtypes

1. U-bottom 96-well microtiter plates, plate covers, and plate sealing tape
2. 0.5% Suspension of chicken erythrocytes (see Section 3.1)
3. Standardized HA subtype-specific reference antigen and antisera (see Section 3.3)
4. Multiple- and single-channel pipettes calibrated to dispense 0.025–0.05 mL
5. Aerosol-resistant and standard pipette tips
6. PBS
7. BSC

2.5. HI Assay for the Identification of HA Subtype Antibody Specificity and Antigenic Comparison

1. U-bottom 96-well microtiter plates, plate covers, and plate sealing tape
2. 0.5% Suspension of chicken red blood cells (see Section 3.1)

3. Standardized HA subtype-specific reference antigen and antisera (see Section 3.2)
4. Multiple- and single-channel pipettes calibrated to dispense 0.025–0.05 mL
5. Aerosol-resistant and standard pipette tips
6. PBS
7. BSC

3. Methods

3.1. Preparation of 0.5% and 10% Erythrocyte Suspension

1. Collect whole blood in an equal volume of Alsever's solution for preservation of erythrocytes (*see* **Note 1**).
2. Wash the erythrocytes to remove Buffy coat and Alsever's solution [3, 4] by adding 20 mL of blood to a 50-mL centrifuge tube and fill the tube with PBS. Gently invert the tube several times to wash the erythrocytes. Centrifuge at 800 Xg for 10 min. Aspirate the PBS and Buffy coat from the tube. Refill the tube with fresh PBS, mix by inversion, and repeat the wash and centrifugation cycle two additional times for a total of three washes. Washed erythrocytes can be stored at 4 °C for up to 1 week. The suspension should be discarded if the erythrocytes show evidence of hemolysis.
3. Prepare a 0.5% suspension of erythrocytes with PBS for use in the HI assay and a 10% suspension for treatment of serum (*see* **Note 2**).

3.2. Preparation of Reference Antigen

1. All work with live virus should be performed in a biological safety cabinet. Prepare dilutions of the AI virus isolate to be used as the antigen source in PBS. The exact virus dilution will vary depending on the virus strain, virus titer, and virus adaptation to growth in chicken embryos. A 1:1000 dilution is a recommended starting place.
2. Inoculate six embryonating chicken eggs with 0.1 mL of diluted virus by the chorioallantoic sac route with a 1-cc syringe with a 26 gauge needle (see Chapter 6). Seal the egg with glue.
3. Incubate the eggs for 4 days at 37 °C with passive humidity and candle daily for mortality. Discard eggs that die within the first 24 hours postinoculation (PI). Store eggs that die between 2–4 days PI at 4 °C until the amnioallantoic fluid (AAF) is harvested.
4. Harvest the AAF from dead and live embryos, and check for virus replication by the HA assay (see Chapter 7).
5. Once virus replication has been confirmed, inactivate the virus-AAF material (the antigen) with BPL with a final BPL concentration of 0.1% in the AAF.

Incubate for a minimum of 4 hr at room temperature. Adjust the pH of the antigen to 7.0 with sodium bicarbonate. Test for inactivation by inoculating six embryos with 0.1 mL of the pH-corrected, BPL-treated AAF preparation as described in step 2. Incubate and harvest as described in steps 3 and 4. Test for virus growth with HA assay. A negative HA assay indicates that the virus has been inactivated.

6. Clarify the antigen by centrifugation at 1500 Xg for 20 min prior to bottling and storage centrifugation prior to bottling and storage.
7. Reference antigens including the positive control must be standardized to a concentration of 8 HAU/50 µL (equivalent to 4 HAU/25 µL). The initial concentration of undiluted reference antigen is determined by the hemagglutination assay (see Chapter 7) using 50 µL of the undiluted specimen. The number of HAU present is equal to the endpoint of the hemagglutination titration, which is the highest dilution of the antigen/virus causing complete hemagglutination; for example, if the 1:128 dilution is the highest dilution to cause complete agglutination in the hemagglutination assay, the hemagglutination titer is 128, and there are 128 HAU/50 µL (if the hemagglutination assay is performed in 50 µL). The virus dilution containing 8 HAU is determined by dividing the total HAU by 8. For this example, that would be 128 ÷ 8 = 16; therefore, for 8 HAU/50 µL, one would make a 1:16 dilution. If the virus isolate does not contain 8 HAU/50 µL (±1 HAU), an additional passage of the virus in embryos should be performed to increase the virus titer.
8. Once the dilution factor has been determined, the reference antigens may be diluted with PBS or BSA-PBS-SA. BSA-PBS-SA will stabilize the virus and extend storage life at 4 °C up to 2–4 weeks.
9. To ensure that the virus has been diluted to the correct concentration, a back-titration is performed by using the diluted virus in an hemagglutination assay where the hemagglutination titer should be 8 HAU.

3.3. Preparation of Reference Antisera

1. In order to remove natural serum agglutinins from reference sera produced in SPF chickens, the serum needs to be treated with chicken erythrocytes. Add 0.1 mL of packed erythrocytes per 1 mL of serum.
2. Incubate for 30 min at room temperature with occasional mixing to keep the erythrocytes suspended.
3. Centrifuge the serum with packed erythrocytes at 800 Xg for 10 min. Decant the serum and discard the erythrocyte pellet. The serum can be stored for several weeks at 4 °C, or –20 °C long-term.
4. Using 8 HAU/50 µL (or 4 HAU/25 µL) of homologous antigen, determine the HI titer of the serum by the HI assay as described (see Section 3.5) and standardize the serum to an HI titer of 1:32-1:64 by dilution in PBS or 0.4% BSA-PBS-SA (*see* **Note 3**).

3.4. HI Assay for the Identification of AI Virus Isolate HA Subtypes

1. Each unknown isolate should be tested against antiserum to each of the 16 known subtypes of the AI virus.
2. The test virus must be standardized to a concentration of 8 HAU/50 µL (equivalent to 4 HAU/25 µL) prior to testing with the HI assay to identify the HA subtype. The procedure is the same as that used to standardize the reference antigen (see Section 3.2).
3. Working in a BSC, dispense 25 µL of standardized test virus for each unknown isolate into a series of three wells (in triplicate) in a U-bottom microtiter plate. Also, dispense 25 µL of the corresponding HA subtype positive-control antigen at 8-HAU/50-µL concentration into the positive-control wells.
4. Add 25 µL of the appropriate standardized antiserum to the first well of an HA subtype series.
5. Serially dilute (25 µL carry-back and discard the excess 25 µL from the final row) the antiserum in the antigen wells beginning with the first well. In this format the serum is diluted in standardized antigen. Each subtype series should be diluted as soon as possible after the addition of the antiserum for that series. After this step there should be 25 µL in each well.
6. Cover plate and incubate for 30 min at room temperature (*see* **Note 4**).
7. Add 50 µL of 0.5% erythrocyte suspension to each well and gently shake/agitate the plate to mix. Mix the erythrocyte solution periodically during this step to ensure that the erythrocytes are evenly suspended during the dispensing process.
8. Cover the plate with microtiter plate sealing tape (the plates can be removed from the BSC after being sealed with plate sealing tape) and incubate it at room temperature until a distinct button has formed in the positive-control wells. This usually takes 20–30 min. The assay plates should be first observed after about 20 min of incubation and checked frequently after that for evidence of hemagglutination. Because some isolates may begin to elute (detach from erythrocytes) in as little as 30 min, the time window where the assay results may be evaluated may be short for some isolates (*see* **Note 5**).

3.5. Analysis of Test Results

1. Wells with complete hemagglutination are recorded as positive for hemagglutination; wells with a distinct button formation are recorded as negative for hemagglutination or positive for HI; wells with partial button formation (fuzzy margins, or doughnut-like appearance) are recorded as incomplete hemagglutination. When interpretation between complete and incomplete inhibition is difficult, tilt the microtiter plate at a 45-degree angle for 20–30 s and look for a "teardrop" of the erythrocytes to form. The teardrop will only form in the wells

with complete inhibition (or that are HI-positive); wells with partial inhibition will not form a teardrop. The assay should not be incubated too long before the result is read (*see* **Note 5**).

2. If the following conditions are not met, the test should be repeated:
   a. The antiserum causing the inhibition of agglutination is void of homologous neuraminidase antibody (e.g., if the antiserum being used in the test has an N2 NA, the antigen in the HI assay should not have an N2 NA).
   b. Positive reference antigen and its homologous antiserum demonstrate the expected HI titer.
   c. The back-titration demonstrates the presence of 8 HAU (±1 HAU).
3. An HI titer of 1:8 is considered positive (*see* **Note 6**). Further confidence of the HA subtype can be obtained by repeating the test with additional reference sera of the same HA subtype, but different neuraminidase subtype.

3.6. HI Assay for the Identification of the HA Subtype Antibody Specificity and Antigenic Comparison

1. This test can be used to identify the HA specificity of AI virus antibodies in a serum sample or can be used quantitatively to evaluate the antigenic relatedness of AI virus isolates. As mentioned previously, when possible, it is recommended, when performing the HI assay, to use antigens and antisera that do not have the same NA specificity to avoid problems with steric inhibition due to the interaction of homologous neuraminidase antibodies and antigen [3, 5]. Serum is the preferred sample for the HI test, although plasma may also be used; under certain conditions, plasma samples coagulate, rendering the sample unusable. Blood samples must be of good quality and free of bacterial contamination and hemolysis. Test samples extracted from egg yolk (*see* **Note 8**) or dried blood on filter paper strips (*see* **Note 9**) can also be used in the HI test [9].
2. Before the HI assay is performed, nonspecific inhibitors of hemagglutination and nonspecific agglutinins must be removed from serum, plasma, and yolk samples since nonspecific agglutinins prevent the buttoning of the erythrocytes and will lead to false-negative results. The presence of natural agglutinins is monitored in the serum control; however, both the reference sera and the diagnostic sera should be routinely pretreated with erythrocytes from the same species from which the serum or plasma originated to remove any nonspecific agglutinins. To remove nonspecific agglutinins, prepare 1 part serum, 2 parts PBS, and 1 part of a suspension of 10% washed erythrocytes (see Section 3.1). Mix thoroughly on a shaker and incubate at room temperature for 30 min, mixing every 10 min to keep the erythrocytes suspended. Centrifuge the plate or test tube at 200xg for 10 min to pellet the erythrocytes. Decant the serum and discard the erythrocyte pellet. Sufficient serum should be treated to test each serum against the 16 subtype-specific antigens selected for the test (at least 0.5 mL total).

3. Dispense 25 µL of standardized HA antigen into three wells (in triplicate) of a U-bottom microtiter plate that has been designated for their respective HA subtype.
4. Add 25 µL of serum that has been treated for nonspecific agglutinins to the first well designated for each sample. Because of the treatment in step 2, this will give an initial serum dilution of 1:8. Prepare a serum control (25 µL of test serum and 25 µL of PBS; i.e., no antigen) for each serum sample to further ensure that the specimen does not contain nonspecific agglutinins.
5. Serially dilute the serum (25 µL carry-back) beginning with the first well (1:8 serum dilution) through the third well (1:32 serum dilution) with a multichannel pipette. Discard the excess 25 µL after the final dilution. After this step there should be a total of 25 µL in each well. Similar to the test for virus subtype identification (see Section 3.4), serum is diluted in antigen. A minimum of three dilutions of each serum can be tested against standardized antigens for subtype specificity testing. When evaluating antigenic relatedness among isolates, further dilutions may be necessary; the end-point will need to be determined empirically (*see* **Note 4**).
6. Include a positive control (standardized reference antigen and antisera) for each HA subtype in the test and a cell or erythrocyte control (50 µL of PBS and 50 µL of 0.5% erythrocytes) in each test. The reference antisera are treated in the same manner as the unknown sera. The control sera are serially diluted in the standardized reference antigen as described in step 5.
7. Cover plate and incubate for 30 min at room temperature.
8. Add 50 µL of 0.5% erythrocyte suspension to each well and gently shake/agitate the plate to mix. Mix the erythrocyte solution periodically during this step to ensure that the erythrocytes are evenly suspended during the dispensing process.
9. Cover the plate with microtiter plate sealing tape (the plates can be removed from the BSC after being sealed with plate sealing tape) and incubate it at room temperature until a distinct button has formed in the positive-control wells. This usually takes 20–30 min. The assay plates should be first observed after about 20 min of incubation and checked frequently after that for evidence of inhibition of hemagglutination (*see* **Note 5**).

3.7. Interpretation of Test Results

1. Wells with complete hemagglutination are recorded as positive for hemagglutination; wells with a distinct button formation are recorded as negative for hemagglutination or positive for HI; wells with partial button formation (fuzzy margins, or doughnut-like appearance) are recorded as incomplete hemagglutination. When interpretation between complete and incomplete inhibition is difficult, tilt the microtiter plate at a 45-degree angle for 20–30 s and look for a teardrop of the erythrocytes to form. The teardrop will only form in the wells with complete inhibition (or that are HI-positive); wells with partial inhibition will not form a teardrop. The erythrocytes in the cell control should button in the same period of time it takes the positive control to form a distinct button (20–30 min) (*see* **Note 6**).

2. The assay should not be incubated too long before the result is read (*see* **Note 5**).
3. Samples are considered positive (i.e., indicate prior infection with AI virus of the respective inhibiting HA subtype) if inhibition of hemagglutination is observed at the 1:8 dilution or higher. When evaluating the antigenic relatedness of virus isolates, the endpoint of the HI titer with homologous serum is compared with that of the heterologous serum (*see* **Note 10**).
4. Endpoints are reported as the highest serum dilution causing complete inhibition of hemagglutination.
5. If the following conditions are not met, the test should be repeated:
    a. The correct number of HAU (8 HAU/50 µL) for each HA antigen subtype is present, as determined by the back-titration.
    b. The serum/plasma/yolk extract sample inhibiting hemagglutination has a different neuraminidase subtype than the antigen used in the test.
    c. The serum control does not produce hemagglutination.
    d. The expected HI titer is observed with homologous antigen and antiserum.

4. Notes

1. Erythrocytes should be collected from specific pathogen-free (SPF) chickens and mixed with an equal volume of Alsever's solution for preservation. Either rooster or hen erythrocytes can be used in the test; however, hormones present in hen's blood can reduce the test's reproducibility. If SPF chickens are not available, blood may be collected from birds that are regularly monitored and shown to be free from antibodies to the AI virus.
2. The HI assay format described here was standardized using 50 µL of 0.5% erythrocytes collected from SPF chickens (the final concentration of erythrocytes—50 µL of 0.5% vs. 25 µL of 1%—is the same per 25 µL of test volume). The HI procedure has been described by a number of diagnostic manuals [1–3, 5, 10–14], and the volume and concentration of the erythrocyte suspension may vary. For example, the Office International des Epizooties (OIE) recommends using 25 µL of a 1% erythrocyte solution [13]. The procedure presented here has been used routinely by the USDA, the National Veterinary Services Laboratories, as well as other diagnostic laboratories and has been shown to be very effective in subtyping AI viruses.
3. Diluting serum with BSA-PBS-SA, instead of plain PBS, will prevent the growth of bacteria and extend the storage life of the reagent at 4 °C.
4. To conserve time and resources and to determine hemagglutinin subtype, each isolate can be tested against just three dilutions (1:8, 1:16, and 1:32) of antiserum for each of the AI virus subtypes. However, when evaluating isolate antigenicity or relatedness, specimens should be endpoint-diluted.
5. In addition to the HA protein, influenza viruses have neuraminidase enzyme or the NA protein on the virion surface, which can remove sialic acid from the ends of oligosaccharide chains. In virus replication, since the HA protein binds to sialic acid on the cell surface during virus attachment, the function of the NA

serves to break the bonds between virus and cells, allowing newly replicated virus to disperse. By this same mechanism, a fully agglutinated sheet of erythrocytes will eventually collapse *in vitro*. The rate of neuraminidase activity varies by virus strain, and some virus strains have a very high neuraminidase activity that may not allow proper agglutination. To avoid this or to compensate for a virus with high neuraminidase activity, the assay may be performed at 4 °C instead of at room temperature [10]. Also, viruses with highly active neuraminidase molecules will not form a stable sheet of agglutinated erythrocytes and should not be selected as seed stock for the production of reference antigen.

6. Troubleshooting:

   a. If the erythrocytes in the cell control do not settle into a well-defined button: (1) The PBS may be incorrect in formulation (pH or salt concentration is incorrect); (2) excessive evaporation of PBS from plates during the test has occurred, as PBS exposed to air or blowing air form a BSC exhaust fan can cause the erythrocytes to button late (greater than 20–30 min); (3) the erythrocytes are too old and have started to deteriorate; or (4) the concentration of erythrocytes is incorrect.
   b. Incomplete or negative inhibition test results; for example, if the unknown virus has been shown to be positive for the AI virus by a type A influenza test (antigen detection, rRT-PCR, or AGID) or the serum has tested positive for type A influenza antibody by the ELISA or AGID test, but the HI test did not identify the HA subtype, one of several situations may be occurring: (1) The HI reference reagents were not adequately antigentically related to the unknown sera or isolate; therefore, repeat the HI test with different reference reagents of the same HA subtypes; (2) the antibody titer of the serum was too low for detection with the HI test; (3) the hemagglutination titer of the reference antigen was too high (i.e., >8 HAU/50 μL ± 1 HAU); therefore, the test serum has an insufficient concentration of antibody to inhibit the elevated hemagglutination activity of the antigen; (4) the titer of the positive-control sera is lower than expected; or (5) the test serum contains natural agglutinins, which have agglutinated the erythrocytes, thus preventing the detection of inhibition, or the erythrocytes in the serum control do not button.
   c. Elevated virus titer in control reagents may mean an incorrect concentration of erythrocytes. Lower concentrations of erythrocytes in the assay will give a falsely high virus titer. It is important that the erythrocyte suspension be thoroughly suspended before use.
   d. False-positive results can be due to (1) nonspecific inhibitors present in the sera tested in the HI antibody test, (2) nonspecific inhibition due to NA steric inhibition in HI antigen test, or (3) the reference antigen is not standardized correctly to 8 HAU/50 μL and/or the reference antiserum is not standardized to 1:32–1:64 HI titer.
   e. False-negative results may occur due to (1) the presence of nonspecific agglutinins in the test serum in an HI antibody test, (2) the reference reagents

are antigentically distinct from unknown specimen(s), or (3) the reference antigen is not properly standardized to 8 HAU/50 µL and/or the reference antiserum is not standardized to a 1:32–1:64 HI titer.

7. Receptor-Destroying Enzyme (RDE) Treatment. The sera of many species contain nonspecific inhibitors that lead to false-positive results. As a rule, sera from the majority of avian species do not contain nonspecific inhibitors and do not need to be treated prior to testing. However, problematic sera should be treated to reduce or destroy nonspecific inhibitors. RDE treatment may lower specific antibody levels.

   a. Add 400 µL of RDE (100 units/mL) to 100 µL of serum and incubate in a 37 °C water bath overnight.
   b. Add 300 µL of sodium citrate and incubate in a water bath at 56 °C for 30 min.
   c. Add 200 µL of PBS (this will result in a final dilution of the serum of 1:10).
   d. Adsorb any nonspecific agglutinins by adding 50 µL of washed undiluted erythrocytes or 10% suspension of erythrocytes and incubate at room temperature for 30 min with periodic mixing.
   e. To remove the erythrocytes, centrifuge the treated serum at 800Xg for 10 min.
   f. Collect treated serum supernatant and proceed with the HI assay as described in Section 3.4 or 3.5.

8. Procedure for the extraction of antibodies from egg yolk by separating the antibody-containing aqueous phase from the lipid phase. The resulting sample can be used in the HI assay to evaluate the immune status of a flock.

   a. Carefully break the test egg, and pour the contents into a shallow bowl without breaking the yolk. Insert a 1-cc syringe without a needle into the yolk sac and withdraw 1.0 mL of yolk material.
   b. Expel the yolk into a tube with 1.0 mL of PBS.
   c. Vortex the yolk-PBS mixture at maximum speed for 10–15 s.
   d. Incubate the yolk-PBS mixture at room temperature for 1 hr. Then repeat the mixing step (step c).
   e. Centrifuge the yolk-PBS mixture at 1,500Xg for 30 min at 4 °C.
   f. Collect the supernatant (aqueous phase) and discard the lipid portion. Note that this procedure results in a 1:2 dilution of the yolk. The supernatant may be used in the HI assay.

9. Procedure for the filter paper method of blood collection: Avian blood samples can be collected and preserved on filter paper. Blood collected in this manner can be used in place of serum in the HI assay. The technique is especially useful in field situations where aseptic collection and adequate preservation of serum and/or plasma may be difficult.

   a. Cut filter paper into 1.2 × 10-cm strips.
   b. Collect the blood sample by puncturing the brachial wing vein, or cut a toenail of the bird to be sampled in accordance with the appropriate animal care and use procedures.

c. Briefly allow blood to pool on the skin at the puncture site. The blood should not be allowed to clot before soaking the filter paper strip.
d. Saturate the distal 1.2 to 2.5 cm of a filter paper strip with blood by soaking the flat side (not the edge) in the blood that has pooled at the puncture site. Collect sufficient blood so that both surfaces of the strip are saturated.
e. Allow the strips to air-dry.
f. Place the dry strips in a plastic bag and seal.
g. Store dried strips at 4 °C until processed.
h. To elute the antibodies, punch three 5-mm discs from a saturated portion of the filter paper strip and transfer the discs with forceps to the microtiter plate well.
i. Dispense 200 µL of PBS into the well containing the dried blood discs.
j. Place the microtiter plate on a shaker or orbital mixer for 1 hr at a high speed, but take care not to displace liquid from wells. Cover plates to prevent evaporation.
k. Incubate the plate at 4 °C overnight to allow the maximum elution of blood. Collect the supernatant. The eluted sample is equivalent to a 1:10 dilution of serum.

10. When numerous viruses or serum samples are being compared in order to evaluate antigenic variation or relatedness, the test sera are standardized to a specific homologous HI titer; for example, each serum sample in a test will have a titer of 256 against the virus it was raised against. This helps to control for variation in antibody levels among different serum samples.

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Chapter 9
Neuraminidase-Inhibition Assay for the Identification of Influenza A Virus Neuraminidase Subtype or Neuraminidase Antibody Specificity

Janice C. Pedersen

Summary The neuraminidase-inhibition (NI) assay is a laboratory procedure for the identification of the neuraminidase (NA) glycoprotein subtype in influenza viruses or the NA subtype specificity of antibodies to influenza virus. A serological procedure for subtyping the NA glycoprotein is critical for the identification and classification of avian influenza (AI) viruses. The macro-procedure was first described in 1961 by D. Aminoff et al. [2] and was later modified to a microtiter plate procedure (micro-NI) by Van Deusen et al. [4]. The micro-NI procedure reduces the quantity of reagents required, permits the antigenic classification of many isolates simultaneously, and eliminates the spectrophotometric interpretation of results. Although the macro-NI has been shown to be more sensitive than the micro-NI, the micro-NI test is very suitable for testing sera for the presence of NA antibodies and has proven to be a practical and rapid method for virus classification. This chapter will provide an overview of the USDA-validated micro-NI procedure for the identification of subtype-specific NA in AIV and antibodies.

Keywords neuraminidase; neuraminidase inhibition.

1. Introduction

Influenza virus membranes contain two subtype-specific glycoproteins on their surface envelope: hemagglutinin (HA) and neuraminadise (NA). The HA mediates cell-surface sialic acid receptor binding to initiate virus infection, while the NA contains a head domain that is enzymatically active [1–4]. The NA is important both for its biological activity in removing N-acetyl neuraminic acid (NANA) or sialic acid from sugar chains of glycoproteins and as a major antigenic determinant [2–4]. After virus replication, NA removes sialic acid from the virus and cellular glycoproteins by catalytic cleavage of the NANA from adjacent D-galactose or D-galactosamine sugar chains, facilitating virus release and the spread of infection to new cells. Nine antigentically distinct neuraminidase subtypes (N1–N9) have

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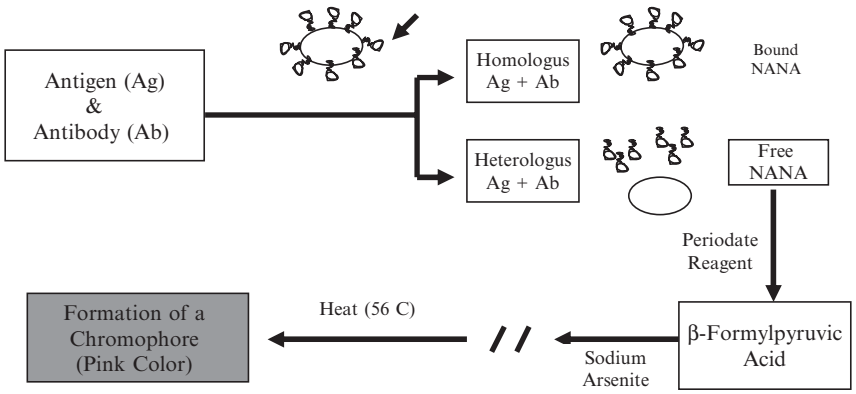

Figure 1 Interaction of free N-acetyl neuraminic acid (NANA) with β-formylpyruvic acid and sodium arsenite for the formation of a pink chromophore

been identified [5, 6]. Despite the antigenic diversity of the NA glycoprotein, all influenza neuraminidases bind the carboxylate of NANA [4].

The inhibition of viral enzymatic activity by specific antibodies forms the basis of the neuraminidase-inhibition (NI) assay (see Figure 1). The micro-NI assay is performed in a microtiter plate and is evaluated based on a colorimetric change where the substrate produces a dark pink color when the NA is active. Specifically, the enzymatic release of NANA is detected when the aldehyde to NANA is produced by reaction with periodate, a test component. A pink chromophore forms when the NANA aldehyde reacts with thiobarbituric acid, another NI test component [1, 2, 4, 7]. When the NA catalytic activity is inhibited, such as with neutralizing antibody, the color change does not occur and a light pink color is observed [4]. The micro-NI procedure is conducted in opaque white 96-well microtiter plates, which allows the color to be more easily visualized and eliminates the need for spectrophotometric interpretation of results [4]. Although both a macro- and micro-NI procedure have been described, the micro-NI procedure is more practical since it permits the testing of several viruses or sera simultaneously and greatly reduces the volume of reagents and time required to conduct the test [2, 4].

The NI assay can be performed directly on AI virus isolates (amnioallantoic fluid or cell culture fluid) with subtype-specific reference antisera for the determination of NA subtype, or the NI assay can be used to identify antibody NA subtype specificity with serum or plasma by using subtype-specific reference antigen instead of reference sera. Antigenic diversity and relatedness must be considered in the selection of reference antigens and antisera, as the NI procedure is directed to an antigentically variant epitope. Using reference sera or antigen from viruses with the same HA subtype as the test materials will improve specificity by allowing the NA subtype to be the only variable in the test [4].

2. Materials

1. White polystyrene 96-well microtiter plates.
2. Fetuin (12.5 mg/mL) from fetal calf serum: Dissolve 250 mg of fetuin in 10 mL of sterile distilled water. Add 10 mL of sterile 0.4 M of phosphate buffered saline (PBS-0.4 M), pH 5.9. Store fetuin at -20 °C.
3. Sodium periodate (Meta). Periodate reagent: Dissolve 4.28 g of sodium m-periodate in 38 mL of distilled water. Carefully add 62 mL of concentrated phosphoric acid and mix. Store at room temperature in an amber or foil-covered bottle in a cool, dark place.
4. Sodium m-arsenite. 50% Arsenite reagent: Dissolve 50 g of sodium arsenite in 100 mL of distilled water. Add 1.5 mL of concentrated sulfuric acid (caution: add slowly) and mix. Store at room temperature.
5. 2-Thiobarbituric acid. 0.6% Thiobarbituric acid: Dissolve 0.6 g of thiobarbituric acid in 100 mL of distilled water by heating in a boiling water bath, stirring frequently, or on a hot plate with a magnetic stirring mechanism. Store at room temperature.
6. Phosphate buffered saline (PBS), 0.01 M, pH 7.2.
7. Phosphate buffered saline (PBS-0.4 M), pH 5.9.
8. Bovine serum albumin (BSA), fraction V.
9. BSA-PBS-SA: 4% BSA in PBS (0.01 M, pH 7.2) containing 0.1% sodium azide: Dispense 10 mL of 4% BSA and 89 mL of PBS into a flask. Add 1.0 mL of 10% sodium azide solution (10 g of sodium azide Q.S. to 100 mL with distilled water) to BSA-PBS.
10. Sodium azide, practical.
11. Sulfuric acid.
12. Phosphoric acid 85%.
13. Microtiter plate sealing tape.
14. Antiserum to each of the nine neuraminidase subtypes and a known negative serum.
15. Inactivated viral antigen for each of the nine neuraminidase subtypes and a negative antigen (see Chapter 8 for inactivation method).
16. Calibrated multiple- and single-channel pipettes (0.025–0.10 mL).
17. Aerosol-resistant and standard pipette tips.

3. Methods

3.1. Preparation and Optimization of Reference Reagents

1. Reference antigens for each of the nine neuraminidase subtypes are propagated in embryonating chicken eggs (see Chapter 6). Either live or inactivated virus can be used as a reference reagent; however, inactivated virus or antigen allows

the antibody test to be conducted outside a Biosafety cabinet. Live virus can be inactivated with beta-propriolactone (BPL) at a final concentration of 0.1% (see Chapter 8). A check for virus viability must be conducted in chicken embryos to determine if the virus has been inactivated.
2. Determine the optimum antigen dilution by titration.

3.1.1. Antigen Titration

1. Dispense 25 μL of PBS into rows 2 through 6 of a white opaque 96-well U-bottom microtiter plate.
2. Add 50 μL of antigen into the first well of row 1.
3. Make serial twofold dilutions (25-μL volumes) of antigen, starting with the first well (undiluted) through well 6 (well 6 will have a 1:32 dilution).
4. Add 25 μL of PBS (0.01 M, pH 7.2) to all wells.
5. Mix on a microtiter plate shaker for 10–15 s and incubate at room temperature for 1 hr (±15 min). Cover the plate to prevent evaporation.
6. Add 25 μL of fetuin to each well.
7. Mix on a microtiter plate shaker for 10–15 s. Cover and incubate at 37 °C for 3 hr. Incubation time may vary depending on the enzymatic activity of the neuraminidase. For some isolates, the incubation time may need to be increased by 1–2 hr.
8. Add 25 μL of periodate reagent to each well.
9. Mix on a microtiter plate shaker for 10–15 s. Cover and incubate at room temperature for 20 min (±2 min).
10. Add 25 μL of 50% sodium arsenite reagent to each well. The addition of sodium arsenite reagent will cause a dark brown precipitate to form.
11. Mix on a microtiter plate shaker until the dark brown color fades (this may take several minutes).
12. Add 100 μL of 0.6% thiobarbituric acid reagent to all wells. Cover plate with plate sealing tape. Puncture the tape (small pinhole) over each well to allow for expansion.
13. Float sealed plate in a 56 °C water bath for 30 min.
14. Read results by tilting the plate to view color through the sealing tape.
15. The optimum antigen dilution is the highest dilution, which has a "medium" pink color or the dilution prior to a reduction in pink color. A uniform color should be selected for all reference antigens. In addition, the light pink positive color should be easily differentiated from the negative-control darker pink. In Figure 2, reference antigen 1 should be diluted 1:4 and reference antigen 2 should be diluted 1:8. Unknown isolates can be standardized before testing by titration, but this is not required in most cases.

3.1.2. Optimization of Reference Antisera

1. The optimum dilution for each reference antiserum is determined by titrating the serum against homologous antigen (see Section 3.1.1 for the titration procedure).

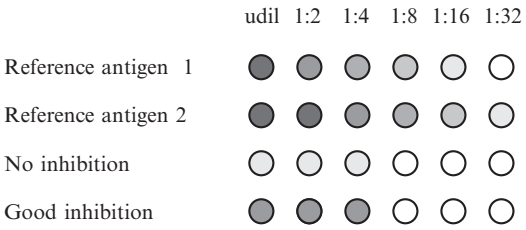

Figure 2 Antigen titration for the neuraminidase-inhibition test. The optimum antigen dilution is the highest dilution, which has a "medium" color, or the dilution prior to a reduction in pink color. The "medium" color should be easily differentiated from the lighter, no-inhibition color, and uniform for all reference antigens. Reference antigen 1 should be diluted 1:4, and reference antigen 2 should be diluted 1:8

The optimal dilution is the highest dilution that provides good inhibition when compared to a positive-control standard. The optimal serum dilution usually ranges from 1:4 to 1:10. The negative-serum dilution should be the same as the lowest dilution used for any one of the antisera; for example, if the dilutions used in the test serum are 1:4, 1:8, and 1:10, the negative-control serum should be used at a dilution of 1:4. Reference antisera and antigens should be diluted with 0.4% BSA-PBS-SA.

2. Heat-inactivate sera at 56 °C for 30 min prior to testing. Diluted sera and antigens can be stored for several weeks at 4 °C or frozen at -20 °C for long-term storage.

3.2. Neuraminidase Inhibition Assay

3.2.1. Subtype Determination of Virus Isolates

1. Preparation of specimens: Virus isolates: Start with a 1:12 dilution of amnioallantoic fluid (AAF) containing the unidentified influenza A virus with 0.4% BSA-PBS (50 µL of AAF and 550 µL of PBS), as this will work with most isolates. Lower or higher dilutions may be needed for some isolates and may be determined empirically. A standardized concentration of the unknown isolate can be determined by titration as described in Section 3.1.1.
2. Add 25 µL of previously standardized neuraminidase antiserum into the corresponding vertical columns labeled N1 through N9 for each isolate and in the positive-control row (see Figure 3 for layout).
3. Dispense 25 µL of negative-control serum in the negative-control column and in the first 10 wells of the negative-control row.
4. Add 25 µL of diluted AAF, for each unknown, into the first 10 wells of the corresponding horizontal row.
5. Dispense 25 µL of each positive-control AAF into the corresponding positive- and negative-control wells.

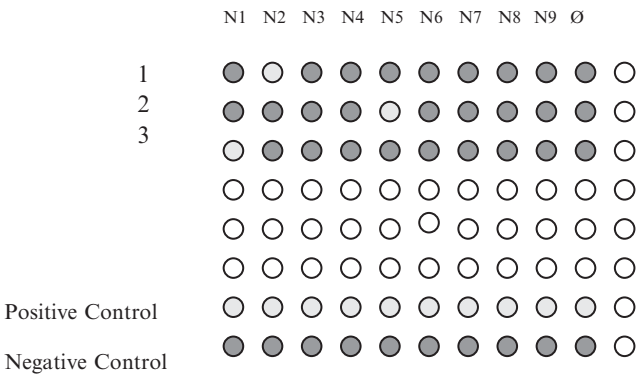

Figure 3 Neuraminidase-inhibition (NI) test plate with three unknown specimens numbered 1–3 and positive and negative controls. Specimens positive for inhibition are light in color, and specimens negative for inhibition are darker in color. Specimen 1 is positive for inhibition for N2, specimen 2 is positive for inhibition for N5, and specimen 3 is positive for inhibition for N1

6. Mix on a microtiter plate shaker for 10–15 s and incubate at room temperature for 1 hr (±15 min). Cover plate during incubation to prevent evaporation.
7. Add 25 μL of fetuin to each well.
8. Mix on a microtiter plate shaker for 10–15 s. Cover and incubate at 37 °C for 3 hr. Incubation time may vary depending on the enzymatic activity of the neuraminidase. The incubation time for some isolates may need to be increased by 1–2 hr.
9. Add 25 μL of periodate reagent to each well.
10. Mix on a microtiter plate shaker for 10–15 s. Cover and incubate at room temperature for 20 min (±2 min).
11. Add 25 μL of 50% sodium arsenite reagent to each well. The addition of sodium arsenite reagent will cause a dark brown precipitate to form.
12. Mix on a microtiter plate shaker until the dark brown color fades (this may take several minutes).
13. Add 100 μL of 0.6% thiobarbituric acid reagent to all wells. Cover plate with plate sealing tape. Puncture the tape (small pinhole) over each well to allow for expansion.
14. Float sealed plate in a 56 °C water bath for 30 min.
15. Read the results by tilting the plate to view color through the sealing tape.

3.2.2. Characterization of Serum Antibody for NA Subtype Specificity

1. Preparation of specimens: Serum, plasma, and processed yolk specimens (see Chapter 8) are suitable for the test. Dilute 0.2 mL of serum/plasma/yolk with 0.25 mL of PBS, and heat-inactivate at 56 °C for 30 min.

2. Add 25 µL of previously standardized neuraminidase antigen into the corresponding vertical columns labeled N1 through N9 for each test serum and in the corresponding well in the positive- and negative-control rows.
3. Dispense 25 µL of negative-control antigen in the negative-control column.
4. Add 25 µL of heat-inactivated test sera into the first 10 wells of the corresponding horizontal row.
5. Dispense 25 µL of each positive-control antiserum into the corresponding positive-control well.
6. Dispense 25 µL of negative-control sera into the negative-control wells.
7. Mix on a microtiter plate shaker for 10–15 s and incubate at room temperature for 1 hr (±15 min). Cover plate during incubation to prevent evaporation.
8. Add 25 µL of fetuin to each well.
9. Mix on a microtiter plate shaker for 10–15 s. Cover and incubate at 37 °C for 3 hr. Incubation time may vary depending on the enzymatic activity of the neuraminidase. The incubation time for some isolates may need to be increased by 1–2 hr.
10. Add 25 µL of periodate reagent to each well.
11. Mix on a microtiter plate shaker for 10–15 s. Cover and incubate at room temperature for 20 min (±2 min).
12. Add 25 µL of 50% sodium arsenite reagent to each well. The addition of the sodium arsenite reagent will cause a dark brown precipitate to form.
13. Mix on a microtiter plate shaker until the dark brown color fades (this may take several minutes).
14. Add 100 µL of 0.6% thiobarbituric acid reagent to all wells. Cover plate with plate sealing tape. Puncture the tape (small pinhole) over each well to allow for expansion.
15. Float sealed plate in a 56 °C water bath for 30 min.
16. Read results by tilting the plate to view color through the sealing tape.

3.2.3. Analysis of Test Results

Results for unknown isolates and test sera are based on the difference in color intensity between the test specimen and the positive-control antigen with the negative serum (negative control) and the positive control with inhibition (*see* **Notes 1–4**). Results are recorded for each well as follows: Pink color is negative (no inhibition); the neuraminidase is not this subtype. Light pink color or greatly reduced color is positive for inhibition; the neuraminidase is the subtype of the corresponding serum (see Figure 3).

The color of the test well is evaluated when compared to the NA-specific positive- and negative-control wells. Quantitative data are not obtained with the NI assay; the results are recorded as either positive or negative. When the NI assay is used to test serum samples, antibody against more than one NA may be detected. When determining the subtype of a virus isolate, reaction against serum of more than one NA is indicative of a mixed infection. Due to the antigenic nature of the

test, cross-neutralization reactions between two different neuraminidase subtypes are possible. The only NA subtypes that are known to cross-react are NA 1 and NA 4. Subtype NA 4 will cross-react with NA 1.

All positive controls should have a similar "medium" pink color. Unknown isolates that have a very dark pink color, as compared to the positive controls, may need to be diluted further and retested if the neuraminidase subtype cannot be determined. Too much neuraminidase can overpower the specific antibody and give a false-negative result (*see* **Note 1**). In contrast, if the unknown is too weak (very light pink color), the sample may have to be repeated at a lower dilution to determine the neuraminidase subtype (*see* **Note 2**).

Column Ø contains negative sera (virus isolate subtype test) or normal AAF (antibody subtype test). The Ø control will be a dark pink color for the isolate subtype test, indicating the neuraminidase is active, and will form an aldehyde when reacted with periodate. The Ø control for the antibody subtype test will be a light pink color, indicating the sera do not contain substances that will nonspecifically cause an enzymatic reaction.

4. Notes

1. False-negative result:
   a. The viral isolate (AAF or cell culture supernatant) is too concentrated. Too much neuraminidase can overpower the specific antibody and give a false-negative result. Antigen titration should be conducted to determine the correct dilution.
   b. The viral isolate represents a previously unidentified AI virus neuraminidase subtype.
   c. The hemagglutinating virus is not an influenza virus. The viral isolate should be tested by a type A influenza test (antigen detection, rRT-PCR, or AGID) to confirm that the isolate is the AI virus.
   d. Reference reagents are not optimized, and the reference antisera (isolate subtyping test) are not concentrated enough to inhibit the neuraminidase activity of the unknown isolate.
2. False-positive result:
   a. The virus is too weak and does not have sufficient neuraminidase activity for production of the medium pink chromophore. Viral titer should be increased by a second passage in chicken embryos.
   b. The normal control (Ø) is a light pink, indicating the neuraminidase is inactive (isolate subtyping test).
   c. Reference reagents have not been optimized, and the reference antigen (antibody subtyping test) is too concentrated. The test serum cannot inhibit neuraminidase activity.

d. The normal control (Ø) for the antibody test is a light pink, indicating the test specimen contains substances that nonspecifically cause an enzymatic reaction.

3. Negative result: The selected reference antigen or antiserum is not antigentically related to the unknown viral isolate or test serum and cannot effectively inhibit neuraminidase activity. The test should be repeated with a different reference reagent that is antigentically related to the unknown specimen.
4. No neuraminidase activity in any test well (N1–N9) for the isolate subtyping test; a light pink color, is in all test wells may indicate inadequate neuraminidase activity for enzymatic reaction. The incubation time may need to be increased by 1–2 hr for some isolates.

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Chapter 10
Immunohistochemical Staining for the Detection of the Avian Influenza Virus in Tissues

Mary J. Pantin-Jackwood

Summary Immunohistochemical methods are commonly used for studying the pathogenesis of the avian influenza (AI) virus by allowing the identification of sites of replication of the virus in infected tissues and the correlation with the histopathological changes observed. In this chapter, the materials and methods for performing immunohistochemical detection of AI virus antigens in tissues are provided. The technique involves the following steps: heat-induced antigen retrieval; binding of a primary antibody to a virus type-specific antigen; antibody-antigen complex binding by a biotinylated secondary antibody; and binding of an enzyme-streptavidin conjugate. The enzyme is then visualized by application of the substrate chromogen solution to produce a colorimetric end product. Demonstration of AI virus antigen in tissues is based on chromogen deposition in the nucleus and/or cytoplasm of infected cells.

Keywords avian influenza virus; immunohistochemistry; biotin–streptavidin detection method.

1. Introduction

Viral antigens in fresh, frozen, or fixed cell suspensions or tissues can be detected by the use of specific antibodies labeled with dyes or enzymes. This detection process is known as immunohistochemistry (IHC). This antigen detection system has allowed the direct morphological localization of avian influenza (AI) virus antigen in tissues from animals infected with the virus, permitting us to correlate virus replication with the corresponding pathological cellular changes. For this reason, IHC is frequently used in AI virus pathogenesis studies and has allowed for greater understanding of the pathogenic mechanisms involved during AI virus infection by the detection of viral antigens in target tissues. Antigen detection can then be related to the lesions observed and the stages of the infection [1–10].

The great variation in the distribution and severity of lesions caused by the AI virus results from differences between AI virus strains and host species. In poultry,

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low pathogenic AI viruses can produce pneumonia, nephritis, and pancreatitis; and viral antigen is commonly demonstrated in necrotic respiratory epithelium, renal tubule epithelium, and pancreatic acinar epithelium [11]. Histological lesions caused by highly pathogenic AI viruses vary in severity and location but typically include necrosis, hemorrhage, and/or inflammation within multiple visceral organs, especially the heart, brain, lung, adrenal glands, pancreas, and lymphoid organs [11, 12]. Lesions in the brain and heart have abundant associated AI virus proteins in neurons and myocytes, respectively. Viral antigen staining is also commonly observed in the respiratory epithelium, skeletal myofibers, kidney tubules, vascular endothelial cells, adrenal corticotrophic cells, and pancreatic acinar cells [11]. Antibodies specifically reacting with AI virus proteins have been developed for IHC, including monoclonal antibodies against the nucleoprotein (type-specific) and the hemagglutinin (H) (subtype-specific) proteins [2, 4–6, 13, 14]. AI virus antigen staining in cells is found in the nucleus and often also in the cytoplasm when using antibodies against the nucleoprotein [2], and in the cytoplasm and on the cell membrane when using antibodies against the hemagglutinin [1].

Viral antigens in tissues can be detected by IHC in a two-stage process: the binding of the primary antibody to specific viral epitopes and the subsequent detection of this binding by a colorimetric reaction. Here we report the procedure for detecting AI virus antigens in formalin-fixed, paraffin-embedded tissue sections using a mouse-derived monoclonal antibody specific for a type A influenza virus nucleoprotein as a primary antibody and a biotin–streptavidin system as the detection method. Similar results can be achieved with monoclonal antibodies directed against other viral proteins with minimal optimization. Subtype-specific tests may be more difficult to optimize, due to antigenic variation, unless the target virus is homologous with the virus to which the antibodies were developed. Because of their higher specificity, monoclonal antibodies are often preferred over polyclonal antibodies. Increased specificity reduces the possibility of cross-reactivity with other antigens, which significantly reduces background staining. The advantage of polyclonal antibodies over monoclonal antibodies is that the former are more likely to identify multiple epitopes of the target protein. However, the likelihood of cross-reactivity with similar epitopes in other proteins increases; therefore, the likelihood of false positives increases as well [15].

2. Materials

2.1. Solutions and Reagents

1. Primary antibody: monoclonal antibody specific for type A influenza virus nucleoprotein
2. CitriSolv (Fisher Scientific, Pittsburg, PA)
3. 100% Ethanol
4. 95% Ethanol

5. Tris-buffered saline (TBS) (DakoCytomation, Carpenteria, CA) with 0.1% Tween 20 (DakoCytomation)
6. Antigen Retrieval Citra (BioGenex, San Ramon, CA)
7. Common Antibody Diluent (BioGenex)
8. Super Sensitive Link-Label Alkaline Phosphatase Detection System (BioGenex)
9. Fast Red Substrate-chromogen (BioGenex)
10. Hematoxylin Gill (Fisher Scientific)
11. SuperMount Permanent Aqueous Mounting Medium (BioGenex)
12. Xylene
13. Permount Mounting Media (Fisher Scientific)

2.2. Equipment/Tools

1. Glass and plastic Coplin jars or slide-staining dishes: vertical and horizontal
2. Slide racks and trays
3. Humidified chamber tray with lid, for antibody application
4. Adjustable pipetters with tips
5. Polyethylene disposable transfer pipettes
6. 60 °C oven
7. Standard domestic microwave oven rated at 700–1000 W, with turntable for constant temperature and electronic digital timer control
8. Microscope slides: charged and precleaned (Fisher Scientific)
9. Glass cover slip (Fisher Scientific)
10. Light microscope

3. Methods

3.1. Slide Preparation

Formalin-fixed, paraffin-embedded tissue sections are cut at 4-μm thickness using a standard microtome. In a 55 °C water bath, floating paraffin tissue sections are gently adhered to positively charged slides. Slides are then dried at 60 °C for 1 hr or at 37 °C overnight.

3.2. Immunostaining Protocol

3.2.1. Deparaffinization and Rehydration of Tissue Sections Slides

1. Place slides of paraffin-embedded, unstained tissues (Section 3.1) in a slide rack into the oven and bake for 15 min at 60 °C.

2. Remove and air-dry for 30 s.
3. Place the slides in the rack into CitriSolv and incubate for 2 min to remove the paraffin. Repeat through two changes of fresh CitriSolv (*see* **Notes 1** and **2**).
4. Place the slides in 100% ethanol for 3 min. Repeat through two changes of fresh ethanol.
5. Place the slides in 95% ethanol for 3 min. Repeat through two changes of fresh ethanol.
6. Move slides into TBS and wash at room temperature for 5 min. Repeat through two changes of TBS, for a total of two washes.

3.2.2. Antigen Retrieval with Citra Buffer

1. Place the deparaffinized, rehydrated slides in plastic slide holders, place the slide holders in a glass staining dish horizontally, and fill with citric acid solution (Citra 1X) until the slides are completely covered. Cover with a glass lid.
2. Microwave for 10 min at near-boiling temperatures with the lid ajar (*see* **Note 3**).
3. Remove the slides from the microwave oven and allow to cool at room temperature in the citric acid buffer for 30 min.
4. Rinse the slides in deionized water for 2 min. Repeat through three changes of water.
5. Rinse the slides in TBS.

3.2.3. Immunohistochemical Staining

1. Lay the slides across horizontal slide trays (*see* **Note 4**).
2. Gently blot the slides with absorbent paper towels without touching the tissue sections.
3. Apply the primary antibody (diluted 1:2000 in Common Antibody Diluent) with enough to cover the tissue section, approximately 100 µL per slide. Incubate for 60 min at room temperature in a humidified chamber (*see* **Notes 5** and **6**).
4. Rinse each slide gently with wash buffer (TBS), by using transfer pipettes or a wash bottle, three times for 5 min each (*see* **Note 7**).
5. Apply 100 µL of multilink to each slide (biotynilated anti-immunoglobulins diluted 1:20 in Common Antibody Diluent). Incubate for 20 min at room temperature in a humidified chamber.
6. Rinse each slide gently with TBS three times for 5 min each.
7. Apply 100 µL of label (Alkaline Phosphatase-conjugated streptavidin diluted 1:20 in Common Antibody Diluent) to each slide. Incubate for 20 min at room temperature in a humidified chamber.
8. Rinse each slide gently with TBS three times for 5 min each.
9. While doing the above incubations, add one Fast Red tablet to a bottle of Naphthol Phosphate buffer and shake until dissolved. Let sit approximately 1 hr before using (**see Notes 8** and **9**).

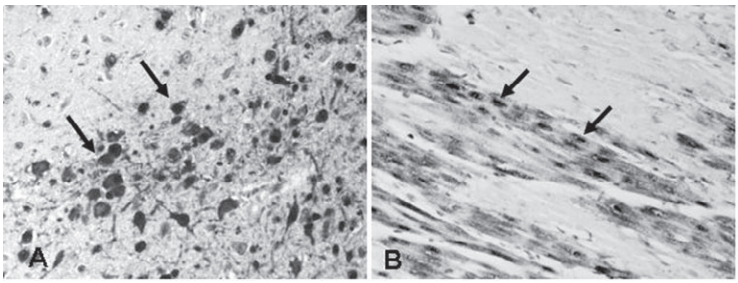

Figure 1 Detection of AI virus antigen in the nucleus and cytoplasm of neurons (a) and myocardial cells (b) by immunohistochemical staining using a monoclonal antibody specific for type A influenza virus nucleoprotein and a biotin-streptavidin system as the detection method. Slides are counterstained with hematoxylin

10. Apply 3 drops of the above-mentioned Fast Red chromogen substrate to each slide, and incubate for 2–10 min at room temperature (preferred time 6 min). The color development may be monitored under the microscope (*see* **Note 10**).
11. Place the slides in racks and rinse well with distilled water or buffer. Three changes, 2 min each.
12. To counterstain, dip slides in and out of a bath of Gills Hematoxylin 15 times (*see* **Note 11**).
13. Rinse the slides thoroughly in tap water for 5 min. Repeat through two changes of water. Rinse the slides in TBS for 2 min.
14. Drain the slides and place them on a horizontal surface. Apply 2–4 drops of Supermount to the tissue sections. Rotate the slides so that the entire section is covered; drain each slide individually to remove any excess Supermount and any bubbles. Do not apply a coverslip on top of the Supermount.
15. Place slides horizontally in a 60 °C oven for 15 to 20 min to allow the Supermount to harden. Remove the slides from the oven, and allow to cool at room temperature.
16. Quickly dip slides individually into xylene and immediately coverslip with Permount permanent mounting media (**see Note 12**).
17. The slides are ready to be evaluated microscopically. Both the cell nuclei and background will stain blue. Avian influenza viral antigen will appear as a red precipitate in the nucleus of affected cells but may also be observed within the cytoplasm (see Figure 1).

4. Notes

1. Deparaffinization and rehydration can also be accomplished by the use of xylene and graded ethanols. CitriSolv is a d-limonene–based solvent used as a safer alternative to xylene; is biodegradable, noncorrosive, and nonflammable; and has low levels of toxicity.

2. Proper deparaffinization is critical. Always use fresh solutions. Each incubation should be done in a fresh batch of reagent.
3. Standardization of the conditions for a specific heat antigen retrieval procedure is recommended, since several factors greatly impact the results. The wattage of the microwave oven, the power setting, the number of slides, the volume of the retrieval solution, and the duration of the treatment are all related [16]. Therefore, well-controlled conditions should be applied in order to obtain reproducible results. Antigen retrieval time may need to be increased for tissues that were fixed in 10% neutral buffered formalin for longer than 48 hours.
4. These steps can be done on the bench, using a humidified chamber, or done with an automated immunostainer (e.g., Dako Autostainer Plus, DakoCytomation). As with all immunological procedures, it is important that the specimens stay hydrated throughout. Drying will result in nonspecific immunoglobulin binding and will consequently cause nonspecific and high levels of background staining.
5. Several primary antibodies against AI viral antigens are commercially available. It is recommended that the manufacturer's instructions be followed for determining the correct dilutions and the selection of secondary antibodies. Dilutions will vary depending on the antibody.
6. Sections of tissues from uninfected and AI virus-infected animals of the same species as the test tissue sections are used as negative and positive controls, respectively.
7. Complete washing is critical to minimize the background. The wash stream should not directly splash the specimen surface, as this may cause the section to dislodge from the slide or dislodge antibodies with low avidity. Washes are the most important aspect of background reduction and should therefore be extended if too much background is a problem [17].
8. The chromogen solution should be shaken until the Fast Red tablet is completely dissolved before use. The solution should be filtered through the filter device provided with the kit when it is applied to the tissue section.
9. To increase reactivity, incubations can be extended in length, or temperatures can be raised. This often increases the background as well. Sometimes reaction intensity can be increased without the background by incubation with the primary antibody overnight at 4 °C instead of at room temperature.
10. Optimal signal development should be monitored closely at the staining step by viewing the slide under a microscope. In our system, alkaline phosphatase staining for AI virus antigen usually takes 5–10 min. Staining beyond this duration leads to high background and inaccurate grading of positively stained cells. In most cases, color development will be completed within 30 min; however, the development time may vary due to the degree of specific antigen/antibody binding in the tissue. If the presence of endogenous pigment is a problem with a particular specimen, or a color other than red is desired as an indicator, different chromogenic compounds can be used.
11. It is better if the counterstain is weak so that it doesn't mask the principal reaction. Ideally, just enough hematoxylin to identify the structure is all that is required.
12. Slides prepared in this manner are permanent and should not fade with time.

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Chapter 11
Wild Bird Surveillance for the Avian Influenza Virus

Justin D. Brown and David E. Stallknecht

Summary Avian influenza (AI) viruses have been isolated from a wide diversity of free-living avian species representing several orders. Isolations are most frequently reported from aquatic birds in the Orders Anseriformes and Charadriiformes, which are believed to be the reservoirs for all AI viruses. Since their first recognition in the late 1800s, AI viruses have been an important agent of disease in poultry and, occasionally, of nongallinaceous birds and humans. However, the recent highly pathogenic avian influenza (HPAI) H5N1 virus epidemics have increased the awareness of AI viruses and their potential implications among the scientific community, politicians, and the general public.

In response to the spread of HPAI H5N1 viruses to Europe and Africa in 2005–2006, many countries developed surveillance plans to detect AI viruses; a large portion of these sampling efforts was targeted at migratory avian species. This chapter is intended to give general concepts and guidelines for surveillance of the AI virus in wild birds. Separate sections are included for low pathogenicity avian influenza (LPAI) and HPAI H5N1 viruses because the unique biological characteristics of HPAI H5N1 require a modified surveillance plan tailored to these viruses.

Keywords avian influenza virus; wild birds; surveillance; sampling; H5N1; highly pathogenic avian influenza.

1. Introduction

1.1. General Background

Wild birds are the primordial reservoir for all known hemagglutinin (H1-16) and neuraminidase (N1-9) subtypes of AIV [1–4]. Naturally occurring infections have been reported from free-living birds, representing more than 90 species in 12 orders [5, 6]. Most of these species are associated with aquatic habitats, and presently anseriforms (ducks, geese, and swans) and charadriiforms (gulls, terns, and shorebirds)

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are considered the most important reservoirs for AI viruses. Species of birds within these two orders are diverse and often occupy very different habitat types, ranging from small freshwater marshes to pelagic salt-water habitats [7–9].

The peak prevalence of AI viruses in North American anseriform populations is in late summer/early fall and is associated with premigration staging [10]. This peak is attributable to high concentrations of susceptible juvenile birds on waterfowl marshalling sites throughout Canada and the northern United States. During this time, AI virus infection rates can exceed 30% in juvenile ducks [10], but prevalence rapidly decreases as the population migrates and in wintering areas is often lower than 1–2% [6].

Much less is known about influenza in charadriiforms, but preliminary data indicate that AI virus infection in these species differs spatially, temporally, and genetically from what is observed in anseriforms. The peak prevalence of the AI virus in North American charadriiforms occurs in the spring when shorebirds migrating along the Atlantic coast of the United States stop over at the Delaware Bay. This is the only site worldwide where consistent AI virus isolations from shorebirds have been reported; in general, prevalence rates from these species at other locations or times are either very low or zero [5, 6, 11].

The transmission and maintenance of AI virus in wild bird populations are dependent on fecal-oral transmission via contaminated water [2, 12–14]. Replication of AI viruses in ducks occurs primarily in the intestinal tract [15], and high concentrations of infectious virus are shed in the feces [1, 16]. Experimentally infected Muscovey ducks (*Cairina moschata*) shed 6.4 g of fecal material per hour, with a viral titer of $1 \times 10^{7.8}$ mean embryo-infectious doses (EID_{50}) per gram [16]. In addition, the duration of viral shedding in ducks can be prolonged, as evidenced by experimentally infected Pekin ducks (*Anas platyrhynchos*), which shed virus for more than 28 days [2].

In 2002, significant mortality associated with HPAI H5N1 virus infection was reported in captive and feral aquatic birds housed within two waterfowl parks in Hong Kong [17]. Mortality was also observed in free-living gray herons (*Ardea cinerea*) and black-headed gulls (*Larus ridbundus*) spatially associated with these outbreaks. Since 2002, mortality in wild birds due to HPAI H5N1 has continued [18], and the subsequent spread of these viruses to Europe and Africa suggests that the long-range movement of these viruses may have occurred through migratory birds [19, 20]. While these events demonstrate a potential for wildlife involvement, the role of migratory birds in the ongoing HPAI H5N1 epidemics throughout Asia, Europe, and Africa is not completely known. However, experimental and field data to date support the inclusion of wild birds in any HPAI H5N1 virus surveillance and response plan.

1.2. Surveillance

Disease surveillance in wild avian populations can be conducted by sampling asymptomatic populations or targeted collections from birds exhibiting morbidity or mortality. These samples provide useful information such as the prevalence of AI virus in a

population, subtype diversity, and differences in infection rates based on host characteristics, including gender, species, and age. In general, it is preferable to collect samples over multiple years in order to evaluate the data for long-term trends and to control for normal temporal fluctuations of AI viruses in the avian population.

Serological testing and virus isolation are two methods commonly used for diagnosing AI virus infection in domestic poultry [21]. Serological tests detect antibodies specific for AI virus and indicate previous exposure to the virus. Two inherent limitations of using serology in the surveillance of any avian population include the inability to identify birds recently exposed to the AI virus (without adequate time for seroconversion) and the fact that a single test will not reflect the time of exposure. In addition, most serological tests are developed for use in domestic poultry and vary in their ability to accurately detect antibodies in nongallinaceous wild birds. In particular, many serological tests are ineffective in ducks, which have a reduced antibody response compared to other avian species [22] and produce antibodies that may not be detectable by hemagglutination-inhibition tests [23, 24]. For these reasons, serological testing should not be the primary method used for AI virus surveillance in free-living avian populations. Virus isolation, however, is the definitive diagnostic test for AI virus infection in any avian species. There is no known avian species-dependent variability in efficacy, as with serological testing, and virus isolation identifies birds that are actively shedding virus and allows for AI virus subtype determination.

2. Low Pathogenicity Avian Influenza Virus

2.1. Introduction

Disease associated with AI virus in wild birds varies depending on several factors, including host species and virus subtype [21]. Prior to the HPAI H5N1 virus epidemics in 2002, reported wild bird mortality associated with AI virus infection had been rare, consisting of sporadic birds usually spatially associated with HPAI virus outbreaks in domestic poultry [25, 26] and a 1961 epornitic in South Africa involving common terns (*Sterna hirundo*) [27]. Aside from these exceptions, neither morbidity nor mortality has been observed with AI virus infections in wild birds. These LPAI viruses follow the traditional paradigm: gastrointestinal replication, asymptomatic infection, and fecal-oral transmission.

2.2. Sample Population

Published reviews summarize the existing data on AI virus isolations in wild avian species [6, 19, 26]. In order to maximize the likelihood of detecting AI viruses, anseriforms and charadriiforms should comprise a large portion of any wild bird sample population. Because the frequency of AI virus infection varies between

species within these orders, sampling efforts should be further targeted toward the species in which influenza is most commonly detected.

Most AI viruses from anseriforms have been isolated from dabbling ducks (*Genus Anas*), which are composed of a diverse group of species including mallards (*Anas platyrhynchos*), blue-winged teal (*Anas discors*), gadwall (*Anas strepera*), American wigeon (*Anas Americana*), and northern pintail (*Anas acuta*). The frequency of AI virus isolations from these birds may be due to their behavior and habitat utilization, which includes the congregation of large numbers of ducks on freshwater habitats, allowing for increased transmission of AI viruses within the avian population.

The vast majority of AI virus isolations from charadriiforms have been from ruddy turnstones (*Arenaria interpres*) at the Delaware Bay [5, 6, 11]. Influenza is rarely isolated from ruddy turnstones at other locations or from other shorebird species or gulls.

Of the remaining avian orders from which AI viruses have been isolated, most are associated with aquatic habitats, including Gaviiformes (loons), Podicepediformes (grebes), Procellariiformes (shearwaters and petrels), Pelecaniformes (pelicans and cormorants), Ciconiiformes (ibis and herons), and Gruiformes (moorhen and coots). This likely can be explained by two factors: (1) sympatric habitats with anseriform and/or charadriiform hosts, and (2) enhanced viral transmission between birds in aquatic habitats where shared water can serve as an efficient viral medium.

2.3. Selecting a Sample Location and Time

The location and time to conduct surveillance should be based on the biology of the waterfowl and the epidemiology of AI viruses within the specific avian population. Surveillance activities should be planned for a time when there are high concentrations of susceptible birds in a focal area and during the seasonal peak prevalence of AI virus in the avian population, if known. If seasonal differences in infection rates are not known for a given species, surveillance should be conducted at a time when high concentrations of birds are found in a focal area to allow for more efficient capture of multiple birds. Preferably, samples should be collected when there are large numbers of susceptible birds in the population. As very little is known about the duration of immunity for AI virus in many birds, immature or juvenile birds—which are more likely to be serologically naïve to AI viruses—should be included in the sample population. There is extensive literature on the habitat use, behavior, and migrational patterns of most avian species, and these data should be utilized in the development of a surveillance plan.

2.4. Samples to Collect

Avian influenza viruses replicate in the gastrointestinal tract of susceptible wild birds and are shed in the feces. Thus, fecal swabs should be collected from these birds for

virus isolation. Sampling individual birds in a population is preferable because it allows the researcher to confirm the species and to collect additional data such as gender, age, and weight. However, capturing and handling birds may be difficult due to a lack of equipment or personnel or the danger in handling the bird. In these situations, it may still be possible to conduct surveillance for the AI virus on a population level by collecting samples from the environment or using sentinel birds.

The two most useful environmental samples for detecting the AI virus in wild birds are water and feces. Avian influenza viruses have been isolated from unconcentrated surface water on lakes with ducks in Canada [2], Minnesota [28], and Alaska [29], but attempts have been unsuccessful using lake water in areas devoid of ducks [30]. For environmental sampling to be successful, the avian species must be present in a focal area in high concentrations, such as gull hatcheries or ducks on marshalling sites prior to fall migration. While routine environmental sampling can provide some useful information on the presence of AI viruses within a population, the data will not indicate which species shed AI viruses or the prevalence of AI viruses in the population.

Sentinel ducks have been successfully used to detect AI viruses in free-living avian populations [15, 31]. Although this surveillance method has the same limitations as environmental sampling, sentinel species have provided information on AI virus prevalence in avian populations that could not have been captured and sampled otherwise, such as pelagic species [31].

2.5. Materials

1. Plastic or wire Dacron swabs; do not use wooden-shafted or calgi swabs (see Chapter 2 for additional information on swabs).
2. Viral transport media: brain-heart infusion broth with penicillin G (10,000 Units/mL), streptomycin (2 mg/mL), kanamycin (0.6 mg/mL), gentamicin (1 mg/mL), and amphotericin B (0.02 mg/mL) (*see* Section 2.7, **Note 1**).
3. 3.0- to 5.0-mL cryogenic vials.

2.6. Bird Sampling

Capture methods are available for most birds [32]. The chosen method for a given surveillance program should be based on its appropriateness for a given species, cost, and the availability of personnel and equipment. The size of bird to be sampled will dictate the type of Dacron cloacal swab to be used. In general, birds the size of a duck and larger can be sampled with plastic-shaft cotton swabs, while smaller birds will require wire-shaft Dacron swabs.

1. Insert the swab into the cloaca, and gently rotate in a circular motion (*see* Section 2.7, **Note 2**).

2. Place the swab in sterile transport media containing high concentrations of antibiotics to reduce bacterial overgrowth. Carefully break or cut the shaft of the plastic or wire swab, respectively, so that the tip of the swab remains in the media. Tightly screw the cap back on the tube and discard the remainder of the shaft. A variety of media compositions are available and acceptable, but a prototype solution is listed in Section 2.5.
3. After sample collection, the swab material should immediately be stored at 4 °C (refrigerator or ice) and may remain at this temperature if the virus isolation is to be performed within 72–96 hr. If virus isolation will be delayed, the swabs should, instead, be stored at −70 °C, where the virus may remain viable indefinitely (*see* Section 2.7, **Note 3**).
4. Virus isolation and/or processing for RNA for RT-PCR (*see* Section 2.7, **Note 4**) can be performed from these samples (see Chapters 3, 4, and 6).

2.7. Notes

1. A variety of media compositions are available and acceptable.
2. As a general rule, the swab should contain visible feces [D. E. Stallknecht, personal communication]. Some of the larger waterfowl species (swans and geese) have a strong sphincter muscle, which may resist insertion of the cloacal swab. Gentle rotation, and possible repositioning of the swab, may be necessary to advance the swab past the sphincter muscle.
3. Field conditions may not permit this ideal protocol. The duration of infectivity of AIV in transport media under various temperatures is not known. Likely, samples may remain at 4 °C for longer than 72–96 hr and still contain detectable virus.
4. If performing RT-PCR on cloacal swabs, it is important to keep in mind that the fecal material may contain RT-PCR inhibitors, which may result in false-negative results. Virus isolation is unaffected by these inhibitors.

2.8. Environmental Feces Sampling

1. When sampling fecal matter, it is best to choose moist droppings, because AI viruses are sensitive to desiccation and attempts to isolate virus from dried feces will be less successful.
2. Either plastic or wire swabs may be used to collect environmental feces (see Section 2.5).
3. Environmental swabs can be stored in the viral transport media listed above, and the sample can be handled with the same protocol (see Section 2.5).
4. Virus isolation and/or processing for RNA for RT-PCR (*see* Section 2.7, **Note 4**) can be performed from these samples (see Chapters 3, 4, and 6).

2.9. Water Sampling

Standardized protocols for collecting water samples for AI virus testing have not yet been validated. However, based on the few studies that have attempted to isolate AI viruses from water, samples should be collected from surface water in aquatic habitats with high concentrations of ducks. The water should be placed in a sealed glass bottle and stored at 4 °C until testing. Water samples should not be stored at −70 °C, because the thawing process will reduce the viral titer. In order to reduce microbial contamination, the samples should be treated with antibiotics prior to virus isolation (Chapter 6), which can be performed using routine techniques [33, 34].

2.10. Sentinel Species

Prior to placing a sentinel species in a selected area, determine that the species will not become invasive or negatively impact the local ecosystem. Domestic waterfowl are usually selected as a sentinel species and are caged or pinioned prior to release to ensure recapture. After allowing the sentinel ducks to integrate with the wild population, the ducks can be recaptured and sampled for serological testing and virus isolation as previously described. The sentinel species should be removed from the site at the end of the study.

3. Highly Pathogenic Avian Influenza H5N1 Virus

3.1. Introduction

The epidemiology of HPAI H5N1 viruses is not completely understood. Unresolved issues that are vital to worldwide surveillance, preparation, and control of HPAI H5N1 viruses include the susceptibility, clinical response, and viral shedding of different wild avian species to HPAI H5N1 virus infection, and the question of the role of migratory wild birds and the movement of domestic poultry in the spread of H5N1 viruses throughout Europe, Asia, and Africa. The guidelines stated in this chapter are based on the experimental and field data available to date. Since first emerging in 1997, HPAI H5N1 viruses have maintained their ability to change. Thus, a successful surveillance program for HPAI H5N1 viruses should be based on the most current field data and experimental studies.

3.2. Sample Population

Experimental studies or field data have reported HPAI H5N1 virus infection in a variety of species from the following avian orders: Anseriformes, Charadriiformes,

Ciconiiformes, Columbiformes, Falconiformes, Galliformes, Gruiformes, Passeriformes, Pelecaniformes, Phoenicopteriformes, Strigiformes, Struthioniformes, Psittaciformes, and Podicipediformes [18]. Field reports from HPAI H5N1 virus outbreaks suggest that these viruses are highly virulent for anseriforms. This susceptibility was particularly evident during the 2005–2006 spread of the HPAI H5N1 virus into Europe, in which high mortality was exhibited by swans, geese, and ducks [35]. Experimentally, the pathogenicity of HPAI H5N1 viruses for anseriforms is variable and species-dependent, with a high lethality for wood ducks, mute swans, whooper swans, trumpeter swans, black swans, and cackler geese [36; J. D. Brown, personal communication]. Neither morbidity nor mortality was exhibited by blue-winged teal, redheads, northern pintails, or mallards.

Since emerging in 1997, HPAI H5N1 viruses have caused mortality in poultry throughout Asia. It has been suggested that the viruses may be endemic in domestic fowl in Southeast Asia [37]. Poultry production in these countries consists largely of small farms in which gallinaceous birds and domestic ducks are raised under semi-confinement or free-ranging conditions [38, 39]. This system allows for a constant interaction between domestic poultry and wild birds, which greatly hinders the control, containment, and eradication of HPAI H5N1 viruses. In addition, the domestic poultry-wildlife interface confuses the question of whether wild birds or domestic poultry are to blame for the persistence of the HPAI H5N1 virus in Asia and the spread of the virus to Europe and Africa. The epidemiology of HPAI H5N1 virus in Europe is markedly different in that most of the outbreaks have been confined to wild birds, with spillover into domestic poultry [35]. This observation is likely attributable both to the confined poultry production systems utilized in Europe and to the rapid and efficient containment policy employed by the affected countries. Very little is known about the epidemiology of HPAI H5N1 viruses in Africa, but field data suggest that the situation may be more similar to that in Asia rather than to that of Europe, thus involving greater opportunities for transmission between domestic poultry and wild birds.

Surveillance programs for HPAI H5N1 viruses should concentrate on anseriforms. Aquatic birds, including ciconiforms (herons, egrets) and charadriiforms (gulls, shorebirds, terns), have also reportedly been infected with the H5N1 virus [17, 18] and should be included in the sample population. Semicaptive gallinaceous birds should be sampled in areas that raise poultry under free-ranging conditions. In addition to routine testing of asymptomatic avian populations, it is critical that surveillance programs should also target sick and dying birds. The detection of the HPAI H5N1 virus in wild birds throughout Europe was based on its detection in sick and dead birds rather than on the sampling of live asymptomatic birds. Testing birds that are exhibiting clinical signs of disease or birds that are found dead is an efficient method for HPAI H5N1 detection in wild birds. Experimentally, ducks and gulls that exhibit morbidity after inoculation with HPAI H5N1 viruses shed higher concentrations of virus for a longer duration than birds that do not become clinically ill (see Figure 1). In addition, sick or dying birds are easy to detect and capture. Devoting a portion of any surveillance program to the targeted sampling of sick and dead birds will save money, personnel, and time.

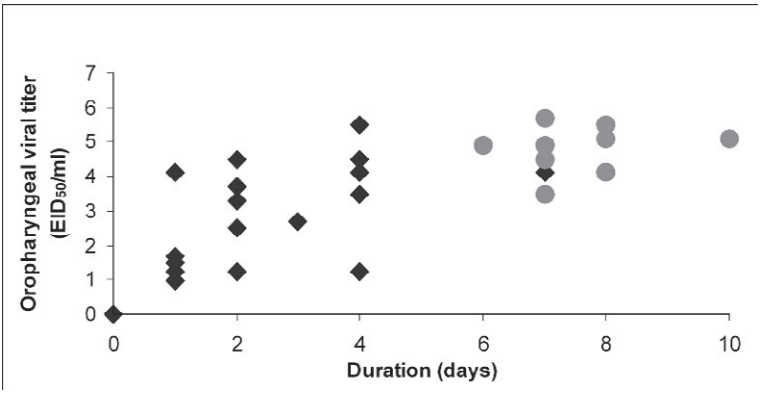

Figure 1 The viral titer and duration of oropharyngeal viral shedding in 3- to 4-month-old ducks and gulls inoculated intranasally with 10^6 EID_{50} of HPAI H5N1 virus [36]. Birds that exhibited clinical signs of disease after inoculation are represented by circles and those that remained clinically normal are indicated by diamonds

3.3. Samples to Collect

As opposed to LPAI viruses, HPAI H5N1 viruses in wild birds are primarily transmitted via the oropharyngeal route [36]. Fecal shedding in experimentally infected ducks and gulls was shorter in duration and at a lower titer than oropharyngeal shedding. Therefore, oropharyngeal swabs should be the primary sample collected from asymptomatic wild birds for HPAI H5N1 virus detection. Inoculated birds that died of HPAI H5N1 virus infection shed moderately high concentrations of virus in both the feces and oropharyngeal secretions, so swabbing either the oropharyngeal cavity or the cloaca should effectively detect the H5N1 virus in dead birds.

Environmental fecal samples can be collected but will be less efficient for HPAI H5N1 virus detection than LPAI virus detection because HPAI H5N1 viruses are primarily transmitted via respiratory secretions. The role of water in the transmission and maintenance of HPAI H5N1 viruses is not completely understood, but, in an experimental model, two H5N1 viruses (with a starting concentration of 10^6 mean tissue-culture infective doses per mL) had an estimated persistence of 94 days for one virus and 158 days for the other virus under freshwater conditions at 17 °C [34]. Water samples may be collected and processed as described for LPAI virus surveillance.

Sentinel species may be an efficient method for HPAI H5N1 virus detection in wild birds, especially in countries that do not have the infrastructure, money, or equipment for a large-scale surveillance program. Only species that are highly susceptible to the HPAI H5N1 virus should be chosen as a sentinel species, and the selection should be based on experimental or field data. Potential sentinel species

for the HPAI H5N1 virus include mute swans, whooper swans, black swans, trumpeter swans, cackler geese, wood ducks, or laughing gulls [36; J. D. Brown, personal communication].

3.4. Selecting a Sample Location and Time

As with the LPAI viruses, the timing of sampling of asymptomatic avian populations should be based on the biology of species (breeding, migration time and routes) and epidemiology of the virus. Sick and dead birds should be sampled as they become available.

3.5. Collection Technique

The same materials (see Section 2.5) listed for LPAI viruses can be used to collect HPAI H5N1 viruses. Cloacal swabs, water samples, and environmental feces, if sampled, should be collected via the same protocols as described for LPAI viruses.

3.6. Bird Sampling

Oropharyngeal samples may be collected with either of the Dacron swabs previously described for LPAI viruses (see Section 2.5). Various techniques are acceptable for collecting the oropharyngeal swab, but it is important to maintain consistency with each sample and swab the entire oropharyngeal cavity (see Figure 2).

1. Insert the swab into the oral cavity with a gentle front-to-back motion under both sides of the tongue.
2. Continue toward the back of the oral cavity, carefully moving the swab over the glottis and caudal oral cavity.
3. Finally, the swab exits the mouth by running along the dorsal oral cavity and into the choanal cleft.
4. The swab can be stored in the same viral transport media from Section 2.5, and the sample can be handled with the same protocol (see Section 2.6).
5. Virus isolation and/or processing for RNA for RT-PCR (*see* Section 2.7, **Note 4**) can be performed from these samples (see Chapters 3, 4, and 6).

3.7. Sentinel Species

Sentinel species should be placed using the same suggestions and precautions as described for LPAI viruses. In addition to collecting samples from these birds for

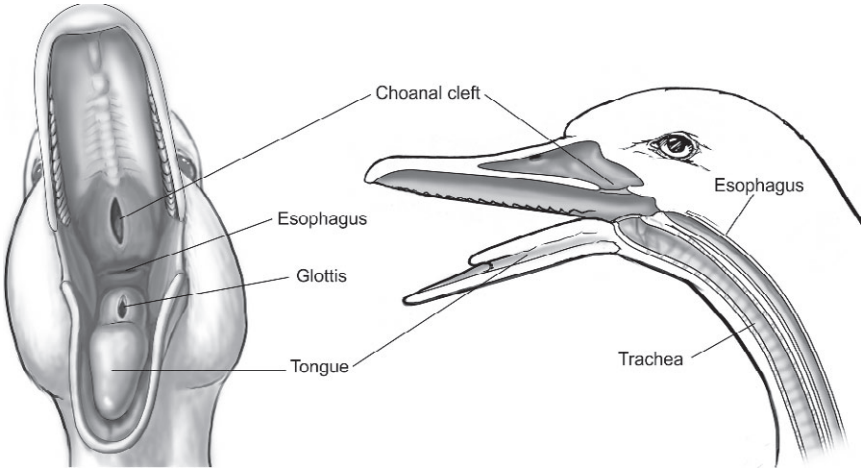

Figure 2 Anatomic landmarks for collection of an oropharyngeal swab (illustration by Kip Carter)

virus isolation and serology, the sentinels can be monitored for morbidity and mortality as an indication of the presence of the HPAI H5N1 virus within the population. The previously suggested species above are all highly susceptible to the HPAI H5N1 virus and will likely become sick and die if infected.

4. Conclusions

Low pathogenicity avian influenza viruses are most frequently isolated from birds in the Orders Anseriforms (duck, geese, and swans) and Charadriiformes (gulls, terns, and shorebirds). The peak prevalence of AI virus infection in North American anseriforms is in the fall and in charadriiforms is in the spring. To date, the only site with a high AI virus infection rate in charadriiforms is the Delaware Bay. Transmission of LPAI viruses in wild birds is via the fecal-oral route; thus, fecal swabs should be collected.

The epidemiology of HPAI H5N1 viruses in wild birds is not completely known. Field and experimental data indicate that some species of anseriforms are highly susceptible to infection and also likely to exhibit clinical disease. Oropharyngeal swabs should be the primary sample collected from wild birds for detection of the HPAI H5N1 virus.

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Chapter 12
Reverse Genetics of the Avian Influenza Virus

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Summary Reverse genetics is the creation of a virus from a full-length cDNA copy of the viral genome, referred to as an infectious clone, and is the most powerful genetic tool in modern virology. The generation of influenza A viruses by reverse genetics has lagged mainly due to the inherent technical difficulties of providing a sufficient amount of all eight viral RNAs from cloned cDNA. A breakthrough was made in 1999 by utilizing the cellular enzyme RNA polymerase I for the synthesis of influenza viral RNAs. Although slightly different methods are being used in different laboratories for the rescue of the influenza virus, the basic concept of synthesizing viral RNA using RNA polymerase I remains the same. Coupled with *in vitro* mutagenesis, reverse genetics can be applied widely to accelerate progress in understanding the influenza virus life cycle, the generation of live-attenuated vaccines, and the use of influenza virus as vaccine and gene delivery vectors.

Keywords influenza; reverse genetics; infectious clone; mutagenesis; cloning.

1. Introduction

Recombinant DNA technology enables us to analyze and manipulate genomes at the molecular level. However, for nonretroviral RNA viruses, the genome does not enter a DNA phase during replication, and direct manipulation of RNA substrates is challenging. For this reason, the study of the molecular biology of RNA viruses has lagged despite the small size of their genome [1]. Genetic manipulation of negative-strand RNA viruses, such as the influenza virus (belonging to the family *Orthomyxoviridae*), is even more demanding because isolated genome or antigenome RNAs are not infectious by themselves, and the virus is segmented. In order to serve as a functional template to initiate transcription/replication, the viral RNA (vRNA) has to be transcribed into a plus-sense mRNA by the polymerase complex, which includes three viral polymerases (PB2, PB1, and PA) and the nucleoprotein (NP) [2]. The influenza virus has a genome comprised of eight RNA gene segments that must be expressed and packaged to complete the virus replication cycle. Furthermore, unlike many other RNA viruses, the influenza virus replicates in the

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nucleus of infected cells, which adds to the complexity of developing a reverse genetics system [3].

The reverse genetics system for the influenza virus was established in 1999 by two different research groups [4, 5], relying on the cellular enzyme RNA polymerase I for the synthesis of influenza viral RNAs. RNA polymerase I is an abundant nuclear enzyme that transcribes ribosomal RNA [6]. RNA polymerase I initiates and terminates transcription at defined promoter and terminator sequences, and the resulting transcript does not contain additional nucleotides at the 5' or 3' end. Thus, this enzyme is ideal for generation of influenza vRNA in the nucleus. Currently, *de novo* synthesis of the influenza virus is achieved by co-transfection of cells with 12 plasmids: Eight plasmids contain the RNA polymerase I promoter, a cDNA encoding one of each of the influenza viral RNAs, and the terminator; the remaining four plasmids express viral proteins required for vRNA replication and transcription (PB1, PB2, PA, NP)(see Figure 1). Although slight modifications are being introduced to improve the rescue system [7, 8], the basic concept of synthesizing vRNA using the RNA polymerase I system remains the same.

2. Materials

2.1. RNA Extraction and RT-PCR, PCR Product Purification

1. RNA extraction kits and reagents, for example: Trizol LS reagent (Invitrogen, Carlsbad, CA), RNeasy Mini Kit (Qiagen, Valencia, CA), or QIAmp Viral RNA Mini Kit (Qiagen), SV Total RNA Isolation System (Promega, Madison, WI)
2. Qiagen OneStep RT-PCR Kit (Qiagen) or equivalent
3. RNase Inhibitor (Promega)
4. Primers (*see* **Note 1**)
   - Primers for the amplification of HA, NA, M, and NS genes
     UNI-RG5' TATTCGTCTCAGGGAGCAAAAGCAGG (26-MER)
     UNI-RG3' ATATCGTCTCGTATTAGTAGAAACAAGG (28-MER)
   - Primers for the amplification of NP, PA, PB1, PB2 genes
     UNI-RG pol 5′ TATTCGTCTCAGGGAGCGAAAGCAGG (26-MER)
     UNI-RG3′ ATATCGTCTCGTATTAGTAGAAACAAGG (28-MER)
5. Qiaquick gel extraction kit (Qiagen)
6. Miscellaneous common laboratory equipment and reagents: microcentrifuge, thermocycler, gel electrophoresis unit, water bath, UV light box, agarose, reagent-grade H_2O (nuclease-free), ethanol, chloroform, isopropanol

2.2. Cloning and Construction of Reverse Genetics Plasmids

1. Vectors: pHH21 vector (contains the human RNA polymerase I promoter and the mouse RNA polymerase I terminator sequences) (kindly provided by

Dr. Gerd Hobom, Institut fur Mikro- und Molekularbiologie, Giessen, Germany), pCR® 3.1 eukaryotic TA expression vector (Invitrogen), TOPO TA Cloning® vector (optional).
2. pHH21 specific primers: pHH21-forward: GGGGGACACTTTCGGACATCT, pHH21-reverse: TGGTGGCGTTTTTGGGGACAG.
3. BsmBI restriction enzyme (New England Biolabs Inc., Ipswich, MA).
4. Shrimp Alkaline Phosphatase (SAP) (Promega).
5. LigaFast™ Rapid DNA Ligation System (Promega).
6. Competent cells (Invitrogen).
7. LB Broth EZMix™ Powder & LB Agar EZMix™ Powder (Sigma, St. Louis, MO).
8. Bluo-Gal (1 g powder, Sigma): Prepare as a 2% (20 mg/mL) solution in dimethylformamide (Sigma). This solution can be stored in a glass tubes with a screw cap wrapped in foil (light sensitive) at −20 °C for at least 6 months.
9. Ampicillin Ready Made Solution (100 mg/mL) (Sigma).
10. PCR reagents: *taq* polymerase, reaction buffer, $MgCl_2$, dNTPs.
11. Qiaprep Spin Miniprep Kit (Qiagen).

2.3. Cell Culture and Transfection

1. Cells: 293T (human renal epithelial cell line into which the gene for the temperature-sensitive Simian virus 40 T antigen has been inserted) (kindly provided by Dr. Yoshihiro Kawaoka, University of Wisconsin), Vero (African green monkey kidney), and Madin-Darby canine kidney (MDCK) cells can be obtained from ATCC (Manassas, VA).
2. Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Grand Island, NY).
3. DMEM/F-12 [Nutrient Mixture F-12 (Ham)] (Invitrogen).
4. Fetal bovine serum (FBS) (Invitrogen).
5. Trypsin-EDTA 10× (0.5% trypsin with EDTA-4Na) (Invitrogen). Working solutions are prepared by 1:10 dilution in sterile PBS.
6. Phosphate buffered saline (PBS) pH 7.2 (Invitrogen).
7. Lipofectamine™ 2000 (Invitrogen).
8. Opti-MEM® I medium (Invitrogen).
9. Miscellaneous equipment: CO_2 incubator, inverted microscope, fluorescent microscope.

2.4. Fluorescent Antibody (FA) Staining

1. Microscope cover glass (12 mm, circle) (Fisher Scientific, Pittsburgh, PA).
2. Bovine serum albumin (Sigma): Prepare as a 1% solution in PBS.
3. Paraform-aldehyde (Sigma): Prepare as a 3% solution in PBS, and adjust pH to 7.6.

4. Triton X-100 (Sigma): Prepare as a 0.1% solution in PBS.
5. Anti-chicken IgG-FITC conjugate or Anti-mouse IgG-FITC conjugate (Sigma) depending on the primary antibody used.

2.5. Virus Propagation in Embryonating Chicken Eggs

1. Specific Pathogen Free (SPF) eggs (SPAFAS, Wilmington, MA) (see Chapter 6 for additional details)
2. 1-mL syringe with 25 gauge, 5/8-in. needle (Becton-Dickinson, Franklin Lakes, NJ)
3. Miscellaneous equipment: egg incubator, egg puncher, egg candler (Lyon Electric Company, Chula Vista, CA)

3. Methods

3.1. Amplification of Full-Length Individual Genes

1. Viral RNAs are extracted using Trizol LS reagent (Invitrogen), RNeasy Mini Kit (Qiagen), or QIAmp Viral RNA Mini Kit (Qiagen) according to procedures recommended by the manufacturer (see Chapter 3). Two hundred fifty microliters (μL) of infectious amnioallantoic fluid [containing 10^6–10^{10} 50% egg infectious doses per mL (EID_{50}/mL) of virus] are typically used for RNA extraction. High viral titer samples are typically required for efficient cloning of the viral genes, and clinical material is usually not a reliable sample for amplification of the full-length genes. The extracted RNAs are eluted or resuspended in a final volume of 50 μL of RNase-free water (*see* **Note 2**).
2. The entire coding and noncoding regions of each of the eight individual genes are amplified by standard reverse transcription (RT)-PCR with the Qiagen one-step RT-PCR kit (Qiagen) (*see* **Note 3**) and primers listed in Section 2.1. Primers are directed to the 12 or 13 conserved bases at the ends of each influenza viral RNA segment and contain the BsmBI restriction site for cloning purposes. The RT step is performed with incubation steps of 45 °C for the PA, PB1, and PB2 genes and 50 °C for the other genes for 30 min. An additional step of 95 °C for 15 min is needed to activate the hot-start *taq* in the Qiagen kit and may not be necessary with other RT-PCR reagents. The PCR incubation steps are 35 cycles of 94 °C denaturation for 30 s, 53 °C annealing for 15 s, and 72 °C extension for 60 s to 135 s, depending on the length of the individual genes (*see* **Note 4**).
3. The PCR products are separated on a 1% agarose gel by electrophoresis, and amplicons of the appropriate size are subsequently excised from the gel and

extracted using the Qiagen gel extraction kit (Qiagen) according to procedures recommended by the manufacturer.

3.2. Cloning

To generate the influenza virus entirely from cloned cDNA, at least 12 plasmids should be constructed (*see* **Note 5**). The RT-PCR product of the NP, PA, PB1, and PB2 genes can be cloned directly into the pCR®3.1 eukaryotic expression vector (Invitrogen) following the manufacturer's protocol (*see* **Note 6**).

Eight transcription plasmids are further constructed by inserting the eight individual viral genomic sequences between the human RNA polymerase I promoter and the mouse RNA polymerase I terminator sequence in the pHH21 vector following the procedure described below.

1. Preparation of inserts:
   a. Digest the RT-PCR products (0.5–2 μg) with BsmBI restriction enzyme by adding 2 μL in a 20-μL reaction. Incubate the reaction mix at 55 °C for at least 2 hr.
   b. Separate on a 1% agarose gel by electrophoresis, and the digested products of the appropriate size are subsequently excised from the gel and purified using the Qiagen gel extraction kit. Although inserts of low concentration have been successfully cloned into the vector, it is recommended to prepare digested inserts of 25–100 ng/μL (*see* **Note 7**).
2. Preparation of pHH21 vector:
   a. Digest the vector (2.5–5.0 μg) with BsmBI enzyme (3 μL) in a 30-μL reaction. Incubate the reaction mix at 55 °C for at least 2 hr. Separate the plasmid by electrophoresis on an agarose gel, and purify the digested vector as described above.
   b. Dephosphorylate the restriction-digested vector (50–100 ng/μL) to prevent self-ligation by treating with shrimp alkaline phosphatase (SAP) (Promega) (1 unit/μg DNA) at 37 °C for 15 min. Then, inactivate the SAP by heating at 65 °C for 15 min. Briefly centrifuge the reaction mix. SAP-treated vectors may be used immediately or stored at −20 °C for later use.
3. Cloning into the pHH21:
   a. Ligate the digested insert and vector using LigaFast™ Rapid DNA Ligation System (Promega). In a 10-μL ligation reaction, add 1 μL of vector (25–100 ng), 3 μL of insert (75–300 ng), 5 μL of 2X ligation buffer, and 1 μL of ligase. Mix the reaction gently and incubate for 5 min at room temperature.
   b. Use 2 μL of the above ligation reaction for transformation into competent cells. Follow the general transformation protocol that can be found elsewhere

[9]. pHH21 vector contains the ampicillin resistance gene, and LB plates containing 100 μg/mL of ampicillin can be used for clone selection.

c. The presence and orientation of the insert can be screened using vector-specific primers in combination with a unique primer for the insert. Select and pick 6–12 colonies and culture them for 4 hr in 200 μL of LB medium containing 100 μL/mL of ampicillin in 96-well tissue culture plate at 37 °C. Use 1 μL of culture from each well as a template for 12.5 μL of PCR reaction to screen for positive clones using the vector-specific primers.
d. Once clones with the correct inserts have been identified, perform a miniprep using the Qiaprep Spin Miniprep Kit (Qiagen) or equivalent to obtain template for sequencing. The vector-specific primers can be used for sequencing.
e. Make a glycerol stock of cultures with the correct inserts for long-term storage of purified and sequence-verified clones.

3.3. Preparation of Cells and Transfection

Due to the species specificity of the human RNA Polymerase I promoter [6], only cells from primate origin with high transfection efficiency (293T or Vero cells) can be used (*see* **Note 8**). In general, lower rescue efficiency is observed using Vero cells compared to the 293T cells. Since MDCK cells support the growth of many influenza viruses and can be grown in the same media as 293T cells, co-cultured 293T-MDCK cells can be used to improve the rescue efficiency of some strains of the influenza virus [10].

1. Prepare cell cultures: 293T cells and MDCK are maintained in DMEM supplemented with 10% FBS at 37 °C in 5% CO_2. Vero cells are maintained in DMEM/F-12 with 10% FBS with the same conditions.
2. The day before transfection, trypsinize the cells and plate approximately 2×10^6 cells/2 mL per well in 6-well cell culture plate. For the 293T-MDCK co-culture, approximately 1×10^6 cells of each cell line are used. Nearly confluent (95–100%) cells at the time of transfection are ideal.
3. Transfection: Cells are transfected with a plasmid using Lipofectamine 2000 according to the manufacturer's instructions.

    a. Dilute 1 μg of appropriate plasmid (or 1 μg of each plasmid), depending on the experiment, in 250 μL of Opti-MEM® I Reduced Serum Medium without serum.
    b. Dilute the 2 μL of Lipofetamine 2000 reagent per 1 μg of DNA used in 250 μL of Opti-MEM® I medium. Mix gently and incubate for 5 min at room temperature.
    c. After the 5-min incubation, combine the diluted plasmid with the diluted Lipofectamine 2000. Mix gently and incubate for 20 min at room temperature to allow the plasmid-Lipofectamine 2000 complexes to form.
    d. Add the 500 μL of plasmid-Lipofectamine 2000 mixture to the wells containing appropriate cells. Mix gently by rocking the plate back and forth.

e. Incubate the cells at 37 °C in a CO_2 incubator for 24–48 hr depending on the experiment.

3.4. Confirmation of the Integrity of RNA Pol I Promoter and Terminator for Influenza cDNA Transcription

The ability of the RNA Pol I promoter in pHH21 vector to drive synthesis of influenza viral RNA can be assessed using different methods. It is useful to generate a cDNA carrying a reporter gene in antisense orientation that is flanked by the 5' and 3' noncoding regions of an influenza viral RNA. This construct can also be used to confirm the integrity of the expression vector (see Section 3.6). In our lab, we constructed the pPolI-NS-GFP plasmid, which contains the GFP gene [derived from pEGFP-N1 (Clonetech, Palo Alto, CA)] in an antisense orientation. The antisense GFP coding sequence, flanked by the 5′ and 3′ ends of the nonstructural gene sequences of the A/turkey/Oregon/71 AI virus isolate, was fused exactly between the human RNA polymerase I promoter and the mouse RNA polymerase I terminator sequence in the pHH21 vector. Transfection of this construct into eukaryotic cells leads to transcription of the reporter gene by cellular RNA polymerase I. Upon influenza virus infection, the artificial vRNAs are replicated and transcribed by the viral polymerase complex, resulting in the expression of the reporter gene.

1. Transfect the nearly confluent 293T or Vero cells with 1 μg of pPolI-NS-GFP plasmid (see Section 3.4).
2. Twenty-four hours after transfection, infect the cells with the parent A/turkey/Oregon/71 virus [multiplicity of infection (MOI) = 1.0].
3. Forty hours after infection, expression of the GFP is evaluated using a microscope with a UV fluorescence lamp. Successful transcription of the artificial RNA will lead to more than 50% expression of the green fluorescence protein.

Transcription of the individual RNA segments can be confirmed by the following method:

1. Transfect 1 μg of transcription plasmid into 293T cells (see Section 3.4).
2. Extract total RNA using SV Total RNA Isolation System (Promega) following the manufacturer's protocol.
3. Any DNA contaminants are removed by digestion with DNase I. Briefly, add 1 unit of RQ1 RNase-Free DNase (Promega) and incubate for 15 min at 37 °C.
4. Re-extract RNA with the RNeasy Mini Kit (Qiagen).
5. Perform the PCR and RT-PCR at the same time with the viral gene sequence-specific primers. Successful transcription of the viral RNA can be confirmed by positive RT-PCR and negative PCR results.

3.5. Confirmation of Expression by the Plasmids That Provide the Polymerase and NP Proteins

Expression of the influenza viral proteins PB2, PB1, PA, and NP that are necessary for functional RNP complex can be checked by testing whether the expression of those plasmids leads to the transcription of an artificial vRNA.

1. Co-transfect 293T cells with plasmids (1 μg per plasmid) expressing the PB2, PB1, PA, and NP proteins together with the pPolI-NS-GFP for intracellular synthesis of reporter gene vRNA.
2. Forty-eight hours after transfection, GFP expression can be determined with a fluorescence microscope. Successful expression of the viral proteins leads to more than 50% GFP expression.

A fluorescent antibody (FA) test can also be performed if the pPolI-NS-GFP or a similar construct is not available, but an antibody specific for the individual influenza viral protein (e.g., monoclonal antibody against HA protein) has to be available.

1. Co-transfect 293T cells with plasmids (1 μg per plasmid) expressing the PB2, PB1, PA, and NP proteins together with the pHH21 vector that contains, for example, the HA gene if that is a protein to which antibody is available.
2. Forty-eight hours after transfection, wash the cells twice with warm PBS. Add 2 mL of PBS into each well with a serological pipette and swirl plate gently. Discard the PBS and repeat washing. Then fix with 4% para-formaldehyde for 10 min at room temperature.
3. Wash cells three times as described above with room-temperature PBS, and permeabilize the cells with 0.1% Triton X-100, 3 min at room temperature.
4. Wash cells three times as described above with PBS, and block with 1% BSA for 30 min at room temperature.
5. Remove the blocking solution and incubate with HA-specific primary antibody for 1 hr at room temperature.
6. Wash the cells three times with PBS as described above but more thoroughly by gently swirling the plate for 3 min, and incubate with diluted FITC-labeled conjugate for 1 hr at room temperature.
7. Wash the cells three times with PBS thoroughly as described above and evaluate the level of expression with a fluorescence microscope.

3.6. Generation of Infectious Influenza Viruses Entirely from Cloned cDNAs

Once all the reverse-genetic plasmids have been generated and tested for transcription and expression, the infectious virus can be generated by co-transfecting all the plasmids in appropriate cell lines [11] (see Figure 1).

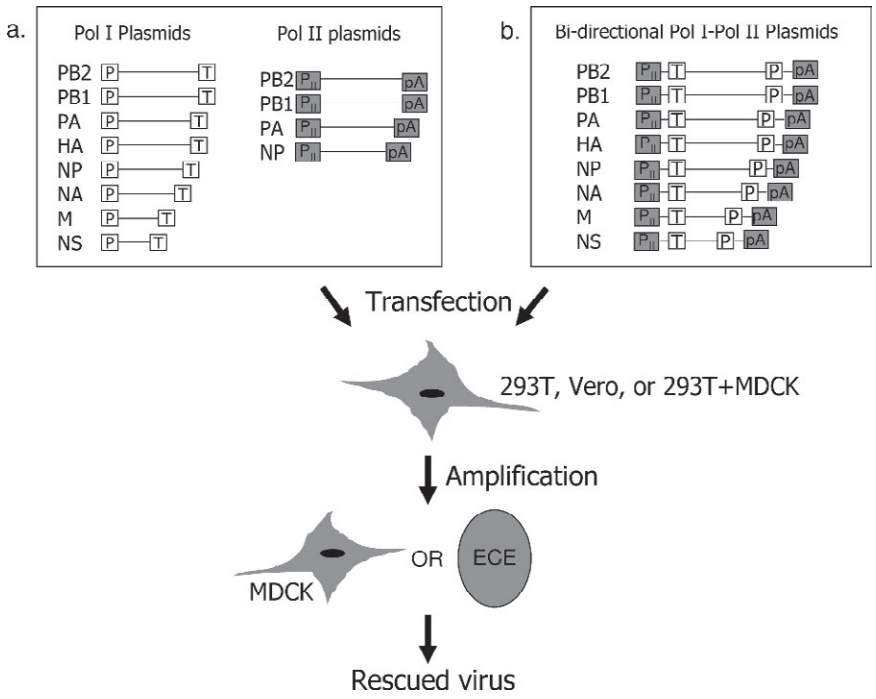

Figure 1 Plasmid-based reverse genetics system. (a) Cells are transfected with eight transcription plasmids (transcribing the eight viral RNA segments) together with four protein (NP, PA, PB1, and PB2) expression plasmids or (b) eight bidirectional plasmids [7]. After 48 hours of transfection, the harvested cells and cell supernatants are inoculated into embryonating chicken eggs or MDCK cells depending on the strains being rescued. After 48 hours of incubation, allantoic fluid or cell supernatant is harvested and tested for the presence of infectious virus

1. Eight plasmids containing the RNA polymerase I promoter, a cDNA for each of the eight viral RNA segments, and the RNA polymerase I terminator (pHH21-PB2, pHH21-PB1, pHH21-PA, pHH21-HA, pHH21-NP, pHH21-NA, pHH21-M, pHH21-NS) are transfected into cells together with at least four protein expression plasmids (pCR3.1-PB2, pCR3.1-PB1, pCR3.1-PA, pCR3.1-NP) (*see* **Note 9**). Use 1 μg of each plasmid and 24 μL of Lipofectamine 2000 reagent for the transfection (see Section 3.4).
2. Forty-eight hours after transfection, freeze the cells. Repeat the freeze-thaw cycle twice.
3. Collected supernatant and disrupted cells are subsequently inoculated into 9- to 11-day-old SPF chicken embryos. After 48 hours of incubation, the amnioallantoic fluid containing infectious virus is harvested and may be stored at –80 °C (*see* **Note 10**).

4. The presence of infectious virus can be confirmed by hemagglutination test (see Chapter 7). Briefly, 50 μL of 0.5% chicken erythrocyte suspension in PBS are added to 50 μL of a twofold dilution of virus in phosphate buffered saline (PBS), and the mixture is incubated at room temperature for 30 min. The hemagglutination titer is calculated as the reciprocal value of the highest virus dilution that caused complete hemagglutination.
5. The identity of the rescued virus should be confirmed by sequencing of each viral gene segment.

3.7. Helper Virus-Based Reverse Genetics

Construction of eight transcription plasmids for every virus to be rescued can be demanding and labor-intensive. This section briefly describes an alternative method of generating virus using only one transcription plasmid and the helper virus. This method, however, requires a strong selection system for the reassortant virus being rescued.

In the helper virus-based reverse genetics, a plasmid containing a cDNA encoding the vRNA to be rescued between the RNA polymerase I promoter and the RNA polymerase I terminator is transfected into cells. Intracellular transcription by RNA polymerase I produces vRNA from a cDNA template, which is then packaged into progeny virus particles upon helper virus infection. With this method, reassortant viruses (i.e., viruses containing RNA derived from cloned cDNA with the remaining genes from a helper virus) are selected from the vast helper virus population and thus require a strong selection system. Here we describe the generation of hemagglutinin (H) gene mutants, which may grow in cell culture without trypsin (an indicator of pathogenicity for poultry) from a low pathogenic parent virus using this system [12] (see Figure 2).

1. Nearly confluent 293T cells are transfected with 1 μg of transcription plasmid that contains desired HA gene sequences, together with the four expression plasmids by using Lipofectamine 2000 reagent (Invitrogen) (see Section 3.4).
2. Twenty-four hours after transfection, the cells are infected with either the parent virus from which the HA gene sequence was derived or low pathogenic AI viruses that do not form plaques in chicken embryo fibroblast (CEF) cells without the addition of trypsin (MOI = 1.0).
3. Forty hours after infection, the supernatants of the infected cells are collected and used to infect CEF cells.
4. Reassortant viruses in the supernatants are screened by sequencing the virus from plaques formed in the CEF cells. Because the helper virus does not form plaques in CEFs, the presence of plaques indicates the reassortant virus that contains the mutated HA gene, which can grow in cell culture without trypsin.

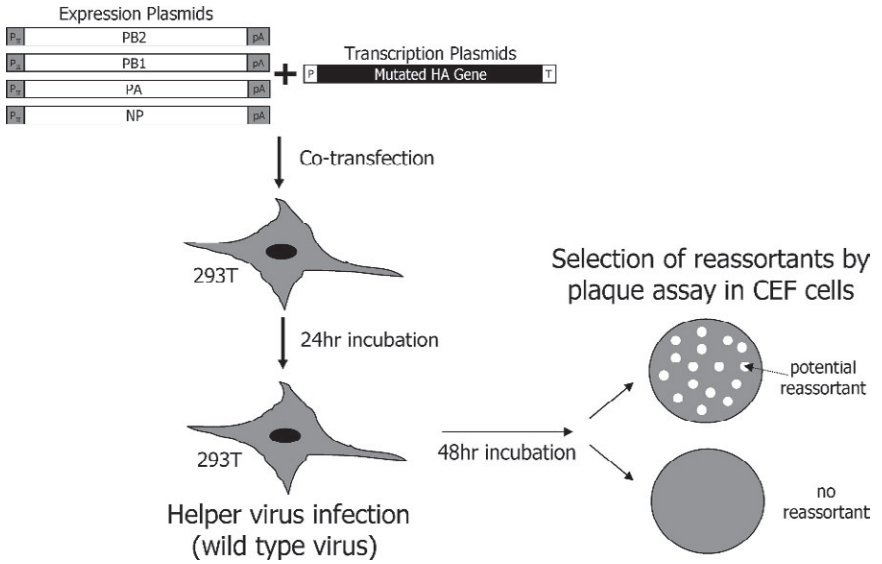

Figure 2 Helper virus-dependent reverse genetics system. Cells are transfected with a plasmid containing a cDNA that encodes a mutated HA gene RNA, flanked by RNA polymerase I promoter and terminator sequences. Transcription of mutated HA gene RNA occurs by cellular RNA polymerase I upon transfection. After infection with influenza helper (parental) virus, newly synthesized mutated HA gene RNA undergoes replication and transcription and is packaged into a progeny virus particle. Because the helper virus does not form plaques in CEF cells, the presence of plaques may indicate a reassortant virus containing the mutated HA gene. Reassortants are further confirmed by purification and sequencing

4. Notes

1. The primers are designed based on the published sequence. However, influenza viral sequence varies in the noncoding region, and it is recommended to sequence the specific isolate to be rescued and modify the primer sequences accordingly.
2. In general, RNA extraction with Trizol reagent yields a higher concentration of RNA than the other two methods. If a high enough titer or high enough concentration of virus is not available, it is recommended to use Trizol reagent and then use less water for the final reconstitution of the RNA pellet.
3. RT-PCR reactions can introduce unwanted mutations, and the product is not always an accurate copy of the template molecule. To reduce the rate of inaccurate synthesis of double-stranded cDNA, the use of high-fidelity RT-PCR kits available from numerous companies is recommended.

4. In many cases, the larger-size genes (PA, PB1, and PB2) are difficult to amplify in full length. In that case, first consider using a two-step RT-PCR reaction, which is, in general, more sensitive than the one-step RT-PCR. If the two-step RT-PCR does not improve the amplification, those gene segments can be amplified in two parts using internal primers and those segments ligated to create a full-length gene.
5. Reverse genetics of influenza virus using 8- or 3-plasmids has also been developed [7, 8]. However, the efficiency of these systems has not been compared side by side.
6. Cloning into the pCR3.1 vector is an easier approach, since subcloning into another vector is not required. We and other researchers [4, 13] have used other eukaryotic expression vectors such as pCI, pCI-neo (Promega), pCAGGS/MCS, and pCDNA3.1.
7. If the concentration of the RT-PCR product is too low, it is recommended to clone the product into vectors such as TA vectors (Invitrogen). Then the insert can be efficiently subcloned into the pHH21 vector.
8. Massin et al. [14] recently cloned the chicken RNA polymerase I promoter sequence and used CEF and QT-6 cells for the generation of the influenza virus.
9. Although infectious viruses can be generated with plasmids expressing just the PA, PB1, PB2, and NP genes, the expression of the remaining structural proteins increases the efficiency of virus production [4].
10. Some mammalian-origin influenza viruses (such as triple-reassortant H3N2 virus from turkeys) do not grow readily in embryonating chicken eggs or 293T cells [15]; therefore, it is recommended to blind-passage the supernatant from transfected cells three to five times in MDCK cells.

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Chapter 13
Evaluating the Cell-Mediated Immune Response of Avian Species to Avian Influenza Viruses

Darrell R. Kapczynski

Summary The measurement of avian cellular immunity is critical to understanding the role and regulation of avian lymphocytes following avian influenza (AI) virus infection. Although the ability to measure avian T cell responses has steadily increased over the last few years, few studies have examined the role of cell-mediated immunity in avian species against the AI virus. Because of the structural and functional differences between mammalian and avian immune systems—including MHC architecture, different modes of somatic recombination for antibody production, and the absence of lymph nodes in birds—the extent to which birds and mammals regulate similar immune responses against the AI virus is currently under investigation. The increasing availability of monoclonal antibodies recognizing avian T cell-associated antigens as well as a number of inbred lines of chickens with genetically defined MHC haplotypes make this an important field of research for the future.

Keywords methods; cell-mediated immunity; avian influenza; avian lymphocytes; proliferation; cytotoxicity; flow cytometry; cytokines.

1. Introduction

The major function of an immune response is to recognize and eliminate invading microorganisms. The immune system of vertebrates is made up of two functional elements, the innate and adaptive, which contrast by their time of response and their mechanisms of pathogen recognition. The early reactions of the innate immune system use germ-line encoded receptors, known as pattern recognition receptors, which recognize evolutionarily conserved molecular markers of infectious microbes, known as pathogen-associated molecular patterns (PAMPs). The latter adaptive immune responses use highly specific antigen receptors on T- (cellular immunity) and B-lymphocytes (humoral immunity) that are generated by random processes by gene rearrangement. There are two well-defined T cell lymphocytes subpopulations: T helper (T_H) and T cytotoxic (T_C), which have been characterized by antigenic glycoproteins found on their surfaces. T cells displaying CD4 markers generally function as T_H cells, while those displaying CD8 generally function as

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T_C cells. Two subpopulations of T_H cells have been described and characterized, as T_H1 and T_H2, based on cytokine profiles secreted following stimulation. T_H1 cells secrete cytokines that support the induction of a cell-mediated immune response, including interferon–γ and interleukin (IL)-12. T_H2 cells produce IL-4, -5, and -13 and are responsible for aiding in the humoral immune response. The innate immune response drives the adaptive immune response and thus defines the nature of the overall immunological response. That influence is predicated by and dependent upon the type of cytokine response generated at the cellular level.

This chapter will describe methods useful for measuring antiviral cell-mediated immune responses in chickens following AI virus challenge. As a rule, all procedures should be carried out in proper biosafety-level containment (BSL-2, BSL-3, or BSL-3Ag) under sterile conditions with proper personal biosafety equipment. Although these techniques may be applied to other avian species through empirical testing, it is necessary to mention that many reagents produced against chicken markers do not necessarily cross-react with other avian species. In addition, some chicken-specific reagents appear to preferentially react with certain genetic lines of birds. These observations underscore the difficulties in researching avian cellular immunity and the need for empirical testing of reagents with birds to determine compatibility. The reader may refer to other sources for further information with the understanding that not all mammalian-based immunoassays work in the avian model.

2. Materials

2.1. Preparation of Lymphocytes

1. Vacutainer tubes filled with either K-EDTA or heparin (BD Vacutainer™ CPT, BD, Franklin Lakes, NJ).
2. Hanks Balanced Salt Solution (HBSS) (Fisher Scientific, Atlanta, GA).
3. Ficoll-Hypaque (F-H) (or Histopaque) 1077 from Sigma-Aldrich, Inc. (St. Louis, MO).
4. Phosphate buffered saline (PBS) containing 0.85% NaCl, pH 7.2.
5. Nylon-mesh cell strainer with a pore size of 70 μm (Fisher Scientific).
6. 1% Methylcellulose (Sigma Chemical Co., St. Louis, MO) in sterile distilled deionized water at a 1.5:1 ratio.
7. HBSS formulated without Ca^{2+} and Mg^{2+} (CMF-HBSS) (Fisher Scientific)
8. RPMI-1640 media (Sigma).
9. CMF-HBSS containing 1 mM of dithiothreitol (DTT) (Sigma).
10. CMF-HBSS containing 5% fetal calf serum (FCS) (Cellgro, Herdon, VA).
11. CMF-HBSS containing 0.5 mM of EDTA (Sigma) and 5% fetal calf serum (FCS) (Cellgro).
12. Nylon wool (Polysciences, Inc., Warrington, PA).
13. Percoll density gradient (GE Healthcare Bio-Science Corp., Piscataway, NJ).

2.2. Lymphocyte Proliferation

1. Proliferation media: 94 mL of RPMI-1640 (Sigma), 4 mL of GGNP. GGNP is 2 parts glutamine (200 mM), 1 part nonessential amino acids, 1 part pyruvate 10 mM) (Fisher), 1 mL of FCS (Cellgro), 1 mL of antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) (Fisher Scientific).
2. Concanavalin A (ConA, 12.5 μg/mL) (Sigma).
3. ^{3}H thymidine (e.g., Amersham Biosciences, Piscataway, NJ, or ICN Pharmaceuticals Inc., Costa Mesa, CA).
4. Automated cell harvester (Skatron cell harvester, Packard Bioscience, Meriton, CT).
5. 96-Well filter plate (Packard Bioscience).
6. MTT [3-(4,5-dimethyethiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Promega, Madison, WI).
7. CMF-HBSS (see Section 2.1).
8. 0.04 N of HCl (Sigma).
9. HBSS or RPMI-1640 (see Section 2.1).
10. AlamarBlue™ (Biosource International Inc., Camarillo, CA).

2.3. Virus-Specific Cytotoxicity

1. Sterile nylon mesh with a pore size of 70 μM (Fisher Scientific).
2. Ficoll-Hypaque (or Histopaque) 1077 (Sigma-Aldrich).
3. 1X PBS.
4. RPMI-1640 (Sigma).
5. T-75 flask (Fisher Scientific).
6. RPMI-1640 (Sigma) supplemented with 10% chicken serum (Sigma-Aldrich), 2 mM of L-glutamine (Sigma), 5×10^{-5} M of 2-mercaptoethanol (Sigma), 0.01 mM of sodium pyruvate (Sigma), and 0.1 mM of MEM nonessential amino acids (Fisher Scientific).
7. Nylon wool columns (Polysciences, Inc.).
8. Rabbit antichicken immunoglobulin G (IgG; 60 mg/mL in PBS, pH 7.2] (Bethyl Inc., Montgomery, TX).
9. Mouse antichicken CD4+, CD8+, TCR1, or TCR2 monoclonal antibody (Southern Biotech, Birmingham, AL) in PBS.
10. PBS containing 0.1% bovine serum albumin (BSA) (Sigma).
11. Magnetic beads for antibody coupling (Miltenyi Biotec, Auburn, CA).
12. Flow cytometer for fluorescence-activated cell sorting (BD, Mountain View, CA, or Beckman Coulter, Miami, FL).
13. ^{51}Chromium ($Na_2{}^{51}CrO_4$) (DuPont NEN, Wilmington, DE).
14. 96-Well round bottom plate (Fisher Scientific).
15. 3% Triton®-X100 (Sigma) solution in sterile ddH_2O.

2.4. Immunophenotyping with Flow Cytometry

1. Mouse antichicken CD4+, CD8+, TCR1, or TCR2 monoclonal antibody (Southern Biotech).
2. 1X PBS.
3. Rabbit antimouse IgG FITC-labeled conjugate antibody (Southern Biotech).
4. Vacutainer tubes filled with either K-EDTA or heparin (BD Vacutainer™ CPT).
5. 1X PBS containing 4 mg/mL of BSA (Sigma) and 1 mg/mL sodium azide (Sigma).
6. A solution of 2% formaldehyde (Fisher Scientific).
7. 1X PBS containing 10% FCS (Cellgro).
8. 96-Well nitrocellulose filter (0.45 μM) (Fisher Scientific).
9. RPMI-1640 (Sigma) containing 5% FCS (Cellgro), 2 mM glutamine (Sigma), 15 mM HEPES (Fisher Scientific), 100 U/mL penicillin, and 100 U/mL streptomycin (Fisher Scientific).
10. Secondary antibody (goat antichicken IgG–horseradish peroxidase conjugate) (Bethyl Inc.).
11. 1X PBS containing Tween 20 (0.01%) (Sigma) with 5% FCS (Cellgro).
12. AEC substrate (Vector Laboratories, Burlingame, CA) or TMB membrane peroxidase substrate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD).

3. Methods

3.1. Preparation of Lymphocytes

The source of lymphocytes used for measuring cellular immunity in poultry and wild bird species can be procured from either peripheral blood or from tissues, including spleen, bursa, and gut. The advantage for using tissues is the large number of cells collected per tissue. On the other hand, collection of peripheral blood lymphocytes (PBL) allows for time–course analysis of the cellular immune response by an individual. For companion avian species, PBLs are the usual source. Lymphocytes can be collected using gradient centrifugation or, in the case of tissues, by the use of filtration through mesh screens depending on the downstream application [1–8].

3.1.1. Isolation of PBLs over a Ficoll-Hypaque (F-H) Gradient

1. Whole chicken blood (5 mL) is collected following venipuncture in syringes and quickly mixed with either K-EDTA or heparin to prevent coagulation. (If necessary, blood may be mixed 1:1 with Hanks Balanced Salt Solution following collection.)
2. The blood is layered onto an equal amount of F-H (or Histopaque) 1077 and centrifuged at 400Xg for 30 min with no brake.

3. Lymphocytes are removed at the mononuclear cell interface (see Figure 1) and washed twice in HBSS to remove any contaminating F-H.
4. Resuspend cells in appropriate media and count using Trypan Blue exclusion. Cell viability should be >95%. Enumerate using a hemacytometer at 400X.

3.1.2. Isolation of Lymphocytes from Avian Tissues by Mesh Screen Filtration

1. Spleens are collected aseptically and placed in sterile cold PBS (0.1 M of phosphate buffer with 0.85% NaCl, pH 7.2).
2. Single-cell suspensions are prepared by gently pushing the splenic pulp through a sterile nylon mesh with a pore size of 70 μM (60–150 μM) (*see* **Note 1**).
3. Cells are washed and resuspended in 3 mL of cold PBS and then layered over 3 mL of F-H.
4. The preparations are enriched for leukocytes by centrifugation (450Xg) for 30 min at 18 °C.
5. Cells are recovered from the interface, resuspended in cold PBS, and washed twice in 3 mL of cold PBS (see Figure 1).

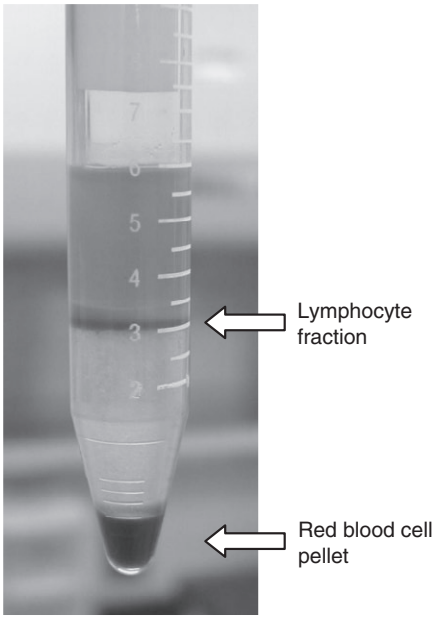

Figure 1 Isolation of splenic lymphocytes over Histopaque 1077 (see Section 3.1.2) following centrifugation (450Xg for 30 min). The purified lymphocyte fraction is harvested at the interface and washed for further application. The red blood cells are found at the bottom of the tube

3.1.3. Isolation of Peripheral Blood Heterophils

1. Collect blood in the presence of anticoagulant (*see* **Note 2**) and mix with 1% methylcellulose at a 1.5:1 ratio.
2. Following centrifugation at 25Xg for 30 min, collect the serum and Buffy coat layers and suspended in Ca^{2+}-free and Mg^{2+}-free HBSS (CMF-HBSS) (1:1).
3. Overlay the suspension on a discontinuous Ficoll–Hypaque (Sigma Chemical Co.) gradient (specific gravity 1.077 over specific gravity 1.119).
4. Centrifuge at 250Xg for 60 min, and collect the band containing the heterophils at the 1.077/1.119 interface.
5. Wash cells twice in RPMI 1640 medium and resuspend in fresh RPMI 1640.
6. Determine cell viability by Trypan Blue exclusion. The purity of the heterophil suspensions obtained by this method is typically >98% pure and >95% viable.

3.1.4. Isolation of Intraepithelial Lymphocytes (IELs)

1. Intestinal tissues (*see* **Note 3**) are collected and pooled from five chickens, and cut open.
2. In a Petri dish full of media, flush out the contents of the gut with ice-cold Ca^{2+}-free and Mg^{2+}-free HBSS (CMF-HBSS) containing 1 mM of dithiothreitol (DTT).
3. Cut tissue into 1-in. (2.5-cm) sections; put into a beaker with 250 mL of CMF-HBSS containing 0.5 mM of EDTA and 5% FCS for 20 min at 37 °C with constant swirling.
4. Cells released into the supernatant are pooled, passed through nylon wool (Robbins Scientific, Sunnyvale, CA), and washed twice with CMF-HBSS containing 5% FCS.
5. The IELs are purified on a discontinuous Percoll density gradient by centrifugation at 600Xg for 25 min at 24 °C and washed three times with CMF-HBSS containing 5% FCS.
6. Cell viability should be greater than 95% and lymphocytes more than 80% viable as determined by Trypan Blue dye exclusion assay.

3.2. Lymphocyte Proliferation

Lymphocyte proliferation is a measurement of cellular memory response to an antigenic stimulus and is considered an *in vitro* correlate of antigen recall response. Only birds previously exposed to the AI virus (either by vaccination or by natural exposure) will react with a proliferative response. To study proliferation, several methods are available, including measurement of DNA synthesis or measurement of the reducing environment of the cells. The methods used to measure DNA synthesis include (1) quantitation of ^{3}H-thymidine incorporation, (2) quantitation of 5-bromodeoxyuridine (BrdU) incorporation, and (3) monitoring DNA quantity

with the fluorescent dye (e.g., Hoechst 33258). There are two methods of monitoring the reducing environment of the cell: (1) quantitation of tetrazolium salt reduction and (2) quantitation of alamarBlue™ reduction.

When added to media of proliferating cells, ^{3}H-thymidine is incorporated into newly synthesized DNA. While providing an accurate indicator of DNA synthesis, measurement of ^{3}H-thymidine incorporation has several disadvantages, including the use of radioactivity, and sample processing and quantitation can be performed at only one predetermined time point. Continuous monitoring of a single sample is not possible with this method. Detection of incorporation of BrdU is through indirect methods (usually antibody conjugate, which is then developed with a substrate). As with ^{3}H-thymidine, this method has some chemical hazard associated with it and is also a single sample time point [9–11]. Quantification with tetrazolium salts depends on the internal environment of proliferating cells being more reduced than that of nonproliferating cells (*see* **Note 4**). This reduced state can be measured using tetrazolium salts. The most frequently used of these are MTT [3-(4,5-dimethyethiazol-2-yl)-2,5-diphenyltetrazolium bromide] [10], XTT (sodium 3'-[1-phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate, and MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]. As with tetrazolium salts, alamarBlue™ monitors the reducing environment of the proliferating cell. alamarBlue™ is soluble, stable in culture medium, and nontoxic, allowing for continuous monitoring of cells in culture [4, 12].

3.2.1. Choice of Avian Influenza Virus Antigen

Different sources of AI virus antigen can be used for measuring the proliferation of lymphocytes following stimulation *in vitro*. Formalin or beta propriolactone-inactivated whole virus (see Chapter 8) is a good starting point for measuring lymphocyte proliferation. Under these conditions, the cells are able to respond to all viral proteins. One consideration is the choice of homologous or heterologous virus. For example, if vaccination or infection of birds occurs with an LP H3N2 virus, the proliferative response to homologous virus should be high, however, to measure the contribution of individual viral proteins; the choice of a heterologous virus (e.g., H3N4 or H9N2) may be employed to examine heterosubtypic cellular immune responses. To determine the immunodominant viral protein, it is necessary to use individually purified viral proteins and/or peptides.

3.2.2. ^{3}H-Thymidine Assay

1. Lymphocytes are prepared as in Section 3.1 using either peripheral blood, bursa, or splenic lymphocytes (10^{4}-10^{6} cells), with three to five duplicates of 100 μL/well for each sample, in 96-well microtiter plates.

2. Lymphocytes populations include unstimulated, stimulated by AI virus, and positive stimulation control with concanavalin A (ConA, 12.5 μg/mL) (or other appropriate stimulator). Incubate for 24 or 48 hr at 41 °C in 5% CO_2.
3. Samples are pulsed 6–18 hr prior to collection with 1–6 μCi 3H thymidine (e.g., Amersham Biosciences, ICN Pharmaceuticals Inc.) per well.
4. Harvest cells on glass fiber or automated cell harvester (e.g., Skatron cell harvester, Packard Bioscience, Meriton, CT) onto a 96-well filter plate (e.g., Packard Bioscience) and air-dry.
5. Transfer filter dots to scintillation vials, add scintillation fluid, and count with β counter. Alternative for 96-well format: Treat each well with 30 μL of scintillation liquid and count in a 96-well plate counter.
6. Cell suspensions without virus or mitogens are included in each assay as the control (e.g., media only). The mean count per minute (cpm) is determined for all duplicates of stimulated and unstimulated cells, and the stimulation indices (S.I.) are calculated from the ratio of mean cpm stimulated to mean cpm unstimulated.

3.2.3. MTT [3-(4,5-dimethyethiazol-2-yl)-2,5-diphenyltetrazolium bromide] Assay

1. Lymphocytes are prepared (see Section 3.1) and stimulated with the AI virus and ConA as above (see Section 3.2.2).
2. After incubation at 41 °C for 24–48 hr, 20 μL of MTT (5–10 mg/mL in Ca^+-/Mg^+-free media) are added to each well and incubated for 3–5 hr in 5% CO_2.
3. Centrifuge the plates at 1000Xg for 10 min at room temperature and remove the supernatant.
4. To each well, 150 μL of 0.04 N HCI in isopropanol are added to dissolve the dark blue crystals.
5. The plates are incubated for 5 min, with occasional shaking at room temperature.
6. The optical density (O.D.) of each well is measured at 570 nm.
7. Cell suspensions without virus or mitogens are included in each assay as the control (e.g., medium only). The stimulation indices (S.I.) are calculated from the ratio of mean O.D. stimulated cells to mean O.D. unstimulated cells.

3.2.4. alamarBlue™ Proliferation Assay

1. Collect lymphocytes (see Section 3.1) in prewarmed cell culture media (HBSS or RPMI-1640) at 10^6 lymphocytes per mL. Add 100 μL of cells to wells of a 96-well microtiter plate and stimulate as in Section 3.2.2.
2. Incubate plate(s) in a CO_2 incubator at 40 °C for 24 hr.
3. Add 100 μL of proliferation media to all wells containing inactivated AI virus, Con A, media alone, etc. in triplicate.

4. Add 22 µL of alamarBlue™ (AB) to all wells (add to rows and discard pipettes after each treatment group), and mix gently by pipetting up and down three times. Final concentration of AB is 1:10. After 24–48 hr, read OD: 565–570 (low) and 595–600 (high).
5. The absorbance of alamarBlue™ in culture medium is measured at both a higher wavelength and a lower wavelength. The absorbance of the medium alone (without AB) is subtracted from the absorbance of medium plus alamarBlue™ at the higher wavelength. This value is called AO_{HW}. The absorbance of the medium alone is subtracted from the absorbance of medium plus alamarBlue™ at the lower wavelength. This value is called AO_{LW}. A correction factor R_0 can be calculated from AO_{HW} and AO_{LW}, where $R_0 = AO_{LW}/AO_{HW}$.
6. The percent alamarBlue™ reduced is then expressed as follows: % reduced = $A_{LW} - (A_{HW} \times R_0) \times 100$, with proliferative cells reducing a higher percentage of AB than nonproliferating cells.

3.3. Virus-Specific Cytotoxicity

Cytotoxic T-lymphocytes (CTLs) constitute a major line of defense against viral infection. CTLs lyse virus-infected cells via MHC class I- or II-restricted manner and, as such, measuring the specific CTL response to infection is an important component to understanding cellular immunity against the AI virus in birds. Recognition of virally infected cells occurs through the T cell receptor (TCR), which can recognize processed vial peptides bound to either MHC class I or II proteins on infected cells. MHC class I molecules are recognized by CD8+ lymphocytes, while MHC class II molecules are recognized by CD4+ lymphocytes. Since influenza viruses grow within cells, most viral antigens are delivered by MHC class I molecules. Thus, the CTL response against influenza is predominantly CD8+ and class I-restricted.

Measuring avian CTL responses has been previously performed using ^{51}chromium (^{51}Cr) release assays; however, nonradioactive reagents (e.g., CytoTox96, Promega), which measure the stable cytosolic enzyme lactate dehydrogenate released from lysed cells, have been produced that reduce the inherent risks associated with radiolabeled molecules [13–15]. These molecules are taken up nonspecifically by live cells; when lysed, the release of these compounds can be measured in the supernatant.

3.3.1. Effector Cell Preparation

1. Splenocytes from infected or uninfected chickens are used as the source of effector cells (see Section 3.1.2). Spleens from chickens are collected at 7–10 days' postinfection, and single-cell suspensions are made by passage through a 70-µM mesh screen to remove connective tissue fragments as described above.

2. Lymphocytes are isolated by centrifuging 5 mL of the diluted spleen cells for 20 min at 1,500 rpm through 5 mL of Ficoll-Hypaque gradient (Histopaque 1077).
3. Lymphocytes are collected at the interface, washed three times with PBS (pH 7.2), and washed once with RPMI 1640.
4. Macrophages are removed by incubating cells in 75-cm^2 tissue culture flasks with RPMI 1640 supplemented with 10% chicken serum, 2 mM of L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 0.01 mM of sodium pyruvate, and 0.1 mM of MEM nonessential amino acids for 4 hr at 41 °C.
5. B-lymphocytes are removed by passing the cell suspension from step 4 through a nylon wool fiber column previously equilibrated with complete medium.

3.3.2. Depletion of CD4+ and CD8+ T Cells

1. The CD4+ and CD8+ antigen-bearing lymphocytes can be separated by a modification of the panning method for assessment of Class I and Class II restriction.
2. Briefly, 75-cm^2 tissue culture flasks are incubated overnight at room temperature with rabbit antichicken immunoglobulin G [IgG; 60 mg/mL in PBS (pH 7.2)].
3. Lymphocytes are purified as described above and labeled with either mouse antichicken CD4+, CD8+, TCR1, TCR2, or TCR3 monoclonal antibody (Southern Biotechnology Associates, Birmingham, AL) in PBS.
4. The cells are washed three times with ice-cold PBS (pH 7.2), and the concentration is adjusted to 10^7 cells per mL in PBS.
5. The antibody-labeled cells are added to goat antimouse IgG-coated plates and incubated at room temperature for 30 min.
6. The suspensions are swirled gently before incubation for an additional 30 min at room temperature.
7. Nonadherent cells are decanted from the plates following gentle agitation and centrifuged at 600Xg for 10 min.

3.3.3. Positive or Negative Selection with Magnetic Beads

1. Purified chicken lymphocytes (5×10^7 in PBS with 0.1% BSA) are incubated with mouse antichicken antibodies (1–2 μg) for 10–15 min at room temperature (alternately for 30 min at 4 °C).
2. Mix cells with magnetically labeled secondary antibodies against mouse IgG for 5–10 min (alternatively for 30 min at 4 °C).
3. Place cells in a magnetic field for 2–5 min (*see* **Note 5**).
4. Pour off or decant supernatant and save as negative selected lymphocytes for downstream analysis.
5. Remove the tube from the magnetic field and add fresh media to tube, mix gently, and reapply magnetic field for 5 min.
6. Pour off supernatant as above and then remove the magnetic field. Resuspend in fresh media to harvest positively selected cells.
7. Assess the purity of fractions by flow cytometry.

3.3.4. Generation of Labeled Virus-Infected Target Cells

1. Primary avian cell culture (MHC matched to the effector cells) including CEFs and chick embryo kidney (CK) cells can be used to replicate the virus to limiting degrees and therefore can be used as target cells for CTL activity.
2. Infect cells with homologous or heterologous AI virus for 10 hr at an MOI of 2.
3. For ^{51}Cr assay, mix 300 μCi of $Na_2{}^{51}CrO_4$ with 2×10^6 cells for 90 min. Wash three times, and resuspend in medium at a final concentration of 2×10^5 per mL.

3.3.5. Cytotoxic T Cell Assay

1. Effector cells (splenic lymphocytes, PBLs, enriched or depleted) cell populations (both stimulated and nonstimulated) are tested for cytotoxic activity against infected and noninfected target cells in each assay.
2. Add 0.2 mL of stimulated and nonstimulated effector cells (5×10^6/mL) to a 96-well round bottom plate. Add 0.1 mL of media to the next three rows.
3. Make three doubling (twofold) dilutions with a multichannel pipette by transferring 0.1 mL of cells into the next row of 0.1 mL of fresh media. This will give four effector-to-target cell ratios: 6.25:1, 12.5:1, 25:1, and 50:1. Set up all dilutions in triplicate.
4. Add target cells (100 μL; 2×10^4 per well) prepared as above to each well and incubate at 40 °C with 5% CO_2 for 4–8 hr.
5. Set up three wells of target cells with 0.1 mL of media alone (spontaneous release) and three wells of target cells with 0.1 mL of 3% Triton X-100.
6. After centrifugation (600Xg for 5 min), remove 100 μL of supernatant for determining ^{51}Cr release using a gamma radiation counter. Percent specific lysis equals (E - S/M - S) x 100, where E is the mean of three test wells, S is the mean of spontaneous release from the three target cells without effector cells, and M is the mean maximal release from the three target cells with 3% Triton X-100.

3.4. Identification of Lymphocyte Subsets

The use of antibodies to recognize cell surface markers has provided a foundation for understanding cellular immunology as well as a tool for purifying cell populations for downstream analysis. Of course, measuring cellular fluctuations during infection can provide useful information about the pathogenesis of individual AI virus isolates [16]. Over the last 10 years, the number of antibodies to chicken cell surface markers has steadily increased, providing tools to measure the avian cellular response [1–3]. One important caveat to the measurement of avian cells with flow cytometry is the differences between mammalian and avian erythrocytes. Whereas mammalian erythrocytes can be removed from samples with the addition of a hypotonic lysing solution, avian erythrocytes are nucleated and are resistant to many lysing solutions used in mammalian immunology. In addition, the glutinous

nature of released chromatin from avian erythrocytes can interfere with the mechanics of "flow cytometry." Two general procedures for immunophenotyping are outlined below: one based on standard density centrifugation and the other using whole blood [17]. In addition, a standard ELISPOT assay is included to quantitate the number of AI virus-specific antibody secreting cells (ASC). This measures antibody production by individual B cells using AI virus antigen bound to a membrane. The nonadherent cells are washed away, and bound cells are detected like a regular ELISA. The ASC are then stained with substrate and enumerated. Different tissues harboring AI virus-specific B cells can be compared with this test.

3.4.1. Flow Cytometry

1. Lymphocytes are purified as above (see Section 3.1) and incubated on ice for 30 min with mouse monoclonal antibodies specific for the desired marker in PBS according to the dilution guidelines provided by the manufacturer.
2. Cells are washed (1800Xg for 5 min) three times with PBS (pH 7.2) and incubated on ice with a 1:50 dilution of fluorescein isothiocyanate-labeled goat anti-mouse IgG (1:1000) in PBS for 30 min.
3. After five washes with PBS, the reaction samples were fixed with 2% formaldehyde in PBS and measured by fluorescence-activated cell-sorting (FACS) analysis.

3.4.2. Single-Step Microflow Cytometric Analysis of Avian Leukocytes

1. Collect blood (0.1–0.5 mL) from the wing vein into potassium-EDTA or heparin syringes.
2. Mix and dilute 100 μL of whole blood in 900 μL of PBSA (PBS with 4 mg/mL of BSA fraction V and 1 mg/mL of sodium azide).
3. Dilute antichicken MAb to 0.4–1.0 μg/mL in PBSA and aliquot in 50 μL to each well.
4. Mix MAbs with 10–20 μL of diluted whole blood (approximately 2×10^4 lymphocytes per well). Negative controls include isotype-matched MAb conjugates mixed with lymphocytes.
5. Incubate for 30 min at 4 °C, and add 60 μL of 2% formaldehyde for 1 hr at 4 °C.
6. Add stained lymphocytes to a microfuge tube with 500–1000 μL of sheath fluid and measure percent staining by FACS analysis.

3.4.3. ELISPOT to Detect AI-Specific Antibody-Secreting Cells

1. Nitrocellulose bottomed 96-well multiscreen filtration plates (0.45 μM) are coated with 100 μL/well of purified AI virus antigen (1–5 mg/mL) and incubated overnight at 4 °C.

2. Plates are then washed with PBS and blocked with 10% FCS in PBS for 1 hr at room temperature.
3. Cell preparations from PBL, spleen, lung, or bursa are added in concentrations of 10^2–10^7 cells/well and incubated overnight at 37 °C with 5% CO_2 in RPMI-1640 containing 5% FBS, 2 mM of glutamine, 15 mM of HEPES, 100 U/mL of penicillin, and 100 U/mL of streptomycin. The cells are dispensed into triplicate wells.
4. The cells are removed by rinsing with PBS, and secondary antibody (goat anti-chicken IgG–horseradish peroxidase conjugate), diluted to 1:2000 in PBS-Tween 20 (0.01%) with 5% FBS, is added for 1 hr at room temperature. Plates are washed three times in PBS-Tween 20.
5. The plates are developed by the addition of AEC substrate (Sigma) or TMB membrane peroxidase substrate to identify the spots representing antibody-secreting cells. The spots are counted under a dissecting microscope or alternatively with an ELISPOT reader according to the manufacturer's directions.
6. The number of spots is averaged for triplicate wells, after which the average number of spots in the three negative-control wells is subtracted. The data are reported as the mean number of antibody-secreting cells per starting concentration.

4. Notes

1. To remove macrophage cells, incubate cell suspensions after step 2 on a plastic dish (e.g., T25) for 2 hr at 37 °C in 5% CO_2. Harvest supernatants and continue with step 3.
2. This procedure is recommended for birds less than 2 weeks of age. When birds are older than 2 weeks of age, double centrifugation times at each step, which will result in increased cell yields.
3. Collection of intestinal IELs has been reported from the duodenum or between the Meckel's diverticulum and the ileac region.
4. Following cell proliferation, the ratios of NADPH/NADP, FADH/FAD, FMNH/FMN, and NADH/NAD increase, resulting in an internal environment that is more reduced than that of nonproliferating cells. Compounds such as tetrazolium salts and alamarBlue™, which can be reduced by these metabolic intermediates, are useful in monitoring cell proliferation because their reduction is accompanied by a measurable shift in color.
5. Because each company supplies its own instrument containing a magnetic field, consult the product insert for more specific details for this step.

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Chapter 14
Measurement of Avian Cytokines with Real-Time RT-PCR Following Infection with the Avian Influenza Virus

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Summary Functional and molecular techniques have both been employed to measure the production of cytokines following influenza infection. Historically, the use of functional or antibody-based techniques was employed in mammalian immunology. In avian immunology, only a few commercial antibodies are available to measure avian cytokines. However, the determination of the genomic sequence of *Gallus gallus* species has made it possible to measure cytokine induction without monoclonal antibody- or functional-based tests, but rather based on molecular techniques. Although these tests do not measure functionally expressed cytokines, the lack of reagents to identify and quantify avian cytokines makes them critical to extend any measure of cytokine response. Measurement of cytokine induction, based on the design of primers and probes for RT-PCR or real-time RT-PCR for the cytokine mRNA, has become one of the more recent technologies reported to measure avian cytokines. It is important to note that small nucleotide polymorphisms between different lines of birds may result in substandard results when using published primer and probe sequences. This requires empirical testing to ensure adequate results.

Keywords avian immunology; cytokines; real-time RT-PCR; avian influenza.

1. Introduction

Cytokines are soluble, low-molecular-weight polypeptides and glycopeptides produced by a broad range of cell types of hematopoietic and nonhematopoietic origin that have suppressive or enhansive effects on cellular proliferation, differentiation, activation, and motility. These molecules not only are produced by diverse cell types but also are pivotal for communication between a variety of cells. Cytokines, for the most part, are not constitutively secreted but are produced in response to stimulation by infectious agents or their derived products (for example, endotoxin), inflammatory mediators, mechanical injuries, and cytokines themselves [1, 2]. The development of recombinant DNA technology has provided a cost-effective means of producing large quantities of cytokines for use in human and veterinary medicine. Additionally, the availability of recombinant cytokines allows investigations

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to elucidate the mechanisms of the immune system in response to infection. However, because of the species-specific nature of most of these recombinant cytokines, the identification and purification of the effector avian cytokines and cloning of these genes for cytokines of avian origin are of utmost importance.

In general, chicken cytokines have only 25–35% amino acid identity with their mammalian orthologues [3]. As a result, there are few, if any, cross-reactive monoclonal antibodies or bioassays, and cross-hybridization or degenerative RT-PCR/PCR approaches were also unsuccessful. Prior to the release of the chicken genome sequence, some progress had been made through a combination of expression cloning from expressed sequence tag (EST) libraries, systematic sequencing of EST libraries, and genomics approaches based on the conservation of synteny. However, the availability of the chicken genome sequence has radically altered the ability to understand the repertoire and the functional activities of the avian cytokines and chemokines [3].

The recent cloning of chicken cytokines has driven the development of a more comprehensive panel of reagents for investigations of innate and acquired immune mechanisms at the cellular and molecular levels. Therefore, at present, there is a great opportunity to consider the role of cytokines in the development of inflammatory and immune responses to microbial pathogens under multiple experimental conditions [1, 2, 4].

Chicken orthologues of the Th1 cytokines, interferon-γ (IFN-γ) and IL-18, and the pro-inflammatory cytokines IL-1β and IL-6 have been cloned and sequenced [5–9, 11, 12, 13, 16]. None of the other IL-1 family members is identifiable in the chicken genome. For the Th2 cytokines, the chicken possesses the canonical Th2 cytokine gene cluster genes IL-3, IL-4, IL-5, IL-13, and GM-CSF [4, 6, 17]. The chicken has four members of the IL-10 family: IL-10, IL-19, IL-22, and IL-26. All four chicken genes are expressed, and the function appears to be conserved where IL-10 acts as an anti-inflammatory cytokine, downregulating the effects of IFN-γ; IL-19 acts as a Th2 cytokine; and IL-22 and IL-26 are involved in inflammatory responses. Three subgroups of type I IFN have been identified so far in the chicken: IFN-α, IFN-β, and IFN-λ. The genes for at least seven IFN-α can be identified in the chicken. Three of these have been cloned and fully sequenced as cDNA [10, 14, 15]. IFN-α and IFN-β both have antiviral activity. Information on gene structure now makes it possible to design probes and primers to specifically quantify cytokine mRNA using real-time quantitative fluorescent RT-PCR and to follow changes in cytokine transcription during inflammation and infections.

The real-time quantitative RT-PCR studies demonstrate that chicken immune cells can be induced to express increased levels of cytokine mRNA. Cytokine mRNA appears to be differentially expressed depending upon recognition and signaling pathway activation during innate and acquired immune responses. However, a word of caution should emphasized: Quantitative RT-PCR does not necessarily equate to bioactive protein. For example, to date, only IFN-γ and IL-6 mRNA levels have been shown to correlate with protein levels. Although an increasing number of reliable bioassays for chicken cytokines are available (type I IFN, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, and IL-18), as yet many of these are not specific. For example, the bioassays for IFNs do not differentiate between type I IFN or IFN-γ without the use of neutralizing antibodies. The bioassay for IL-1β is nonspecific in

that it also measures IL-2, as is the bioassay for IL-4, which also measures IL-13. Even fewer monoclonal antibodies are available (type I IFN, IFN-γ, IL-2). Therefore, real-time quantitative PCR is currently the most highly sensitive method available to reliably quantify a wide variety of avian cytokines and chemokines, particularly in the absence of suitable bioassays for most of them [1, 2].

Chemokines are small, structurally related chemoattractant molecules that regulate movement of various leukocytes. They can be divided into subfamilies based on the position of the two amino terminal cysteine residues, i.e., adjacent [CC] or separated by a single amino acid [CXC]. In mammals, CXC chemokines mainly attract polymorphonuclear leukocytes, while CC chemokines primarily induce an influx of mononuclear phagocytes. In mammals, both CCL and CXCL chemokines can be broadly subdivided further by function into those that have homeostatic roles and those that are involved in inflammation. The homeostatic chemokines have a single ligand-single receptor relationship. On the other hand, an inflammatory chemokine receptor can have multiple ligands. Further, inflammatory chemokines are present in three multigene families: the MIP-like and MCP-like CCL chemokines, and a family of CXCL inflammatory chemokines (including human IL-8). Several chicken chemokines and chemokine receptors have been identified and cloned [18–25]. Recently, analysis of the chicken genome sequences showed that the chicken possesses all three multigene families but seems to have evolved a different repertoire of members of these families compared to those in mammals. For this reason, much of the nomenclature for chicken inflammatory chemokines present in the literature is at best premature—and at worst misleading and incorrect [1, 3]. A new nomenclature has been proposed. The inflammatory chemokine genes are designated as either CCLi or CXCLi, the "I" standing for presumed inflammatory function, and numbered in the order in which they occur in the genome. Ten CCLi and only three CXCLi chemokine genes were identified. Of these, CXCLi1 corresponds to the previously described K60, CXCLi2 to the previously described CAF/IL-8, and CCLi4 to the previously described MIP-1β.

2. Materials

2.1. Isolation of RNA

1. Purified lymphocytes or tissues as previously described (see Chapter 13).
2. Concavalin A (ConA, 12.5 μg/mL) (Sigma Chemical Co., St. Louis, MO).
3. Qiagen Mini Prep Kit for RNA isolation (Qiagen, Valencia, CA) or Trizol™ RNA extraction reagent (Invitrogen, Carlsbad, CA).

2.2. Production of Gene-Specific RNA

1. Cloned cytokine gene in eukaryotic expression plasmid (pcDNA 3.1, pCI-Neo, etc.).

2. Cell line (e.g., Vero, COS, MDCK) and growth media.
3. RNA extraction kit (see Section 2.1).

2.3. Real-Time RT-PCR

1. RNA from stimulated cells or gene-specific RNA.
2. Primers and probes specific for gene of interest.
3. Real-time RT-PCR machine (e.g., Applied Biosystems, Foster City, CA; Roche Applied Science, Indianapolis, IN; Bio-Rad, Hercules, CA).

3. Methods

3.1. Production of Positive-Control RNA for Generating a Standard Curve for 28S and Cytokine

1. Isolate avian spleen cells (see Chapter 13) and place in 24-well plate at 40 °C in 5% CO_2.
2. Stimulate cells with either ConA or LPS for 24 hr.
3. Harvest the total RNA using a standard RNA extraction kit.

3.2. Gene–Specific RNA Preparation

1. Using RT-PCR, clone cytokine of choice into a eukaryotic expression vector (e.g., pCI-Neo, pcDNA3.1, etc.). Confirm insertion by sequence analysis.
2. Transfect cells (e.g., Vero, COS, etc.) with purified plasmid DNA for 24-72 hr (see Chapter 12).
3. Harvest total RNA from cells using standard RNA extraction kit.

3.3. Real-Time Quantitative RT-PCR

1. Tissue (e.g., spleen, bursa, thymus, or lung) from avian influenza (AI) virus-infected birds is processed for RNA using standard RNA extraction techniques. For low pathogenic strains, tissues collected 2–10 days' postinfection can be used; for highly pathogenic strains, tissues should be taken between 1–4 days' postinfection. Conversely, if individual cell populations are isolated as above,

inoculate with the AI virus at a multiplicity of infection (MOI) of 1:1. Take aliquots between 8–72 hours postinoculation.

2. Total RNA is prepared from tissues or lymphocyte preparation using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Purified RNA is eluted in 50 mL of RNase-free water and stored at −70 °C.
3. For both cytokine and 28S rRNA-specific amplification, primers and probes should be designed from the sequence of the relevant genes, to lie across intron/exon boundaries. Cytokine and 28S rRNA probes can be labeled with the fluorescent reporter dye 5-carboxyfluorescein (FAM) at the 5' end and with the quencher N,N,N,N'-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3' end (*see* **Note 1**).
4. Real-time RT-PCR is performed using the standard reverse-transcriptase techniques and will require optimization of reagents and platform specifics. Amplification and detection of specific products can be performed using a number of different commercially available systems in a 96-well microtiter plate format (e.g., Roche, Applied Biosystems, Bio-Rad) or tube format.
5. Quantification is based on the increased fluorescence detected by hydrolysis of the target-specific probes by the 5' nuclease activity of the DNA polymerase during PCR amplification. The cycle where the signal increases above the significance threshold is relative to the amount of starting material; the lower the Ct value, the greater the amount of starting material.
6. Results are expressed in terms of the cycle threshold (Ct) value, the cycle at which the change in the fluorescence level of the reporter dye passes a significance threshold.
7. To generate standard curves for the cytokine and 28S rRNA-specific reactions, total RNA, extracted from cells expressing the cytokine mRNA of interest, is serially diluted (10−1–10−5) in sterile RNase-free water.
8. Each RT-PCR experiment is run in triplicate with no-template controls and test samples and a log10 dilution series of standard RNA (step 7). Regression analysis of the mean values of three replicate RT-PCR reactions for the log10 diluted RNA is used to generate standard curves.
9. To correct for differences in RNA levels between samples within an experiment, the difference factor (DF) for each sample is calculated by dividing the mean Ct value for 28S rRNA-specific product of a sample by the mean C_t value for 28S rRNA-specific product from all samples. Adjusted C_t values are then produced by multiplying the C_t value of the cytokine by the DF. Once the adjusted C_t values have been determined, subtract 40 from each sample to obtain the corrected 40 C_t value (*see* **Note 2**). The corrected 40 C_t values are then used to determine the fold changes in mRNA expression (see Tables 1 and 2).
10. Data are expressed as fold change in cytokine mRNA levels in AI virus-treated groups compared with those from age-matched uninfected groups. No difference in mRNA levels is derived as a fold change of 1. The fold change in cytokine mRNA expression is derived by the following equation: [(Experimental 40 C_t) values - experimental control 40 C_t value) × 10] divided by the cytokine slope (see Figure 1).

Table 1 Standard Curve Data from Real-Time Quantitative RT-PCRs on Total RNA Extracted from COS-7 Cells Transfected with pCIneo Expressing the Relevant cDNA[a]

	ΔRn[b]	Log Dilutions	C_t[c]	R^2[d]	Slope
28S	0.03	10^{-3}–10^{-7}	7–21	0.9181	2.7097
IL-1β	0.03	10^{-1}–10^{-5}	13–23	0.9628	3.1335
IL-6	0.03	10^{-1}–10^{-5}	11–26	0.9829	4.453
IFN-α	0.03	10^{-1}–10^{-5}	15–21	0.9876	4.141

[a]For 28S, RNA from chicken splenocytes was used.
[b]ΔRn = change in the reporter dye.
[c]C_t = threshold cycle level: the cycle at which the change in the reporter dye levels detected passes the ΔRn.
[d]R^2 = coefficient of regression.

Table 2 Corrected 40 C_t Basal Levels of Cytokine mRNA Expression in Heterophils Isolated from Day-Old Chickens

Line	IL-1β	IL-6	IFN-α
A	22.85 ± 1.65*	23.79 ± 1.85*	21.58 ± 1.29
B	17.66 ± 1.04	13.94 ± 0.89	23.52 ± 0.66

*Asterisks indicate significant differences ($p < 0.05$) between line A and line B birds.

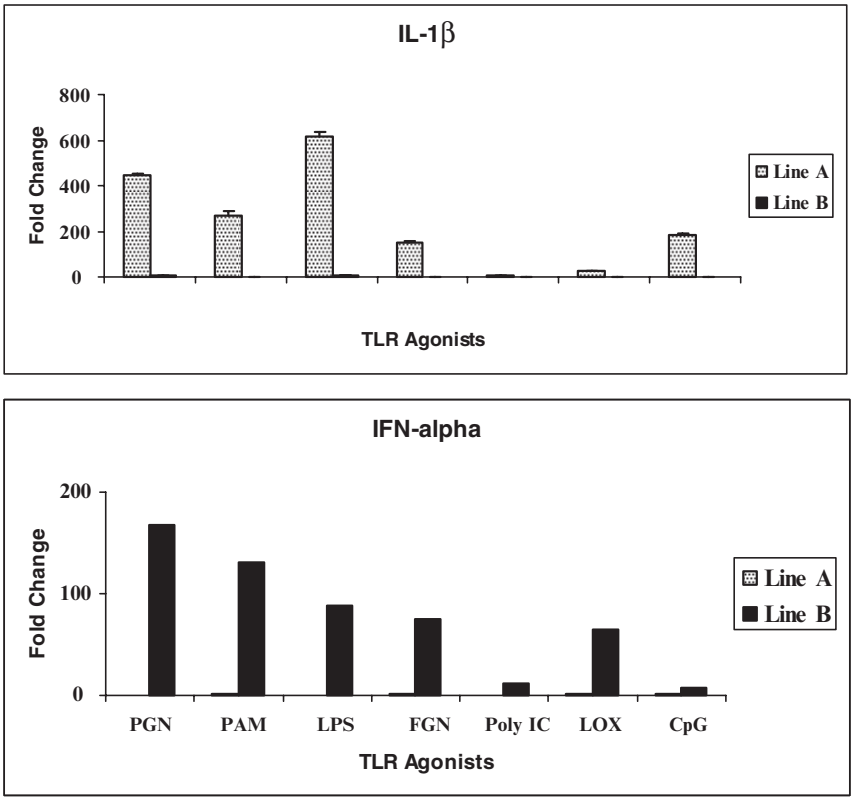

Figure 1 Quantitation of innate cytokine IL-1β and IFN-α mRNA expression induced in heterophils from line A and line B following stimulation by different concentrations (μg/mL) of various toll-like receptor agonists. Data are expressed as the fold change in cytokine mRNA levels when treated samples were compared to nonstimulated control heterophils from age-matched chickens from each line

4. Notes

1. The exact reporter and quencher dyes used will depend on the available real-time PCR instrumentation. Consult with the manufacturer of your instrument.
2. Because samples with lower C_t values represent increased mRNA expression, the C_t values obtained from individual reactions are subtracted from 40 to demonstrate (numerically) increased mRNA expression.

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