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Thomas Lion *Editor*

Human Fungal Pathogen Identification

Methods and Protocols

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Human Fungal Pathogen Identification

Methods and Protocols

Edited by

Thomas Lion

Children's Cancer Research Institute (CCRI), St.Anna Kinderkrebsforschung, Vienna, Austria; LabDia Labordiagnostik GmbH, Vienna, Austria; Department of Pediatrics, Medical University of Vienna, Vienna, Austria

Editor
Thomas Lion
Children's Cancer Research Institute (CCRI)
St.Anna Kinderkrebsforschung
Vienna, Austria

LabDia Labordiagnostik GmbH
Vienna, Austria

Department of Pediatrics
Medical University of Vienna
Vienna, Austria

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Preface

Fungal infections constitute an ever-growing healthcare problem worldwide. Invasive fungal disease (IFD) is a leading cause of morbidity and mortality in severely immunocompromised individuals, including a variety of critically ill patients. High-risk conditions for IFD include hematologic malignancies, hematopoietic stem cell or solid organ transplantations, primary or acquired immunodeficiencies such as AIDS, long-term treatment at intensive care units, preterm birth, long-term immunosuppressive therapy, broad-spectrum antibiotic therapy, and long presence of central indwelling catheters. Despite the availability of a number of potent antifungal drugs, successful treatment of IFD is often hampered by the limited diagnostic options which neither permit rapid and reliable identification of systemic, acute, or latent fungal infections nor facilitate assessment of host susceptibility. The resulting delay in the onset of antifungal therapy is an important factor contributing to the poor overall clinical outcome of IFD. Moreover, the widespread use of prophylactic or empirical treatment without firm evidence of IFD leads to a high rate of overtreatment associated with considerable toxic side effects, and broad-spectrum antimycotic therapy administered against unidentified fungal pathogens can promote the evolution of clinical drug resistance.

The vast majority of IFDs are caused by *Candida* and *Aspergillus* species. However, changes in the epidemiology have occurred over the last decades, with a number of newly emerging fungal pathogens affecting even immunocompetent patients. Additionally, rare but pronounced antifungal drug resistance of some major fungal pathogens (e.g., *C. glabrata, C. krusei, A. terreus*) poses a serious challenge for treatment. Despite current evidence that the innate immune surveillance is critical for defence against invasive fungal infections, host predisposition to IFD, which would permit targeted preventive treatment, is barely considered in diagnostic procedures. Microbiological diagnostic criteria of IFD provided by the EORTC/MSG (European Organization for Research and Treatment of Cancer/Mycoses Study Group) include the detection of fungal pathogens by cytology, direct microscopy, or culture and serological detection of antigens or cell wall constituents, such as galactomannan or ß-d-glucan. By contrast, molecular detection of fungal nucleic acids is currently not accepted as microbiological evidence of IFD due to insufficient clinical evidence and lack of standardized and validated assays.

Rapid, reliable, and species-specific diagnosis of fungal pathogens causing IFD is a prerequisite for cost-effective and successful therapy but remains one of the great challenges. The changing epidemiology, the increasing variety of fungal pathogens, and the rising number of affected patients create a growing demand for broad-range and cost-effective clinical diagnostic tests, also permitting the prediction of pathogenicity and resistance to antifungal agents. The knowledge of host-related predisposing factors and stratified treatment options facilitating timely onset of adequate antifungal therapy are critical for successful clinical management and outcome of IFD. This requires not only rapid diagnosis of a fungal infection and identification of the causative species but also

assessment of pathogen/host factors related to pathogenicity, susceptibility, and response to treatment. The present book responds to the great need for timely and authoritative information offering a comprehensive overview of the current state of the art in fungal diagnostics. Moreover, it addresses ongoing developments expected to provide a basis for targeted treatment strategies resulting in improved outcome of invasive mycoses.

Vienna, Austria ***Thomas Lion***

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Contributors

SANI H. ALIYU • *National Infection Service, Public Health England, Cambridge Microbiology and Public Health Laboratory, Addenbrooke's Hospital, Cambridge, UK*
OLIVER BADER • *Institute for Medical Microbiology, University Medical Center Göttingen, Göttingen, Germany*
ROSEMARY A. BARNES • *Public Health Wales Microbiology Cardiff, University Hospital of Wales, Heath Park, Cardiff, UK; Department of Infection and Immunity, Cardiff University, Heath Park, Cardiff, UK*
KARSTEN BECKER • *Institute of Medical Microbiology, University Hospital Münster, Münster, Germany*
ANKE BURGER-KENTISCHER • *Fraunhofer Institute for Interfacial Engineering and Biotechnology, Stuttgart, Germany*
ELENA DE CAROLIS • *Institute of Microbiology, Università Cattolica del Sacro Cuore, Rome, Italy*
AGOSTINHO CARVALHO • *Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal; ICVS/3B's—PT Government Associate Laboratory, Braga/Guimarães, Portugal*
CRISTINA CUNHA • *Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal; ICVS/3B's—PT Government Associate Laboratory, Braga/Guimarães, Portugal*
STEFAN CZURDA • *Children's Cancer Research Institute (CCRI), St.Anna Kinderkrebsforschung, Vienna, Austria; LabDia Labordiagnostik GmbH, Vienna, Austria*
HEIDE-MARIE DANIEL • *Laboratory of Mycology, Applied Microbiology, Earth and Life Institute, Mycothèque de l'Université Catholique de Louvain (BCCM/MUCL), Université Catholique de Louvain, Louvain-la-Neuve, Belgium*
DAVID A. ENOCH • *National Infection Service, Public Health England, Cambridge Microbiology and Public Health Laboratory, Addenbrooke's Hospital, Cambridge, UK*
SAMUEL M. GONÇALVES • *Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal; ICVS/3B's—PT Government Associate Laboratory, Braga/Guimarães, Portugal*
ANDREAS H. GROLL • *Department of Pediatric Hematology/Oncology, Infectious Disease Research Program, Center for Bone Marrow Transplantation, University Children's Hospital Münster, Münster, Germany*
CHRISTIAN GRUMAZ • *Fraunhofer Institute for Interfacial Engineering and Biotechnology, Stuttgart, Germany*
MOHAMMAD T. HEDAYATI • *Invasive Fungi Research Center (IFRC) & Department of Medical Mycology and Parasitology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran*
UWE HIMMELREICH • *Biomedical MRI Unit/MoSAIC, Department of Imaging and Pathology, Faculty of Medicine, University of Leuven, Leuven, Belgium*

MACIT ILKIT • *Division of Mycology, Department of Microbiology, Faculty of Medicine, University of Çukurova, Adana, Turkey*
FABIAN ISTEL • *Max F. Perutz Laboratories, MFPL—Department of Medical Biochemistry, Medical University of Vienna, Vienna, Austria*
RENATE KAIN • *Clinical Institute of Pathology, Medical University of Vienna, Vienna, Austria*
PHILIPP KIRSTAHLER • *Fraunhofer Institute for Interfacial Engineering and Biotechnology, Stuttgart, Germany*
IVA KOCMANOVA • *Department of Internal Medicine—Hematology and Oncology, Masaryk University and University Hospital Brno, Brno, Czech Republic; Department of Microbiology, University Hospital Brno, Brno, Czech Republic*
KARL KUCHLER • *Max F. Perutz Laboratories, MFPL—Department of Medical Biochemistry, Medical University of Vienna, Vienna, Austria*
ANDREAS KÜHBACHER • *Fraunhofer Institute for Interfacial Engineering and Biotechnology, Stuttgart, Germany*
JACLYNN KURPEWSKI • *Division of Infectious Diseases, Department of Medicine, Miriam Hospital/Alpert Medical School, Brown University, Providence, RI, USA*
MICHAELA LACKNER • *Division of Hygiene and Medical Microbiology, Medical University of Innsbruck, Innsbruck, Austria*
CORNELIA LASS-FLÖRL • *Division of Hygiene and Medical Microbiology, Medical University of Innsbruck, Innsbruck, Austria*
THOMAS LEHRNBECHER • *Pediatric Hematology and Oncology, Hospital for Children and Adolescents, Johann Wolfgang Goethe-University, Frankfurt, Germany*
SALOMÉ LEIBUNDGUT-LANDMANN • *Section of Immunology, Institute of Virology, University of Zürich, Zürich, Switzerland*
MARINA LINOW • *Molzym GmbH & Co. KG, Bremen, Germany*
THOMAS LION • *Children's Cancer Research Institute (CCRI), St.Anna Kinderkrebsforschung, Vienna, Austria; LabDia Labordiagnostik GmbH, Vienna, Austria; Department of Pediatrics, Medical University of Vienna, Vienna, Austria*
JÜRGEN LÖFFLER • *Medizinische Klinik II, Universitätklinikum Würzburg, Würzburg, Germany*
MICHAEL G. LORENZ • *Molzym GmbH & Co. KG, Bremen, Germany*
MICHAEL LUSTIG • *Molzym GmbH & Co. KG, Bremen, Germany*
JIRI MAYER • *Department of Internal Medicine—Hematology and Oncology, Masaryk University and University Hospital Brno, Brno, Czech Republic; CEITEC–Central European Institute of Technology, Masaryk University, Brno, Czech Republic*
CHRISTIANNE MICALLEF • *National Infection Service, Public Health England, Cambridge Microbiology and Public Health Laboratory, Addenbrooke's Hospital, Cambridge, UK*
ELEFTHERIOS MYLONAKIS • *Infectious Diseases Division, Rhode Island Hospital, Warren Alpert Medical School of Brown University, Providence, RI, USA*
FILOMENA NOGUEIRA • *CCRI—Children's Cancer Research Institute, Vienna, Austria; Labdia—Labordiagnostik GmbH, Vienna, Austria; Max F. Perutz Laboratories, MFPL—Department of Medical Biochemistry, Medical University of Vienna, Vienna, Austria*
LEONEL PEREIRA • *CCRI—Children's Cancer Research Institute, Vienna, Austria; Labdia—Labordiagnostik GmbH, Vienna, Austria; Max F. Perutz Laboratories, MFPL—Department of Medical Biochemistry, Medical University of Vienna, Vienna, Austria*
BRUNELLA POSTERARO • *Section of Hygiene, Institute of Public Health, Università Cattolica del Sacro Cuore, Rome, Italy*

Zdenek Racil • *Department of Internal Medicine—Hematology and Oncology, Masaryk University and University Hospital Brno, Brno, Czech Republic; CEITEC–Central European Institute of Technology, Masaryk University, Brno, Czech Republic*
Haleh Rafati • *Department of Biochemistry, Erasmus University Medical Center, Rotterdam, The Netherlands*
Volker Rickerts • *FG 16: Erreger von Pilz-, und Parasiteninfektionen und Mykobakteriosen, Robert Koch-Institut, Berlin, Germany*
Steffen Rupp • *Fraunhofer Institut für Grenzflächenund Bioverfahrenstechnik, Stuttgart, Germany; Institut für Grenzflächenverfahrenstechnik und Plasmatechnologie, Universität Stuttgart, Stuttgart, Germany*
Maurizio Sanguinetti • *Institute of Microbiology, Università Cattolica del Sacro Cuore, Rome, Italy*
Seyedmojtaba Seyedmousavi • *Department of Medical Microbiology, Radboud University Medical Center, Nijmegen, The Netherlands; Molecular Microbiology Section, Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA; Invasive Fungi Research Center (IFRC) & Department of Medical Mycology and Parasitology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran*
Ilka McCormick Smith • *FG 16: Erreger von Pilz-, und Parasiteninfektionen und Mykobakteriosen, Robert Koch-Institut, Berlin, Germany*
Kai Sohn • *Fraunhofer Institute for Interfacial Engineering and Biotechnology, Stuttgart, Germany; Institute of Interfacial Process Engineering and Plasma Technology, University of Stuttgart, Stuttgart, Germany*
Tania C. Sorrell • *Westmead Millennium Institute, Centre for Infectious Diseases and Microbiology, University of Sydney, Sydney, NSW, Australia; Department of Infectious Diseases, Westmead Hospital, Westmead, NSW, Australia*
Florian Sparber • *Section of Immunology, Institute of Virology, University of Zürich, Zürich, Switzerland*
Jan Springer • *Medizinische Klinik II, Universitätklinikum Würzburg, Würzburg, Germany*
Michael Tscherner • *Max F. Perutz Laboratories, MFPL—Department of Medical Biochemistry, Medical University of Vienna, Vienna, Austria*
Ali Tolooe • *Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran*
Antonietta Vella • *Institute of Microbiology, Università Cattolica del Sacro Cuore, Rome, Italy*
Paul Verweij • *Department of Medical Microbiology, Radboud University Medical Center, Nijmegen, The Netherlands*
Barbora Weinbergerova • *Department of Internal Medicine—Hematology and Oncology, Masaryk University and University Hospital Brno, Brno, Czech Republic*
P. Lewis White • *Public Health Wales Microbiology Cardiff, University Hospital of Wales, Heath Park, Cardiff, UK*
Birgit Willinger • *Division of Clinical Microbiology, Department of Laboratory Medicine, Medical University of Vienna, Vienna, Austria*
Huina Yang • *National Infection Service, Public Health England, Cambridge Microbiology and Public Health Laboratory, Addenbrooke's Hospital, Cambridge, UK*
Ioannis M. Zacharioudakis • *Infectious Diseases Division, Rhode Island Hospital, Warren Alpert Medical School of Brown University, Providence, RI, USA*
Fainareti N. Zervou • *Infectious Diseases Division, Rhode Island Hospital, Warren Alpert Medical School of Brown University, Providence, RI, USA*

Part I

Introductory Chapters

Chapter 1

Current Challenges in the Diagnosis of Fungal Infections

Cornelia Lass-Flörl

Abstract

Diagnosing fungal infections is a challenge, particularly in the immunocompromised host. Signs and symptoms are nonspecific, colonization is difficult to distinguish from invasive disease, blood cultures are commonly negative, and patients are often unable to undergo invasive diagnostic procedures. Culture and microscopic examination remain the "gold standard" but are insensitive. Antigen assays such as the galactomannan and glucan detection systems are frequently used, yet these tests vary in sensitivity and specificity, depending on the patient population involved. Molecular-based assays are not yet clinically validated.

Key words Fungal infections, Galactomannan assay, Glucan assay, PCR, Microscopic examination, Culture

1 Introduction

Moulds and yeasts are widely distributed in the environment, and of the 100,000 fungal species present, only 300 have been linked to diseases in humans and animals. Human mycoses are caused by true or opportunistic fungal pathogens [1]. True or primary fungal pathogens are distributed in predictable geographic areas, and usually invade healthy, non-immunocompromised hosts. The most striking adaptation in the human host is the ability to switch from hyphal to yeast cells. This phenomenon is called thermal dimorphism, and allows fungi to grow as moulds at 30 °C and as yeasts at 37 °C. By contrast, opportunistic fungal pathogens display little or no virulence, are ubiquitous, and host defense usually is impaired once fungal disease occurs. Fungal infections vary from superficial to systemic and are an emerging medical concern. Presently, fungal infections account for 10% of all nosocomial infections. Deep, systemic infections are less common but can affect any organ. Immunocompromised hosts such as organ transplant recipients and humans with underlying conditions such as diabetes or lung diseases are at an increased risk of suffering from fungal infections. The most important fungal pathogens are *Aspergillus* species, *Candida* species,

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Cryptococcus species, *Pneumocystis jirovecii*, and Mucormycetes (formerly Zygomycetes), with *Rhizopus* species, *Mucor* species, and *Lichtheimia* species being the main representatives. True pathogens are *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis*, and *Paracoccidioides brasiliensis* [1].

Fungal diseases are a concern in the medical and public health community because of the increasing number of people with weakened immune systems and the advances in health care practices. Fungal infections are difficult to diagnose because clinical symptoms, signs, and radiographic manifestations are unspecific. Therefore, a definitive diagnosis requires direct identification of fungi from the site of infection [2]. Genus or species identification is not possible based on microscopic characteristics and culture techniques are often insensitive and time-consuming. Fungi are also part of the human microbiome which limits the role of antigen and antibody detection based assays; fungal colonization may lead to false positive test results. For some emerging fungi (Mucormycetes), no routine serologic tests are currently available. Knowing the fungal pathogen guides appropriate antifungal treatment, dose and duration of therapy [1, 2].

Diagnosing fungal diseases in the laboratory typically includes microscopic examination, culture, antifungal susceptibility testing, antigen or antibody testing, or molecular assays (*see* Table 1).

Table 1
Overview of laboratory methods available for the diagnosis of invasive fungal diseases

Conventional methods
Direct microscopy (KOH/Calcofluor white stains, Giemsa)
Histopathology
Culture
Serology
Galactomannan test for *Aspergillus* species
Mannan test for *Candida* species
Antibody test for aspergillosis in immunocompetent hosts
ß-1-3 D glucan panfungal test
Lateral device flow assays for *Aspergillus* and *Cryptococcus* species
Antigen and antibody based assays for non-European mycoses
Molecular methods
Fluorescence *in situ* hybridization test
PCR assays

Specimen collection depends on the infection site involved and may include skin scrapings, body fluids, blood, and/or tissue biopsies. Test interpretation often requires experience in mycology, and results must be carefully considered along with signs and symptoms as well as the medical history (*see* Table 2).

Table 2
Specimens applied for diagnosing fungal infections

Assay	Specimens	Goal	Results	Time
Potassium hydroxide solution (KOH)	Skin scrapings, hair or nail clippings, tissue, vaginal secretions, body fluids, sputum	KOH dissolves non-fungal elements and reveals fungi on the microscope slide	Screening tool which detects fungal elements; does not inform about genus or species	Rapid, 40 min
Calcofluor white stain (CWS)	Skin scrapings, hair or nail clippings, body fluids, sputum, BALs	CWS binds to fungal elements and fluoresces under ultraviolet light; allows visualization sensitive visualizing fungal elements	Screening tool which detects fungal elements; does not inform about genus or species	Rapid, 40 min
Cultures	Skin, nail, hair, body fluids, tissue, swabs, sputum, blood	The specimen is placed on culture media	Tool to diagnose fungal infections, to perform species identification and antifungal susceptibility testing	Days to weeks
Antifungal susceptibility testing	Fungal cultures	Follow-up to fungal culture; susceptibility testing may be ordered to guide antifungal therapy	To guide antifungal treatment, to monitor fungal epidemiology	3–4 days
Antigen testing	Blood, urine, CSF, body fluids	Detects antigens associated with specific fungi, a variety of tests is available	To diagnose and monitor infection	Rapid
Antibody testing	Blood, CSF, other body fluids	Detects immune response to a specific fungus; may be ordered on a single sample or on acute and convalescent samples collected 2–3 weeks apart	Diagnose current or recent infection by specific fungus; to monitor treatment	Days
Molecular tests for DNA and RNA	Fungus isolated in culture, or blood, CSF, body fluids, tissue	Detects genetic material of a specific fungus	Detects fungal DNA or RNA; not yet widely available, not yet validated, preferable in research settings	Days

2 Materials and Methods

Proof of diagnosis usually requires microscopic or cultural evidence of fungi at sterile body sites [1]. For many superficial skin and yeast infections, a clinical examination of the patient and a microscopic examination of the sample may be sufficient to determine a fungal infection. For deep or systemic infections, additional diagnostic tests need to be applied. Fungal cultures support specific identification but many fungi are slow-growing, and it may take weeks until results become available. Susceptibility testing may support antifungal treatment options. The detection of fungal antigens and antibodies is used to determine whether a patient has or had a fungal infection [1, 2]. These tests are more rapid than fungal cultures but are indicated for specific fungal pathogens only. Many people produce fungal antibodies from any exposure to the organism so that a single antibody test may not confirm the presence of a current infection. Molecular tests may be performed to specify fungi grown in culture and/or directly from the specimens collected. Table 1 gives an overview of specimens and tests available for sensitive diagnosis. Getting high quality samples is most important, and specimen collection should be done before antifungal therapy is given.

A Little Fungal ABC

1. The microscopic presence of fungal elements in tissue is indicative of a fungal infection. Microscopy does not permit identifying the fungal species, but may provide information whether yeasts or moulds are present; in some cases an etiologic diagnosis may be possible, e.g., for *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis/posadasii*, *Paracoccidioides brasiliensis*, *Pneumocystis jirovecii*, *Penicillium marneffei*, or *Sporothrix schenckii*. Because the presence of fungi in tissue is diagnostic for proven infection, biopsy should be attempted whenever possible.
2. Positive cultures must be interpreted with caution, as the clinical significance depends on the type of fungus detected and the type of specimen investigated.
   (a) For non-sterile sites, such as the skin, fungi identified may be disease-causing (pathogenic) or may be part of the normal skin flora; a mixture may be present.
   (b) Sterile samples, such as blood or tissues that are properly collected will not be contaminated with normal flora, and a positive culture identifies the causing pathogen.
3. A positive antigen test is suspicious for the presence of fungal disease, however, at least two positive results should be obtained.
4. A positive antibody test of a single blood sample indicates exposure to a specific fungus, but does not indicate whether

the exposure occurred recently or in the past. A rise in the level of antibody-titer between two serum samples collected over 2 or 3 weeks indicates an active fungal infection. Individuals with weakened immune systems may not produce antibodies as expected.

5. Several molecular tests are not validated yet; a positive signal is indicative of a likely infection when the test was performed on a sterile body specimen such as blood or tissue biopsy. Some molecular tools have been developed for the detection and identification of fungi, but several assays need to overcome major limitations, as (a) tests are not commercialized, (b) restricted in their availability, (c) lacking clinical validation, (d) hardly tested in prospective clinical studies, (e) applied only on pure cultures, (f) showing cross-reactivity, and (g) displaying a limited spectrum of species detection [3].

2.1 Direct Microscopic Examination and Histopathology

Direct examination implies the use of 10% potassium hydroxide (KOH) or optical brighteners such as Fungi-Fluor™ (Calcofluor white staining solution) or Blancophor® [1]. Dyes binding to the fungal cell wall chitin will emit blue-white or green fluorescence, thus providing rapid detection of fungi. Microscopic examination may be less sensitive than culture, and hence, a negative result does not rule out fungal infection. Positive direct microscopy from a sterile site must be considered significant, even if the laboratory is unable to culture the fungus. The morphological characteristics seen on microscopy are diverse and depend on the pathogens involved; yeast cells (blastoconidia), budding cells, hyphae (septated, rarely septated and non-septated) and pseudohyphae may be present. *Candid*a represents polymorphic yeasts (10–12 μm in diameter) and shows species-related variations, e.g., *Candida glabrata* lacking hyphal elements. *Aspergillus* proliferates with septated, 2.5–4.5 μm broad hyphae and branching dichotomously (approximately 45 °C angle). Mucormycetes typically display thick-walled, refractile hyphae, 6–15 μm in diameter, swollen cells (up to 50 μm), and distorted hyphae. India ink staining of cerebrospinal fluid for diagnosing cryptococcal meningitis has a sensitivity of 60% and shows the characteristic capsules. *P. jirovecii* is easily detected by microscopy; Giemsa staining demonstrates the nuclei of trophozoites and intracystic stages, and silver stains display the cyst walls. Immunofluorescence microscopy using monoclonal antibodies can identify the organisms with higher sensitivity than conventional microscopy [1].

By hematoxylin–eosin (H&E) staining, fungi can easily be missed or misinterpreted as artifacts, fibrin, or necrotic masses. Hyphae are best visualized with periodic acid Schiff reaction (PAS), Gridley's fungus stain, or the silver-methenamine technique. Fontana-Masson stain is used to visualize fungal cell wall melanin

(pigmented dematiaceous fungi), and aids in differentiating capsule-negative *C. neoformans* (melanin-positive) from other yeasts (melanin-negative) in tissue. Muricarmine identifies *C. neoformans* by staining the polysaccharide capsule [1].

Immunohistology staining with monoclonal and polyclonal fluorescent-antibody reagents have been developed for differentiating the genera of *Aspergillus*, *Fusarium*, and *Scedosporium in situ*. Unfortunately, the high degree of antigenic relatedness among these and other fungal pathogens, such as *Paecilomyces* species, has resulted in significant cross-reactivity and low specificity [1].

In principle, microscopic examination does not permit fungal genus or species identification, as several fungi have similar microscopic and histopathologic appearance (e.g., *Aspergillus* and *Fusarium* species); however, it allows differentiation between infections caused by septate moulds (*Aspergillus*) or non-septate moulds (Mucorales), which affects the choice of antifungal treatment [1, 4].

2.2 Culture Techniques

Cultures represent the gold standard in diagnosing fungal diseases. They facilitate identification of the specific etiological agent and support antifungal susceptibility testing. Fungi grown from sterile body sites should be identified at the species level. Detection of fungemia is useful in diagnosing opportunistic infections caused by *Candida* species, *C. neoformans*, *Trichosporon* species, *Malassezia* species, *Fusarium* species, and, occasionally *Acremonium* species, *Paecilomyces* species, *Scedosporium* species, and *Aspergillus terreus*. Blood cultures have a low sensitivity for diagnosing candidemia (~50 %), and several days may be required until they become positive. Identification of *Candida* species from non-sterile body sites is not specific. Blood cultures may test negative even when disseminated disease is present [1–4].

Aspergillus recovery from the respiratory tract usually represents colonization in immunocompetent patients but indicates invasive disease in immunocompromised hosts. However, cultures of respiratory tract secretions provide low sensitivity, as *Aspergillus* can be grown from sputum in only 34 %, and from bronchoalveolar lavages (BAL) in 62 % of patients with active infection. Blood and cerebrospinal fluid specimens rarely reveal *Aspergillus* species. Disseminated fusariosis can be diagnosed by blood cultures in 40 % of patients, and the rate of positive blood cultures increases to 60 % in the presence of disseminated skin lesions [5]. Eighty percent of disseminated infections due to *Scedosporium prolificans* display positive blood cultures but the proportion is much lower with *S. apiospermum* infections [6].

The diagnosis of mucormycosis is usually made microscopically by examination of infected human body sites. The poor sensitivity of respiratory tract cultures (25 %) makes the diagnosis of pulmonary mucormycosis challenging.

3 Serological Tests

Noninvasive diagnostic tests include the detection of fungal cell wall components and antibodies. However, the detection of serum antibodies is ineffective, as immunocompromised patients lack specific antibody response. The detection of anti-*Candida* antibodies fails to discriminate between dissemination and superficial infection; moreover, antibodies may be positive in colonized but non-infected hosts. Table 3 provides an overview of various assays including the advantages and disadvantages.

Table 3
Advantages and disadvantages of the various methods available

Method	Indication	Advantages	Disadvantages
Galactomannan (GM) Lateral flow assay	Early detection of invasive aspergillosis 2 Serum samples/ week Positive cutoff index > 0.5 BAL, positive cutoff index > 0.5 Detection of invasive aspergillosis Immunochromatographic dipstick assay for the qualitative and semiquantitative detection of *Aspergillus* antigen	A screening test to accompany conventional diagnostic methods in patients at high risk of IA: For neutropenic adults and children Consistent serum value > 1 is a sign of therapeutic failure Easy to handle, sensitivity and specificity is similar to GM tests	No good data for non-neutropenic patients Mould-active antifungal drugs have an impact on sensitivity Persistent GM antigenemia during therapy is a poor prognostic sign and should prompt a reassessment In some cases the stick is not easy to read, hence lack of result
B-D-glucan (BG)	Diagnosis of invasive fungal infections 2 Serum samples/ week (minimum)	Panfungal marker in critically ill patients Covers *P. jirovecii* pneumonia Does not cover Mucormycetes and *Cryptococcus neoformans* 37 % false positive result: 1 × 80 pg/mL 23 % false positive results: 2 × 80 pg/mL Increases the specificity but decreases the sensitivity The site of infection may be important: patients with tissue infections failed to show a significant drop in BG levels despite successful outcomes	False-(+) results when bacteremia Limited experience (less widely used than GM) The threshold for positive results depends on the test that is used: Fungitell > 80 pg/mL Wako > 70 pg/mL Declines slowly in most invasive aspergillosis and candidiasis in patients with appropriate antifungal therapy May persists above the usual threshold for positivity long after clinical resolution of the original infection Less accurate in hematological patients

(continued)

Table 3 (continued)

Method	Indication	Advantages	Disadvantages
Mannan plus anti-mannan	Candidemia	Good sensitivity and specificity when combined in ICU patients Early diagnosis prior to blood culture results ESCMID Diagnostic & Management Guideline for Candida Diseases 2012 recommend this test-combination because of high negative PV	Limited experience Non-mycological criterion The sensitivity and specificity were 87.5 and 85.5 % for (1 → 3)-β-D-glucan and 89.3 and 63.0 % for mannan antigen plus anti-mannan antibody *C. parapsilosis* and *C. guilliermondii* fungemias were not detected by the Platelia *Candida* Ag Plus assay
Molecular methods (polymerase chain reaction; PCR) Most experience of in-house tests	DNA detection mainly of *Aspergillus* species less experience for Candida species Suitable for blood, BAL, tissue	Early diagnosis (rapid techniques) with a high NPV High sensitivity (multicopy genes), capacity for rapid speciation and ability to quantitate fungal burden Low burden of organisms during bloodstream infections: <10 CFU/mL (in 25 % <1 CFU/mL) and intermittent nature of candidemia due to hepatic clearance of fungal cells and/or periodic release of cells from deep organ sites into circulation	Non-mycological Limited to reference laboratories (low availability) High costs, improve technical equipment Technical difficulties of efficient fungal DNA extraction from complex clinical samples

3.1 Antigen Assays for Yeasts

The detection of *Candida* species antigens has been shown to have limited value. The Cand-Tec latex agglutination test (Ramco Laboratories, Houston, TX) detects not specifically defined antigens, and the sensitivity and specificity vary among reports. False positive reactions due to the rheumatoid factor and renal insufficiency have been observed. The double sandwich enzyme immunoassay Platelia *Candida* Antigen (Bio-Rad, Marnes, France) has been shown to be somewhat more sensitive. The combined testing of antigen and antibody permits improved diagnosis, in comparison to antigen testing alone. However, mannan is rapidly cleared from the blood and occurs at low levels, necessitating frequent sampling for sensitive detection. Enolase is another antigen holding promise for the diagnosis of invasive candidiasis. Sensitivity ranges from 54 to 75 %, and improvement may be achieved by

serial testing. The enolase antigen seems to be highly specific for invasiveness, as it is not present in *Candida* colonization, but the test is not commercially available yet [1, 7, 8].

The detection of cryptococcal capsular polysaccharide of *C. neoformans* and *C. gattii* allows rapid and sensitive diagnosis of serum and cerebrospinal fluid (CSF) infection. The tests are available as Latex agglutination assay, ELISA, or lateral device flow assay (LDF). False positive reactions have been reported in few patients with disseminated trichosporonosis, *Capnocytophaga canimorsus* septicemia, malignancy, and positive rheumatoid factor. The lateral device flow assays show high sensitivity (90 %) and specificity (95 %). The cryptococcal antigen titer is useful for monitoring of the response to therapy in CSF, but is less useful in serum. Titers fall slowly, and variations in testing may not reveal a uniform decrease, unless earlier samples are tested together with the current sample [1, 4, 7].

The commercially available tests for detection of *H. capsulatum* antigen in urine, blood or BAL have proven value for rapid diagnosis of histoplasmosis with a sensitivity of 81 % and a specificity of 99 %. There is cross-reactivity with *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Paracoccidioides brasiliensis*. Antigenuria and antigenemia was detected in 92 and 100 %, respectively, in patients with disseminated histoplasmosis. Lower frequencies of positivity are found in hosts with subacute and chronic pulmonary histoplasmosis [1, 9].

3.2 Galactomannan (GM) Immunoassay for Acute Aspergillosis

Galactomannan is a cell-wall polysaccharide detectable in serum and other body fluids during invasive aspergillosis. The commercially available sandwich ELISA (Platelia *Aspergillus*, Bio-Rad) detects GM by using a rat monoclonal antibody. The test has been validated for serum and BAL specimens with a detection limit of ~1 ng/mL. Circulating GM may be detected 5–8 days before clinical manifestations, and the circulating GM corresponds with the fungal load. Studies evaluating the role of the GM assay have largely been conducted in leukemia patients or hematopoietic stem cell transplant (HSCT) recipients, and suggested a high sensitivity and specificity. Cross-reactivity with *Bifidobacterium bifidum* and false-positive results were observed in patients treated with ampicillin–sulbactam, piperacillin–tazobactam, or amoxicillin–clavulanic acid, and in patients with autoreactive antibodies. To improve the test performance, serial sampling and 0.5 as cutoff value are recommended. The detection of GM in BAL fluid provides better evidence than detection in serum. GM may also be detected in CSF, with a sensitivity and specificity of 80 and 100 %, respectively. The prevalence of invasive aspergillosis and the application of antifungal prophylaxis affect the accuracy. So far, the GM test also detects other invasive infections, and is not uniformly specific for invasive aspergillosis, since researchers demonstrated the detection of circulating GM in patients with penicillinosis [1, 2, 5].

3.3 Beta Glucan (BG) Panfungal Assay

Beta glucan is a cell-wall constituent of many pathogenic fungi, and is detectable in patient serum during infections with *Candida*, *Aspergillus, Fusarium*, *Trichosporon*, *Saccharomyces*, *Acremonium* species, and *P. jirovecii*. The test does not detect *Cryptococcus* species or Mucormycetes. Commercially available assays (Fungitec-G; Glucatell (Fungitell); [1–3]-ß-D-glucan Test Wako-WAKO-WB003; B-G Star; beta-Glucan Test Maruha) display various sensitivities and specificities. The absence of a positive test result has a high negative predictive value, and serial testing is necessary. BG is ubiquitous in the environment, and false-positive results may be caused by poor specimen handling, hemodialysis using certain cellulose membranes, exposure to certain types of gauze, and infusion of albumin or immunoglobulin products. For clinical practice it is still unclear which cutoff value is best for which test and which patient population [1, 4, 5, 7].

3.4 Antibody Testing

Antibody testing is limited to the diagnosis and monitoring of treatment of coccidioidomycosis, chronic pulmonary aspergillosis, allergic bronchopulmonary aspergillosis, allergic, chronic, and granulomatous *Aspergillus* rhinosinusitis. It is supportive for the diagnosis of *Aspergillus* bronchitis in non-immunocompromised patients, for the diagnosis of acute (seroconversion) and chronic histoplasmosis, and diagnosis of paracoccidioidomycosis [5].

4 Molecular Detection of Fungi in Clinical Specimens

4.1 *C. albicans* Peptide Nucleic Acid (PNA) Fluorescence In Situ Hybridization (FISH) Test

The FISH method uses PNA probes for the identification of *C. albicans* directly from positive blood culture. The *C. albicans* PNA FISH test (Advandx) is based on a fluorescein-labeled PNA probe that targets *C. albicans* 26S rRNA. Recent single-center and multicenter studies have documented the excellent sensitivity (99–100%) and specificity (100%) of this test in the direct identification of *C. albicans* from blood cultures. The FISH results are unaffected by the type of blood culture system or broth formulation. This approach may provide a time saving of 24–48 h, compared with conventional laboratory identification methods used [8].

4.2 Polymerase Chain Reaction (PCR)

Detection of fungal DNA by means of PCR has been studied in detail for candidemia and invasive aspergillosis. The potential usefulness of DNA detection is evident, given that many opportunistic fungal pathogens grow slowly or are difficult to isolate. Clinical specimens include serum or whole blood for *Candida* and *Aspergillus* species, and respiratory samples (e.g., sputum, BAL fluid or tissue) for invasive aspergillosis. Target sequences vary and most often include ribosomal genes (18S rRNA, 28S rRNA) or internal transcribed spacer regions. Real-time detection techniques like LightCycler, TaqMan or molecular beacons are rapid, reproducible, automated, and sensitivities range from 78 to 100% for candidiasis and from 33 to 100% for invasive aspergillosis (*see* Table 4). In invasive aspergillosis, the combination of

Table 4
Overview of commercially PCR test assays available

Name	Affigene *Aspergillus* tracer	MycAssay *Aspergillus*	*Aspergillus* spp. Q-PCR alert kit	MycoReal *Aspergillus*
Company	Cepheid	Myconostica	Nanogen	Inogenetix
IVD accredited	Yes	Yes	Yes	No
Detection	Genus *Aspergillus*	Genus *Aspergillus*	Genus *Aspergillus*	*A. fumigatus, A. terreus, A. flavus, A. niger, A. nidulans*, and others
Species identification	No	No	No	Yes
Sample materials	Full blood, serum, plasma	BAL, sputum	BAL, sputum	Blood, liquor, BAL, puncture specimen, tissues, paraffin
DNA extraction kits	No kit/protocol	MycXtra	EXTRAcell	Protocol
Technology	Scorpions (FAM) amplification graphs	Molecular Beacons (FAM) amplification graphs	TaqMan-MGB (FAM) amplification graphs	HybProbes (LC640, 705) amplification and melting graphs
Target	18S-rRNA-gene	18S-rRNA-gene	18S-rRNA-gene	ITS-region
Internal control	Plasmid (ROX)	Plasmid (HEX)	Beta-globin-gene (VIC)	Plasmid (LC610)
PCR-plattform	Mx3000P and Mx300P iQ and iQ5 Rotor-Gene 3000	Light Cycler 2.0 ABI 7500, smart cycler MX3000	ABI 7500	Light Cycler 2.0
Analysis	Automatically	Automatically	Manually	Manually
Sensitivity	0.54 Genome equivalents/μl	1.3 Copies of genome/PCR	10 Copies of target/PCR	3CFU/PCR
Specificity	Cross reaction *(Penicillium)*	Cross reaction *(Penicillium)*	Cross reaction (*Penicillium*)	Specific for *Aspergillus*

PCR and GM antigen testing has recently been shown to improve sensitivity and specificity, when compared to either test alone. A systematic review and meta-analysis was done on the use of PCR tests for the diagnosis of invasive aspergillosis (IA). Data from more than 10,000 blood, serum, or plasma samples obtained from 1618 patients at risk for IA were retrieved from 16 studies. A single PCR-negative result is thus sufficient to exclude diagnosis of proven or probable IA. However, two positive tests are required to confirm the diagnosis

because the specificity is higher than that attained from a single positive test. Populations at risk varied and there was a lack of homogeneity of the PCR methods used in the years before. Efforts are underway to devise a standard for *Aspergillus* PCR for screening which will help enabling formal validation of the PCR, and identify patients who are most likely to benefit from its implementation. For diagnosis of pneumocystis pneumoniae, PCR may be helpful, and is more sensitive than staining methods [5, 8].

5 Concluding Remarks

A precise mycological diagnosis is only possible with the help of laboratory testing. In previous years, only a positive culture could reliably define a fungal infection. With currently available improved diagnostics, confirmation of the cause of fungal infection is possible by serological and molecular testing. Conventional microbiological and microscopic techniques remain the cornerstone of diagnosis but lack sensitivity. Combined implementation of other available tools is therefore mandatory for proper diagnosis. The choice of appropriate diagnostic tests depends on local fungal epidemiology, implemented treatment guidelines, availability of a laboratory, and prevalence of fungal infections (*see* Table 5). DNA-

Table 5
Diagnostic performance based on fungal diseases present

	Diagnostic performance					
Fungal diseases	Microscopy	Culture	Xray/CT scan	Antigen	Antibody	DNA detection
Candida bloodstream	–	+++	+	+	+	+++
Cryptococcal meningitis	++	+++	+	+++	–	–
Invasive (pulmonary) aspergillosis	+	+	+++	++	–	++
Chronic aspergillosis	+	+	+++	–	+++	++
Allergic aspergillosis	+	+	++	–	+++	+
Coccidioidomycosis	+	++	++	–	+++	–
Histoplasmosis	+	++	+	++	–	–
Mucormycosis	+++	+	++[a]	–	–	–
Pneumocystis jirovecii	+++	–	++	–	–	++
Unknown fungi in various specimens	+++	+++	–	–	–	–[b]

[a]Usually no differentiation between *Aspergillus* and other moulds
[b]A panfungal PCR may help in fungus identification

and RNA-based methods hold promise for improved sensitivity and specificity, but these methods will require extensive validation in clinical studies.

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Chapter 2

The Changing Epidemiology of Invasive Fungal Infections

David A. Enoch, Huina Yang, Sani H. Aliyu, and Christianne Micallef

Abstract

Invasive fungal infections (IFI) are an emerging problem worldwide with invasive candidiasis and candidemia responsible for the majority of cases. This is predominantly driven by the widespread adoption of aggressive immunosuppressive therapy among certain patient populations (e.g., chemotherapy, transplants) and the increasing use of invasive devices such as central venous catheters (CVCs). The use of new immune modifying drugs has also opened up an entirely new spectrum of patients at risk of IFIs. While the epidemiology of candida infections has changed in the last decade, with a gradual shift from *C. albicans* to non-albicans candida (NAC) strains which may be less susceptible to azoles, these changes vary between hospitals and regions depending on the type of population risk factors and antifungal use. In certain parts of the world, the incidence of IFI is strongly linked to the prevalence of other disease conditions and the ecological niche for the organism; for instance cryptococcal and pneumocystis infections are particularly common in areas with a high prevalence of HIV disease. Poorly controlled diabetes is a major risk factor for invasive mould infections. Environmental factors and trauma also play a unique role in the epidemiology of mould infections, with well-described hospital outbreaks linked to the use of contaminated instruments and devices. Blastomycosis is associated with occupational exposure (e.g., forest rangers) and recreational activities (e.g., camping and fishing).

The true burden of IFI is probably an underestimate because of the absence of reliable diagnostics and lack of universal application. For example, the sensitivity of most blood culture systems for detecting candida is typically 50%. The advent of new technology including molecular techniques such as 18S ribosomal RNA PCR and genome sequencing is leading to an improved understanding of the epidemiology of the less common mould and dimorphic fungal infections. Molecular techniques are also providing a platform for improved diagnosis and management of IFI.

Many factors affect mortality in IFI, not least the underlying medical condition, choice of therapy, and the ability to achieve early source control. For instance, mortality due to pneumocystis pneumonia in HIV-seronegative individuals is now higher than in seropositive patients. Of significant concern is the progressive increase in resistance to azoles and echinocandins among candida isolates, which appears to worsen the already significant mortality associated with invasive candidiasis. Mortality with mould infections approaches 50% in most studies and varies depending on the site, underlying disease and the use of antifungal agents such as echinocandins and voriconazole. Nevertheless, mortality for most IFIs has generally fallen with advances in medical technology, improved care of CVCs, improved diagnostics, and more effective pre-emptive therapy and prophylaxis.

Key words Candida, Cryptococcus, Aspergillus, Mucor, Fusarium, Scedosporium, Pneumocystis, Dimorphic fungi, Paracoccidioides, Histoplasma, Dermatophytes

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1 Invasive Candidiasis

1.1 Introduction

Invasive candidiasis is the most common fungal disease among hospitalized patients in the developed world. Invasive candidiasis consists of deep-seated tissue candidiasis (candida in a sterile site) and candidemia (candida in the bloodstream). Deep-seated candidiasis may be a consequence of either direct inoculation or hematogenous spread. Most epidemiological studies describe candidemia. When candida disseminates, multiple organs are often involved with the kidney, liver and spleen, myocardium, eye, and brain most commonly involved. Involvement of the liver and spleen is often present, especially in neutropenic patients

1.2 Incidence

There are several problems with studying the incidence of invasive candidiasis and candidemia. One is the sensitivity of most blood culture systems which is typically 50% [1, 2] and depends on blood culture volume, blood culture system, and whether the patient is receiving antifungal agents. Other diagnostic tests (e.g., β-D-glucan and mannan/anti-mannan) recommended in the same European guidelines [1] are being increasingly used but are by no means universally employed so candidemia rates underestimate the true burden of disease. These guidelines do not currently recommend nucleic acid amplification tests (e.g., PCR) due to a lack of data.

Another limitation is the use of different denominators in studies; some studies are population-based (e.g., cases per 100,000 persons), while others are hospital based (e.g., cases per 1000 admissions or cases per 10,000 patient days).

Candidemia is often cited as the fourth most common cause of bloodstream infection [3, 4]. While this applies to intensive care units, most population-based studies report candidemia as the seventh to tenth most common bloodstream infection [5].

The incidence of candidemia varies in population-based studies from between 1 and 14 cases per 100,000 inhabitants [6]. The reasons for this variation and recent changes are described below.

1.3 Changes in Epidemiology Over Time

It is generally accepted that the incidence of candidemia have increased over the last two to three decades [7]. This has been attributed to an increase in the use of more aggressive therapy practices (e.g., chemotherapy, transplantation, and intensive care use). However, some recent studies suggest that this rise in incidence is being reversed in some areas [5, 6, 8]. Cleveland and colleagues found that the declines noted were probably related to health care delivery improvements; the biggest falls were among cases with health care exposure rather than among the small number of community-associated cases, specifically those occurring in the presence of central venous catheters (CVCs) [6]. Improvements in the care of CVCs were thought to be the likely explanation for a drop in candidemias in a pediatric study [9]. This may explain

why pediatric cases in the UK rose throughout the 2000s, especially in neonates but then fell after a peak in 2007 [10]. Another possible explanation for the falling incidence of candidemia in some studies is the use of biomarkers and prediction tools (colonization index, candida score, etc.) which allow preemptive therapy which may prevent candidemias [11].

Candida albicans was always the organism most frequently isolated from clinical specimens but this has evolved over time with a rise in non-albicans Candida (NAC). Trick and colleagues described the rise in candidemias as a being due solely to a rise in NAC; *C. albicans* remained stable during their study [12]. NAC are of concern since some are highly virulent and are associated with treatment failure due to reduced susceptibilities to some antifungal agents. Prophylaxis with azole antifungal agents may account for this change in epidemiology. *C. albicans* was responsible for 79% of candidemias in intensive care patients but only 37% in hematology patients [13], with similar results seen in other studies [14].

1.4 Changes in Epidemiology in Different Geographical Regions

The distribution of *Candida* spp. causing candidemia varies in population-based studies carried out around the world; indeed there can be differences between hospitals in local areas and even between different units in the same hospital. This variation occurs as a result of the different predisposing factors of the patients, the antifungal use in those hospitals and other factors.

Falagas and colleagues performed a systematic review involving candidemia studies between 1996 and 2009 [15]. *C. albicans* was the predominant species in almost all the studies. The highest proportion of *C. albicans* was found in North/Central Europe and the USA. NAC were more common in South America, Asia, and South Europe. *C. glabrata* was commonly isolated in the USA and North/Central Europe; *C. parapsilosis* in South America, South Europe, and several parts of Asia; and *C. tropicalis* in South America and Asia.

Studies of candidemia published and available on PubMed for the period 2012–2015 are summarized in Table 1 [6, 9, 10, 16–104]. These confirm these findings.

1.5 Predisposing Factors

The risk factors for invasive candidiasis are well recognized and are summarized in Table 2. Increasingly, invasive candidiasis is now more likely to occur in non-neutropenic patients in critical care units; 40% of the estimated 5000 cases of invasive candidiasis occurring in the UK were thought to be on intensive care [105], which is comparable to other studies [106, 107]. Risk factors of invasive candidiasis in critical care patients have been addressed in a systematic review and are summarized in Table 2 [106, 108]. Other risk factors recently described include alterations in innate immunity such as defects in cytokine receptors and toll-like receptors [109–111].

Table 1
Candidemia and invasive candidiasis studies published from 2012 to 2015 by geographical area

Sample	Type of study		Number of centers	Country (ies)	Population	Number in study	*C. albicans*	*C. glabrata*	*C. tropicalis*	*C. parapsilosis*	*C. krusei*	Other	Data collection period	Years	Mortality	Incidence	Microbiology	Reference
Europe																		
Blood	Observational study	Retrospective	1	Turkey	Hospital-wide	381	58%	7%	13%	15%	Not stated	Not stated	2001–2010	10	Not stated			[16]
Blood	Surveillance	Not stated	31	Denmark	Hospital-wide	334	53%	22%	5%	5%	8%	9%	2006	1	37% at 30d			[17]
Blood	Observational study	Not stated	14	Iceland	Hospital-wide	199	56%	16%	13%	5%	Not stated	10%	1990–2011	12	30% at 30d	Increase		[18]
Blood	Observational study	Retrospective	1	Italy	Hospital-wide	270	54%	9%	10%	23%	Not stated	4%	2010–2014	5	35% at 30d		Increase NAC	[19]
Blood	Surveillance	Retrospective	1	Italy	Liver	16	88%	6%	0%	6%	Not stated	Not stated	2008–2012	5	56% at 30d			[20]
Blood	Surveillance	Prospective	1	Italy	Hospital-wide	348	29%	10%	7%	28%	4%	5%	2008–2010	3	43% at 30d			[21]
Blood	Surveillance	Not stated	1	Italy	Hospital-wide	204	60%	12%	6%	17%	Not stated	Not stated	2009–2014	5	47% at 30d			[22]
Blood	Observational study	Not stated	5	Spain and Italy	Hospital-wide	955	58%	8%	9%	20%	Not stated	Not stated	2008–2010	3	40% at 30d			[23]
IAI	Cohort study	Retrospective	13	Spain, Italy, Greece, Brazil	Hospital-wide	481	64%	16%	7%	5%	Not stated	5%	2011–13	3	26% at 30d			[24]
Blood	Cohort study	Prospective	18	Israel	Hospital-wide	450	25%	15%	17%	17%	Not stated	Not stated	2005–2007	2	48% inhospital mortality			[25]
Blood	Cohort study	Retrospective	1	Norway	Adults	112	76%	6%	9%	9%	Not stated	Not stated	2002–2012	11	49% at 30d			[26]
Blood	Population study	Not stated	Country wide	France	Hospital-wide	15559	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2001–2010	10	40%	Increase		[27]
Blood	Observational study	Not stated	1	Italy	Hospital-wide	394	44%	6%	5%	35%	5%	14%	1998–2013	16	28% at 20d	Various	Increase NAC	[28]
Blood	Case-control study	Not stated	1	Turkey	NICU	28	43%	0%	0%	57%	0%	0%	2000–2007	8	43%			[29]

Blood	Cohort study	Prospective	13	Germany, Italy, France, UK, Turkey, Netherlands, Belgium, Switzerland	Adults with cancer	267	40 %	10 %	13 %	10 %	9 %	7 %	2005–2009	5	35 % at 30d			[30]
Blood	Cohort study	Retrospective	2	Italy	Hospital-wide	779	57 %	11 %	7 %	17 %	1 %	2 %	2004–2008	5	45 % at 30d			[31]
Blood	Cohort study	Retrospective	1	Poland	Children	118	40 %	5 %	4 %	36 %	0 %	7 %	2000–2010	11	Not stated			[32]
Blood	Observational study	Prospective	28	Sweden	Hospital-wide	403	61 %	20 %	8 %	9 %	1 %	7 %	2005–2006	1	Not stated			[33]
Blood	Cohort study	Retrospective	1	Austria	Burns	20	60 %	10 %	5 %	15 %	5 %	5 %	2007–13	7	30 %			[34]
Blood	Case-control study	Retrospective	1	Spain	Hospital-wide	419	43 %	13 %	5 %	34 %	2 %	5 %	2000–2009	10	37 % at 30d	Increase	Increase NAC	[35]
Blood	Population study	Prospective	9	Greece	Hematological malignancy	40	13 %	10 %	15 %	50 %	5 %		2009–2012	4	45 % at d28			[36]
Blood	Surveillance	Prospective	1	Spain	Hospital-wide	226	47 %	12 %	17 %	17 %	6 %	2 %	2004–2009	6	Not stated			[37]
Blood	Cohort study	Retrospective	1	Turkey	Children	208	53 %	1 %	8 %	26 %	0 %	10 %	2004–2012	9	29 % at 30d			[38]
All	Observational study	Prospective	72	Austria, Italy, Czech Republic, Netherlands, Finland, Portugal, France, Germany, Sweden, Greece, Turkey, Hungary, UK, Spain	ICU	807	54 %	14 %	6 %	19 %	3 %	6 %	2006–2008	3	39 %			[39]
Blood	Surveillance	Prospective	101	France	ICU	136	57 %	18 %	6 %	9 %	5 %	8 %	2005–2006	1	Not stated			[40]
Blood	Surveillance	Prospective	24	France	ICU	2507	56 %	18 %	9 %	12 %	3 %	Not stated	2002–2010	9	41.5-56.9 % at 30d			[41]
Blood	Surveillance	Not stated	682	Germany	ICU	575	66 %	Not stated	Not stated	Not stated	Not stated	Not stated	2006–2011	6	Not stated	Stable		[42]
Blood	Cohort study	Retrospective	Country wide	UK	Children	1473	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2000–2009	10	Not stated			[10]

(continued)

Table 1 (continued)

Sample	Type of study		Number of centers	Country (ies)	Population	Number in study	*C. albicans*	*C. glabrata*	*C. tropicalis*	*C. parapsilosis*	*C. krusei*	Other	Data collection period	Years	Mortality	Incidence	Microbiology	Reference
Europe																		
Blood	Surveillance	Prospective	1	France	Hospital-wide	182	54 %	17 %	7 %	11 %	4 %	7 %	2009–2010	2	29 % at 30d	Increase	Increase NAC	[43]
Blood	Cohort study	Retrospective	1	Hungary	Hospital-wide	129	63 %	13 %	9 %	10 %	4 %	1 %	2010–2014	5	51 % at 30d	Decrease		[44]
Blood	Population study	Prospective	29	Spain	Adults with cancer	238	42 %	18 %	10 %	19 %	3 %	8 %	2010–2011	1	30 % at 30d			[45]
All	Cohort study	Prospective	10	Germany, Portugal, Belgium, Czech republic, Romania, Italy, France, UK, Greece, Spain	ICU	216	56 %	15 %	8 %	10 %	0 %	6 %	Not stated	na	40 % at 60d			[46]
Blood	Surveillance	prospective	34	Italy	Hospital-wide	464	45-52 %	20 %	8 %	15-24 %	1 %	2 %	2009	1	Not stated	Increase	Increase NAC	[47]
Blood	Observational study	Retrospective	1	Germany	Children	35	46 %	14 %	6 %	17 %	0 %	17 %	1998–2008	11	11 % at 30d			[48]
Blood	Case-control study	Retrospective	1	Italy	Hospital-wide	222	59 %	5 %	10 %	23 %	Not stated	3 %	2005–2007	3				[49]
Blood	Cohort study	Retrospective	1	Finland	ICU	82	73 %	18 %	Not stated	6 %	Not stated	3 %	2000–2009	10	16 % at 30d	Stable		[50]
Blood	Cohort study	Retrospective	1	Germany	Adults with cancer	21	29 %	10 %	19 %	19 %	14 %	10 %	2003–2009	7	56 % at 30d	Increase	Increase NAC	[51]
						29298												
North America																		
Blood	Surveillance	Retrospective	1	Canada	Hospital-wide	266	49 %	30 %	5 %	8 %	Not stated	Not stated	2000–2009	10	40 %			[52]
All	Observational study	Prospective	19	USA	NICU	137	63 %	4 %	1 %	30 %	Not stated	5 %	2004–2007	4	34 % versus 14 %			[53]
Blood	Surveillance	Prospective	398	USA	NICU	1407	50 %	Not stated	3 %	35 %	Not stated	2 %	1999–2009	11	Not stated	Decrease		[54]
Blood	Population study	Prospective	39	USA	Hospital-wide	2675	38 %	29 %	10 %	17 %	1 %	5 %	2008–11	4	Not stated			[55]

Blood	Population study	Prospective	40	USA	Hospital-wide	3848	36%	27%	9%	15%	1%	5%	2008–2013	5	Not stated	Decrease	Increase NAC	[6]
Blood	Cohort study	Retrospective	1	USA	Adults with cancer	10	30%	20%	10%	30%	10%	Not stated	2002–11	10	57% at 90d			[56]
Blood	Surveillance	Prospective	1	USA	Hospital-wide	108	47%	29%	6%	12%	0%	Not stated	2004–2007	4	31% inhospital mortality	Increase	Increase NAC	[57]
Blood	Surveillance	Retrospective	1	USA	Children	406	49%	Not stated	Not stated	24%	Not stated	Not stated	1997–2007	13	Not stated			[58]
Blood	Cohort study	Retrospective	1	USA	Children	180	56%	3%	3%	25%	Not stated	Not stated	2000–2006	7	Not stated			[59]
Blood	Cohort study	Retrospective	1	USA	ICU	18	26%	Not stated	Not stated	Not stated	Not stated	Not stated	1998–2003	5	76%			[60]
Blood	Cohort study	Retrospective	1	USA	ICU	224	54%	25%	6%	13%	3%	2%	2002–2010	9	68% inhospital mortality			[61]
Blood	Surveillance	Retrospective	41	USA	Hospital-wide	2329	38%	29%	10%	17%	1%	4%	2008–2011	4	Not stated		Increase NAC	[62]
Blood	Surveillance	Retrospective	14	USA	Hospital-wide	163	42%	20%	11%	7%	2%	5%	2011–2012	2		Decrease	Increase NAC	[63]
Blood	Cohort study	Retrospective	1	USA	Children	1350	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2001–2010	11	Not stated	Decrease		[9]
Blood	Surveillance	Prospective	23	Canada, USA	Hospital-wide	3648	42%	27%	9%	16%	3%	3%	2004–2008	5	40% at 90d			[64]
Blood	Surveillance	Not stated	Not stated	USA	Hospital-wide	2,070	43%	28%	9%	18%	Not stated	4%	2008–2012	5	45% at 30d	Decrease		[65]
Blood	Surveillance	Retrospective	1	USA	NICU	37	59%	0%	8%	24%	0%	1%	2000–2010	11	14% inhospital mortality			[66]
Blood	Case-control study	Retrospective	Not stated	USA	Hospital-wide	113	37%	25%	12%	19%	Not stated	6%	2009–2013	5	29% at 30d			[67]
Blood	Surveillance	Retrospective	1	USA	Adults with cancer	71	1%	20%	23%	32%	2%	14%	2008–2012	5	52% at 30d			[68]
Blood	Surveillance	Prospective	38	USA	Hospital-wide	2029	51%	17%	10%	17%	Not stated	5%	1998–2006	9	Not stated			[69]
						20926												

(continued)

Table 1 (continued)

Sample	Type of study		Number of centers	Country (ies)	Population	Number in study	*C. albicans*	*C. glabrata*	*C. tropicalis*	*C. parapsilosis*	*C. krusei*	Other	Data collection period	Years	Mortality	Incidence	Microbiology	Reference
South & Central America																		
Blood	Surveillance	Retrospective	9	Peru	Hospital-wide	153	40%	5%	24%	28%	Not stated	Not stated	2009–2011	3	Not stated			[70]
Blood	Surveillance	Retrospective	22	Brazil	Hospital-wide	1392	42%	9%	20%	19%	Not stated	Not stated	2003–2012	9	61% at 30d	Increase	Increase NAC	[71]
Blood	Observational study	Not stated	7	Colombia	Hospital-wide	131	66%	2%	11%	15%	Not stated	3%	2008–2009	1	36% inhospital mortality			[72]
Blood	Cohort study	Retrospective	1	Brazil	Hospital-wide	108	29%	8%	31%	24%	2%	7%	2006–7 and 2010–11	4	Not stated			[73]
Blood	Cohort study	Retrospective	14	Brazil	Hospital-wide	987	39%	7%	24%	21%	2%	7%	1994–2004	11	57% at 30d			[74]
Blood	Observational study	Retrospective	2	Brazil	Hospital-wide	180	35%	Not stated	Not stated	38%	Not stated	Not stated	2006–2011	6	49% at 30d	Increase	Increase NAC	[75]
Blood	Surveillance	Retrospective	1	Brazil	Hospital-wide	313	44%	5-23%	22%	14%	4%	Not stated	2006–2010	5	Not stated	Increase	Increase NAC	[76]
Blood	Surveillance	Prospective	20	Chile, Argentina, Ecuador, Honduras, Mexico, Colombia, Venezuela	Hospital-wide	672	38%	6%	18%	27%	3%	9%	2008–2010	2	40% at 30d			[77]
Blood	Surveillance	Not stated	1	Brazil	Children	104	38%	2%	24%	22%	3%	7%	2007–2010	4	Not stated			[78]
Blood	Surveillance	Retrospective	1	Brazil	Hospital-wide	388	42%	4%	27%	22%	1%	Not stated	2000–2004	5	55%			[79]
						4275												
Asia																		
Blood	Surveillance	Retrospective	1	Saudi Arabia	Hospital-wide	258	34%	9%	16%	12%	Not stated	Not stated	2002–2009	8	50% at one year	Increase	Increase NAC	[80]
Blood	Observational study	Prospective	27	India	ICU	1400	21%	7%	42%	11%	2%	10%	2011–12	2	Not stated			[81]
Blood	Surveillance	Retrospective	1	India	Hospital-wide	27	30%	19%	41%	Not stated	Not stated	Not stated	2009	1	41%			[82]
Blood	Surveillance	Not stated	1	Taiwan	Hospital-wide	152	52%	9%	20%	15%	0%	5%	2004–2006	3	Not stated			[83]
Blood	Cohort study	Retrospective	1	Taiwan	Hospital-wide	504	54%	18%	23%	9%	Not stated	Not stated	2002 and 2010	2	45% at 30d	Increase	Increase NAC	[84]

All	Cohort study	Prospective	175	Pakistan	Hospital-wide	188	20%	10%	33%	15%	2%	18%	2006–2009	4	Not stated			[85]
Blood	Case control	Retrospective	1	South Korea	ICU	49	65%	1%	27%	6%	Not stated	Not stated	2000–2006	6	96% inhospital mortality			[86]
Blood	Cohort study	Retrospective	1	Taiwan	Hospital-wide	209	44%	22%	22%	16%	1%	4%	2009–2012	4	52% at 30d	Increase	Increase NAC	[87]
Blood	Surveillance	retrospective	1	Japan	Hospital-wide	75	37%	5%	3%	30%	not stated	25%	2007–2013	7	27% at 30d			[88]
Blood	Surveillance	Retrospective	1	Taiwan	Hospital-wide	474	38%	18%	22%	11%	2%	2%	2009–10	2	Not stated			[89]
Blood	Cohort study	Retrospective	1	South Korea	Liver	19	47%	5%	26%	5%	11%	5%	2005–11	6	Not stated			[90]
Blood	Cohort study	Prospective	1	Pakistan	Hospital-wide	145	9%	4%	21%	36%	0%	30%	2012	<1	23%			[91]
Blood	Cohort study	Retrospective	1	China	Adults with cancer	41	49%	10%	10%	24%	0%	8%	2006–2010	5	Not stated	Increase	Increase NAC	[92]
Blood	Cohort study	Not stated	1	Taiwan	ICU	126	57%	14%	17%	11%	Not stated	1%	2009–2012	4	Not stated			[93]
All	Surveillance	Retrospective	1	China	Hospital-wide	257	48%	2%	16%	12%	2%	4%	2011–2013	3				[94]
Blood	Surveillance	Not stated	1	India	Hospital-wide	212	14%	11%	39%	20%	0%	16%	2009–2012	4	43%			[95]
Blood	Surveillance	Not stated	25	Singapore, India, China, Thailand, Taiwan, Hong Kong	Hospital-wide	1601	41%	14%	25%	12%	2%	6%	2010	1	Not stated	Various		[96]
Blood	Surveillance	Retrospective	1	Taiwan	Older people	175	55%	13%	22%	14%	0%	3%	2009–2012	4	50% inhospital mortality			[97]
Blood	Case control	Retrospective	4	Taiwan	Older people	147	43%	11%	25%	14%	6%	2%	2008–2010	3	31% inhospital mortality			[98]
Blood	Cohort study	Retrospective	1	China	Hospital-wide	238	30%	5%	11%	28%	Not stated	26%	2009–2011	3	20% at 30d			[99]
All	Cohort study	Retrospective	11	China	PICU	223	57%	19%	1%	9%	Not stated	7%	2009–2011	3	19%			[100]
Blood	Surveillance	Retrospective	1	China	Hospital-wide	121	37%	6%	7%	15%	2%	9%	2008–2012	5	28% at 30d			[101]
Blood	Surveillance	Retrospective	1	China	Hospital-wide	270	36%	13%	22%	8%	Not stated	21%	2000–2009	10	67% inhospital mortality	Stable		[102]
						6911												
Africa																		
Blood	Surveillance	Prospective	1	Egypt	PICU	66	40%	8%	17%	25%	6%	4%	Not stated	1	42% at 30d			[103]
Blood	Cohort study	Retrospective	1	South Africa	Hospital-wide	266	46%	23%	3%	25%	3%	0%	1990–2007	9	60%	Increase	Increase NAC	[104]
						332												

d days, *ICU* intensive care unit, *NAC* non-albicans Candida, *NICU* neonatal intensive care unit, *PICU* pediatric intensive care unit

Table 2
Risk factors for invasive candidiasis

Risk factors for invasive candidiasis	Risk factors for invasive candidiasis	Risk factors for invasive candidiasis in intensive care patients [106, 108]
Critical illness	Y	Y
Abdominal surgery, with particular risk among patients who have anastomotic leakage or have had repeat laparotomies	Y	Y
Acute necrotizing pancreatitis	Y	
Hematological malignancy	Y	
Neutropenia		Y
Solid organ transplantation	Y	
Solid organ tumors	Y	
Neonates, particularly low birth weight	Y	Y
Broad spectrum antibiotics	Y	
Central venous catheter	Y	Y
Total parenteral nutrition	Y	Y
Hemodialysis	Y	Y
Urinary catheter		Y
Glucocorticoid therapy	Y	
Fungal colonization		Y
Infection or sepsis		Y
Mechanical ventilation		Y
Diabetes		Y
Acute physiology and chronic health evaluation II (APACHE II) or APACHE III score		Y
Gastrointestinal bleed		Y
Age		Y

APACHE acute physiology and chronic health evaluation

1.6 Mortality

Candidemia is associated with significant mortality. Even recent studies give a 30-day mortality of up to 60 % (Table 1), while mortality in septic shock was almost 90 % in a retrospective case series from the USA [60]. Candidemia increases mortality rates in the range of 20–49 % [17]; the attributable mortality has been calculated to be around 15 % in several studies [17, 112].

Factors affecting mortality vary by study include increasing age, underlying medical conditions, presence on intensive care, choice of therapy (appropriate therapy is protective; echinocandin use is typically associated with lower mortality than azoles; [113]), prophylactic therapy [30] and whether the CVC can be removed (if present) [17, 19, 39, 71, 101]. A recent study found that candidemia in patients with peripherally inserted central catheters was associated with higher mortality in comparison with central venous catheters and no CVC use [22]. Failure to promptly remove a line was associated an increased risk of persistent candidemia in one study [66]. Echinocandin use may be associated with improved outcomes since they are recognized to have more activity against biofilm-producing organisms, and infection with these is associated with a poorer outcome [49]. Source control is also associated with improved outcome [61]. Care on a medical ward (rather than a surgical ward or intensive care unit) was seen as a risk factor for death in one study [101].

There are differences in the literature when it comes to species however. Arendrup and colleagues suggested *C. krusei* had the highest mortality (36%) compared to 25% for *C. parapsilosis* and 14% for other *Candida* species [17]. Barchiesi agreed that *C. krusei* had the highest mortality but found that *C. parapsilosis* had the lowest mortality. Other studies suggest *C. albicans* is associated with the highest mortality [19], while Gamelatsou et al. found that *C. glabrata* had the highest mortality and *C. parapsilosis* had the lowest mortality [36]. These conflicting findings could be due to different populations or differences in study design.

1.7 Drug Resistance

Fluconazole resistance has been recognized for several years, especially in *C. glabrata* (dose dependent) and *C. krusei* (intrinsically resistant to fluconazole). Of note, however, was the emergence of azole resistance in *C. parapsilosis* in Brazil [73]. While most studies describe a rise in fluconazole resistance over time, one study noticed a decrease in resistance [6]. Predictors associated with fluconazole-resistant *Candida* spp. include neutropenia, chronic renal disease, and previous fluconazole exposure [37]. Interestingly, infection with a fluconazole-resistant isolate was associated with exposure to antibacterial agents in another study (notably carbapenems, trimethoprim-sulfamethoxazole, clindamycin, and colistin) [25].

Echinocandin resistance is increasingly recognized. Resistance is mediated primarily through mutations in hot-spot regions of FKS genes, which encode the echinocandin target enzyme (1,3-β-D-glucan synthase) [114]. Resistance can emerge on therapy [115]. Between 1 and 10% of isolates were resistant to echinocandins in most studies [6, 62–64]. However, some studies report higher, and increasing resistance. Echinocandin resistance increased from 4.9 to 12.3% and fluconazole resistance increased from 18 to 30% in a study

of 313 *C. glabrata* isolates performed between 2001 and 2010 [114]. Among the 78 fluconazole resistant isolates, 14.1 % were resistant to one or more echinocandins. Twenty-five (7.9 %) isolates harbored the FKS mutation. The predictor of the FKS mutant strain was prior echinocandin therapy [114]. One study noted an increase in cases of *C. glabrata* resistant to echinocandins which was related to a tenfold increase in echinocandin use over the preceding decade [115]. A reduction in echinocandin use was followed by a reduction in echinocandin resistance [115].

A recent study found echinocandin resistance in 20 % of isolates (*C. glabrata* but also four of 5 *C. kefyr* isolates). Of concern was the associated increased mortality; 29 % were fluconazole resistant, so 19 % were considered "multidrug resistant" [68].

2 Cryptococcus

Cryptococcus neoformans and *C. gattii* are encapsulated, heterobasidiomycetous fungi that are now a common systemic mycosis, particularly in immunosuppressed individuals. Cryptococcosis is the third most prevalent disease in HIV-positive patients [117] and an AIDS-defining condition. *Cryptococcus neoformans* is not considered to be a constituent of the normal human flora. Infection occurs via inhalation to cause a pulmonary infection which may then disseminate to cause meningitis, encephalitis or meningoencephalitis. The vast majority of patients with symptomatic disseminated

Table 3
Conditions associated with predisposition to infection with *Cryptococcus* spp

HIV infection
Lymphoproliferative disorders
Sarcoidosis
Corticosteroid therapy
Hyper-IgM syndrome
Hyper-IgE syndrome
Monoclonal antibodies (e.g., infliximab)
Systemic lupus erythematosus
HIV-negative CD4+ T-cell lymphopenia
Diabetes mellitus
Organ transplantation
Peritoneal dialysis
Cirrhosis

cryptococcosis have an underlying immunocompromised condition (Table 3). The most common underlying conditions worldwide include HIV infection, receipt of corticosteroids, organ transplantation, malignancy, diabetes and sarcoidosis, though treatment with immune-modifying monoclonal antibodies (e.g., alemtuzumab, infliximab) is an increasingly recognized risk factor [118, 119].

Cryptococcosis peaked in the USA in 1992 at the height of the HIV epidemic but this fell following the introduction of fluconazole (as treatment for oral candidiasis and then as prophylaxis for cryptococcosis) and antiretroviral therapy. In areas with continuing high levels of HIV transmission such as sub-Saharan Africa, cryptococcosis has reached a high prevalence, with almost one million new cases per year and 600,000 deaths [120]. *Cryptococcus* spp. is the most frequent cause of culture-confirmed meningitis in some studies [121]. Interestingly, hematology patients are at risk, especially those with defects in cell-mediated immunity (e.g., lymphoproliferative disorders and chronic lymphocytic leukemia) but not particularly stem cell transplant recipients, while solid organ transplantation (especially kidney and liver) is a particular risk factor.

At present, nine major molecular types have been recognized: VNI, VNII, VNB, VNIII, and VNIV among *C. neoformans* isolates, and VGI, VGII, VGIII, and VGIV among *C. gattii* isolates. Classically, *Cryptococcus neoformans*, the most frequent species isolated, is found predominantly in soil contaminated by bird droppings and has a universal distribution, whereas *C. gattii* is typically found in tropical and subtropical regions (its ecological niche is the eucalyptus tree). *C. neoformans* was isolated from 89 of 100 isolates from a study performed in Brazil, Chile, and Venezuela [122]. The remainder were due to *C. gattii*; 60 % of patients were HIV-positive.

There is evidence that *C. gattii* is spreading from tropical and subtropical regions to other regions such as the Pacific north-west of the USA [123], Canada [124], and Europe [125, 126].

Case reports of infections due to *C. laurentii* [127, 128] and *C. albidus* have also been described. All patients had impaired immunity.

Excellent reviews of the epidemiology of cryptococcal disease include those by Chen et al. [129] and Cogliati [130].

3 Invasive Aspergillosis

3.1 Introduction

Aspergillus spp. is a hyaline mould that is ubiquitous in nature. Inhalation of the infectious conidia is a frequent event and can cause a range of clinical syndromes from aspergillomas and allergic bronchopulmonary aspergillosis to semi-invasive or invasive conditions such as chronic pulmonary aspergillosis, cutaneous aspergillosis and invasive aspergillosis. Invasive aspergillosis is a potentially life-threatening opportunistic infection affecting the immunocompromised.

3.2 Diagnosis

While the diagnosis of invasive aspergillosis is described in greater detail elsewhere [131], it is important to realize the impact this has on the study of the epidemiology of this disease. In 2002, the European Organisation for Research and Treatment of Cancer (EORTC) and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (MSG) devised criteria for the classification of potential cases according to the likelihood of underlying invasive fungal disease into possible, probable or proven [132]. In 2008, the criteria were revised again to minimize the number of cases previously classified as "possible" invasive fungal disease [133].

Mainly designed as a research or epidemiological tool, most current studies in the literature base their classification of proven or probable invasive aspergillosis on the revised criteria in 2008. A single center retrospective analysis demonstrated an 80.6% reduction of cases previously classified as "possible" invasive aspergillosis, and a similar reduction of cases of "probable" invasive aspergillosis. Hence the data from studies using 2002 and 2008 criteria may be incomparable for anything other than proven infections [134].

3.3 Epidemiology of Invasive Aspergillosis

Invasive aspergillosis is a major cause of invasive fungal disease which tends to affect the immunocompromised. A large population-based analysis study from France over 10 years demonstrated an incidence of invasive aspergillosis of 4.4% per year, with rates of 1.1–1.8 per 100,000 population [27]. This is less than that observed by Dasbach and colleagues in 1996 in the USA [135].

Recently published studies on invasive aspergillosis available PubMed in 2012–2015 are listed in Table 4 [27, 56, 136–184].

The underlying disease tends to determine the risk of a patient for developing invasive aspergillosis [183]. In a large study looking at 960 patients with invasive aspergillosis, 48.3% had an underlying hematological malignancy, 29.2% were solid organ transplant recipients, 27.9% were hematopoietic stem cell transplant (HSCT) recipients. The remainder had an associated underlying disease such as a solid tumor, HIV/AIDS or an inherited immunodeficiency [183].

In the intensive care setting, critically ill patients are also highly susceptible to invasive fungal infections. In this group, the incidence of invasive aspergillosis has been shown to be around 1.7–6.31 per 1000 adult admissions [165, 167]. In children, the annual incidence of invasive aspergillosis was 0.4% in the USA in 2006, with three quarters of these patients being immunosuppressed or having a malignancy [185]. Mortality attributable to invasive aspergillosis was estimated to be 33.1% at 30 days in adult ICU patients [169] and 37.5% in children, contributing to 60% of overall deaths [186]. The incidence and impact of invasive aspergillosis varied according to the underlying disease, but other associations include the use of corticosteroids, prolonged and profound neutropenia, the use of broad spectrum antibiotics and mechanical ventilation [168–170, 187].

Table 4

Invasive aspergillosis studies published from 2012 to 2015 by risk type

Hematology/HSCT recipients																		
Diagnostic criteria	**Type of study**		**Number of centers**	**Country(ies)**	**Age range**	**Population**	**Number in study**	***A. fumigatus***	***A. flavus***	***A. niger***	***A. terrus***	***A. spp***	**Mixed**	**Data collection period**	**Years**	**Mortality**	**Incidence of IA**	**Reference**
Modified EORTC/MSG	Autopsy study	Retrospective	1	USA	All	Hematological malignancies	195	12%	9%	1%	8%	1%	Not specified	1989–2008	20		Decreased incidence	[136]
2008 EORTC/MSG	Observational study	Retrospective	1	France	Adult	Hematological malignancies	55	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2005–07	2			[137]
2008 EORTC/MSG	Observational study	Retrospective	1	France	Pediatrics	Hematological malignancies	19	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2001–10	0	42%	Decrease in mortality over time	[138]
2002 EORTC/MSG	Observational study	Prospective	1	Tunisia	Adult	Hematological malignancies	9	0%	0%	0%	0%	0%	22%	2009–11	2			[139]
2008 EORTC/MSG	Observational study	Retrospective	22	Japan	All	Hematological malignancies	23	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2006–08	3			[140]
2008 EORTC/MSG	Surveillance	Retrospective	1	France	All	Hematological malignancies	29	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2004–07	3			[141]
2008 EORTC/MSG	Surveillance	Prospective	9	Italy	All	Hematological malignancies	10	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2007–08	2	45%		[142]
2002 EORTC/MSG	Observational study	Retrospective	13	Czech Republic, Slovakia	All	Hematological malignancies	176	11%	1%	1%	1%	1%	Not stated	2005–09	5	58% at 12 weeks		[143]
2008 EORTC/MSG	Cohort study	Prospective	8	Brazil	All	HSCT recipients	19	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2007–09	2	37% at 6 weeks		[144]
2002 EORTC/MSG	Observational study	Prospective	37	France	All	HSCT recipients	73	4%	1%	0%	0%	0%	0%	2007–08	1	18% at 12 weeks		[145]
2008 EORTC/MSG	Cohort study	Prospective	8	Brazil	Adult	HSCT recipients	6	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2007–09	2	Not stated		[146]
2008 EORTC/MSG	Cohort study	Retrospective	1	USA	Adult	HSCT recipients	33	78%	3%	0%	3%	6%	15%	2002–11	10	52% at 12 weeks		[56]
2008 EORTC/MSG	Cohort study	Retrospective	1	Netherlands	Pediatrics	HSCT recipients	25	Not specified	Not specified	Not specified	Not specified	60%	Not specified	2004–12	9			[147]

(continued)

Table 4 (continued)

Diagnostic criteria	Type of study	Number of centers	Country(ies)	Age range	Population	Number in study	*A. fumigatus*	*A. flavus*	*A. niger*	*A. terrus*	*A. spp*	Mixed	Data collection period	Years	Mortality	Incidence of IA	Reference
Hematology/HSCT recipients																	
2008 EORTC/MSG	Observational Retrospective study	10	Spain	Adult	HSCT recipients	69	12 %	6 %	4 %	3 %	Not stated	Not specified	1997–2009	12	Attributable mortality 27 % versus 42 %, trend for lower mortality over time (cut off 2002)	Increase incidence, decrease in mortality over time	[148]
2008 EORTC/MSG	Cohort study Retrospective	1	France	All	HSCT recipients	80	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	1997–2008	12	40 % with vorico nazole versus 64 % in non-voriconazole		[149]
2008 EORTC/MSG	Cohort study Prospective	1	South Korea	Pediatrics	Pediatric oncology and HSCT recipients	23	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2007–10	3	44 % at 12 weeks		[150]
2008 EORTC/MSG	Observational Retrospective study	1	South Korea	Pediatrics	Pediatric oncology and HSCT recipients	37	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2009–13		27 % at 12 weeks		[151]
2008 EORTC/MSG	Observational Retrospective study	1	USA	Adult	HSCT or SOT recipients	69	65 %	10 %	10 %	3 %	12 %	Not specified	2000–09	10	43 % at 12 weeks in HSCT 12 % in lung transplant, 40 % in kidney transplant, 55 % in liver transplant 33 % in heart transplant at 12 weeks		[152]
Solid organ transplant recipients																	
Modified EORTC/MSG	Observational Retrospective study	1	Spain	Adult	Heart transplant recipients	31	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	1988–2011	23	46 %		[153]

2008 EORTC/ MSG	Observational study	Retrospective	1	Spain	Adult	Kidney transplant recipients	7	86%	0%	0%	0%	0%	0%	1979–2012	33	43%	[154]
2008 EORTC/ MSG	Observational study	Retrospective	1	Portugal	Adult	Kidney transplant recipients	11	82%	18%	0%	0%	0%	0%	2003–13	11	45%	[155]
Positive culture	Observational study	Retrospective	1	Turkey	Adult	Liver transplant recipients	8	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	1990–2012	22		[156]
Histological diagnosis	Observational study	Retrospective	1	China	Adult	Liver transplant recipients	25	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2003–10	7	12%	[157]
Positive culture	Observational study	Retrospective	1	Italy	Adult	Liver transplant recipients	4	50%	0%	0%	50%	0%	0%	2003–12	9	50%	[158]
2008 EORTC/ MSG	Cohort study	Retrospective	1	USA	Adult	Lung transplant recipients	19	45%	0%	0%	5%	20%	17%	2002–11	10	23% at 1 year	[159]
2008 EORTC/ MSG	Observational study	Retrospective	1	Spain	Adult	Lung transplant recipients	22	45%	50%	12%	23%	9%	36%	2003–13	11	83% at 12 weeks	[160]
2008 EORTC/ MSG	Cohort study	Retrospective	1	Denmark	Adult	Lung transplant recipients	68	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2002–06	5		[161]
Modified 2008 EORTC/ MSG	Observational study	Retrospective	1	Spain	Adult	SOT recipients	8	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2008–11	4	60%	[162]
2008 EORTC/ MSG	Observational study	Retrospective	1	Spain	Adult	SOT recipients (no lung/ heart transplants at the center)	27	66%	Not stated	Not stated	Not stated	Not stated	Not stated	2003–10	7	52%	[163]
Intensive care patients																	
Modified 2008 EORTC/ MSG	Observational study	Prospective	1	Iran	Adult	ICU	9	33%	33%	0%	0%	0%	0%	Not stated	1	66%	[164]
Positive culture, clinical and biomarkers	Observational study	Not stated	18	Italy	Adult	ICU	12	42%	Not stated	Not stated	Not stated	Not stated	Not stated	2007–08	2		[165]
Modified 2008 EORTC/ MSG -	Observational study	Retrospective	30	Spain, Belgium, Portugal, France, Greece, China, Brazil India	Adult	ICU	94	88%	2%	2%	Not stated	8%	Not stated	2000–11	12	72% at 12 weeks	[166]
Positive biomarkers or culture	Surveillance	Prospective	27	Italy	Adult	ICU	57	82%	9%	2%	0%	0%	7%	2006–08	2	Crude mortality 63% at 30 days	[167]

(continued)

Table 4 (continued)

Diagnostic criteria	Type of study		Number of centers	Country(ies)	Age range	Population	Number in study	*A. fumigatus*	*A. flavus*	*A. niger*	*A. terrus*	*A. spp*	Mixed	Data collection period	Years	Mortality	Incidence of IA	Reference
Hematology/HSCT recipients																		
Modified 2008 EORTC/ MSG	Observational study	Retrospective	2	Belgium	Adult	ICU	9	89 %	0 %	0 %	0 %	0 %	0 %	2009–11	2			[168]
ICD code on database	Cohort study	Retrospective	National	USA	Adult	ICU (SOT/ hematology excluded)	412	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2005–08	4	33.1 % at 30 day		[169]
Other patient groups																		
Modified EORTC/ MSG	Case-control study	Retrospective	1	China	Adult	COPD exacerbation	39	74 %	3 %	0 %	0 %	0 %	0 %	2006–09	4			[170]
2008 EORTC/ MSG	Observational study	Retrospective	National	France	Adult	HIV positive adults	242	92 %	Not stated	Not stated	Not stated	Not stated	Not stated	1992–2011	20	79 %, 50 % , 57 % at 12 weeks	Decreased incidence, decrease in mortality over time	[171]
2008 EORTC/ MSG	Cohort study	Retrospective	1	China	Adult	Liver failure	19	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2008–12	3	80 % at 12 weeks		[172]
2008 EORTC/ MSG	Cohort study	Retrospective	1	China	Adult	Liver failure	39	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2008–12	3	Not stated		[173]
Histological diagnosis + clinical records	Autopsy study	Retrospective	2	Japan	All	All	113	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	1955–2006	50		Increased incidence	[174]
ICD code on database	Observational study	Retrospective	National	France	Adult	All	8563	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2001–10	10	29 %	Increased incidence, decrease in mortality over time	[27]
2008 EORTC/ MSG	Cohort study	Retrospective	1	China	Adult	All	52	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2000–2010	10	45 % with underlying disease 11 % without underlying disease		[175]

2008 EORTC/MSG	Observational study	Retrospective	1	France	Adult	All	5	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	2003–09	7	49 % at 12 weeks		[176]
2002 EORTC/MSG	Observational study	Prospective	2	Canada	Adult	All	50	46 %	12 %	8 %	2 %	32 %	Not specified	2004–08	5	33 % at 12 weeks		[177]
Modified EORTC/MSG	Cohort study	Retrospective	1	Taiwan	All	All	96	41 %	41 %	3 %	5 %	5 %	Not specified	2000–09	10	62.5 % at 12 weeks	Increased incidence	[178]
2008 EORTC/MSG	Observational study	Retrospective	1	Sweden	Adult	All	104	56 %	9 %	3 %	0 %	6 %	Not specified	2005–09	5	52 % at 12 weeks		[179]
Modified 2008 EORTC/MSG	Surveillance	Prospective	23	Italy	Adult	All	153	30 %	17 %	5 %	3 %	1 %	3 %	2009–11	2	44 % in hematology patients 35 % in non-hematological patients		[180]
Clinical and positive culture	Observational study	Retrospective	1	Japan	Adult	All	194	46 %	4 %	15 %	Not stated	2 %	Not stated	1997–2011	14	50 %		[181]
Clinical symptoms, radiological findings and positive culture	Cohort study	Retrospective	1	Japan	All	All	42	68 %	4 %	14 %	Not specified	13 %	Not specified	2001–09	8	50 % (43 % attributable mortality)		[182]
2002 EORTC/MSG	Observational study	Prospective	25	USA, Canada	Adult	All	361	73 %	10 %	9 %	4 %	5 %	Not stated	2004–08	5	35 % at 12 weeks		[183]
2008 EORTC/MSG	Observational study	Prospective	22	USA, Switzerland, Australia, Denmark, Brazil, India, Sweden, Germany, Colombia, Estonia, Greece, UK, Chile	Pediatrics	All	98	27 %	7 %	6 %	5 %	5 %	8 %	2007–11	4	30 % at 12weeks		[184]

COPD chronic obstructive pulmonary disease, *EORTC-MSG* European Organization for Research and Treatment of Cancer/Mycoses Study Group, *HIV* human immunodeficiency virus, *HSCT* hematopoietic stem cell transplantation, *ICU* intensive care unit, *SOT* solid organ transplant

The majority of Aspergillus isolates causing invasive aspergillosis is often attributed to *Aspergillus fumigatus*, with isolation rates of up to 92% [171], followed by *A. flavus*, *A. niger*, and *A. terreus*. A significant proportion of invasive infections may also be mixed (up to 36%; [160]). Interestingly, these isolation rates are not in proportion with the rates seen in patients colonized with *Aspergillus* spp.

3.4 Changes in Epidemiology Over Time: Hematology Patients

The increase in the incidence of invasive fungal infection is largely attributed to the prolonged neutropenia in hematological regimes due to the intensification of cytotoxic chemotherapy, the use of corticosteroids, the increased use of allogeneic hematopoietic stem cell transplantation, the increased incidence of graft-versus-host disease (GvHD) and the widespread use of immunosuppressive agents for GvHD [152, 187, 188]. Aspergillus was the most frequent causative pathogen in invasive fungal infections seen in many studies worldwide [137, 140, 143, 145], except in Brazil where fusariosis was most commonly seen [144].

The overall incidence of invasive aspergillosis is estimated to be 0.8–2.3% in hematological malignancies, but this varied according to the underlying disease [140, 142]. Nucci and colleagues showed that the one year cumulative incidence of invasive aspergillosis was 13.4% in acute myeloid leukemia/ myelodysplastic syndrome, 2.3% in allogeneic HSCT, and 0% in autologous HSCT [144]. Attributable mortality varied from 18 to 57.8% [56, 143, 145].

Predictors for poorer prognosis in hematological patients included CMV infection, grade II–IV acute GvHD, severe chronic GvHD, and age >35 years [148]. Despite the increase in incidence, most centers report a trend towards an improvement in attributable mortality.

3.5 Changes in Epidemiology Over Time: Solid Organ Transplantation Recipients

In a 10 years study in the USA, Neofytos et al. demonstrated that the overall incidence rates were 49% in lung transplant, 11% in liver transplant, 10% in heart transplant, and 2% in kidney transplant recipients. Except in lung and liver transplant recipients, invasive aspergillosis tends to be a late diagnosis. As a result, 12-week mortality after diagnosis was 47.1% for liver, 27.8% for kidney, 16.7% for heart, and 9.5% for lung transplant recipients respectively [189].

Due to the high incidence in lung transplant recipients and their underlying risk factors (prior colonization, idiopathic pulmonary fibrosis, corticosteroid use), several studies have concluded that antifungal prophylaxis in lung transplantation is effective in decreasing the incidence of invasive aspergillosis. However, the optimal strategy for this remains unclear, with some centers using long term azole prophylaxis and others using nebulized amphotericin B. Both strategies appear to be effective in reducing the incidence of invasive aspergillosis [159, 160].

3.6 Changes in Epidemiology Over Time: Intensive Care Unit Patients

The incidence of invasive aspergillosis in intensive care has been estimated to be around 1.7–6.3 per 1000 admissions to ICU [165, 167, 169]. However, the frequency of invasive aspergillosis is often difficult to determine in this group of patients as the classical radiographical signs are not always present, Aspergillus colonization may be problematic, and molecular and serological tests may not have been adequately validated in non-neutropenic patients. As the diagnostic methods for invasive aspergillosis improve, together with improvements in supportive and intensive care and more aggressive therapies in patients with greater comorbidities, an increase in the incidence of invasive aspergillosis in the ICU setting should be expected.

Mortality of invasive aspergillosis in non-neutropenic patients has been estimated to be approximately 63–72 %, primarily due to delays in recognition and diagnosis [164, 166, 167]. Poor prognostic factors associated with invasive aspergillosis include age, corticosteroids prior to ICU admission, mechanical ventilation, septic shock, and hemodialysis [166, 169].

4 Agents of Mucormycosis and Entomophthoramycosis

The agents of mucormycosis are ubiquitous fungi in the environment that are commonly found in decaying organic matter, such as bread, compost bins, and animal excreta. Diagnostic limitations remain (i.e, culture and histology) and recognized fungal biomarkers such as β-D-glucan and galactomannan remain negative in infections caused by these organisms. However, the epidemiology of these infections is increasingly better understood with the introduction of molecular methods for diagnosis (e.g., 18S rRNA PCR) and genomic sequencing studies. These molecular studies, along with improvements in culture-based morphological identification, have led to changes in terminology for this group of organisms [190]. The organisms described in this section are listed in Table 5.

Infection is typically by inhalation of spores, though ingestion or via the cutaneous route (e.g., trauma or burns) is also recognized.

The largest study of the epidemiology of invasive mucormycosis described all cases in the reported literature from 1885 to 1999 and included over 900 cases [191]. *Rhizopus* spp. (47 %) was the most common cause of mucormycosis followed by *Mucor* spp. (18 %), *Cunninghamella* spp. (7 %), *Apophysomyces elegans* (5 %), *Lichtheimia* spp. (5 %), *Saksenaea* spp. (5 %), and *Rhizomucor* spp. (4 %). Most (80 %) had an underlying disease. These included diabetes (36 %), malignancy (17 %), solid organ transplantation (7 %), deferoxamine therapy (6 %), injection drug use (5 %), bone marrow transplantation (5 %), renal failure (4 %), low birth weight infant (3 %), diarrhea and malnutrition (3 %), HIV infection (2 %), and systemic lupus erythematosus (1 %). The sinuses were the most

Table 5
Organization of the most common agents of mucormycosis

Rhizopus arrhizus
Rhizopus microsporus
Rhizomucor pusillus
Rhizopus stolonifer
Cunninghamella bertholletiae
Apophysomyces elegans
Saksenaea vasiformis
Lichtheimia corymbifera
Mucor circinelloides
Mucor velutinosus
Syncephalastrum racemosum
Cokeromyces recurvatus
Mortierella wolfii

frequent site of infection (39 %) followed by pulmonary (24 %), cutaneous (19 %), cerebral (9 %), gastrointestinal (7 %), and disseminated (3 %). The site varied by underlying condition; the majority of patients with malignancy (60 %) had pulmonary disease, whereas the majority of patients with diabetes (66 %) had sinus disease. Rhinocerebral disease was seen more frequently in patients with diabetes (33 %), compared with patients with malignancy (4 %). Mortality also varied with site and underlying disease.

Several prospective multicenter studies performed since this review has been published. A European prospective study, involving patients from 13 countries, performed between 2005 and 2007 involved 230 patients [192], while a prospective US study (performed between 2004 and 2008) included 121 cases [193]. A study from six countries in Europe and Asia (performed between 2006 and 2009) included 41 patients from 15 centers [194].

Skiada found that *Rhizopus* spp. (34 %), *Mucor* spp. (19 %), and *Lichtheimia* spp. (19 %) were the most frequently isolated species [192]; *Rhizopus* spp. (52 %) was the most commonly isolated species, followed by *Mucor* spp. (23 %), other or unknown (14 %), *Rhizomucor* spp. (7 %), and *Lichtheimia* spp. (3 %) in the US study [193]. *Mycocladus corymbifera* was the most frequently identified species (24 %) in another study [194].

Hematological malignancies (44 %), trauma (15 %), hematopoietic stem cell transplantation (9 %), and diabetes mellitus (9 %) were the most common underlying conditions in one study [192] whereas Kontoyiannis found hematological malignancy (61.2 %) and diabetes mellitus (23 %) were the most frequent conditions [193]. Among patients with a hematological malignancy, 43 % patients had received a hematopoietic stem cell transplant and 60 % were neutropenic at baseline. Malignancies (63 %), diabetes mellitus (17 %), and solid organ transplantation (10 %) were the most common predisposing factors in the study by Ruping et al. [194]. Studies of hematological malignancy cohorts include risk factors such as acute leukemia, prolonged neutropenia and lymphocytopenia [195].

The most common infection sites were the lungs (30 %), rhinocerebral (27 %), soft tissue (26 %), and disseminated disease (15 %) [192] which compared to the lungs (46 %), sinuses (29 %), and skin/soft tissue (22 %). Twenty five (21 %) had evidence of disseminated infection as multiple anatomic sites were reported [193]. The main sites of infection were the lungs (59 %), soft tissues (20 %), rhino-sinu-orbital region (20 %), and brain (15 %). Disseminated infection was seen in six patients (15 %).

Numerous single center studies have also been performed in Europe [196], Asia [197–199], and Africa [200]. Diabetes mellitus is the predominant risk factor for infection in India, Pakistan and Egypt [198–200]. Some studies have concentrated on hematology patients [195, 201–203], transplant recipients [204, 205], and pediatrics [206, 207].

A cluster of 13 cases (5 of whom died) occurred following a tornado in the USA [208]. A case control study found that penetrating trauma was associated with infection. Another case was described in a previously healthy 17-month-old boy who developed pulmonary mucormycosis after a near-drowning incident in a goose pond [209]. He survived with aggressive therapy.

There are also reports of cases related to outbreaks. These were reviewed by Antoniadou [210]. Twelve hospital outbreaks and two pseudoepidemics caused by mucormycosis were cited in the English literature between 1977 and 2008 in the USA and Europe. Cases have included cutaneous, disseminated, pulmonary, and rhinocerebral disease. Species identified have included *Rhizopus arrhizus*, *Rhizopus rhizopodiformis*, *Rhizopus microsporus*, *Rhizopus* spp., *Absidia corymbifera*, and *Rhizomucor pusillius*. Sources of infection have included Elastoplast adhesive bandage rolls, ventilation systems, wooden tongue depressors, karaya (plant-derived adhesive) ostomy bags, and water damage to a linen store and patient shower room. Patients have included cardiac surgery patients, renal transplant recipients, orthopedic patients, adult leukemia patients, intensive care unit neonates, immunocompromised hematology patients, and burn unit patients. Three cases of cutaneous mucormycosis due to *Lichtheimia* spp. in the intensive care

and orthopedic units were recently reported [211]. Environmental and epidemiological investigations suggested a possible cross-transmission of *L. ramosa* between two patients in intensive care. Another report included five cases of *Rhizopus delemar* in the USA (all fatal) related to linen [212].

Construction works have also been implicated in several cases of invasive fungal infection including infection with mucoraceous moulds [213].

The number of cases reported has increased over time in a number of studies [191, 196, 214], which could reflect an increased population at risk (e.g., due to immunosuppression, increases in patients with diabetes) or increased awareness/improved recognition/improved diagnostics.

Some cases have presented as breakthrough infection in patients receiving prophylaxis or treatment with echinocandins and azoles that have activity against Aspergillus but not mucormycosis [214–216].

Mortality approaches 50% in most studies [191–193, 195, 196, 198, 204, 214].

Roden described changes in mortality was related to underlying diseases, site of infection (disseminated had 100% mortality), and organism (infection with *Cunninghamella* spp. had the highest mortality rate [191]. Outcome varied by infecting organism in the study by Kontoyiannis et al. [193]. It was better for infections caused by *Lichtheimia* (0.5) rather than other organisms (*Rhizopus* spp. (0.47), *Mucor* spp. (0.40), unknown Mucormycetes species (0.40), other Mucormycetes species (0.17), and *Rhizomucor* spp. (0.15).

Other factors associated with survival include trauma as an underlying condition [192], treatment with amphotericin B and surgery [192, 196, 204, 214] and treatment of the underlying disease [202]. In one study, however, age, diabetes mellitus, transplant status, or antifungal therapy was not associated with mortality [217].

Factors associated with death were higher age [192], malignancy or neutropenia at enrolment [217]. Patients treated with deferasirox had a higher mortality rate at 90 days in a small Phase II study of 20 patients [218] and the review by Roden et al. [191]. The prior administration of caspofungin [192] or voriconazole [193] has also been associated with increased mortality.

5 Fusarium and Scedosporium

The frequency of rare pathogenic fungi commonly resistant to amphotericin B (such as *Fusarium* spp. and *Scedosporium* spp.) has significantly increased over the past 20 years among patients with hematologic malignancies. The role of selective antifungal pressure possibly contributes as much to the emergence of these pathogens as environmental exposure and underlying immunosuppression.

Fusarium is a ubiquitous environmental filamentous fungus and a major plant pathogen that is now emerging as an opportunist human pathogen. *Fusarium* spp. cause a range of infections in humans, from superficial localized nail, skin, and corneal infections in the immunocompetent, to more deep seated invasive disease in the immunocompromised. In one retrospective study in Israel, the majority of patients (69/89; 76 %) with infections due to *Fusarium* spp. were immunocompetent and had predominantly locally invasive [34] or superficial infections [48] occurring mainly in the lower limbs [219]. Seven patients had disseminated disease. The presence of chronic renal failure, hematological malignancy, burns, and disseminated infection were independently associated with mortality.

There have been an increasing number of case reports of invasive fusariosis among patients with hematological malignancies, including stem cell and solid organ transplant recipients. Fusarium infections have been described in one series as the second most common mould pathogen in this patient group [220].

Scedosporium are filamentous fungi that are widespread in the environment and can be found in soil, sewage and polluted waters. There are two main human pathogens—*S. apiospermum* and *S. prolificans. S. prolificans* is found commonly in temperate climes, while *S. apiospermum* is confined to the northern part of the Iberian peninsula, Australia, California, and parts of the southern USA [221, 222]. They are frequent colonizers of the respiratory tract and can be isolated from asymptomatic patients with cavitary lung disease due to tuberculosis, cystic fibrosis, and bronchiectasis. In immunocompetent patients, they have been associated with sino-pulmonary infections, secondary pneumonia following near-drowning incidents, and infections of the skin, soft tissue, and bones.

Scedosporium is increasingly recognized as an important emerging pathogen among immunocompromised patients with invasive pneumonia, brain abscess, and fungemia responsible for most deaths. Cortez and colleagues have published a detailed review of infections caused by *Scedosporium* spp. [223]. In one single center retrospective study of scedosporiosis among 27 solid organ transplant recipients, the majority (59 %) were colonized with *Scedosporium* and had no active disease (exclusively seen in the lung transplant recipients) [224]. *S. apiospermum* was the dominant strain (67 %) followed by *S. prolificans* (33 %), but there was no significant clinical difference among strains. In addition to lung transplant recipients, patients with multivisceral, heart, liver, and small intestine transplants were also at risk and presented with pneumonia (64 %), mediastinitis (18 %), and fungemia/disseminated infections (18 %). Mortality was high at 6 months (55 %) and linked to earlier onset scedosporiosis post transplant, mediastinitis or disseminated infections, and failure to treat with a voriconazole-containing regimen.

Fusarium and Scedosporium infections are difficult to differentiate from other mould infections such as Aspergillus and Mucorales because they affect similar organ sites and have a similar progressive and aggressive course with inadequate treatment. In one of the largest series of solid organ and stem cell transplant patients with probable or confirmed IFI, Park et al. identified 37 cases of Fusarium and 27 cases of Scedosporium infections, making them second only to Mucorales as a cause of non-*Aspergillus* IFI [225]. The lower respiratory tract was the most common site of involvement (38.9% of fusariosis and 25.9% of scedosporiosis), followed by disseminated disease (22.2% of fusariosis, 25.9% scedosporiosis). In both instances, onset of infections was mostly soon after transplant, although a large number occurred more than 6 months after transplant.

6 Pneumocystis Species

Pneumocystis is a genus of unicellular fungi found in the lungs of mammals, including humans. Infection in humans is caused by *Pneumocystis jirovecii*. Infection is mainly acquired by inhalation of cysts. Primary infection may occur in childhood and may be asymptomatic or cause a mild upper respiratory tract infection [226, 227]. It has long been recognized that the majority of children throughout the world have detectable antibodies by 2–4 years of age [228]. However, it is increasingly recognized that Pneumocystis pneumonia (PCP) may be transmitted between individuals or from the environment. One study suggested that a history of gardening or hiking and camping was associated with increased risk [229]. Outbreaks have been described in oncology patients [230–233], transplant recipients [233–240], and patients receiving rituximab [241]. Risk factors described in these outbreaks include lack of chemoprophylaxis, frequent interpatient contact, and lack of adherence to isolation precautions [242]. Pneumocystis organisms were detected from 80% of air samples at 1 m from the bedside and 33% of sample taken 8 m from the bedside in one study [243].

Colonization with *P. jirovecii* is associated with immunosuppression (HIV, cancer, autoimmune disease, organ transplantation), immunosuppressive drugs (steroids, tumor necrosis factor α inhibitors), and chronic lung diseases. Colonization may or may not proceed to infection [244].

PCP was first reported in HIV patients in 1981 [245] and its incidence rose dramatically. PCP was the leading cause of morbidity and mortality in people with HIV for many years. Numbers declined once antiretroviral (ARV) therapy and anti-PCP prophylaxis were introduced, particularly in high resource countries [246]. In these countries, PCP typically now only occurs among people who are unaware of their HIV status, who do not seek

medical care or who are do not comply or respond to ARV therapy or anti-PCP prophylaxis [247].

PCP typically occurs in patients with a CD4+ count of less than 200 cells/μl. Previous PCP, oral candidiasis, and persistent fevers are also risk factors for PCP, though a US study found that a greater proportion of black people, women, and people from southern states were affected over time [248].

Mortality has also fallen with advances in medical technology [249]. Mortality was 10.1 % for the period from 1985 to 1989, 16.9 % for the period from 1990 through June 1996, and 9.7 % for the period from July 1996 to 2006, though this was not related to ARV therapy; no patients were receiving ARV therapy prior to their diagnosis. Similar figures were shown in a US study [248]. Mortality remains high in patients requiring intensive care; older age, low serum albumin, need for mechanical ventilation, pneumothorax, lower hemoglobin, and a greater alveolar–arterial gradient were associated with increased mortality in intensive care patients [248].

There are, however, geographic differences. Early reports from sub-Saharan Africa suggested that PCP was a less important cause of morbidity and mortality [250–252], and other AIDS-associated opportunistic infections such as tuberculosis and enteric pathogens predominate [253, 254]. The apparently low burden of PCP among African adults may be explained by early mortality from other more virulent infections, a genuine low infection rate [255, 256], geographic/environmental/climatic factors, genetic factors [257] or limited access to sensitive diagnostic tests. Recent studies suggest it may be more common than previously thought; it was the second most common cause of pneumonia in patients admitted to a high dependency unit in Malawi in a study, of whom 94 % were coinfected with HIV [258], while a review suggested an increase in reported cases between 2002 and 2010 [259]. Of note, PCP was also associated with increased deaths after starting ARV therapy in a study from East Africa [260] due to immune reconstitution. It is, however, more common in India [261, 262].

PCP in HIV-seronegative patients in the developed world has continued to increase in recent years, though numbers remain small. This may reflect increases in the number of immunosuppressed patients at risk for PCP such as organ transplant recipients (solid organ and hematopoietic stem cell), cancer patients and patients receiving anti-TNF-α agents or steroids. These patients often receive prophylaxis, which is highly effective [263].

Mortality due to PCP in HIV-seronegative individuals is higher than that in HIV-seropositive individuals [264]. Patients were older and had higher APACHEII scores in the HIV-seronegative cohort. Patients without HIV are thought to have a greater inflammatory response than HIV-seropositive patients. In-hospital mortality was 67 % in another study of HIV-seronegative patients with PCP [265]. Poor prognostic factors included a high APACHE III scores,

intubation delay, longer duration of positive pressure ventilation and development of pneumothorax. None of the patients in this series received PCP prophylaxis prior to the development of pneumonia. They also suggested that CD4 count was less helpful in determining the risk of developing PCP in these patients.

7 Dimorphic Fungi

7.1 *Blastomycosis*

Blastomycosis is a systemic mycosis with an increased prevalence in the mid-West of the USA [266] and was originally described in 1894 in one patient, with the causative agent *Blastomyces dermatitidis* isolated in 1898 in a second patient. For many years it was known as Gilchrist`s disease, Chicago's disease, or North American blastomycosis [266, 267].

B. dermatitidis is a thermally dimorphic fungus which inhabits the soil. Human infection occurs primarily via inhalation. *B. dermatitidis* is the asexual form of this holomorphic fungus and the sexual phase is termed *Ajellomyces dermatitidis*. It occurs in the mycelial form at 30 °C and as a yeast at 37 °C (tissue phase). This transition is also affected by nutrients [266].

A number of virulence factors can be produced and the dimorphic nature of this fungus, as well as the tolerance to temperature aid pathogenicity (Table 6).

Blastomycosis occurs mainly in two clinical forms: pulmonary and extra-pulmonary [266]. It affects mostly immunocompetent male adult patients in the fourth decade of life. It has been suggested that this is probably due to longer exposure to this organism as the individual ages. It is also associated with occupational

Table 6
***Blastomycetes* spp. virulence factors [266]**

Virulence factor	Description/comments
BAD1 (Blastomyces adhesion factor)	Thought to be involved in the start of the infection and also blocks the release of TNFα
Yeast component: 95 % α-3 glucan Mycotic component: α-glucan and β-glucan in similar proportions	
Melanin production	Protects fungus from leukocytes and their oxidative reactions
Large amount of yeasts (tissue phase) produces a late hypersensitivity reaction that causes tissue damage, e.g., abscesses, hemorrhagic lesions and also chronic forms, e.g., fibrosis and granulomas	

exposure (e.g., forest rangers) or with recreational activities such as camping and fishing. Infections in immunocompromised patients have been reported and the organism tends to cause more aggressive disease exhibiting a higher case fatality rate, when compared to immunocompetent cases i.e, 30–40% versus 2–20% [267]. Although not normally associated with children, a 30 year report from Canada found 34 cases of blastomycosis with 59% male and a mean age at diagnosis of 10 years of age. Disease manifestations also included a large number of central nervous system infections in addition to pulmonary and extrapulmonary manifestations [268]. The children were otherwise healthy and only two were immunocompromised [268].

B. dermatitidis has been reported in Canada [269], Africa and India [270], Lebanon, Israel, and Saudi Arabia [266]. Although this disease does not often cause outbreaks, a large community outbreak was reported in Wisconsin during 2009–2010. Community outdoor activities did not correlate with the increase in cases, though Hmong ethnicity (people from the mountainous regions of China, Vietnam, Laos, and Thailand), age, and having a chronic medical condition were independently associated with cluster case status [271].

For the majority of blastomycosis cases, the incidence depends on reporting of clinically diagnosed cases of infection, since there are no simple and reliable markers of previous infection. Only a few American states/provinces endorse mandatory reporting of clinical cases (i.e, Wisconsin, Illinois, Mississippi) in the US and Ontario and Manitoba in Canada which may indicate potential under-reporting [272].

7.2 Coccidioidomycosis

Coccidioidomycosis was the first major mycosis to be recognized. There are two species: *Coccidioides immitis* and *C. posadasii* (267, 273). This disease is also known as San Joaquin Valley fever or Valley fever [274, 275].

Coccidioides immitis and *C. posadasii* are thermally dimorphic fungi, nearly identical species and appear in the environment as a mould (producing fungal hyphae) and in the host, which typically includes small mammals, produces endosporulating spherules [267, 273, 275].

Asexual reproduction of the mould form produces arthroconidia which are environmentally resistant. These are then inhaled by the host, when the soil is disturbed, to produce a primary pulmonary infection. This is also the main mode of infection in humans [267, 273, 275]. The arthroconidia enlarge and become spherules which give rise to endospores and when these are released, the endospores disperse and disseminate and enlarge giving rise to the next generation of spherules and the cycle continues. Genetic studies have also identified that sexual reproduction does occur though has not been observed [267, 273, 275].

Coccidioidomycosis occurs in the Western Hemisphere at 40° latitudes north and south and this zone includes south western USA which includes the deserts and north-west Mexico. The climate here is arid with little rainfall, very hot summers and mild winters and an alkaline, sandy soil. *C. immitis* is found in California and *C. posadasii* found in Texas, Arizona and areas of endemicity in Mexico, Central and South America. *C. posadasii* was also known as non-California *C. immitis* [267, 273].

Risk factors for developing coccidioidomycosis include dust exposure (via the aerosolized arthroconidia), male sex, race (Filipinos, Asian and African Americans), pregnancy, smokers, increased age, diabetes mellitus, immunosuppression, and hemodialysis [267, 273].

Infection occurs via inhalation and ranges from a self-limiting infection, without needing any medical intervention, to chronic pulmonary or disseminated disease. Those with self-limiting infection recover spontaneously and retain lifelong immunity. Extrapulmonary infection is almost always due to hematogenous spread via an initial pulmonary focus [273]. Coccidioidomycosis is also known to affect HIV sero-positive patients [276].

7.3 Paracoccidioidomycosis

Paracoccidioidomycosis (PCM) is also referred to as South American blastomycosis, Lutz disease or Pb mycosis and caused by a thermo-regulated dimorphic fungus called *Paracoccidioides brasiliensis* [267, 277].

Paracoccidioides brasiliensis is exists as a mould producing a mycelium in soil and as a yeast in the host which includes armadillos, penguins as well as humans [278].

Paracoccidioidomycosis has a restricted geographical niche which ranges from Central to South America, from Mexico to Argentina [279]. This fungus is the most important systemic mycosis in Brazil with 85 % of cases occurring here. Overall, in Latin America, ten million individuals are infected and approximately 2 % of these going on to develop the disease [278].

Paracoccidioides brasiliensis is found in areas of abundant rainfall, mild temperatures (17–24 °C) and tropical and subtropical regions of Latin America [279], including coffee and tobacco-growing areas [267]. The micro-niche of this organism remains unknown, although it is thought to be a soil-inhabiting organism and has been isolated rarely from soil and also found in the internal organs of armadillos known to inhabit endemic areas [279].

Paracoccidioidomycosis is caused by inhalation of spores but the organism can remain dormant for a long time following primary pulmonary acquisition with the infection thought to be acquired in the first 2 decades of life. Although paracoccidioidomycosis is uncommon in children, 5–10 % of all paracoccidioidomycosis infections occur in children and testing of children in Brazil, living in rural, endemic areas revealed that one-third have

subclinical infection [267]. Patients with tuberculosis and AIDS can become co-infected with paracoccidioidomycosis [280].

Infection is generally self-limiting and restricted to either the site of contact with the fungal hyphae or to a single organ affecting both sexes. Paracoccidioidomycosis generally affects males and can evolve into benign disease or disseminate systemically, causing severe damage to the host [278]. Oestriol is thought to offer some protection to females [279].

7.4 Histoplasmosis

In 1905, Samuel Taylor Darling, a North American pathologist first described histoplasmosis but thought it was due to a protozoan. Histoplasmosis is also known as Darling disease [281] and is a systemic endemic mycosis caused by the thermally dimorphic fungus: *Histoplasma capsulatum*. This mycosis is also cited as classic histoplasmosis, Caver's and Miner's disease, Cave disease, Bat disease, Ohio Valley disease, Tingo Maria fever, reticuloendotheliosis, and cytomycosis [281].

Histoplasma capsulatum is normally found in soil as a mould and has been often recovered from soil contaminated with bat or bird droppings, especially those found under bat caves or bird roosts. Birds do not become infected with this organism, whereas bats do. Bird droppings act as a source of nutrients for the mould. Even after roosts have cleared, it has been reported that the soil site remains contaminated with *H. capsulatum* for prolonged periods [267].

It is also found in the guano of domestic fowl (e.g., turkeys and chicken) as well as migratory birds. Occupational exposure both from professions such as guano collectors, geologists, archaeologists, and recreational activities which includes cave and ecotourism enthusiasts are at a greater risk of acquiring the infection. Immunocompromised patients are at risk of invasive, disseminated disease [281]. Disseminated histoplasmosis is being increasingly reported in patients on anti-TNFα agents for rheumatoid arthritis and other conditions with a reported case fatality rate of 20%, mostly from places where this fungus is endemic [267]. Histoplasmosis is a known opportunistic disease affecting HIV patients since 1987. However, with ARV therapies and better CD4+ level control the incidence of this disease and other fungal infections has declined [276].

Histoplasma occurs as two pathogenic varieties: *H. var capsulatum* and *H. var duboisii* [267]. Once in the host tissue, it forms a small round, budding yeast. While the mould forms for *H. var capsulatum* and *H. var duboisii* are identical, the yeast forms are different. Yeast cells of *var duboisii* are larger with thicker walls. *Var duboisii* is also referred to as African histoplasmosis [267].

This mycosis is endemic in many regions, including the Americas, Asia and Africa but infection can occur worldwide. The prevalence can be estimated using the histoplasmin test, with the largest concentration of positive skin reactors in central

USA. Positive skin reactors in Brazil range from 2.6 to 93.2 % with areas in Rio de Janeiro considered endemic and even hyper-endemic [276, 282]. An *in vitro* study found that exposure of an avirulent *H. capsulatum* strain to the amoeba, *Acanthamoeba castellani*, produced a phenotype of *H. capsulatum* that caused a persistent murine lung infection. This is consistent with the theory that soil amoebae may contribute to the selection pressure of certain phenotypes that enhance the potential of pathogenic dimorphic fungi to produce virulent disease in humans and other mammals [283].

Histoplasma infections range from subclinical to severe, disseminated disease but most infections tends to be self-limiting and resolve spontaneously. However, the organism can remain latent within the host and could still cause infection even years after initial infection [267].

Infection occurs through the direct inhalation of microconidia (spores) from the environment which passes through the bronchioles and end up in the alveoli, giving rise to a primary pulmonary infection. The spectrum of clinical disease is described as acute pulmonary, chronic cavitary, and disseminated [267, 281].

8 Dermatophytes

Dermatophytes are fungal pathogens responsible for a wide range of superficial skin, hair, and nail infections. They have a worldwide distribution and account for a large number of hospital visits in the developed world [284]. There are three genera of dermatophytes; *Microsporum* spp., *Trichophyton* spp. and *Epidermophyton* spp. *Trichophtyon tonsurans* is the most common cause of tinea capitis in N. America, Canada, and Latin America, while *T. violaceum* accounts for most cases of tinea capitis in Africa. *Trichophyton rubrum* is the most common dermatophyte worldwide [285], accounting for nearly 70 % of tinea cases and is responsible for most cases of invasive disease.

Invasive dermatophytosis is rare but increasingly reported as a result of changes in host pathogenesis and advances in medical care leading to a rise in patients that are highly susceptible to invasive fungal disease. The presence of an impaired innate immune response (e.g., functional neutropenia or NK cell dysfunction) or defective T cell immunity is the main driver for invasive disease [286]. Risk factors include lymphoproliferative disease, atopy, blood and solid organ transplantation, poorly controlled diabetes, HIV/AIDS, and autoimmune disease [287]. Patients with liver failure and iron overload conditions are also at increased risk of invasive dermatophytosis. The most frequently implicated drugs are steroids (both systemic and topical), combination chemotherapy, azathioprine, tacrolimus, cyclosporine, cyclophosphamide, and infliximab [287].

Most patients presenting with invasive disease have a prior history of preexisting dermatophytosis. The fungi are usually limited to the stratum corneum in immunocompetent individuals due to the ideal nutritional and temperature requirements of this skin layer. The three main types of invasive disease are: (1) Majocchi's granuloma, a nodular infiltrative folliculitis that is self-limiting in immunocompetent individuals but can progress to more extensive lesions in the immunocompromised; (2) more invasive dermatophytosis following minor trauma or topical steroid use and involving dermal and subcutaneous tissue; and (3) disseminated disease through lymphatics and blood to regional lymph nodes and distant sites. The most common invasive sites are lymph nodes (17%), bone (7%), brain (7%), and liver (5%). Spread to muscle, testes, and spleen have also been described [287].

Transparency Declarations

DAE has received funding to attend conferences from MSD, Gilead, and Astellas. SHA has served on UK Advisory Boards for liposomal amphotericin B (Gilead), caspofungin (MSD) and posaconazole (MSD) and has received sponsorship to attend international meetings from Schering-Plough, Gilead, and Wyeth. CM has received travel grants to attend scientific conferences from Astellas, Gilead, Pfizer and Novartis, educational grants from Pfizer and Novartis, attended a Pfizer Advisory Board Meeting and consulted for Astellas. HY has no conflicts of interest.

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Chapter 3

Current Algorithms in Fungal Diagnosis in the Immunocompromised Host

Thomas Lehrnbecher, Karsten Becker, and Andreas H. Groll

Abstract

Invasive fungal diseases (IFDs) are a major cause of morbidity and mortality in immunocompromised patients such as patients with hematological malignancies or allogeneic hematopoietic stem cell transplant recipients. Whereas the definite diagnosis of IFD requires invasive diagnostic procedures, imaging and noninvasive diagnostic assays may help in decision making with regard to the institution and the choice of antifungal agents, the duration of therapy, surgical intervention, and monitoring of fungal manifestations.

Unfortunately, signs and symptoms of IFD are often nonspecific in the immunocompromised patient. Therefore, in immunocompromised patients with suspected IFD, all samples collected need to be cultured for fungi, and, in the case of specimens obtained by invasive diagnostic procedures, also microscopically examined. For high sensitivity of the cultural and microscopic approaches, specific media and stains, respectively, are crucial. Non-culture based method such as the detection of galactomannan or ß-D-glucan and molecular tools such as polymerase chain reaction may help in the early diagnosis of IFD. Imaging studies may be indicative for IFD, but invasive diagnostics such as bronchoalveolar lavage and/or biopsy should be pursued in order to identify the causative pathogen. This chapter summarizes the current knowledge on diagnosing IFD and proposes practical help in the use of diagnostic tools.

Key words Invasive fungal disease, Culture, Galactomannan, ß-D-glucan, Polymerase chain reaction, Computerized tomography, Magnetic resonance imaging, Brochoalveolar lavage, Biopsy

1 Background and General Considerations

Invasive fungal diseases (IFDs) are a major cause of morbidity and mortality in immunocompromised patients such as patients with hematological malignancies or allogeneic hematopoietic stem cell transplant (HSCT) recipients [1, 2]. The most common fungi causing invasive infections in this setting are *Aspergillus* and *Candida* spp., but other organisms such as mucormycetes or *Fusarium* spp. are increasingly found. The definite diagnosis of IFD requires invasive diagnostic procedures, but imaging and noninvasive diagnostic assays may help in decision making with regard to the institution and the choice of antifungal agents, the duration of therapy, surgical intervention, and monitoring of fungal manifestations.

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Early diagnosis of IFD is difficult, in particular in invasive mould infection. On the other hand, early institution of therapy results in an improved outcome. There are different antifungal strategies, which depend on a number of factors, including but not limited to populations at risk for IFD and the local epidemiology. For example, in patients at high-risk for IFD such as patients with acute myeloid leukemia (AML) or undergoing allogeneic hematopoietic stem cell transplantation (HSCT), who are neutropenic, afebrile and do not have symptoms suggesting IFD, screening with non-culture based methods is a potential strategy, in particular when these patients do not receive mould-active prophylaxis (Fig. 1). In neutropenic patients who develop fever which does not respond to broad spectrum antibiotics, there are two potential strategies: in the empirical antifungal therapeutic approach, antifungal therapy is started in all persistently febrile neutropenic patients, whereas in the preemptive strategy, antifungal therapy is only instituted when imaging and/or non-culture based diagnostics indicate IFD. The results of the diagnostic workup will also help to modify and guide antifungal therapy. Current methods for diagnosing systemic fungal infections include assessment of clinical signs and symptoms, mycological examination by microscopy and cultural as well as non-cultural techniques (e.g., antigen and antibody detection, molecular methods), and imaging procedures.

In this chapter, the different diagnostic tools are described and potential differences between children and adults are highlighted. In addition, practical help in the use of these diagnostic tools is presented.

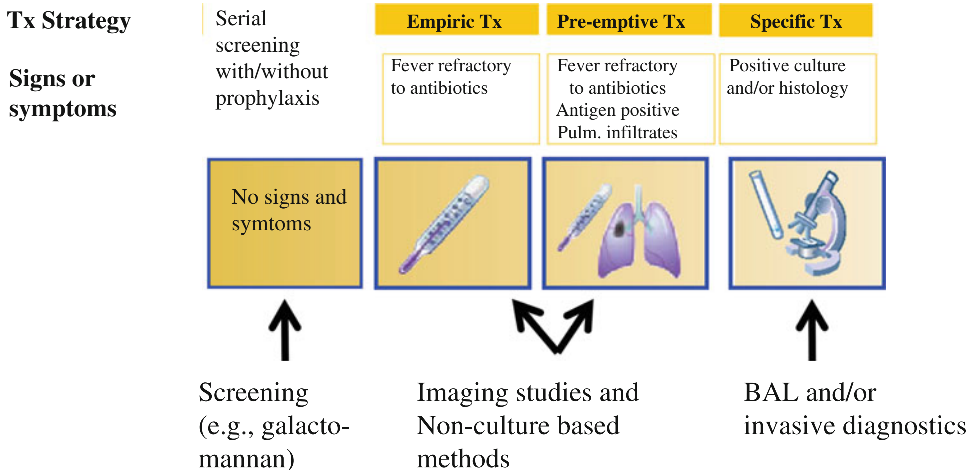

Fig. 1 Potential diagnostic and therapeutic strategies in immunocompromised patients at high risk for invasive fungal disease

2 Signs and Symptoms of Invasive Fungal Infection

Signs and symptoms of IFD are often nonspecific, in particular in early stages of the disease. Unexplained fever despite broad-spectrum antibiotics or recurring febrile episodes after initial defervescence in the neutropenic host may be the only symptom of invasive fungal infection, but fever may also be caused by bacteria or viruses. Table 1 shows possible symptoms of invasive fungal infection depending on the site involved.

2.1 Microscopy

In the case of IFD suspicion, microscopy is a key investigation because it is rapidly to perform within a few hours and the diagnostic yield is more than that for culture alone [3]. Thus, samples from invasive diagnostic procedures such as bronchoalveloar lavage (BAL), biopsies, or specimens from surgical procedures should always be microscopically examined by an experienced microbiologist and the treating clinician must be informed promptly in the case of a positive result [4]. Based on morphologic characteristics, the occurrence of fungal structures per se could be demonstrated and a rough differentiation of several groups of fungi such as such as hyaline moulds, mucorales, "black yeasts (dematiaceous fungi)" or yeasts could be possible. Of note, pretreatment with antifungal agents may influence fungal morphology.

Table 1
Potential clinical symptoms of invasive fungal disease in different organ/organ systems

Organ/system	Features	Likely infection
Skin	Scattered lesions, maculopapular, progressing to pustular lesions with central necrosis	Acute disseminated candidiasis, disseminated aspergillosis, or *Fusarium* infection
Sinus	Facial pain, black or bloody nasal discharge, nasal eschars	Invasive aspergillosis or mucormycosis
Palate	Ulceration	Rhinocerebral mucormycosis
Chest	Unspecific symptoms such as cough, pleural pain, or back pain	Invasive pulmonary aspergillosis, PcP, or other fungal pneumonia
Central nervous system (CNS)	Headache, neck stiffness, altered mental status, seizures, other focal neurologic signs	CNS aspergillosis or mucormycosis, cryptococcal or *Candida* meningitis
Liver	Right upper quadrant pain Hepatosplenomegaly	Chronic disseminated candidiasis
Eyes	Visual disturbances up to sudden blindness Funduscopy may reveal "cottonwool ball" lesions in *Candida* choroidoretinitis	Acute disseminated candidosis

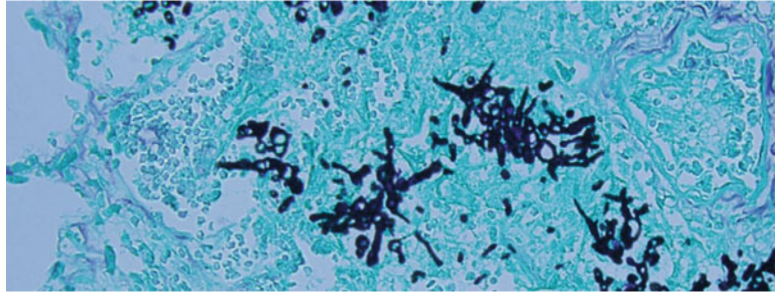

Fig. 2 Grocott's methenamine silver stain of lung tissue of a patient with chronic graft-vs-host disease and augmented immunosuppression who died due to disseminated invasive *A. fumigatus* aspergillosis. Note the visualization of fungal elements on the background of lung tissue altered by inflammation and hemorrhage

When direct preparations are stained with Gram or hematoxylin–eosin, fungal elements may be insufficiently stained and/or misinterpreted as artifacts. Whereas hyphae are best visualized by "special fungal" stains, such as periodic acid–Schiff (PAS) stain, Grocott's methenamine silver stain or optical brighteners (fluorescent whitening agents, such as Blankophor of Calcofluor white), Grocott staining is the best method to demonstrate nonvital fungal structures (Fig. 2). In patients with suspected infection with *Cryptococcus neoformans*, cerebrospinal fluid (CSF) should be examined by direct India ink preparation or mucicarmine stain in addition to antigen testing (see below). Although direct microscopic examination of filamentous fungi can provide important morphologic information (e.g., the presence of septa, the hyphal diameter, or ramification pattern), immunohistochemical examination should be performed in particular to differentiate between *Aspergillus* spp. and Mucoraceae [5]. Similarly, polyclonal antibodies may be used to detect *Candida* spp. in biopsy specimens. In the past years, fluorescent *in situ* hybridization (FISH) was shown to offer significant advantages that allow for the rapid and specific identification of fungal structures [6].

2.2 Culturing Techniques

All clinical samples from patients at high risk for IFD must be cultured for fungi to ensure species identification and antifungal susceptibility determination. In general, moulds are more difficult to isolate from clinical samples than *Candida* spp. [4]. Of note, yeasts and moulds do not grow well on conventional media used for the cultivation of bacteria and may require also other deviant culture conditions, thus, to ensure a high level of sensitivity, the use of specific agar and broth media and respective culture conditions is crucial. Consequently, the microbiological laboratory must be informed that there is a suspicion of an IFD and/or general diagnostic practices for defined patient groups and specimens should be established between the clinical partners and the laboratory.

Early detection of candidemia is very important because it is an indicator of disseminated disease. According to recently published European guidelines for the diagnosis of *Candida* infection, the number of blood cultures to be recommended in a single session is 3 [2–4], with a total volume varying according to the age of the patient, 40–60 mL for adults, 20 mL for children between 12 and 36 kg, 6 mL between 2 and 12 kg, and 2–4 mL for children under 2 kg [7]. The frequency recommended is daily when candidemia is suspected, and the incubation period must be at least 5 days. Sensitivity of blood cultures to detect *Candida* is up to 75 %, but sensitivity rates seem to be lower in neutropenic patients and in those undergoing antifungal treatment. Notably, blood cultures are usually negative in patients with chronic invasive *Candida* infections such as hepatosplenic candidiasis and for most medically important hyaline mould infections (e.g., *Aspergillus* spp.). Bactec Mycosis IC/F medium apparently detects growth of *Candida* spp. faster than the fully automated and commonly used BacT/Alert system but requires a third blood culture bottle [8]. Special methods such as lysis centrifugation (Dupont Isolator) may improve the detection of fungi but can lead to false-positive results. Novel technologies have been suggested to accelerate yeast identification from positive blood cultures including peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH®) and procedures based on matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry [9, 10]. As blood cultures cannot be considered as early diagnostic techniques, alternative procedures based on the detection and quantification of fungal biomarkers and metabolites have been developed to improve and anticipate the detection of invasive fungal infection (see below) [4, 7].

Samples from sterile sites (e.g., blood, CSF) have to be processed promptly to avoid multiplication of organisms. If not possible, storage at 4–5 °C is recommended. Biopsy and tissue specimens for culture should not be placed in histopathology fixatives and must be kept moist. Materials should be cultured on special media supplemented with antibiotics, preferably at 26 and 37 °C, over a prolonged period of time. The use of enriched media (e.g., Sabouraud with 2 % glucose) may be helpful for isolating fungi. Notably, negative culture results do not exclude invasive fungal infection.

All fungal isolates, which may have an important impact on the therapeutic approach, should be identified down to the species level [11].

The reference procedures for antifungal susceptibility testing of yeasts and moulds (e.g., the microdilution method) have been described by the Clinical Laboratory and Standards Institute in the USA and by the European Committee for Antimicrobial Susceptibility Testing in Europe [4, 12, 13].

2.3 Detection of Antigen and Antibody

Often, culture-based positive evidence for IFD is not available to the clinician. To improve the diagnostic accuracy, some surrogate markers such the detection of antigens and antibodies have been developed for the diagnosis of invasive fungal infections in patients at high risk for IFD over the last decades. Those non-culture based assays may assist the clinician to approve the diagnosis.

2.3.1 Galactomannan

Galactomannan (GM) is a polysaccharide cell-wall component that is released by most *Aspergillus* spp. during its hyphal growth. GM can be detected by an FDA-approved enzyme immunoassay that uses EB-A2 rat monoclonal antibodies (Platelia™ Aspergillus Enzyme Immunoassay, Bio-Rad). Most studies evaluated the usefulness of GM as screening test in adults, and results demonstrated a 90–100 % specificity and 80–100 % sensitivity of the assay in granulocytopenic patients [14–18]. In addition, its negative predictive value for excluding invasive aspergillosis is very high (>90 %). The value of the assay does not seem to be different in children compared to adults [19, 20]. As circulation of GM in serum is transient, testing should be carried out at least twice a week [4]. The GM assay may be positive before clinical suspicion of an infection and may also be useful in monitoring the therapeutic response [4]. Based on these studies, testing GM is included in a number of guidelines for the diagnosis and management of invasive aspergillosis in children and adults [4, 19–22]. GM positivity in serum, bronchoalveolar lavage (BAL) fluid and cerebrospinal fluid are included as a mycological criterion in the revised definitions of invasive fungal disease from the EORTC/MSG consensus group [23]. The manufacturer recommends a cutoff of 0.5 for the Platelia Aspergillus in serum, measured in two consecutive samples. A cutoff of ≥1.0 might be more appropriate for BAL [21].

It is important to consider a number of causes for false-positivity of the GM test, such as cross-reactivity with fungal species of other fungal genera such as *Alternaria*, *Paecilomyces*, *Penicillium*, or *Histoplasma*, cross-reactivity with transfused blood or antiglobulin sera and cyclophosphamide. Since batches of fungus-derived ß-lactam antibiotics (e.g., ampicillin, amoxicillin clavulanate, and piperacillin/tazobactam) might be GM-contaminated, collection of samples before administration of these antibiotics are recommended in patients receiving these drugs. In children in particular, milk-based diet and nutrient supplement containing soybean proteins may cause false-positive GM tests as well as the lipoglycan of *Bifidobacterium bifidum* in neonates. On the other hand, the rate of false-negative results may be higher in patients receiving antimould prophylaxis [24], and a recent analysis concluded that, due to the low pretest risk of invasive aspergillosis in the context of effective antimould prophylaxis, serum GM surveillance of asymptomatic patients is unreliable, as all results would be either negative or false positive [25].

2.3.2 1→3-ß-D-Glucan (BG)

With the exception of the *Zygomycetes* (*Mucor* spp. and *Rhizopus* spp.), which do not produce BG, this marker is a cell wall polysaccharide component of many pathogenic fungi such as *Aspergillus* spp., *Candida* spp., *Fusarium* spp., *Trichosporum* spp., *Saccharomyces* spp. or *Pneumocystis jirovecii*. In contrast, BG concentrations are usually low or absent in patients with cryptococcal infections since *Cryptococcus* spp. produce only low levels of BG.

Similar to GM, BG is included as mycological criterion in the revised definitions of invasive fungal disease by the EORTC/MSG consensus group [23]. A recent meta-analysis in adult patients reported that (1) similar performance was observed among the different BG assays (e.g., the Fungitell™ assay (Associates of Cap Cod, Inc Falmouth, MA), which is widely used in Europe and the USA and is approved by the FDA; Fungitec-G (Seikagaku)), (2) for the cutoff recommended by the manufacturer, two consecutive positive test results increase the diagnostic performance of the BG assay in proven or probable IFD, and (3) for two consecutive tests, sensitivity and specificity were 50 and 99 %, respectively, and estimated positive and negative predictive values for an IFD prevalence of 10 % were 84 and 95 %, respectively [26].

Unfortunately, occurrence of BG is not limited to fungi, but found also in bacteria, algae, and higher plants leading to false-positive results. Therefore, BG contamination from the environment is frequently observed and this marker can be detected during various bacterial infections (e.g., *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*) and, to a certain extent, in healthy individuals [27]. Importantly, BG does not differentiate between fungi (e.g., *Aspergillus* or *Candida*). In children, there are limited data on the assay, and the clinical value of the test is less clear. A recent prospective study on BG screening in children undergoing allogeneic hematopoietic stem cell transplantation reported on a limited usefulness of BG due to a very low positive predictive value (<15 %) [28]. It was speculated that this limitation is due to multiple factors which may lead to false-positive results of the BDG assay, such as bacteremia, severe mucositis, the administration of albumin and immunoglobulins, thrombocyte infusion with leukocyte-removing filters, or the administration of antibiotics such as amoxicillin-clavulanate or piperacillin-tazobactam [28]. In addition, transient candidemia has been reported as a cause of persistent false-positive BDG levels, and also mucosal colonization with *Candida* species may result in higher BDG level than the recommended cutoff. In addition, as immunocompetent uninfected children have a higher BG levels than adults, the optimal cutoff for BG testing in children is unknown [29], and current guidelines do not recommend the routine use of this assay in children [19, 20].

2.3.3 Cryptococcal Antigen

In immunocompromised patients, the detection of cryptococcal antigen in blood and/or CSF is highly indicative (>95 %) of cryptococcal meningitis [4]. The commercially available latex antigen

detection assays detect all cryptococcal serotypes (A, B, C, D, and AD hybrids), i.e., antigens of *Cryptococcus neoformans* and *C. gattii* will be found. It is recommended to test CSF and serum in parallel. Although most studies on diagnosing cryptococcal meningitis focus on AIDS patients and not on those with underlying malignancies, this rapid approach is most likely to be useful in both settings. Poorly encapsulated strains, prozone phenomenon and early infection may lead to false-negative results.

2.3.4 Antibody Detection

Although the detection of antibodies in the serum does not seem to play a major role in the diagnosis of most fungal infections in the immunocompromised patient [4], the combined detection of mannan, a major polysaccharide component of the *Candida* cell wall, and anti-mannan antibodies in the blood is considered to be a method for specific detection of *Candida* spp. in serum samples of both patients with hematologic malignancies and patients in the intensive care unit [7, 30]. There is a combination of tests commercially available [Platelia Candida Antigen Plus (Ag Plus™) and Antibody Plus (Ab Plus™; Bio-Rad Laboratories)]. In the setting of suspected candidemia and chronic disseminated candidiasis, the accuracy has been reported to be 50–70 %, but more importantly, due to a high negative predictive value (>85 %), the assay can be used to rule out infection. However, because the clearance of mannan from the blood is fast, several serum samples should be investigated.

2.4 Polymerase Chain Reaction

Molecular methods such as polymerase chain reaction (PCR) are promising diagnostic tools for IFD. However, sensitivity and specificity widely vary across the reports, and the method, which is not standardized to date, is not included as criterion in the revised definitions of IFD [23, 31]. In this regard, major methodological issues include optimal clinical sampling, an optimized DNA extraction method and an optimal PCR (in particular, primer target selection) design [32]. There are a various approaches such as the use of nested PCR, which detects genus-specific genomic sequences as well as single-copy genes or the use of panfungal PCR primers, which amplify multiple-copy genes identifiable in almost all fungal species. This latter method is followed by hybridization with species-specific probes or by sequencing (ribosomal ITS-1 and -2 regions).

Newer assays successfully employ real-time PCR technology (e.g., LightCycler™, TaqMan™), which potentially allow quantification of the fungal burden. The SeptiFast assay (Roche Diagnostics, Mannheim, Germany), a multiplex PCR designed for detection of most common pathogens causing sepsis covers also *A. fumigatus* and several common species of the *Candida* genus (*C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*). Also for *A. fumigatus*, the PCR approach has impact on the antimicrobial

treatment in febrile neutropenia as shown in a randomized controlled study [33].

When testing blood samples, sensitivity of Aspergillus PCR using plasma seems to be superior to that of serum [34]. A recent randomized study demonstrated that the combined use of GM and PCR to direct treatment in high-risk hematology patients resulted in reduced use of empirical antifungal treatment [35].

However, testing BAL samples seems to achieve higher sensitivity and specificity rates than testing blood samples [4]. A high negative predictive value in most studies makes invasive disease less likely in patients with repeatedly negative PCR results. In contrast, a positive PCR test may identify patients with breakthrough infections or those with an insufficient response to antifungal treatment, thus characterizing a patient population requiring intensification or modification of antifungal treatment. Again, recent data suggest that molecular diagnostic methods should be combined with other non-cultural tests such as GM [36, 37].

When evaluating lung biopsy specimens, PCR added to histopathology and culture may improve the detection and specification of pathogens [38, 39]. Recent studies suggest that molecular methods should be included as complementary tool in the diagnostic program for fresh tissue samples or cerebrospinal fluid obtained in patients with suspected infection [40]. Therefore, specialized laboratories should be contacted for these tests prior to invasive diagnostic procedures.

2.5 Imaging

2.5.1 Lungs

About half of neutropenic patients persistently febrile for more than 48 h of broad-spectrum antibiotic therapy show pathological findings in high-resolution CT scans [41]. Compared to conventional chest radiographs, CT scans reveals fungal pneumonia earlier, thus facilitating the early institution of antifungal therapy which is associated with a better outcome (Table 2).

Table 2
Recommendations for imaging diagnostic procedures (modified according to [21])

In febrile neutropenic patients with signs or symptoms of lower respiratory tract infection, computed tomography (CT) scan of the lungs is the diagnostic method of choice
Conventional chest radiographs are not recommended for the routine diagnosis of lung infiltrates in febrile neutropenic patients
In most cases, thoracic CT scan can be done without contrast media
Whereas CT findings such as consolidation, “halo sign,” and “air-crescent sign” are indicative for pulmonary aspergillosis in adults, radiographic findings in children with IFD are often unspecific
If infiltrates are detected on pulmonary CT scans, bronchoalveolar lavage should be considered at a segmental bronchus supplying an area of radiographic abnormalities
The role of routine sinus imaging scans in otherwise asymptomatic patients during prolonged febrile neutropenia is uncertain

CT findings such as consolidation, “halo sign,” and “air-crescent sign” may be important signs of filamentous fungal disease and are included in the revised definition of invasive fungal disease (Fig. 3a) [23, 42]. Whereas the “halo sign” is typically found in neutropenic patients, other CT findings indicative of pulmonary aspergillosis are comparable in neutropenic and in non-neutropenic patients [43]. Recently it has been demonstrated that a “reversed halo sign,” showing a focal rounded area of ground-glass opacity surrounded by a crescent or complete ring of consolidation, is relatively specific for fungal pneumonia due to mucorales [44]. Although the findings of pulmonary CT scans may be suggestive for invasive fungal disease, it is important to note that differential diagnoses such as pneumonia due to other microorganisms as well as lung involvement by underlying malignancies have always to be considered. In contrast, diffuse bilateral perihilar infiltrates, patchy areas of ground-glass attenuation, cysts and septal thickening, consolidation, and centrilobular nodules may indicate Pneumocystis pneumonia (PcP) [21]. Therefore, in addition to the institution of preemptive therapy, invasive diagnostic procedures should be pursued (Fig. 4).

In children, the limited data on CT findings of invasive pulmonary fungal disease suggest that radiographic findings are often unspecific (Table 2). In contrast to adults, typical signs of IFD (e.g., halo sign, air crescent sign, and cavities) are not seen in the majority of children, in particular in the younger age group (e.g., <5-year of age), whereas even atypical pulmonary infiltrates such as multiple nodules or fluffy masses and infiltrates which look like mass lesions may support the diagnosis of invasive pulmonary fungal disease [45].

As in patients with pulmonary aspergillosis the volume of pulmonary infiltrates may increase during the first week despite effective antifungal therapy [46], follow-up thoracic CT scans should not be performed earlier than 7 days after start of treatment, in particular when the neutrophil count increases. Reduction of the “halo” and the development of an “air-crescent” sign typically indicate favorable response [47].

2.5.2 Gastrointestinal Tract

Chronic disseminated candidiasis, such as hepatosplenic candidiasis, is the most common form of gastrointestinal involvement by invasive fungal disease. In these patients, lesions with hyperechogenic center and a hypoechogenic rim (“bull’s-eye sign,” size 5–20 mm) may be detected by ultrasound, CT, or magnetic resonance imaging (MRI) (Fig. 5) [4]. These lesions may persist for months after successful therapy, but MRI may be particularly helpful for monitoring the response to treatment since signal changes in T2- and T1-weighted postgadolinium images may suggest inactivity [4]. 2-[fluorine-18]-fluoro-2-deoxy-D-glucose positron emission

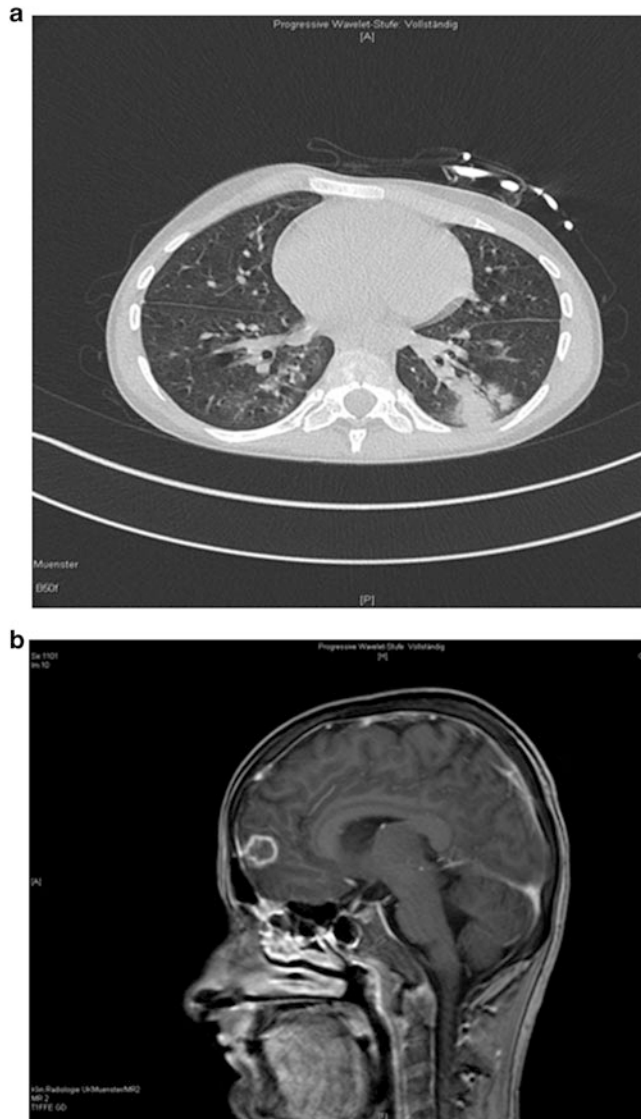

Fig. 3 Invasive pulmonary and cerebral aspergillosis with documentation of *A. fumigatus* in bronchoalveolar lavage fluid from a patient with chronic recurrent myeloid leukemia (CML), long-term treatment with glucocorticosteroids and intermittent granulocytopenia. (**a**) Pulmonary computerized tomography (CT) scan demonstrating multiple pulmonary nodules. The peripheral, rounded lesion has a small surrounding halo; note the anatomical relationship of the lesion to a smaller bronchus that was targeted for the diagnostic BAL procedure. (**b**) Cerebral magnetic resonance imaging (MRI; T1 sagittal after contrast) demonstrating a ring-enhancing lesion in the frontal region with meningeal reaction that is highly suggestive of a metastatic lesion from infected lung tissue, allowing for a diagnosis of probable cerebral aspergillosis

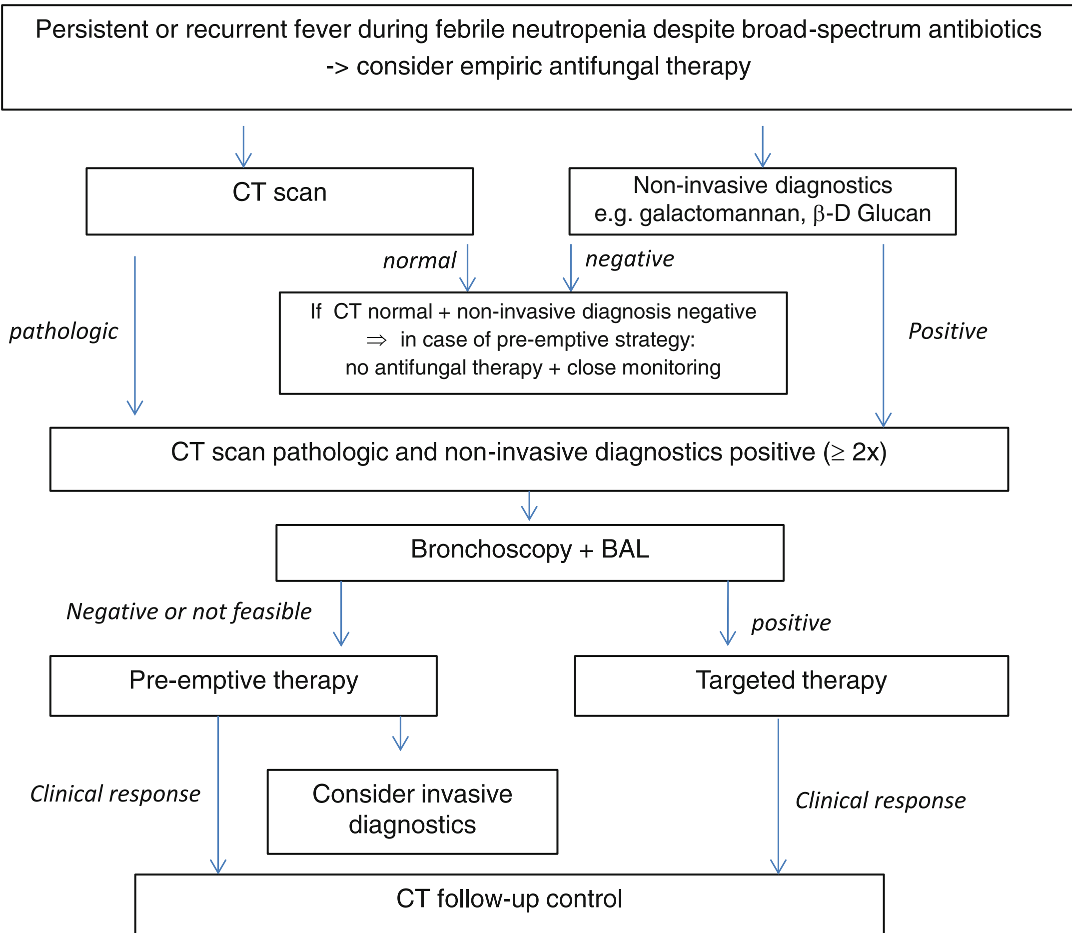

Fig. 4 Diagnostic procedures in neutropenic patients with persistent fever (modified according to [21]). If defervescence cannot be achieved by broad-spectrum antibacterial treatment within 48–72 h, empiric antifungal therapy should be initiated (if chosen as strategy) and diagnostic procedures should include pulmonary computerized tomography (CT) scan and non-culture based methods (e.g., galactomannan, ß-D-glucan). In case of pulmonary infiltrates, bronchoscopy with bronchoalveolar lavage (BAL) is recommended, and/or invasive diagnostics including biopsy should be considered

tomography (FDG-PET+/−CT) scans may be helpful in individual patients, but cannot discriminate between malignancy and infection [48]. CT and particularly MRI scanning may be helpful in all other fungal infections of the gastrointestinal tract.

2.5.3 Central Nervous System and Paranasal Sinuses

In patients with new neurological symptoms, such as seizures, hemiparesis, change in mental status, or persistent headache, imaging is always indicated. Whereas cerebral CT scan is rapidly being performed, MRI is preferred since it detects subtle pathological changes of the brain parenchyma and meninges (Fig. 3b) [4]. In invasive aspergillosis, complications such as aneurysm formation, hemorrhage, infarction, abscess formation, granulomas, meningitis, and cerebritis occur. Typical findings in MRI are (1) multiple lesions

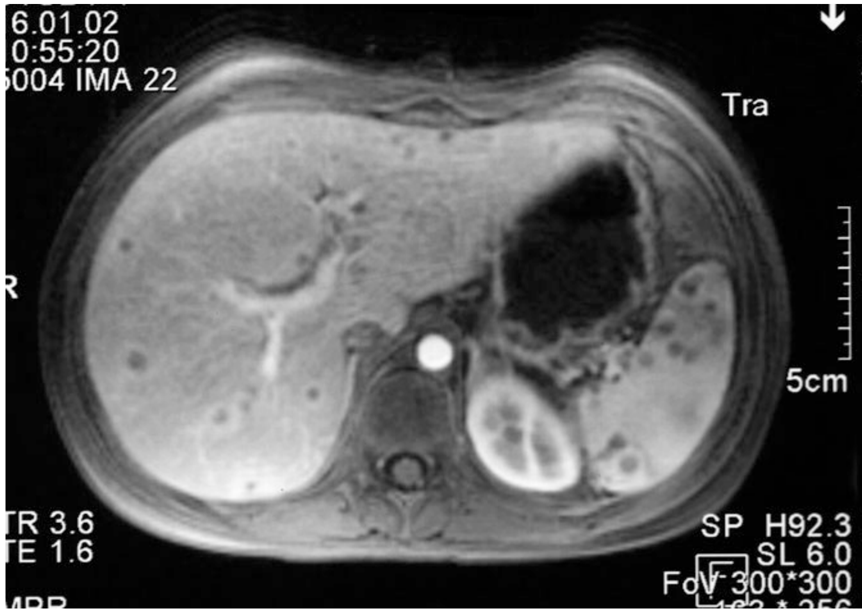

Fig. 5 Magnetic resonance imaging (MRI) of chronic disseminated candidiasis (CDC) in a 4-year-old girl with acute lymphoblastic leukemia (ALL) with multiple hypointense lesions in liver and spleen. The clinical hallmark of this condition is persistent fever despite granulocyte recovery in a patient with leukemia, right upper quadrant pain, and elevated liver function tests

in the cerebral hemispheres, basal ganglia, and cortico-medullary junction, (2) areas of petechial hemorrhage and paramagnetic substances, (3) ring enhancement, (4) grossly hemorrhagic lesions, and (5) early vascular enhancement [49]. Aspergilloma shows hypointense signal on T1-weighted image (WI), and mixed signal on T2WI [49]. If the rhinocerebral region is involved, arterial occlusion can rapidly result in tissue infarction. In order to explore the paranasal sinuses, CT has proven to be more reliable than MRI as it is superior in detecting bone destruction [50]. Hemorrhage and/or infarction (tissue necrosis) irrespective of the site of infection is highly suggestive of angiotropic fungal invasion. However, the role of routine sinus imaging scans in otherwise asymptomatic patients during prolonged FN is uncertain [19]. Notably, children younger than 2 years have not had sufficient pneumatization of all the sinus cavities and thus sinus imaging is rarely informative in this age range. Plain radiography of the paranasal sinuses is of limited value for diagnosing acute invasive fungal sinusitis because it may miss progressive lesions including bone erosion.

2.6 Invasive Diagnostic Procedures

2.6.1 Endoscopic Methods

Bronchoscopy with BAL may be useful for workup of pulmonary infiltrates, although studies indicate that the outcome of critically ill febrile cancer patients with severe pulmonary infiltrates has not been improved by these diagnostic procedures (Table 3) [51]. A recent study demonstrated a diagnostic yield of 30% in

Table 3
Recommendations for bronchoscopy and bronchoalveolar lavage (BAL) (modified according to [21])

Bronchoscopy and bronchoalveolar lavage should be carried out using a standardized protocol
Microbiological workup of BAL samples should follow a standardized protocol and include histological, microbiological and molecular workup
Bronchoscopy and BAL should only be carried out in patients without critical hypoxemia
Urgent need to start or modify antimicrobial therapy should not be postponed by bronchoscopy and BAL

Table 4
Clinical assessment of microbiological findings in febrile neutropenic patients with lung infiltrates (modified according to ref. [21])

The following findings may indicate pathogens causative for lung infiltrates: • Isolation of pneumococci, alpha-hemolytic streptococci, *Bacillus cereus*, or gram-negative aerobic pathogens from blood culture • Positive *Aspergillus* galactomannan in blood (threshold 0.5) • Positive *Legionella pneumophila* serogroup 1 antigen in urine • *P. jirovecii*, gram-negative aerobic pathogens, pneumococci, *Nocardia*, *M. tuberculosis*, or *Aspergillus spp*. or *Aspergillus* galactomannan (threshold 1.0) or Mucorales obtained from bronchoalveolar lavage or sputum samples; positive rapid culture for CMV, detection of CMV "immediate early antigen"
"Potentially relevant" findings include: • Common respiratory viruses, isolation of *Staphylococcus aureus*, *Legionella* spp. or atypical mycobacteria in respiratory secretions, positive CMV-or non-quantitative *Pneumocystis*-PCR (without conformation by other methods) from BAL
The following findings are unlikely to represent pathogens causative for lung infiltrates: • Isolation of enterococci from blood culture, swabs, sputum, or BAL • Coagulase-negative staphylococci or *Corynebacterium* spp. obtained from any sample • Isolation of *Candida* spp. from swabs, saliva, sputum, or tracheal aspirates • Findings from surveillance cultures, feces, and urine cultures

hematological patients, which was defined as identification of a true pathogen or an underlying malignancy [52]. However, this rate clearly depends on the risk profile of patients and the experience of the investigator. Although a "gold standard" is lacking, recovery of moulds from sputum in patients with prolonged neutropenia and clinical signs suggestive for invasive fungal disease should be regarded as a possible indicator of fungal pneumonia (Table 4) [4]. In contrast, yeast found in sputum or BAL fluid as well as in the upper and lower gastrointestinal tract should be regarded as contamination or colonization until invasive disease is proven (e.g., lung biopsy), since yeast belong to the physiological flora of the mucous membranes (Table 4). As detailed above, the highest sensitivity and specificity of diagnosing invasive pulmonary fungal infection are obtained by a combination of different culture- and non-culture based methods.

The performance of other endoscopic procedures should be considered according to the clinical symptoms, e.g., paranasal sinus endoscopy in patients with suspected invasive fungal sinusitis or esophago-gastro-duodenoscopy in patients with esophagitis not responding to preemptive antifungal therapy.

2.6.2 Organ Biopsy

Cultural isolation of fungi and histological proof from lung tissue are regarded as diagnostic "gold standard" for invasive pulmonary aspergillosis. Unfortunately, there are no quality standards for diagnostic procedures, and patient populations undergoing biopsy are highly selected. A recent meta-analysis analyzed the diagnostic yield of BAL and lung biopsy [53]. Although this was not a head-to-head comparison of the two methods, the authors reported that an infectious diagnosis was more common with BAL compared with lung biopsy, whereas lung biopsy more commonly led to a noninfectious diagnosis and was more likely to change how the patient was managed. Again, these patient populations are highly selected, and no prospective study to date has compared different methods for invasive approaches or invasive diagnostic to no diagnostic procedure. The results of the meta-analysis also showed that compared to BAL, complications significantly occur more often in lung biopsies, in which also the procedure-related mortality was fourfold higher, but the analysis did not include important and potentially confounding facts such as the location of the infiltrate and comorbidities. Transbronchial biopsy (often performed during the BAL procedure) is not recommended in severely thrombocytopenic patients with lung infiltrates [21]. Similarly, percutaneous biopsy, which may be guided by ultrasound or CT requires platelet counts >50,000/μL plus sufficient coagulation indices [54]. The latter approach should be limited to patients without an obvious risk of respiratory failure in case of complications such as a pneumothorax. When these diagnostic approaches are not feasible or do not yield a result and a specific diagnosis is important, open-lung biopsy, mini-thoracotomy, or video-assisted thoracoscopic surgery may be safely carried out in patients with treatment-refractory lung infiltrates. As described above, the diagnostic yield of invasive diagnostic procedures increases when a combined mycological, molecular and histopathological sample processing is performed (e.g., microscopy, culture, antigen testing, PCR etc).

Liver biopsies for presumed hepatosplenic candidiasis may be important to identify the causative pathogen in order to guide therapy. Earlier reports suggest that a liver biopsy during the first 3 weeks after recovery from granulocytopenia provides the highest diagnostic yield [55]. Again, combined culture-based and non-culture based methods should be performed of biopsy specimens, which should be diluted in isotonic saline for further workup.

3 Conclusions and Perspectives

In immunocompromised patients, IFD is often difficult to diagnose. Available diagnostic tools include the assessment of clinical signs and symptoms, mycological examination by microscopy and cultural as well as non-cultural techniques (e.g., antigen and antibody detection, molecular methods), and imaging procedures, which should be combined and performed according to current recommendations. The development of new diagnostic tools will hopefully improve the early and reliable diagnosis of IFD.

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Chapter 4

Commercial Molecular Tests for Fungal Diagnosis from a Practical Point of View

Michaela Lackner and Cornelia Lass-Flörl

Abstract

The increasing interest in molecular diagnostics is a result of tremendously improved knowledge on fungal infections in the past 20 years and the rapid development of new methods, in particular polymerase chain reaction. High expectations have been placed on molecular diagnostics, and the number of laboratories now using the relevant technology is rapidly increasing—resulting in an obvious need for standardization and definition of laboratory organization. In the past 10 years, multiple new molecular tools were marketed for the detection of DNA, antibodies, cell wall components, or other antigens. In contrast to classical culture methods, molecular methods do not detect a viable organisms, but only molecules which indicate its presence; this can be nucleic acids, cell components (antigens), or antibodies (Fig. 1). In this chapter, an overview is provided on commercially available detection tools, their strength and how to use them. A main focus is laid on providing tips and tricks that make daily life easier. We try to focus and mention methodical details which are not highlighted in the manufacturer's instructions of these test kits, but are based on our personal experience in the laboratory. Important to keep in mind is that molecular tools cannot replace culture, microscopy, or a critical view on patients' clinical history, signs, and symptoms, but provide a valuable add on tool. Diagnosis should not be based solely on a molecular test, but molecular tools might deliver an important piece of information that helps matching the diagnostic puzzle to a diagnosis, in particular as few tests are *in vitro* diagnostic tests (IVD) or only part of the whole test carries the IVD certificate (e.g., DNA extraction is often not included). Please be aware that the authors do not claim to provide a complete overview on all commercially available diagnostic assays being currently marketed for fungal detection, as those are subject to constant change. A main focus is put on commonly used panfungal assays and pathogen-specific assays, including *Aspergillus*-specific, *Candida*-specific, *Cryptococcus* specific, *Histoplasma*-specific, and *Pneumocystis*-specific assays. Assays are categorized according to their underlying principle in either antigen-detecting or antibody-detecting or DNA-detecting (Fig. 1). Other non-DNA-detecting nucleic acid methods such as FISH and PNA FISH are not summarized in this chapter and an overview on test performance, common false positives, and the clinical evaluation of commercial tests in studies is provided already in a previous book series by Javier Yugueros Marcos and David H. Pincus (Marcos and Pincus, Methods Mol Biol 968:25–54, 2013).

Key words Real-time PCR, Rt-PCR, Polymerase chain reaction, ELISA, Antigen test, Antibody test, *Candida*, *Aspergillus*, *Cryptococcus*, Fungi, *Pneumocystis*

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1 Basic Requirement Before Getting Started

1.1 Clinical Specimen Collection

Samples which should be analyzed with molecular tests need to be taken under sterile conditions, and any contamination during sampling should be avoided. The collection vessels often themselves represent a contamination risk, as shown in previous study; also blood collection vessels are often not free of fungal DNA [1]. Moreover, it is essential to transport the samples as fast as possible to the routine laboratory and chill them at 4 °C, as human DNAses and RNAses will degrade free pathogen-DNA, lowering the change for sufficient DNA quality for pathogen detection. This is of particular importance for the detection of fungal pathogens, which occur compared to viral and bacterial pathogens with relatively low cell loads [2]. Concerning blood, the operator needs to be aware that heparin-treated blood inhibits the PCR reaction and thick sputum needs to be pretreated with hyaluronidase [3]. Glassware should be avoided for sampling as (a) sterile glass equipment is not necessarily free of contaminating DNA, and (b) glassware is often a source of highly resistant RNA-degrading enzymes, a major source are the hands of the investigator. RNase can only be inactivated through high temperatures [4]. Instant inactivation of DNases and RNases is reliably achieved by chaotropic substances (especially guanidinium isothiocyanate, GITC) [5].

1.2 Quality Standards

Before getting started, it is important to first check the quality requirements for molecular diagnostics laboratories. These are highlighted for example in the Q4 document on "Good Laboratory Practice when Performing Molecular Amplification Assay" [5]. An overview on quality requirements from pre-analytic to test evaluation is provided by Neumaier et al. [3]. Fulfilling these basic quality requirements is essential to avoid amplicon contaminations [6] and cross-contaminations [7] or sample-to-sample contaminations [8, 9]. Avoiding amplicons contamination is one of the greatest challenges in molecular diagnostic laboratories, these are especially serious if the same DNA sequence is amplified repeatedly, or if series of amplifications are run, as given in seminested or nested PCRs. Nested PCRs are used to increase the specificity of amplification by using a second set of internal primers. In contrast in "one-step" PCRs have an excessive number of amplification cycles and therefore have a higher risk for generating nonspecific signals [3]. Independent of the amplification method, the possibility of spreading amplicons by aerosols is a general concern, and therefore, real-time PCR reactions are preferable used, as amplicons are not further processed. To avoid amplicon contaminations careful planning of preanalytical and analytical steps is essential. Quality requirements and precautions taken should always reflect the needs of the most critical assay. A well trained and instructed laboratory

staff that is aware of the consequences of inadequate performance and quality control is essential to guarantee quality of molecular analysis. Laboratory staff needs to be constantly trained to ensure that they fulfill the high skills needed for molecular analysis. A contamination of stock solutions, reagents, and machineries in molecular diagnostic laboratories is not just time consuming and difficult to localize, but also cost intensive as reagents need to be discarded or replaced. One of most important ways to avoid contaminations is a well-structured workflow (Figs. 1 and 2).

Structure your workflow as follows and physically separate Pre-PCR and Post-PCR areas [10]:

Pre-amplification area

- Master Mix room or reagent preparation clean room.

  In this room PCR reagents [11], primers, probes, and other essentials are stored and PCR master mixes are prepared, please note that no DNA extracts, biological material, or amplicons are allowed in this room [12]. Plates and tubes are filled with the master mixes [11].

- Nucleic acid extraction room [8, 9].

  In this room the nucleic acids are extracted from the clinical samples. Here internal controls and positive controls are

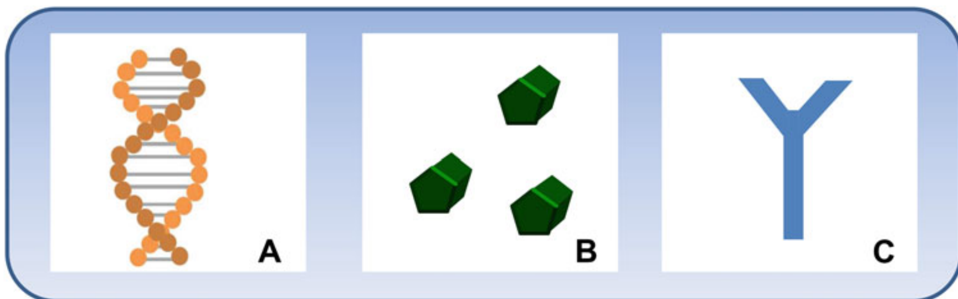

Fig. 1 Target structures detected in patients samples: DNA (**a**), antigen (**b**), and antibodies (**c**)

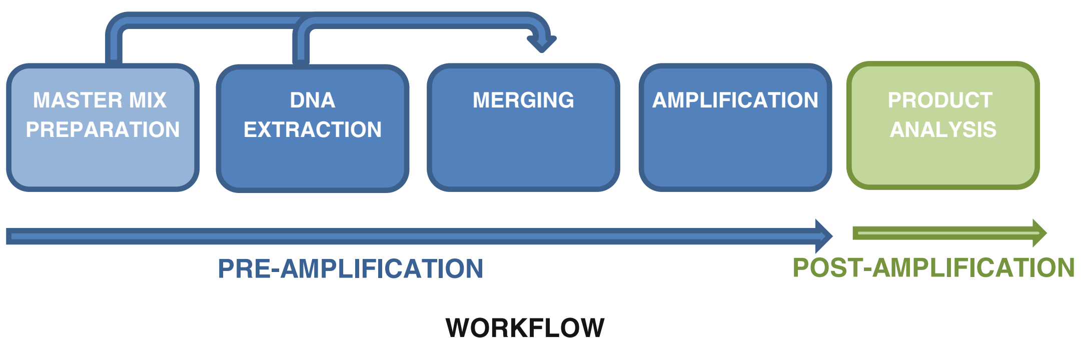

Fig. 2 Workflow of a molecular diagnostic laboratory

added to the master mix. Moreover, master mix and DNA extract are merged together.

– PCR cycler room or amplification room.

  This is the room where the cyclers are housed. The tubes are already locked and the plates sealed when they enter this area and these are exclusively opened in the post amplification area.

Post-amplification area

– Post-PCR room or product analysis room.

  This is the room with high loads of amplicons contaminations, the room should be frequently decontaminated with hypochlorine solution, UV-light and/or enzymatic reagents. All materials that entered this room do not leave the room or are not transferred to any other area. In this room, sequencing, agarose gels, and other assays are run.

In short, the following points should be kept in mind:

– Do not store amplicons longer than necessary.
– Gloves should be changed frequently.
– Use a different lab coat for each laboratory; do not carry on racks or potential contaminated materials from room to room.
– PCR reagents should be aliquot to avoid contamination of big stocks.
– Uncap and close tubes carefully to prevent aerosols.
– Decontaminate your working space with UV light [12] and/or hypochlorite solution [1].
– Use a "post-PCR sterilization" to decontaminate the post-PCR room photochemical by the combination of isopsoralen and long-wave UV light [11].
– Touch nothing with naked hands, as RNAse and DNAse cross-contaminations can lead to false negative results.

1.3 Research Use-Only Kit

On the packages and test inserts of almost all commercially available tests for fungal diagnostics the following terms can be found: "for research use-only," "only for research purposes," or "no diagnostic test." The validity of molecular tests in routine diagnostics for fungal pathogens is still under investigation and by far not as good validated as for viral tests. Currently all tests can only be used as add on tests not as stand-alone tests, and therefore, their results need to be seen with caution. Please carefully check what certificate is indicated on the test and whether the certificate covers the whole molecular procedure from DNA extraction to PCR result or only a part of the process. Many companies sell only the PCR assay alone, but not the kit that is necessary for DNA extraction from the clinical specimen, and therefore, only the PCR

is certificated. Do consider the certification status (CE, IVD, research only) of the products when making your choice. Important to note is that a negative result does not preclude a fungal infection.

1.4 Inhibition of PCR

Direct detection of pathogen DNA from clinical specimens is always challenging due to the imbalance of human and pathogen DNA. Therefore, choosing the right DNA extraction procedure is a key element for a successful PCR. Most companies recommend DNA extraction procedures in their manuals, but to not provide an IVD certificated DNA extraction in the frame of their own test kit. To overcome, PCR inhibition by an overload of human DNA and to avoid inhibition of the PCR reaction by inhibitory substances [13] in patient samples (e.g., heparin [14], cytostatic substances), samples are tested pure and as 1:3 dilution (sample diluted with ultrapure PCR grade water). A patient sample with particular high human DNA yields are biopsies. In general, attention should be paid to the recommended DNA extraction procedure in respect to their suitability for extracting fungal DNA from human specimens. A mechanical lysis step using glass beads (e.g., SeptiFast Lys Kit, M-Grad) [15] is needed for the disruption of ridged fungal cell walls, in particular when those are impeded in human tissues.

1.5 Not Included

Please be aware that only few kits are "all inclusive" kits. This means that usually tips, Pasteur pipettes, Falcon tubes, and other consumables are not included in delivery, so these need to be ordered separately. The user needs to make sure that additional material ordered fulfills the quality requirements of the assay, meaning that all materials should be free of DNAses, RNAses, free of DNA, free from PCR-inhibitors, and that all products used fulfill at minimum "PCR-grade." Free from DNA usually has different meanings and often is restricted to "free from human DNA" or "free from viral DNA." Providers usually do not guarantee that their products are free of microbial DNA. There are also several products on the market from Eppendorf®, Sarstedt, and others that have DNA/RNA low binding properties.

1.6 Improving Performance of ELISA Tests

The authors suggest to immediately aliquot serum/plasma samples for ELISA testing in Low Protein binding Micro Tubes (Sarstedt) or a similar product to avoid that antigen attached to the plastic surface. Aliquots are frozen at −20 °C until processing to avoid settling of antigen. In particular, Fungitell (Associated of Cape Code) is critical for contaminations, and therefore, it is very important to constantly clean lamina flow bench, to work in a room were no fungal pure cultures are handled, to frequently change gloves and to precisely pipette to avoid cross-contaminations between samples negative controls, standard curve wells, and positive controls.

2 Panfungal: Commercially Available Tests That Allow the Detection of Fungi in General

Currently there is no internationally agreed on standard for panfungal assessment of clinical specimens, most widely used in microbiological laboratories are (a) specific-PCRs for *Candida*, *Aspergillus, Cryptococcus and Pneumocystis*, (b) specific-antigen tests for *Candida*, *Aspergillus*, *Cryptococcus*, and *Histoplasma*, or (c) panfungal approaches such as beta-D-glucan test and DNA-sequencing of panfungal products.

2.1 DNA-Detecting

Important to note is that panfungal PCR is generally prone to contamination with fungal DNA [16], only samples revealing BLAST results matching to the overall picture and other test results should be regarded as positive.

Ingenetix offers a panfungal, real-time assay for the detection of a broad-range of fungal DNA. As most panfungal, in-house assays, this assay is based on the partial internal transcribed spacer region (ITS2) [17], one of the most commonly used and sequenced gene region for fungi. The assay is designed on LC™-FastStart DNA Master Kit Hybridization Probes. The product is validated and designed for the LightCycler® 2.0 (Roche) [18]. According to the manufacturer MycoReal Fungi has a LoD 95% (limit of detection) of 15CFU/PCR when used together with the LC™ FastStart DNA Master Kit Hybridization Probes (Roche), no cross-reactivity with human or bacterial DNA was observed. The results are blasted against an ITS database such as NCBI (www.ncbi.nlm.nih.gov) or the ISHAM-ITS database (http://its.mycologylab.org/) [14]. Qiagen and Renshaw Diagnostics (Table 1) offer not panfungal assays, but assays which are able to detect all clinical relevant *Aspergillus* and *Candida* species,

Table 1
Overview on DNA-, antibody-, and antigen-detecting methods for the detection of fungal biomarkers in clinical specimens

Method	Assay name	Company
DNA-detecting	Microbial DNA qPCR Assay for Pan *Aspergillus/Candida*	Qiagen
	MycoReal Fungi (panfungal PCR)	Ingenetix GmbH
	RenDx Fungiplex assay	Renshaw Diagnostics
Antigen-detecting	Fungitec G-Test MK	Seikagaku
	B-G Star kit	Maruha
	Fungitell	Associates of Cape Cod
	turbidimetric β-Glucan Test Wako	Wako Pure Chemical Industries

even though their pathogen spectrum is narrower than the one of MycoReal, great care is necessary to avoid contaminations, this point is discussed more detailed under the header DNA-detecting assays for *Aspergillus*. Using the UMD Universal (Molzym) kit the authors modified the procedure for extracting fungal pathogens from clinical specimens as follows. MolDNAse is not used when fungi are extracted from human tissue, as in case of mucormycetes [3], it might be that free fungal DNA is present rather than intact fungal cells in particular when tissue was homogenized. Instead of the standardized DNA Purification as indicated in the manual, the authors use as alternative procedure QIAquick PCR Purification Kit (Qiagen) and eluted DNA in 30 μL ultrapure PCR grade water instead of elution puffer. Also the second purification after Sequencing PCR is modified as follows: 10 μL eluate is used together with 45 μL SAM Solution and shaken for 30 min on a horizontal shaker

2.2 Antigen-Detecting

Similar to the panfungal PCR assay also panfungal antigen-detecting test are prone to contaminations, and therefore, the usage of β-glucan-free plastic material is essential for assay performance this includes also the usage of β-glucan-free filter tips [19]. In the clinical setting the following materials are known to cause false positives: cotton or rayon surgical swabs (persisting for a few days after surgery), blood transfusion, hemodialysis, albumin, and immunoglobulin infusions. In contrast to the panfungal PCR (including *Candida* spp., *Aspergillus* spp., *Fusarium* spp., *Trichosporon* spp., *Saccharomyces cerevisiae, Acremonium* spp., *Coccidioides immitis, Histoplasma capsulatum, Sporothrix schenckii*, and *Pneumocystis jirovecii)*, the β-glucan tests are panfungal with some important limitations, as *Cryptococcus* species and members of the Mucorales (former zygomycetes). In Europe, among the most frequently used β-glucan ELISA tests is Fungitell for patient serum. An overview on alternative glucan assay kits is given in Table 1. The dynamic range and positive cutoffs differ between kits. Detection of 1,3-β-D-glucan (glucan) in serum is useful in the diagnosis of *Pneumocystis jirovecii* pneumonia (sensitivity 96% and specificity 84% [4] and some invasive fungal infections (sensitivity 80% and specificity 82%) [4]. Glucan is a fungal cell wall component circulating in the blood of patients and is detected using assays which detect activation of factor G of the coagulation cascade of the remarkably ancient horseshoe crabs.

3 *Aspergillus*-Specific: Commercially Available Tests That Allow the Detection of *Aspergillus*

Aspergilli, in particular *A. fumigatus*, are the most frequently found mould species in invasive human infections. Due to its frequency, multiple molecular assays are also commercially available. An overview of commonly used systems (without the claim of

Table 2
Overview on DNA-, antibody-, and antigen-detecting assays for the detection of *Aspergillus* markers in patient samples

Method	Assay name	Company
DNA-detecting	Artus *Aspergillus* diff. RG PCR	Qiagen
	AsperGenius	Pathonostics
	Aspergillus Real-time PCR Panel	ViraCor-IBT Laboratories
	Aspergillus spp. Q-PCR Alert	Nanogen
	Aspergillus tracer	Cepheid
	Gb MICRO *Aspergillus*	Generi Biotech
	LightMIX® *Aspergillus fumigatus*	Roche
	MycoReal *Aspergillus*	Ingenetix GmbH
	MycAssay *Aspergillus*	Myconostica
Antigen-detecting	Lateral flow device (LFD) Platelia TM *Aspergillus* Ag	Chris Thornton of Exter University UK Bio-Rad
Antibody-detecting	*Aspergillus* IgG EIA Test	MiraVista
	Aspergillus fumigatus IgG ELISA	GENWAY Biotech Inc
	Aspergillus fumigatus Ab, IgG by ELISA SERION ELISA classic *Aspergillus* IgG/IgM/IgA	ARUP Laboratories Serion
	ImmunoCap *Aspergillus* IgG	Phadia

being complete) is provided in Table 2. A comprehensive review on the comparison of *Aspergillus* PCR versus antigen tests was recently published by White et al. 2015 [4]. They concluded that for the diagnosis of invasive aspergillosis galactomannan-EIA (sensitivity 81.6% and specificity 91.6%) and β-D-Glucan (76.9 and 89.4%) had similar sensitivity, specificity and comparable utility as *Aspergillus* PCR (sensitivity 76.8–88.0% and specificity 75.0–94.5%,). In contrast to Platelia TM *Aspergillus* Ag (Bio-Rad), *Aspergillus* PCR is not part of the EORTC/MSG definitions for invasive aspergillosis, but PCR was recently proposed by White et al. to be up taken as a criterion.

3.1 DNA-Detection

Everyone performing *Aspergillus*-specific PCRs need to be aware of the fact that this is an airborne fungus which is present in high frequencies in the environment. A current problem in molecular diagnostic of fungi is that currently plastic material supplying companies cannot guarantee that their products are free of fungal DNA, and therefore, DNA contaminations might also originated from used consumables [1, 16]. Positive *Aspergillus*-PCR results of

respiratory trace samples need to interpret with caution, as samples containing only resting stages (conidia) will yield also positive results, even though their presents do not indicate an infections. Also none of the commercial assays at hand can differentiate between inhalted *Aspergillus* conidia and the presence of *Aspergillus* hyphae. This problem is partially overcome by using *Aspergillus* antigen tests such as Platelia TM Aspergillus Ag (Bio-Rad) which is able to quantify the load of *Aspergillus* antigen and moreover is postulated to be formed mainly by actively growing hyphae. An overview on currently marketed test is given in Table 2.

3.2 Antigen-Detecting

Genus specific assays are less prone to contaminations than panfungal assays, but still have a rather high contamination risk, in particular for *Aspergillus* species, as those are airborne fungi and ubiquitously present in the environment. Therefore, especially weak or borderline positive results need to be interpreted with caution. The manufacturer of Platelia *Aspergillus* EIA (Bio-Rad) recommends trash hold values for certain patient groups and clinical specimens to avoid false positive results. A wide variety of interfering factors were reported [8, 19, 20] that are known to affect the performance of Platelia™ *Aspergillus* EIA among those are: host conditions, concomitant treatments (beta-lactam antibiotics [20]), presence of other fungal species, handling and processing of biological specimens (Table 3). Galactomannan is secreted by all pathogenic *Aspergillus* species [21], although some isolates are poor producers. One commercial ELISA format assay is available, namely Platelia™ *Aspergillus* EIA and one lateral flow device called *Aspergillus* LFD which was invented by Prof Chris Thornton of Exeter University, UK (Table 2). The major difference between the two systems is the ELISA delivers a quantification of antigen present in the clinical specimen, while the lateral flow device just differentiates between positive and negative results. In particular, for respiratory tract samples (BAL) higher trash hold values apply for the Platelia Aspergillus EIA Bio-Rad, these higher trash hold values cannot be set with the LFD, which might result in false positives. Both systems can be applied on serum samples and liquid respiratory tract samples such as bronchoalveolar lavages. The generally accepted cutoff in serum for positives is 0.5. No official cutoff has been defined for BAL fluids, but different authors have recommended cutoffs from 1.0 to 2.0. This variation partly reflects different patient populations and different dilution factors during bronchoalveolar lavage.

3.3 Antibody-Detecting

An overview on currently available antibody tests is provided in Table 2. Antibody tests are less prone to environmental contaminations as the antibodies and not the antigen itself is detected and those are not occurring in the environment. But, these tests cannot be used on immunocompromised patients as they might fail to produce

Table 3
Most common variables associated with false-positive and false-negative results for the Platelia™ *Aspergillus* EIA test (Bio-Rad)

Factor and/or situation that can lead to false-positive results	
Host related	Renal failure
	Mucositis
	Food intake of galactofuranose[a]
	Gut colonization and potential translocation of *Bifidobacterium*
	Gastrointestinal microflora of neonates
Iatrogenic	Blood derivatives
	Intravenous solutions containing gluconate
	Treatment with antibiotics derived from the fermentation of *Penicillium* species (e.g., piperacillin-tazobactam, amoxicillin-clavulanic acid)
	Use of cyclophosphamide in cancer patients
Sample collection and/or processing	Use of materials such as cotton swabs and cardboard
	Inappropriate cutoff value (too low)
Environmental	Presence of other non-*Aspergillus* fungi such as *Penicillium*, *Alternaria*, *Paecilomyces*, *Geotrichum*, *Histoplasma*, and even *C. neoformans*[b]
Food	Pasta and yoghurt
Factor and/or situation that can lead to false-negative results	
Host conditions	Chronic granulomatose disease
Iatrogenic	Treatment with antifungals
Sample collection and/or processing	Long-term storage of samples Inappropriate cutoff value (too high)

[a]Stabilizing agent commonly used in some cereal-derived products and some cream desserts
[b]Epitope contained within its galactoxylomannan has been shown to cross-react [8]

sufficient amounts of antibodies. Another advantage of the antibody tests compared with the antigen test is that these work with smaller sample volumes, as for example for the *Aspergillus* IgG EIA test from MiraVista a minimum quantity of 0.1 mL serum or plasma is sufficient. The underlying method of this test is a two phase indirect microplate immunoenzymatic assay. Comparable assays that also detect the presence of Anti-*Aspergillus* IgGs in serum or plasma are *Aspergillus fumigatus* IgG ELISA (GENWAY Biotech Inc) and *Aspergillus fumigatus* AB IgG by Elisa (ARUP Laboratories). Different to those is the ELISA from Serion which is called SERION ELISA classic *Aspergillus* IgG/IgM/IgA (Serion), which is able to detect in addition the presence of IgM and IgA antibodies. The additional markers provide the following information, IgM antibodies are

only formed when the body faces a new pathogen, and these molecules are formed by lymphocytes. IgA is mainly formed in external body fluids such as urine, milk, pleura fluid, making the test also applicable to other clinical specimens than serum or plasma. The antibody titers gained with the various tests and patient cohorts vary widely and need to be seen in reflection with the radiological findings and the clinical presentation of the patient. The best IgG assays have a 90–95 % sensitivity for chronic pulmonary aspergillosis and aspergilloma caused *by A. fumigatus*, much more sensitive than culture [4]. Detection of Aspergillus IgG antibodies is important in the diagnosis of chronic pulmonary aspergillosis and allergic bronchopulmonary aspergillosis. The performance of two commercially available enzyme immunoassays (EIA) namely Phadia ImmunoCap Aspergillus IgG and Bio-Rad Platelia Aspergillus IgG was evaluated recently [6]. Comparing counterimmunoelectrophoresis with ImmunoCap and Platelia Aspergillus IgG, both EIA assay yield good sensitivities of 97 and 93 %, respectively. The level of agreement between the two EIAs for positive results was good, but the concentration of antibodies was not correlated between the tests.

4 *Candida*-Specific: Commercially Available Tests That Allow the Detection of *Candida*

The gold standard tests to diagnose invasive *Candida* infections are based on blood culture and cultures from sterile body sites, as well as histology from biopsies.

4.1 DNA-Detecting

Candida species are among the main agents causing sepsis and blood stream infections in immunocompromised hosts; moreover they cause infections of prosthesis, the upper respiratory tract and the urine tract. Therefore, for this important pathogen multiple real-time PCR assays and antigen assays are commercially available. The contamination risk for samples through the environment is limited, as *Candida* species are yeasts that are distributed via smear contact rather than aerogenously. Still attention needs to be paid to positive PCR results gained from lower respiratory tract samples as those are in most cases caused by sampling procedure rather than being true positives. *Candida* species are also occurring on the skin and hands of healthcare workers, but disinfection before blood sampling avoids contamination of specimens. For the detection of *Candida* species different systems are available either systems that detect only *Candida* species, or assays that detect *Candida* species among other agents of sepsis. One commonly used test is SeptiFast (Roche). When applied for fungal specimens a mechanical lysis step is added [15]. DNA extraction is automatic with MagNA Pure Compact (Roche) using MagNA Pure Compact Nucleic Acid Kit (Roche).

4.2 Antigen-Detecting

Similar to the *Aspergillus* antigen, also a *Candida*-specific mannan exists which is found in the bloodstream of patients suffering from candidemia or *Candida* sepsis circulating in the blood of many patients with candidemia. The most commonly used antigen test is Platelia™ Candida (Bio-Rad). In invasive candidiasis, for patients suffering from candidemia mannan sensitivity was 58 % and specificity 93 % (Bio-Rad), resulting in a diagnostic odds ratio (DOR) of 18. Concurrent detection of anti-mannan antibodies (see next header) increased the performance of the test to a sensitivity of 83 % and a specificity of 86 % and DOR of 58 [4]. Recently the, Serion *Candida* mannan kit was evaluated for the diagnosis of ICI in a clinical study [10]. The test performed moderately with a specificity of 51 % and sensitivity of 77 %.

4.3 Antibody-Detecting

Like all antigens originating from fungal cell walls, also mannan is immunogenic and antibodies are formed over time. Several test kits are available with differing performance. These anti-mannan tests should be used in addition to the Platelia™ Candida test to improve sensitivity and specificity. While as single tests anti-manna tests are of limited values as for example sensitivity and specificity of the Cand-Tec kit (Ramco) were only 52.6 and 50.5 % respectively in patients with candidemia [4]. The sensitivity and specificity of the Cica Fungi Test (Kanto Chemical) were 63.2 and 95.5 % respectively in patients with candidemia (Table 4) [4].

Table 4
Overview on DNA-, antibody-, and antigen-detecting assays for the detection of *Candida* markers in patient samples

Method	Assay name	Company
DNA-detecting	SeptiFast[a]	Roche
	SepsiTestTM CE IVD[a]	Molzym
	Vyoo®[a]	Analytikjena
	Candida albicans genesig® Advanced Kit *Candida* DNA, Qualitative real-time PCR, serum *Candida* real-time PCR Panel HumPCR™ *CANDIDA* Detection Kit LightMIX Kit Candida albicans MycoReal Candida UMD Universal	Genesig Quest Diagnostics™ Viracore IBT™ BioinGentech Human PCR Kits Roche Ingenetix Molyzym
Antigen-detecting	*Platelia™ Candida*	Bio-Rad
Antibody-detecting	*Cand-Tec*	Ramco
	Cica Fungi	Kanto Chemical

[a]Sepsis diagnostic kits

5 *Cryptococcus*-Specific: Commercially Available Tests That Allow the Detection of *Cryptococcus*

Cryptococcus neoformans neither is present in high quantities in the air nor is it a human colonizer, and therefore, contaminations during sampling or from the environment can be widely excluded. Infection with *C. neoformans* is known as cryptococcosis and is the cause of the most common life-threatening meningitis in patients with weakened immune systems, particularly in advanced HIV/AIDS and patients with T cell deficiencies. *Cryptococcus neoformans* is commonly found in soil throughout the world. Human infection of *C. neoformans* occurs via inhalation of aerosolized spores. From the lungs, *C. neoformans* is spread hematogenously to the central nervous system (CNS), resulting in meningoencephalitis. *Cryptococcus* antigen tests combined with fungal positive cultures form the gold standard in diagnosing cryptococcus meningitis and pneumonia, while PCR is less important.

5.1 DNA-Detecting

Specific-PCR assays exist for the detection of *Cryptococcus neoformans* from clinical specimens, in particular for the detection of *Cryptococcus* in human liquor. AmpliSens® *Cryptococcus neoformans*-FRT PCR kit is diagnostic assay that is suitable for the qualitative detection of *Cryptococcus neoformans* DNA in the clinical specimens such as cerebrospinal fluid, bronchoalveolar lavage, sputum, blood, skin lesions aspirate, viscera biopsy, and autopsy materially using real-time PCR.

5.2 Antigen-Detecting

Cryptococcus antigen tests detect glucuronoxylomannan, a long branched polysaccharide found in the capsular of *Cryptococcus* species. The antigen is released into body fluids (liquor, serum) by all common pathogenic species. A great variety of antigen tests exist for *Cryptococcus*, due to its former relevance in AIDS patients (Table 5). The most widely used test is the Cryptococcal Antigen Latex Agglutination System (CALAS®) (Meridian Bioscience) in which serum or CSF can be tested. The prozone phenomenon was described with the Cryptococcal Antigen Latex Agglutination System latex in testing serum and so the serum is first boiled and pronase treated, in most kits, to abolish this problem, as well as releasing any bound antigen. Recently a new dipstick point-of-care test IMMY CrAg® LFA (Cryptococcal Antigen Lateral Flow Assay) was introduced, for this test the serum treatment step is unnecessary and serum, urine, and respiratory tract samples can be tested. This new lateral flow assay delivers an analytical sensitivity that is up to 200× more sensitive than other commercial assays. The major advantage from a practical point of view is its easy and rapid performance.

Table 5
Overview on DNA- and antigen-detecting assays for the detection *of Cryptococcus markers* in patient samples

Method	Assay name	Company
DNA-detecting	AmpliSens® Cryptococcus neoformans-FRT	Ecoli
	Cryptococcus neoformans genesig® Standard Kit	Genesig®
	Cryptococcus neoformans PCR Kit	NORGEN
Antigen-detecting	Cryptococcal Antigen Latex Agglutination System (CALAS®)	Meridian Bioscience
	RemelTM Cryptococcus Antigen	Test Kit Thermo Scientific
	Crypto-LA®	Wampole Laboratories
	Eiken Latex test	Eiken
	Latex-Cryptococcus Antigen Test	IMMY
	CrAg® LFA	IMMY
	Pastorex Crypto Plus	Bio-Rad
	Premier™ Cryptococcal Antigen	Meridian

A recent study [21] including 207 patients, compared Latex-Cryptococcus Antigen Test (IMMY) tests performed on fingerstick whole blood, with CSF and plasma/serum, showed 100 % agreement of CrAg® LFA performed on fingerstick blood with other body fluids—suggesting that fingerstick CrAg® LFA is a reliable bedside diagnostic test. In HIV/AIDS, the cryptococcal antigen is positive in CSF in >99 % of cases of cryptococcal meningitis and almost all of these patients also have detectable serum antigen positives. Using the new dipstick antigen test, almost all are also positive in urine, although the amount of antigen detectable is 20-fold lower. Serum and possibly urine screening may identify *Cryptococcus* in an early disease stage and may improve clinical outcome. Approximately 30 % of immunocompromised patients (with the exception of AIDS/HIV) suffering from cryptococcal meningitis have negative Latex-*Cryptococcus* Antigen Test results, also the CSF titers tend to be lower than those of HIV/AIDS patients. In immunocompetent patients, with cryptococcal disease (pneumonia or meningitis) the serum Latex-*Cryptococcus* Antigen Test is often negative, but CSF or BAL are yielding positive results. The Latex-*Cryptococcus* Antigen Test titer can be used for therapy response in CSF. But in serum titers are of limited value, as the fall slowly and vary between tests.

6 *Histoplasma*-Specific: Commercially Available Tests That Allow the Detection of *Histoplasma*

6.1 DNA-Detecting

As *Histoplasma capsulatum* is a relatively slow growing fungus (approximately 10 days), early diagnosis of disseminated infection required visualization of the organism in blood smears, bone marrow, lymph node, skin, or lung biopsies. Quest Diagnostics offers a real-time PCR assay that functions on whole blood, BAL, CSF, urine, and even tissue. Antigen tests are more commonly used for the detection of *Histoplasma capsulatum* than PCR assays.

6.2 Antigen-Detecting

Most *Histoplasma* detection test use urine as specimen, although some tests can also be applied on serum or BAL. The ALPHA Histoplasma Antigen EIA is an immunoenzymatic, sandwich microplate assay using rabbit anti-Histoplasma IgG antibodies for capturing histoplasma antigen and biotinylated rabbit anti-Histoplasma IgG antibodies as detect antibodies. This test has been approved for diagnostic use by the US Food and Drug Administration in urine specimens only. A reference EIA unit value of less than 2.0 has been established for urine. Clinical studies performed in other laboratories support use of the same reference interval in serum samples for *Histoplasma* antigen EIA assays. A negative result does not preclude diagnosis of histoplasmosis.

The commercially available test Histoplasma Antigen from (IMMY) has a sensitivity of 81% and a specificity of 99% (Immy). Similar cross activities were found as with Alpha Histoplasma AG EIA. Other tests marketed are shown in Table 6. Patients with severe disease and in particular, immunocompromised patients are more likely positive, while patients with acute, subacute, and chronic pulmonary histoplasmosis have lower frequencies of positivity.

6.3 Antibody-Detecting

Few antibody kits are available for detecting anti-*Histoplasma* antibodies. Antibody detection is useful for the chronic forms of histoplasmosis, notably chronic pulmonary histoplasmosis and some

Table 6
Overview on DNA-detecting and antigen-detecting assays for the detection of *Histoplasma* markers in patient samples

Method	Assay name	Company
DNA-detecting	*Histoplasma capsulatum* DNA, Real-Time PCR	Quest Diagnostics™
Antigen-detecting	ALPHA *Histoplasma* Antigen EIA	Viracor IBT™
	Histoplasma Antigen by EIA, Serum	ARUP® Laboratories
	Histoplasma Antigen	IMMY
	Histoplasma Quantitative EIA TEST	MiraVista Diagnostics

patients with progressive (subacute) disseminated histoplasmosis. Seroconversion is often documented after acute pulmonary histoplasmosis. Cross-reaction is common with sera from patients with blastomycosis, penicilliosis, and paracoccidioidomycosis and so the test cannot be used to differentiate these infections.

7 *Pneumocystis jirovecii*-Specific: Commercially Available Tests That Allow the Detection of *Pneumocystis jirovecii*

Pneumocystis pneumonia (PCP) is a serious infection that causes inflammation and fluid buildup in the lungs and is commonly caused by a fungus called *Pneumocystis jirovecii*. *Pneumocystis jirovecii is* a fungus which can also occur in the lungs of healthy humans and the presence of *Pneumocystis* DNA does not always indicate a *Pneumocystis* infection, and therefore, positive results need to be interpreted with caution [18]. Recently a review on the role of beta-D-glucan detection and PCR for the diagnosis of PCP was published by Alanio et al. [2]. Commonly PCP is diagnostic by microscopy of sputum and bronchio-alveolar lavage or by immunofluorescent or histochemical staining, but for non-HIV immunocompromised patients fungal load in lower respiratory tract is low, and therefore, diagnostic sensitivity is lacking for those patients. Particularly for these cohorts specific PCRs and beta-glucan testing was induced. Molecular tests have very high negative predictive values and can help precluding a PCP. Quantitative PCR (qPCR) is current gold standard for PCP, but as currently no consensus for the threshold levels has been set a combined strategy of qPCR and beta-D-glucan testing is most promising. An overview on commercially available molecular *Pneumocystis* diagnosis kits is given in Table 7.

7.1 DNA-Detecting

A quantitative PCR assay should be chosen to avoid false positive results and to be able to set a threshold depending on investigated patient group and underlying disease [16]. Such a quantitative assay is PCR *Pneumocystis jirovecii* (Bio-Evolution), a cutoff value of 2.8×10^5 copies/mL was defined resulting in 72% and 82% of sensitivity and specificity, respectively [16]. Another commercial assay that was clinically evaluated is MycAssay *Pneumocystis*, the assay targets the mitochondrial large subunit (analytical detection limit, ≤3.5 copies/μL of sample). Thirteen of 14 subjects with PCP and 9/96 patients without PCP had positive PCR results; resulting in a sensitivity of 93%, specificity of 91%, positive predictive value of 59%, and negative predictive value of 99%. Of the 9 PCR-positive subjects without PCP, 1 later developed PCP. The PCR diagnostic assay compares well with clinical diagnosis using non-molecular methods [13]. An overview on commercial PCR tests is provided in Table 7.

Table 7
Overview on DNA-detecting and antigen-detecting assays for the detection of *Pneumocystis* markers in patient samples

***Pneumocystis* markers**		
DNA-detecting	AmpliSens® *Pneumocystis jirovecii (carinii)*-FRT	InterLabService/Amplisense®
	LightMix® Kit Pneumocystis jiroveci	Roche
	MycAssay Pneumocystis	Myconostica
	PCR *Pneumocystis* jirovecii	Bio-Evolution
	Pneumocystis	Ingenetics
	Pneumocystis carinii Real Time PCR Kit	Mobitec
	Pneumocystis jirovecii FTD	Fast-Track Diagnostics
	Progenie kits	Progenie Molecular
	RIDA-GENE Pneumocystis jirovecii	R-Biopharma AG

7.2 Antigen-Detecting

See panfungal antigen-detecting tests and Table 1.

7.3 Antibody-Detecting

Pneumocystis jirovecii is an intracellular pathogen; therefore, no antibodies are formed against this pathogen.

8 Notes

1. Split the sample for in two portions one for regular routine tests (such as microscopy, culturing) and molecular tests and chill the portion for molecular tests at 4 °C. Concerning biopsies do not homogenize tissue samples. Correspond with your plastic supplier and ask for DNA-free, DNase-free, and RNase plastic materials which are produced under clean room conditions, to keep contaminations from plastic material at a minimum. Buy tubes and PCR plates that are individually sealed or packed in small quantities. The smaller the package size the smaller the risk of environmental contaminations.
2. Baking at 250 °C for 4 h or more [9].
3. GITC has been increasingly used in concentrations of 4 mol/L. Organic solvents, e.g., phenol, may be added in parallel. Extraction systems based on these additives are now commercially available, e.g., RNAzol, TRIzol. The appropriate concentration leading to an irreversible denaturation of RNases has recently been determined to be 5 mol/L. Use of GITC concentrations, 4 mol/L, leads to very rapid RNA degradation [17].

4. Amplicon contaminations are a nightmare for very molecular diagnostic lab, as they generate false positives and are very difficult to get shot of. PCR reactions to have the ability to produce large copy numbers of target sequences, in sum these are called amplicons. As PCR is highly sensitive only small "carry-over" contaminations of previous amplifications can cause false positive reactions, and therefore, extreme care needs to be taken to avoid those. Avoid "amplicons contaminations" by using as many real-time PCRs as possible were you do not need to open the reaction tube with the amplicons for further processing. The greatest threat of contamination lies in assays that necessitates manipulation of the amplified product or cloned DNA, such as sequencing and gel electrophoresis.
5. One origin of contamination is cross-contamination with unamplified DNA during the simultaneous preparation of many specimens. Most contaminations, however, will consist of the specific PCR fragments generated during previous amplifications.
6. Sample to sample contaminations are relatively rare in routine laboratories, when people are trained in pipetting aerosol free. Essential for avoiding cross-contamination from sample to sample are tips in sufficient length for blood collection tubes and other tubes with large volumes (for example SafeSeal-Tips Professional Line from Biozym Diagnostik GmbH or Biosphere® Filter Tips). What the authors found very useful are single use sleeve guards, these avoid contamination of the laboratory coat and they can be decontaminated with hypochlorite solution (0.1% HCl) and disinfectants, so that all materials placed under the lamina flow including works arms are disinfected. The latter are worn in the extraction room and in the Mastermix preparation room. Moreover nitrile gloves should be decontaminated with chlorine and disinfectants before entering the lamina flow. Change gloves when materials are touched outside the lamina air flow.
7. Use exclusively filter tips to avoid contamination of the pipette. Decontaminate your pipettes on a regular basis appropriate to the number of samples handled in your laboratory. Keep in mind that decontamination of surfaces with ethanol is not sufficient to remove DNA, DNAses, RNA, and RNAses, but hypochlorite solution (0.1% HCl) or enzymatic anti-contamination solutions.
8. Separate sets of laboratory equipment, consumables, and laboratory coats are required for the separated areas. Workflow needs to run from amplicon free areas to amplicons contaminated areas. Use different colored rack and mark laboratory coats and racks with "PRE" and "POST" to avoid the transportation of material between the rooms.

9. Primers, probes, ultrapure water should be aliquoted to avoid contamination of large stocks.
10. As sensitive and expensive materials such as primers and probes are stored in this room; we recommend from or own experience to provide laboratory coats exclusively for this room to avoid contaminations with biological and/or nucleic acids.
11. Master mixes should be chilled during pipetting and also plates should be chilled. Real time master mixes that contain fluorophores should not be exposed to light. The ITS region represents the internationally agreed on primary barcode for fungi, first of all because it has a very high amplification efficiency and second of all because large databases are available for a large variety of fungi [14].
12. 100 mL/L sodium hypochlorite.
13. PCR is a biological reaction based on Taq-polymerases that is susceptible to inhibitory substances. In the past years multiple modified and engineered DNA polymerases were marketed which are claimed to be more robust and less susceptible for inhibition. The authors found that this claim is valid for majority of modified DNA polymerases and are better in performance.
14. Heparin inhibits amplification in concentrations as low as 0.05 IU per reaction volume (routinely 14.3 IU/mL of whole blood) [7].
15. Samples are frozen at −20 °C, then samples are disrupted using Disrupter Genie (Scientific Industries) for 35 s at 7000 rpm, then samples are disrupted using Disrupter Genie (Scientific Industries) for 35 s at 7000 rpm, and chilled down by transferring the sample again for 1 min −20 °C. The chilling steps were introduced to avoid degradation of DNA by human DNAses.
16. These contaminations with fungal DNA can originate from blood collection tubes, other plastic materials used in the clinics or in the routine diagnostic lab, from the environment (air), or reagents used in the laboratory.
17. Important to notice is that the kit is licensed only for research use and not for diagnostics, even though the state that fungal DNA can be recovered from DNA samples purified from bronchioalveolar lavages (BAL), blood, aspirates, cerebrospinal fluid, tissue, and paraffin-embedded tissue. The manufacturers recommend combining their assay with a modified protocol of the High Pure PCR Template Preparation Kit (Roche Diagnostics).
18. Mucormycetes are filamentous fungi, which are sparsely septated, and therefore, when sample is cut, cytoplasma and cell cores easily leak out of the fungal cell and distribute in human tissue.

19. Please be aware that not all plastic material necessary for assay performance is included the scope of supply, please make sure that additional plastic material used is free of 1,3-β-D-glucan.
20. Piperacillin/tazobactam (Tazocin) and amoxicillin/sulbactam (Augmentin), but also some cephalosporins and meropenem
21. First of all it needs to be mentioned that GM is not only produced by human-pathogenic *Aspergillus* species, but also by environmental isolates. Moreover, *Penicillium* spp., and *Histoplasma capsulatum* are known to produce a similar molecule that can result in false positive results.
22. It is important to check the viscosity of the respiratory tract sample before you apply it on the sample area of the lateral flow device, as to viscous samples fail to run along the lateral flow device.
23. Pretreatment of BAL fluid with Sputasol (dithiothreitol) greatly reduces galactomannan levels.
24. Endoscopes are often contaminated during samples with microbiota of the upper respiratory tract.
25. *Cryptococcus neoformans* is actually not a single species but a species complex with at least three variants that can be found in geographically varied locations. Variant grubii and neoformans can be found globally whilst variant gattii is found mostly in the tropics, but has also on the south-eastern coast of Canada.
26. The hook effect or the prozone effect is a false negative result with certain immunoassays due to very high concentrations of a particular analyte. The hook effect mostly affects one-step immunometric assays.
27. The Alpha Histoplasma AG EIA was found to cross-react with *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Paracoccidioides brasiliensis* in urine and serum. Although not tested in the Alpha *Histoplasma* AG EIA, *Penicillium marneffei* is known to cross-react with *Histoplasma* antibodies. The assay was not cross-reactive with *Candida albicans*, *Cryptococcus neoformans* or *Aspergillus* spp. in urine or serum.
28. Collect 2 mL sample in a sterile urinalysis container, then transfer to sterile, screw top tube for shipment.

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Chapter 5

Systemic Antifungal Agents: Current Status and Projected Future Developments

Seyedmojtaba Seyedmousavi, Haleh Rafati, Macit Ilkit, Ali Tolooe, Mohammad T. Hedayati, and Paul Verweij

Abstract

By definition, an antifungal agent is a drug that selectively destroys fungal pathogens with minimal side effects to the host. Despite an increase in the prevalence of fungal infections particularly in immunocompromised patients, only a few classes of antifungal drugs are available for therapy, and they exhibit limited efficacy in the treatment of life-threatening infections. These drugs include polyenes, azoles, echinocandins, and nucleoside analogs. This chapter focuses on the currently available classes and representatives of systemic antifungal drugs in clinical use. We further discuss the unmet clinical needs in the antifungal research field; efforts in reformulation of available drugs such as Amphotericin B nanoparticles for oral drug delivery; development of new agents of known antifungal drug classes, such as albaconazole, SCY-078, and biafungin; and new drugs with novel targets for treatment of invasive fungal infections, including nikkomycin Z, sordarin derivatives, VT-1161 and VT-1129, F901318, VL-2397, and T-2307.

Key words Antifungal agents, Systemic fungal infections, Amphotericin B nanoparticles, Albaconazole, SCY-078, Biafungin-Nikkomycin Z, Sordarins, VT-1161, VT-1129, F901318, VL-2397, T-2307

1 Introduction

Fungal infections are currently among the most difficult diseases to manage in humans [1]. Furthermore, invasive and life-threatening fungal infections are an important cause of morbidity and mortality in patients who are immunocompromised, hospitalized with severe underlying diseases (e.g., acute myelogenous leukemia), require complex surgical procedures (e.g., for trauma), and need support in intensive care units [2–5]. Overall, more than 300 million people are believed to suffer from a serious fungal infection, resulting in approximately 1.4 million deaths annually [6] with most infections being caused by *Candida* and *Aspergillus* species (spp.). The remaining infections are largely due to a variety of fungal pathogens including *Cryptococcus* species, *Pneumocystis jiroveci*, *Fusarium* spp., *Scedosporium* spp., *Penicillium* spp., and numerous

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others [6–9]. The data notwithstanding, it must be recognized that invasive fungal infections (IFIs) only represent part of the magnitude of fungal diseases [10]. The incidence of superficial and cutaneous fungal infections of the hair, skin, and nails, such as dermatophytosis, vaginal candidiasis, allergy, and mycotoxicosis, considerably exceeds that of IFIs. However, the contribution of these diseases to morbidity worldwide is unrecognized [11].

Despite the increase in fungal infections over the past decades, there are very few classes of antifungal drugs available for therapy, which have limited efficacy, particularly in the treatment of IFIs. These drugs include polyenes, azoles, echinocandins and nucleoside analogs. Thus, this chapter focuses on the currently available classes and representatives of systemic antifungal drugs in clinical use, as well as unmet clinical needs in the antifungal research field, efforts in reformulation of available drugs, development of new agents of known antifungal drug and new drugs with novel targets for treatment of invasive fungal infections.

2 Systemic Antifungal Drugs in Current Use

Antifungal therapy is a central component of patient management for IFIs. Depending on the strategy chosen, different drugs can be used [12, 13]. It is noteworthy that treatment options for IFIs are highly limited because very few options are available in the existing antifungal chemical drug classes [14]. As shown in Fig. 1, systemic antifungal agents can be grouped into four classes based on their site of action in pathogenic fungi, and include the polyenes, azoles, echinocandins, and nucleoside analogs [15, 16].

Table 1 shows the recommended label indications of systemic antifungal drugs currently used in the USA and Europe. In addition, a wide variety of topical agents belonging to different classes of antifungals are available as creams, ointments, gels, lotions, powders, shampoos, and other formulations. Topical antifungals can be applied to the skin, nails, or mucous membranes, or could be used vaginally to kill or inactivate fungi. Regardless of the actual mechanism of action of the drug or the viscosity, hydrophobicity, and acidity of the formulation, the drug's ability to penetrate or permeate deeper skin layers is an important property impacting the therapeutic efficacy of topical antifungals [17–19]. Systemic antifungal agents available for routine clinical use are classified in the following sections.

2.1 Polyenes

Polyenes constitute the oldest class of systemic antifungal drugs. More than 200 polyene macrolides have antifungal activity, and most are produced by the soil actinomycete *Streptomyces*. Polyenes bind to ergosterol, the main component of fungal membrane sterols, and form large pores that disrupt cell function [20, 21]. This interaction results in the formation of transmembrane pores,

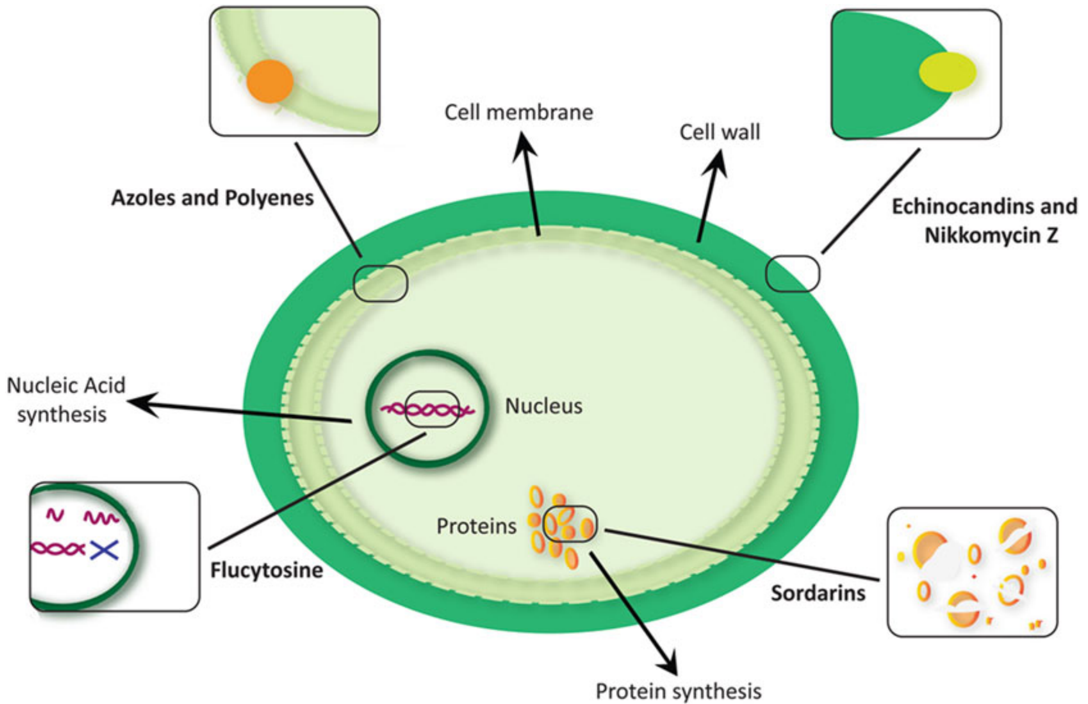

Fig. 1 Targets of systemic antifungal agents

which disrupt cell permeability and results in rapid cellular damage or death [22].

2.1.1 Amphotericin B (AmB)

Amphotericin B (AmB) is a polyene antifungal that contains a macrolide lactone ring with a series of conjugated double bonds. Its antifungal activity was discovered in 1953, and it was approved for clinical use in the US in 1957 [23]. The chemical structure of AmB deoxycholate is shown in Fig. 2.

AmB is insoluble in water but forms soluble salts under both acidic and basic conditions [24]. In the current clinical formulation, it is available for parenteral administration as a colloidal suspension using sodium deoxycholate (41 mg) as a dispersing agent and 25.2 mg sodium phosphate as a buffer for each 50-mg dose.

The primary drawbacks of AmB deoxycholate use are its dose-limited toxicity and significant side effects in various patients. The significant toxicities of AmB include infusion-related adverse effects (fever, chills, arrhythmia, hypotension, and respiratory distress), nephrotoxicity, neurotoxicity, hematological side effects, and allergic reactions [25].

Overall, two approaches have been used to improve the clinical response to AmB: the development of less toxic preparations in the 1990s and direct delivery of AmB to target organs (intranasal, aerosolized, intracavitary, and intraperitoneal administration).

Table 1
Recommended label indications of systemic antifungal drugs currently used

Class of antifungals	Name	Year approved by the US FDA	Brand name	Route of administration	Dosage form	Indication	Recommended dose
Polyens	Amphotericin B	1957	Fungizone	IV, PO, Nebulized	IV formulation (50 mg vial), Suspension (100 mg)	Treatment of life-threatening fungal infections (aspergillosis, cryptococcosis, blastomycosis, systemic candidiasis, coccidioidomycosis, histoplasmosis, and zygomycosis including mucormycosis due to susceptible *Absidia*, *Mucor*, and *Rhizopus* spp., and infections due to related susceptible *Conidiobolus* and *Basidiobolus* spp., and sporotrichosis)	0.25–1 mg/kg, once a day. Under no circumstances should a total daily dose of 1.5 mg/kg be exceeded
	Amphotericin B-lipid complex	1995	ABLC, Abelcet	IV	IV formulation (100 mg vial)	Treatment of IFIs in patients who are refractory to or intolerant of conventional amphotericin B therapy	5 mg/kg once a day
	Amphotericin B colloidal dispersion	1996	ABCD, Amphocil, Amphotec	IV	IV formulation (50 mg vial)	Treatment of invasive aspergillosis in patients where renal impairment or unacceptable toxicity precludes the use of amphotericin B deoxycholate at effective doses, and in patients with invasive aspergillosis who did not respond to prior amphotericin B deoxycholate therapy	3–4 mg/kg once a day

	Liposomal amphotericin B	1997	AmBisome	IV	IV formulation (50 mg vial)	Empirical therapy for presumed fungal infection in patients with febrile neutropenia	3 mg/kg once a day
						Treatment of infections caused by *Aspergillus*, *Candida*, and *Cryptococcus* spp. refractory to amphotericin B deoxycholate, or for patients in whom renal impairment or unacceptable toxicity precludes the use of amphotericin B deoxycholate	3–5 mg/kg once a day
						Treatment of cryptococcal meningitis in patients with HIV infection	6 mg/kg once a day
	Nystatin	1976	Fungicidin	PO	Suspension (100,000 U/ml)	Prevention and treatment of *Candida* infections of the oral cavity and esophagus	Neonates (from birth to 1 month): 1 ml (100,000 U) four times daily Infants (1 month–2 years): 2 ml (200,000 U) four times daily (1 ml for each side of the mouth). Children (over 2 years) and adults: 4–6 ml (400,000–600,000 U) four times daily (half dose on each side of the mouth)
Azoles	Ketoconazole	1981	Nizoral	PO	Tablet (200 mg)	Treatment of systemic fungal infections (blastomycosis, coccidioidomycosis, histoplasmosis, chromomycosis, and paracoccidioidomycosis) in patients who have failed to respond or are intolerant to other therapies	200–400 mg/day (the US FDA completely banned the use of ketoconazole tablets for *Candida* and dermatophyte infections)

(continued)

Table 1
(continued)

Class of antifungals	Name	Year approved by the US FDA	Brand name	Route of administration	Dosage form	Indication	Recommended dose
	Fluconazole	1990	Diflucan	PO, IV	Suspension (350 or 1400 mg), Tablet (50, 100, 150, or 200 mg), Solution for injection (200 mg/100 ml, 400 mg/200 ml)	Vaginal candidiasis	150 mg single PO dose
						Oropharyngeal candidiasis	200 mg on day 1, followed by 100 mg once daily. Treatment should continue for at least 2 weeks to decrease the likelihood of relapse
						Esophageal candidiasis	200 mg, first day, followed by 100 mg once daily. Doses up to 400 mg/day may be used. Treatment should continue for at least 2 weeks to decrease the likelihood of relapse. Patients should be treated for a minimum of 3 and at least 2 weeks following resolution of symptoms
						Systemic *Candida* infections	400 mg daily
						Urinary tract infections and peritonitis	Daily doses of 50–200 mg
						Cryptococcal meningitis	400 mg, first day, followed by 200 mg once daily for 10–12 weeks after the cerebrospinal fluid culture becomes negative

						Prophylaxis in patients undergoing bone marrow transplantation	400 mg, once daily. Patients who are anticipated to have severe granulocytopenia (less than 500 neutrophils/cu mm) should start diflucan prophylaxis several days before the anticipated onset of neutropenia and continue for 7 days after the neutrophil count rises above 1000 cells/cu mm
	Itraconazole	1992	Sporanox	PO, IV	Capsule (100 mg), Solution (10 mg/ml), Injection ampule (10 mg/ml)	Empiric therapy of patients with febrile neutropenia who have suspected fungal infections	200 mg twice a day for four doses, followed by 200 mg once daily for up to 14 days. Treatment should be continued with PO solution 200 mg (20 ml) twice a day until resolution of clinically significant neutropenia
						Treatment of pulmonary and extrapulmonary blastomycosis, chronic cavitary pulmonary and disseminated non-meningeal histoplasmosis, pulmonary and extrapulmonary aspergillosis in patients who are intolerant of or refractory to amphotericin B therapy	200 mg two times a day for four doses, followed by 200 mg once daily
	Voriconazole	2002	Vfend	PO, IV	Tablet (50 and 200 mg), Suspension (200 mg/5 ml), Injection (200 mg/vial)	Treatment of invasive aspergillosis	Loading dose of 6 mg/kg IV every 12 h (first 24 h), followed by maintenance dose of 4 mg/kg IV every 12 h or 200 mg PO every 12 h

(continued)

Table 1
(continued)

Class of antifungals	Name	Year approved by the US FDA	Brand name	Route of administration	Dosage form	Indication	Recommended dose
						Candidemia (nonneutropenic) and disseminated candidiasis in skin, abdomen, kidney, bladder wall, and wounds	Loading dose of 6 mg/kg IV every 12 h (first 24 h), followed by maintenance dose of 3–4 mg/kg IV every 12 h or 200 mg PO every 12 h
						Treatment of esophageal candidiasis	200 mg PO every 12 h
						Serious fungal infections caused by *Scedosporium apiospermum* (asexual form of *Pseudallescheria boydii*) and *Fusarium* spp. in patients intolerant of or refractory to other therapies	Loading dose of 6 mg/kg IV every 12 h (first 24 h), followed by maintenance dose of 4 mg/kg IV every 12 h or 200 mg PO every 12 h
	Posaconazole	2006	Noxafil	PO, IV	Suspension (40 mg/ml), Tablet delayed release (100 mg), Injection 300 mg/16.7 ml (18 mg/ml)	Prophylaxis of invasive *Aspergillus* and *Candida* infections	IV injection, 300 mg loading dose twice on day 1 followed by 300 mg maintenance dose once a day after that Delayed-release tablets, 300 mg loading dose, then three 100 mg tablets twice daily on day 1, followed by maintenance dose of 300 mg (three 100 mg delayed-release tablets) once a day, starting on day 2). PO suspension 200 mg (5 ml) three times a day

						Oropharyngeal candidiasis	PO suspension, loading dose of 100 mg (2.5 ml) twice a day on day 1, and then maintenance dose of 100 mg (2.5 ml) once a day for 13 days
						Oropharyngeal candidiasis refractory to itraconazole and/or fluconazole	PO suspension, 400 mg (10 ml) twice a day
	Isavuconazole	2015	Cresemba	PO, IV	Capsule (186 mg), Injection (372 mg)	Invasive aspergillosis, invasive mucormycosis	Loading dose of 372 mg isavuconazonium sulfate (equivalent to 200 mg of isavuconazole) every 8 h for 6 doses (48 h); PO (two capsules) or intravenous administration (one reconstituted vial), followed by maintenance dose of 372 mg isavuconazonium sulfate (equivalent to 200 mg of isavuconazole) once daily PO (two capsules) or IV administration (one reconstituted vial) starting 12–24 h after the last loading dose

(continued)

Table 1
(continued)

Class of antifungals	Name	Year approved by the US FDA	Brand name	Route of administration	Dosage form	Indication	Recommended dose
Echinocandins	Caspofungin	2001	Cancidas	IV	Lyophilized powder for injection (50 and 70 mg/vials)	Treatment of invasive candidiasis in adult or pediatric patients	Adult patients, a single 70 mg loading dose on day 1 followed by 50 mg daily. Pediatric patients (12 months–17 years), a single 70-mg/m^2 loading dose (patient's body surface area) administered on day 1 followed by 50 mg/m^2 daily after that. Antifungal therapy should continue for at least 14 days after the last positive culture
						Treatment of invasive aspergillosis in adult or pediatric patients who are refractory to or intolerant of other therapies	Treatment should continue for at least 7 days after resolution of symptoms
						Empirical therapy for presumed fungal infections (such as *Candida* or *Aspergillus*) in febrile adult or pediatric patients with neutropenia	Patients should be treated for a minimum of 14 days. Therapy should be continued until up to 72 h after resolution of neutropenia (ANC $\geq$ 500) or at least 7 days after clinical symptoms are resolved

	Anidulafungin	2006	Eraxis (in USA and Russia), Ecalta (in Europe)	IV	Lyophilized powder for injection (50 and 100 mg/vials)	Candidemia and other forms of *Candida* infections (intra-abdominal abscess and peritonitis)	200 mg loading dose on day 1, followed by 100 mg daily for at least 14 days after the last positive culture
						Esophageal candidiasis	100 mg loading dose on day 1 followed by 50 mg daily for a minimum of 14 days and for at least 7 days following resolution of symptoms
	Micafungin	2005	Mycamine	IV	Lyophilized powder for injection (50 and 100 mg/vials)	Treatment of candidemia, acute disseminated candidiasis, *Candida* peritonitis and abscesses	100 mg (once daily for 10–47 days)
						Treatment of esophageal candidiasis	150 mg (once daily for 10–30 days)
						Prophylaxis of *Candida* Infections	50 mg (once daily for 6–51 days)
Nucleoside analogs	Flucytosine	1971	ANCOBON, Ancotil	Oral, IV	Capsule (250 and 500 mg), Solution for Infusion (2.5 g/250 ml)	In combination with amphotericin B for the treatment of systemic candidiasis and cryptococcosis	50–150 mg/kg/day administered in divided doses at 6-h intervals

IV intravenous, *PO* oral, *ANC* absolute neutrophil count, *US FDA* US Food and Drug Administration, *ABLC* AmB-lipid complex, *ABCD* AmB colloidal dispersion, *IFIs* invasive fungal infections

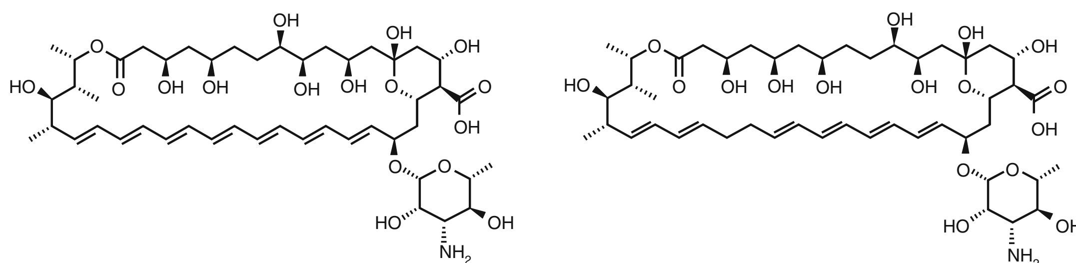

Fig. 2 Chemical structures of amphotericin B (*left*) and nystatin (*right*)

There are three available lipid preparations of AmB, which are AmB-lipid complex (ABLC), AmB colloidal dispersion (ABCD), and liposomal AmB (AmBisome). The lipid formulations have an improved therapeutic index and significantly reduced side effects compared to AmB deoxycholate and, therefore, can be administered at doses 3-5 times higher with significantly comparable efficacy [26]. However, these products are quite expensive and not available in some regions of the world.

In vitro, AmB is active against most common pathogenic yeasts that cause disseminated mycoses in humans. *Cryptococcus* [27] and *Candida* spp. [28, 29] are quite sensitive, except *Candida lusitaniae* [30] and *Trichosporon beigelii* [31], which show decreased susceptibility. *Aspergillus* spp. are sensitive, except *A. terreus* [32]. Most *Zygomycetes* spp. are susceptible [33]. Dimorphic fungi such as *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, and *Coccidioides immitis* are also sensitive [34].

AmB in combination with 5-flucytosine is recommended for the treatment of cryptococcal meningitis [35, 36], as well as a wide range of life-threatening IFIs such as blastomycosis, systemic candidiasis, coccidioidomycosis, histoplasmosis, zygomycosis, and sporotrichosis. The The current clinical guidelines recommend lipid formulations of AmB for the treatment of aspergillosis as an alternative to voriconazole or as salvage therapy in patients who are refractory or intolerant to other antifungal therapies [13].

2.1.2 Nystatin

Structurally, nystatin is an amphoteric tetraene originally isolated from *Streptomyces noursei* [37]. It is a polyene antifungal agent, which was first approved by the US Food and Drug Administration (FDA) in 1955 for the treatment of vaginal candidiasis. Nystatin is not absorbed by intact mucosal surfaces and following oral administration, it is passed unchanged in the feces; therefore, it is only active against yeasts present in the gastrointestinal tract [38]. Oral or topical nystatin is well tolerated; however, patients with renal insufficiency receiving oral therapy with conventional dosage forms may experience toxicity occasionally. Of note, an investigational

lipid formulation of nystatin, liposomal nystatin, showed a significantly reduced incidence of toxicity [39] with expanded antifungal activity against moulds [40, 41].

The spectrum of activity of nystatin includes *Candida* spp., *Cryptococcus neoformans* [42], *Trichosporon* spp., and *Rhodotorula* spp. [43]. Most dimorphic fungi, such as *B. dermatitidis Paracoccidioides brasiliensis, C. immitis*, and *Histoplasma capsulatum,* are sensitive to nystatin; however, it is inactive against dermatophytes and most *Aspergillus* spp. [41].

Topical and oral nystatin are recommended for the treatment of superficial *Candida* spp. infections of the skin, oral cavity, and esophagus including diaper dermatitis, angular cheilitis [44], and oral or vaginal candidiasis [45]. Oral nystatin has also been used for the prevention of systemic candidiasis in patients who are specifically at risk such as those with hematologic malignancies and those undergoing induction of chemotherapy [45]. However, the response has often been disappointing. Furthermore, it is noteworthy that currently available oral azoles have been found to be more effective [46, 47].

2.2 Azoles

Azoles are cyclic organic molecules characterized by a core 5-member azole ring, which can be divided into two groups based on the number of nitrogen atoms on the azole ring. These are the imidazoles and triazoles, which contain two and three nitrogen atoms, respectively within the azole ring [48]. Overall, the azoles are the most widely used class of antifungal drugs [49]. Figure 3 shows the chemical structure of systemic azoles currently used.

The azoles inhibit the synthesis of ergosterol from lanosterol in the fungal cell membrane by the binding of the free nitrogen atom of the azole ring to the iron atom of the heme group of a fungal enzyme [50, 51]. Their target enzyme is the cytochrome P450 (CYP)-dependent 14-α-demethylase (CYP51 or Erg11p), which catalyzes the targeted synthetic reaction. The action of azole antifungals depletes the ergosterol and methylated sterols accumulate in the cell membrane, which either inhibit the growth or induce the death of fungal cells, depending on the species and antifungal compound involved.

2.2.1 Ketoconazole

Ketoconazole is a member of the class of imidazole antifungals and is structurally similar to clotrimazole and miconazole. It was the first broad-spectrum oral antifungal treatment for systemic and superficial fungal infections and was released in the early 1980 [48]. Prior to the introduction of the triazoles, ketoconazole was regarded as the standard and only available oral agent for the treatment of chronic mucocutaneous candidiasis for over a decade, and an effective alternative to AmB in less severe (nonimmunocompromised) cases of blastomycosis, histoplasmosis, paracoccidioidomycosis, and coccidioidomycosis [48, 52]. However, within

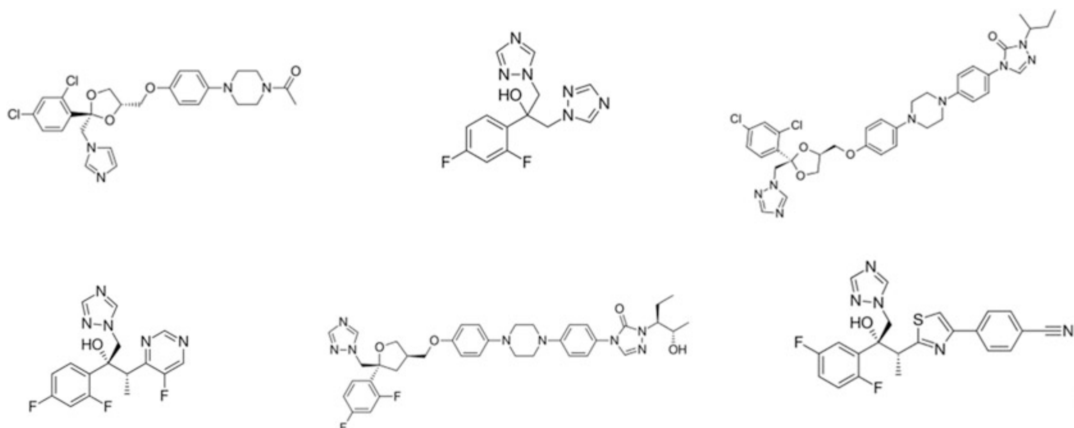

Fig. 3 Chemical structures of ketoconazole (*top-left*), fluconazole (*top-middle*), itraconazole (*top-right*), voriconazole (*bottom-left*), posaconazole (*bottom-middle*), and isavuconazole (*bottom-right*)

the first few years of its approval, numerous clinically relevant shortcomings of this compound became evident such as hepatotoxicity, several drug interactions, considerable interindividual variation influenced by gastric pH, and poor penetration of the blood-brain barrier, which rendered it ineffective and unsuitable for the treatment of fungal meningitis. In addition, ketoconazole is largely fungistatic and has been proved to be less effective in immunocompromised patients [48, 52]. Due to its significant side effects, systemic ketoconazole is currently only indicated for the treatment of endemic mycoses (blastomycosis, coccidioidomycosis, histoplasmosis, chromomycosis, and paracoccidioidomycosis) where alternatives are not available or feasible [52].

2.2.2 Triazoles in Clinical Practice

The poor response rates and frequent recurrences of major fungal infections as well as the toxicity associated with ketoconazole therapy led to the development of a second group of azole derivatives namely the triazoles [48]. Five triazole compounds (fluconazole, itraconazole, voriconazole, posaconazole, and isavuconazole) have been clinically approved and are currently widely used for the prevention and treatment of several life-threatening fungal diseases [53, 54]. The triazoles have different affinities for the CYP-dependent 14-α-demethylase, which in turn results in variability on the susceptibilities of fungi, side effects, and drug–drug interactions [55].

In general, fluconazole has broad activity against clinically relevant yeasts including *Candida* spp. and *Cryptococcus*; however, no clinically relevant activity against moulds. In contrast, itraconazole, voriconazole, posaconazole and isavuconazole all have activity against yeasts and moulds [48]. Triazoles are fungistatic, although itraconazole, voriconazole, posaconazole, and isavuconazole have

been shown to be fungicidal against *Aspergillus* spp. such as *Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, Aspergillus nidulans, Aspergillus terreus, Aspergillus versicolor*, and *Aspergillus sydowii* [51].

Fluconazole

Fluconazole exhibits antifungal activity against most common clinical isolates of *Candida* and *Cryptococcus* spp. and the endemic moulds *B. dermatitidis*, *C. immitis*, *H. capsulatum,* and *P. brasiliensis.* However, fluconazole lacks efficacy against moulds such as *Aspergillus* spp. and, therefore, it does not cover targeted prophylaxis or treatment of aspergillosis [56]. In addition, *Candida glabrata* and *Candida krusei* are inherently less susceptible to fluconazole. Fluconazole is approved for the treatment of vaginal, oropharyngeal, and esophageal candidiasis, as well as systemic *Candida* infections, fungal infections of the urinary tract, peritonitis, cryptococcal meningitis, and prophylaxis of patients undergoing bone marrow transplantation.

Itraconazole

Itraconazole is a high molecular weight, highly hydrophobic, and water-insoluble compound, which is structurally similar to ketoconazole. Itraconazole exhibits a broader range of antifungal activity including activity against most *Aspergillus* spp. than fluconazole does. Furthermore, itraconazole has virtually no permeability into the cerebrospinal fluid, probably due to its high protein binding. It is commonly used for the treatment of chronic and allergic fungal infection and for the empiric therapy of patients with febrile neutropenia who have suspected fungal infections [49]. In addition, it is used for the treatment of pulmonary and extrapulmonary blastomycosis, chronic cavitary pulmonary and disseminated nonmeningeal histoplasmosis, and pulmonary and extrapulmonary aspergillosis in patients who are intolerant or refractory to AmB therapy [49].

Voriconazole

Voriconazole is a low molecular weight water-soluble triazole, which is structurally similar to fluconazole. Voriconazole has a broad spectrum of antifungal activity (except against the Zygomycetes) and is the recommended first choice treatment for invasive aspergillosis [54]. Voriconazole is also indicated for the treatment of candidemia in patients without neutropenia; serious, invasive fluconazole-resistant *Candida* infections (including *C. krusei*); and serious fungal infections caused by *Scedosporium* and *Fusarium* spp. However, several case reports and studies in preclinical animal models suggest a unique susceptibility to breakthrough mucormycosis in patients receiving voriconazole [57, 58].

Posaconazole

Posaconazole is a lipophilic triazole, which is structurally similar to itraconazole. Posaconazole is distinguished from the other azoles by its potent *in vitro* activity against *Mucor* spp., which show reduced

susceptibility to other triazoles. In addition, posaconazole has improved activity against *Aspergillus* spp. compared to itraconazole [59]. Posaconazole is approved only for the following groups of patients. Those who are aged 18 years or older [53], receiving remission-induction chemotherapy for acute myelogenous leukemia (AML) or myelodysplastic syndromes (MDS) expected to result in prolonged neutropenia and those at high risk of developing invasive fungal infections. It is also indicated for prophylaxis in recipients of hematopoietic stem cell transplant (HSCT) who are undergoing high-dose immunosuppressive therapy for graft versus host disease, and are at high risk for developing IFIs; salvage therapy of invasive aspergillosis in patients with a disease condition that is refractory to AmB or itraconazole; and in patients who are intolerant of the other medicinal products [13, 60–62].

Isavuconazole

Isavuconazole is a new broad-spectrum triazole developed for the treatment of severe invasive and life-threatening fungal diseases [63–66]. The prodrug isavuconazonium sulfate (BAL8557) [67], is a highly potent water-soluble triazole suitable for both oral and intravenous administration. BAL8557 consists of a triazolium salt linked to an aminocarboxyl (*N*-[3-acetoxypropyl]-*N*-methylamino-carboxymethyl) via an ester moiety. After oral and intravenous administration, it rapidly and almost completely (>99 %) undergoes enzymatic activation followed by spontaneous chemical degradation to release the active drug isavuconazole (BAL4815, formerly RO-094815) and an inactive cleaved product, BAL8728 [68–70].

Isavuconazole has a broad spectrum of *in vitro* activity and *in vivo* efficacy against a wide range of yeasts and moulds including *Aspergillus* spp., *Fusarium* spp., *Candida* spp., *Mucorales*, *Cryptococcus* spp., melanized and dimorphic fungi, dermatophytes that can reproduce in culture by unilateral budding), and their filamentous co-spp. [70–79].

In 2015, isavuconazole has been approved for the treatment of invasive aspergillosis and mucormycosis by the US FDA and European Medicines Agency (EMA) of European Commission [80].

2.3 Echinocandins

The echinocandins are the only class of antifungal agents that directly target the fungal cell wall [81, 82]. They are semisynthetic amphiphilic lipopeptides formed during the fermentation of some fungi such as *Zalerion arboricola* or *A. nidulans* var. *echinulatus* [83]. The echinocandins inhibit β-1,3-D-glucan synthase, which catalyzes the biosynthesis of glucan, a key component of the fungal cell wall [84]. Of note, mammalian cells do not contain this polysaccharide target (1, 3-β-D-glucan) and, therefore, direct human cell toxicity is minimal [85].

The echinocandins are highly active (fungicidal) against *Candida* spp. including isolates that are resistant to triazoles [86] and *Candida* strains that form biofilms [87]. These agents have modest activity (fungistatic) against *Aspergillus* spp. [88] as well as dimorphic [89] and melanized fungi [90]. However, the echinocandins have weak activity against the Zygomycetes, *Fusarium* spp., *Scedosporium prolificans*, *Cryptococcus* spp., and *Trichosporon* spp. [85].

The echinocandins that are currently approved for clinical use are not orally bioavailable and, therefore, must be administered by slow intravenous infusion (1–2 h). The chemical structures of the echinocandins are shown in Fig. 4. Furthermore, a fourth echinocandin, aminocandin, is still undergoing early stages of clinical evaluation [63].

2.3.1 Caspofungin

Caspofungin is derived from pneumocandin B_o, the fermentation product of *Glarea lozoyensis*. It was the first echinocandin approved by the FDA in 2001, and is recommended for adult and pediatric patients for the primary treatment of invasive *Candida* infections, salvage therapy of invasive aspergillosis infections in patients who are refractory or intolerant to other therapies, and empiric antifungal therapy for presumed fungal infections (such as those caused *Candida* or *Aspergillus*) in persistently febrile patients with neutropenia [91].

2.3.2 Micafungin

Micafungin was the second marketed echinocandin synthesized the chemical modification of a fermentation product of *Coleophoma empetri*. Micafungin is approved for the treatment of esophageal candidiasis and prophylaxis of invasive *Candida* infections in patients undergoing hematopoietic stem cell transplantation. The indication for its use was later expanded to include candidemia, acute disseminated candidiasis, *Candida* abscesses, and peritonitis [92].

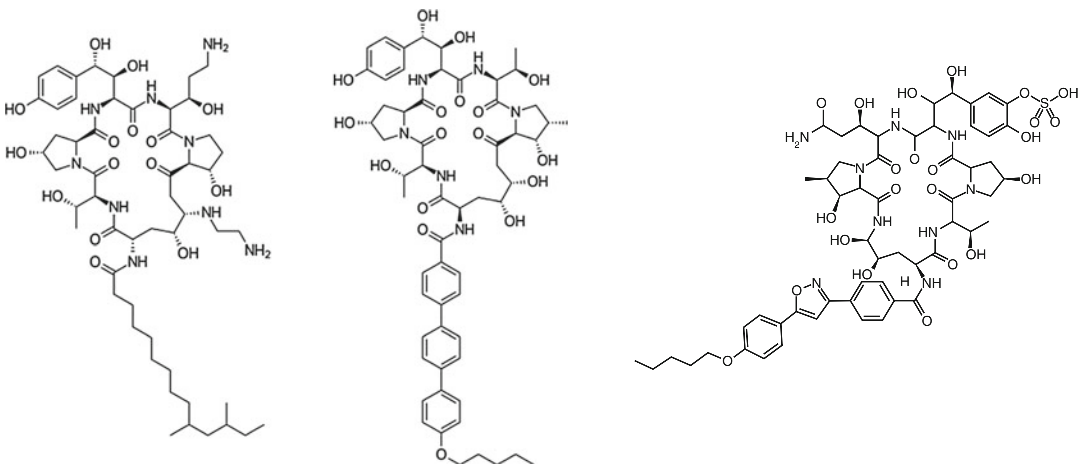

Fig. 4 Chemical structures of caspofungin (*left*), anidulafungin (*middle*), and micafungin (*right*)

2.3.3 Anidulafungin

Anidulafungin was derived from hexapeptides produced by *A. nidulans* and was the third echinocandin antifungal agent to receive approval from the FDA. It is approved for the treatment of candidemia and other *Candida* infections (intra-abdominal abscess and peritonitis) and esophageal candidiasis [93].

2.4 Nucleoside Analogs

Flucytosine (5-fluorocytosine or 5-FC) is the only systemic antifungal agent belonging to the class of nucleoside analogs. It was the first agent used for the treatment of invasive mycoses in 1968 [94]. Flucytosine is the fluorinated analog of cytosine and was discovered in 1957 as an analog of the cytostatic chemotherapeutic agent 5-fluorouracil (5-FU) used for antitumor therapy [95]. After it penetrates the cell wall, which is controlled by the enzyme cytosine permease, 5-FC is converted to 5-FU by the enzyme cytosine deaminase and then further to 5-fluorouridine [96].

After three phosphorylation steps, it is incorporated into RNA instead of uracil, which results in the blockade of protein synthesis. This pathway leads to reduced DNA synthesis because of a reduction in the available nucleotide pool [97]. Therefore, fungi that lack this enzyme are not susceptible to 5-FC [98]. It is noteworthy that cytosine deaminase is also absent in mammalian cells. Figure 5 shows the chemical structures of cytosine and two fluorinated pyrimidines (5-fluorocytosine and 5-fluorouracil).

Flucytosine can be administered both orally and intravenously, and is well distributed in almost all body fluids including the lacrimal fluid, urine, and cerebrospinal fluid (CSF) [97]. Flucytosine is well tolerated but at high concentrations above 100 mg/L may induce liver and bone marrow toxicity including bone marrow suppression, myocardial toxicity, and renal failure [99].

5-FC is active against most clinically important yeast such as those of the *Candida* [100] and *Cryptococcus* genera [101] but has limited activity against melanized fungi and *Aspergillus* spp. [102]. The *in vitro* activity of 5-FC is affected by a variety of test conditions such as incubation time, test medium, medium pH, and endpoint determination criteria [34]. In addition, dimorphic fungi are resistant to flucytosine [103].

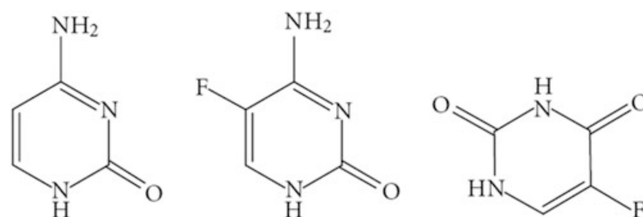

Fig. 5 Chemical structures of cytosine (*left*), 5-fluorocytosine (*middle*), and 5-fluorouracil (*right*)

Flucytosine combined with AmB is primarily recommended for the treatment of cryptococcal infections of the central nervous system [36]. In addition, flucytosine in combination with an azole (fluconazole) may have a role in the treatment of disseminated *Candida* infections that are refractory to first-line antifungal agents (triazoles and lipid formulations of AmB) [104]. Rapid resistance occurs if flucytosine is used as monotherapy [105] and, therefore [173], should never be used alone.

3 Unmet Clinical Needs in the Antifungal Research Field

Despite the recent advances in antifungal pharmacology, the clinical outcome of IFIs is frequently suboptimal, and the associated morbidity and mortality remain unacceptably high [6–9]. It is important to consider that a multitude of factors influence patient outcomes including the host immune status, pathogen variables including drug susceptibility, and effective administration of the appropriate dose of the most potent and safe antifungal drug [106]. Here, we briefly discuss the problem of antifungal drug resistance and the application of a promising tool, "therapeutic drug monitoring (TDM)."

3.1 Challenges of Antifungal Drug Resistance

Antifungal agents differ in their spectrum of activity and their *in vitro* sensitivities are often determined to assess the resistance patterns of the fungal isolate. Recently, major advances have been made in drug susceptibility testing of yeasts and filamentous fungi. Both the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing Subcommittee on Antifungal Susceptibility Testing (EUCAST-AFST) have developed and standardized phenotypic methods that enable the reliable and reproducible determination of the minimal inhibitory concentration (MIC) for yeasts and conidia-forming moulds [107, 108]. Antifungal drug resistance is normally quantified by MIC determination, and it has been shown that treatment success rates fall with increasing MIC values [109–111].

The mechanisms of antifungal drug resistance are either primary or secondary and are related to the intrinsic or acquired characteristics of the fungal pathogen that interfere with the antifungal mechanism of the respective drug/drug class or that lower target drug levels [112]. Furthermore, resistance can occur when environmental factors lead to colonization or replacement of a susceptible spp. with a resistant one [113]. Acquired resistance appears to develop primarily when a large number of microorganisms are exposed to a fungistatic drug for a relatively long period. This scenario is particularly relevant for *A. fumigatus* infections due to the increasing prevalence of azole-resistant isolates in the environment [114].

3.2 TDM

TDM is a promising tool used to tailor an individual patient's exposure to a drug by assessing their serum or plasma drug concentration and subsequently adapting the dosing regimen [106, 115]. Recently, measuring the trough concentrations of voriconazole and posaconazole has been considered as one of the most useful additional parameters for facilitating patient management [106, 116]. TDM will be useful in ensuring adequate drug exposure when using a specific dose in a particular patient.

4 Development of Old and New Antifungal Agents

Antifungal research and development are unfortunately particularly challenging, and no considerable advancements in antifungal therapies have been achieved recently [11, 117, 118]. It is noteworthy that the newest class of antifungal drugs, the echinocandins, was discovered in the 1970s and took 30 years to gain acceptance into clinical practice. Similarly, the gold standard therapy for cryptococcosis, one of the most prevalent invasive, life-threatening fungal infections worldwide, consists of two drugs (AmB and flucytosine) that were discovered over 50 years ago [118].

Compared to the development of new antimicrobials targeting bacteria, antifungal drug development faces a key fundamental challenge in that fungal pathogens are more closely related to the host. Fungi are eukaryotes and apart from their cell wall are metabolically similar to their mammalian hosts on a cellular level and, therefore, offer few pathogen-specific targets [117, 118]. Furthermore, the clinical development of new antifungals agents and their evaluation also present numerous challenges especially with clinical trial designs, which further complicate development [119].

Despite these issues, some novel products are progressing in development [11, 117, 118]. Several approaches to discovering new solutions have been developed over the last few years. Researchers and scientists aim to discover new antifungal drugs either by testing already existing medicinal compounds including those from natural sources such as plants and sea microorganisms or by systematic screening of chemical compound libraries. Researchers also strive to elucidate the underlying biology of fungal microorganism both *in vitro* and *in vivo*. Host–fungal interactions play a critical role in the survival of all fungal pathogens and targeting this interaction could also provide novel therapies, which could be used alone or in combination with existing antifungal drugs [120].

In addition, several efforts have been made to reformulate available antifungal drug classes into better oral and intravenous dosage forms in order to address many of the drug's pharmacokinetic shortcomings.

4.1 Reformulation of AmB

Although lipid-based formulations of amphotericin B exist, which are highly effective and exhibit lower toxicity when compared with AmB-deoxycholate, the cost of these formulations is a barrier to its widespread use. In addition, administration of AmB-deoxycholate has historically been hampered by toxicity, bioavailability, and solubility issues; therefore, many attempts have been made to reformulate this agent [117]. These include formulation of AmB nanoparticles for oral drug delivery that have shown good *in vitro* and *in vivo* activity with decreased toxicity [121, 122], and design of aerosolized AmB for direct delivery to target organs [123]. Moreover, various nanoparticle formulations for itraconazole have also been invstigated [124].

4.2 Development of New Agents of Known Antifungal Drug Classes

4.2.1 Albaconazole

Albaconazole (ALBA, UR-9825) is an investigational triazole agent with broad-spectrum antifungal activity against resistant and emerging pathogens as compared with fluconazole and itraconazole, favorable pharmacokinetics, and excellent oral bioavailability [125]. It has shown *in vitro* activities against pathogenic yeasts, dermatophytes, and other filamentous fungi and has been shown to be effective in animal models of systemic aspergillosis, candidiasis, cryptococcosis, and scedosporiosis [126, 127].

Albaconazole is currently under clinical development in phase I/II studies of candidal vulvovaginitis, tinea pedis, and onychomycosis (www.clinicaltrials.gov: NCT00199264, NCT00509275, and NCT00730405). Notably it was well tolerated and effective in women with vaginal candidiasis [128–130]; however, phase III studies are not available until now. As a drawback, the lack of an intravenous form makes albaconazole studies in the acute infection setting very difficult [131].

4.2.2 SCY-078 (MK-3118)

Due to the large molecular weight of the three approved echinocandins (each ~1200 kDa), they have poor oral bioavailability, and therefore, only intravenous formulations are available for clinical use. The development of glucan synthase inhibitors that can be administered orally would represent major step forward by providing a simple therapy transition to the ambulatory setting [132].

SCY-078 (formerly MK-3118) is a semisynthetic derivative of the natural product enfumafungin—a structurally distinct class of beta-1,3-D-glucan synthase inhibitors, being developed for the treatment of invasive fungal infections. It has shown *in vitro* and *in vivo* potency against the most common *Candida* spp., including *C. albicans, C. glabrata, C. tropicalis, C. parapsilosis*, and *C. krusei* [133–135]. In addition, SCY-078 also demonstrated excellent *in vitro* activity against wild-type and azole-resistant strains of *Aspergillus* spp. [136], *Paecilomyces variotii*, and *Scedosporium prolificans*; however, poor activity was observed against *Mucoromycotina* and *Fusarium* spp. [132].

SCY-078 is currently under phase 2 clinical studies and is designated as a Qualified Infectious Disease Product (QIDP) for oral use by the US FDA, for the indications of invasive candidiasis, including candidemia, and invasive aspergillosis.

4.2.3 Biafungin (CD101)

Biafungin (formerly SP3025) is a highly stable echinocandin in development for weekly intravenous administration. It has a long half-life (~3-fold longer) and slower clearance (~7-fold slower) relative to other echinocandins [137], which enables once-weekly dosing, and thus might decrease costs with long-term hospitalizations. The compound displayed comparable *in vitro* activity to approved echinocandins against *Candida* and *Aspergillus* spp. [138]. Biafungin is currently under phase 1 clinical development for the treatment of candidiasis.

4.3 New Antifungal Drugs with Novel Targets

4.3.1 Nikkomycin Z

Chitin is a major structural component of the fungal cell wall biosynthesized from uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) by chitin synthases [139]. Since mammalian cells do not contain chitin synthases, it is a promising target for the development of antifungal drugs [140]. Nikkomycin Z is an experimental chitin synthase inhibitor currently under development [141].

It consists of a pyrimidine nucleoside linked to a short peptide moiety (Fig. 6) and inhibits chitin synthesis by acting as a competitive analog of the chitin synthase substrate UDP-N-acetylglucosamine [142]. Therefore, it interferes with the fungal cell wall construction [143]. Nikkomycin Z has shown *in vitro* and *in vivo* activity against dimorphic fungi such as *C. immitis*, *H. capsulatum*, and *Blastomyces dermatitidis*, which have a high content of chitin in their cell wall [144, 145]. It has only limited effect against *Candida* spp. and *C. neoformans* and is inactive

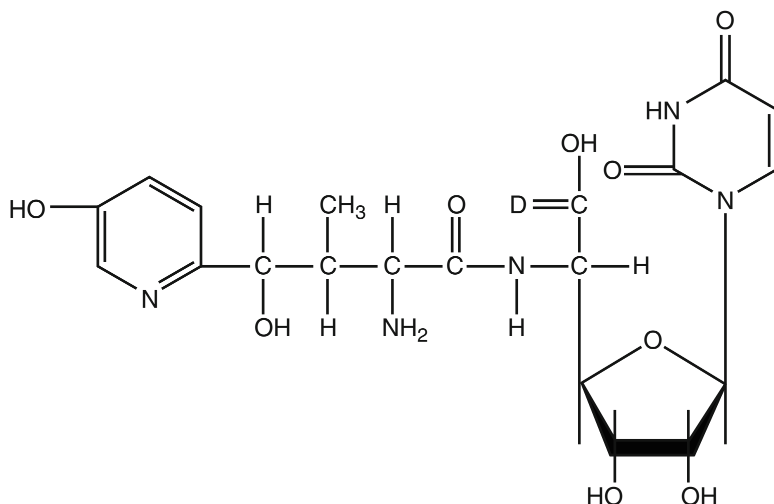

Fig. 6 Chemical structures of nikkomycin Z

against filamentous fungi such as *Aspergillus, Fusarium*, and *Zygomycete* spp. [146–148].

It is noteworthy that the combination of nikkomycin Z and echinocandins has a synergic effect against *A. fumigatus* [149]. This agent is under development as an orphan product for treatment of coccidioidomycosis (https://clinicaltrials.gov/ct2/show/NCT00614666) [150].

4.3.2 Sordarins

Sordarins derivatives represent a novel class of naturally occurring and semisynthetic products first discovered in early 1970 [151, 152], which are characterized by a unique tetracyclic diterpene core including a norbornene system [153]. The sordarin family of compounds were isolated from the fungus *Sordaria araneosa* and found to inhibit fungal protein synthesis through their interaction with the elongation factor 2 (EF2) in the ribosome [152, 154]. Numerous sordarin-based molecules have demonstrated potent antifungal activity *in vitro* and *in vivo* against a wide range of pathogenic yeasts including *Candida albicans* and *C. neoformans* [155, 156].

The unique mode of action of the sordarins, which targets the cell membrane, is different from that of typical antifungals. Their high specificity for the fungal target with no effect on the mammalian hosts as well as the relative ease with which new sordarin variants can be synthesized makes them promising candidates for potential future development [153, 157].

Interestingly, *in vivo* experimental studies have suggested that two sordarin derivatives, R-135853 and R-135853, are promising antifungal agents for the treatment of disseminated or mucosal candidiasis, including fluconazole-refractory infections [158, 159]. The chemical structures of these molecules are shown in Fig. 7.

4.3.3 VT-1161 and VT-1129

VT-1161 is a novel fungal-specific 14α-lanosterol demethylase (CYP51) inhibitor that was rationally designed to selectively target the fungal enzyme rather than human CYP450 enzymes while maintaining high potency for the fungal target [160]. VT-1161

Fig. 7 Chemical structures of sordarin (*left*) and sordaricin (*right*)

has a broad spectrum activity against yeast and dermatophytes [12], as well as invasive fungal species [161]. *In vivo* studies also showed show that VT-1161 was effective in treating experimental mucormycosis [162], invasive candidiasis, murine coccidioidomycosis [163], and dermatophytosis [164]. VT-1161 is currently under Phase 2 clinical studies for the treatment of superficial fungal infections in patients with moderate to severe acute vulvovaginal candidiasis.

Furthermore, VT-1129 in another orally available inhibitor of fungal CYP51, which blocks the production of ergosterol in the fungal cell membrane. This compound is currently under preclinical development targeting the treatment of cryptococcal meningitis [165].

4.3.4 F901318

F901318 is the most advanced member of a new class of antifungal agents, the orotomides, which have a novel mechanism of action distinct from those of currently available clinical antifungal agents [166]. The orotomides class of antifungals act via inhibition of dihydroorotate dehydrogenase (DHODH), which is an important enzyme in pyrimidine biosynthesis. Thus, F901318 arrest pyrimidine biosynthesis via inhibition of DHODH. This agent demonstrated potent *in vitro* activity against a spectrum of filamentous fungi including clinical *Aspergillus* spp, *Fusarium* spp, and *Mucorales. In vivo*, F901318 has demonstrated excellent antifungal activity against disseminated mouse model of aspergillosis [167].

4.3.5 VL-2397

VL-2397 (formerly ASP2397) is a novel second generation echinocandin under development. Active transport of this agent into fungal cells occurs via siderophore iron transporter 1 (sit1), which triggers antifungal effects. Importantly, lack of this siderophore transporter in surface of mammalian cells can be responsible for higher selectivity [168].

This compound demonstrated potent and rapid hyphal growth inhibition against *A. fumigatus* (including azole-resistant siolates), *A. terreus, A. flavus,* and *A. nidulans in vitro* and significantly improved MIC against *Candida parapsilosis* and echinocandin resistant-*Candida* spp. [169]. Preclinical studies also showed that VL-2397 was effective as monotherapy in an established guinea pig model of invasive aspergillosis [168].

4.3.6 T-2307

T-2307 is an investigational arylamidine structurally similar to a class of aromatic diamidines that includes pentamidine [170]. Its mechanism of action is unknown; however, it appears to cause the collapse of fungal mitochondrial membrane potential in yeasts [171]. T-2307 has shown potent activity against *Candida* species, including azole or echinocandin-resistant *Candida* spp., *Cryptococcus neoformans*, *Malassezia furfur*, and *F. solani* [170, 172]. Moreover, T-2307 was active against *Aspergillus* species, and

in vitro activity against these species was shown to be comparable to the activities of micafungin and voriconazole [172]. For this potential antifungal drug, no clinical data are available thus far.

5 Conclusions and Future Directions

The increased risk of fungal diseases particularly in patients who are immunocompromised, emerging fungal pathogens, limited repertoire of antifungal drugs, and the development of resistance to the available antifungal drugs have increased the demand for the development of new and effective antifungal agents. Therefore, there is a need to intensify the current antifungal drug discovery efforts to develop more clinically effective and safer agents, especially against antifungal drug-resistant pathogens.

Conflicts of Interest

S.S. received research grant from Astellas Pharma B.V. P.E.V. has served as a consultant and received research grants from Astellas, Basilea, Gilead Sciences, Merck, and Pfizer. All other authors have no conflict of interests.

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Chapter 6

Fungal-Grade Reagents and Materials for Molecular Analysis

Michael G. Lorenz, Michael Lustig, and Marina Linow

Abstract

Fungal DNA is present at very low loads in clinical specimens. Molecular detection by amplification assays generally is a challenge because of a potentially multiple input of contaminating DNA from exogenous sources. Besides airborne, handling and cross-contamination, materials and reagents used in the molecular laboratory can contain microbial DNA which is a long underestimated potential source of false positive results. In this contribution decontamination procedures of materials and reagents and the selection of certified microbial DNA-free components for sample collection, DNA extraction, and PCR amplification are discussed with respect to the aim of building up a reliable molecular system for the diagnosis of fungal organisms at the limit of detection.

Key words Fungal DNA, Contamination, Decontamination, DNA-free, Fungal PCR reagents, Fungal DNA extraction, Fungal assays

1 Introduction

Microorganisms can be present at very low loads in clinical samples. For instance, Wain et al. [1] cultured *Salmonella typhi* from blood at a median load of 1 cfu/ml (range, <0.3–387 cfu/ml). In another, more broadly focused study Phillips and Bradley [2] plated blood samples from neonates on chocolate agar. Cell counts determined for gram-positive bacteria, gram-negative bacteria, yeasts of the genera *Candida* and *Malassezia* and mixed infections of *Candida* and gram-positives ranged from 1 to >100 cfu/ml, 3 to 8 cfu/ml, 18 to 96 and 66 to >100 cfu/ml, respectively.

Low loads of microorganisms are challenging to be analyzed by molecular methods. For PCR or Real-Time PCR diagnosis, a highly sensitive assay is therefore necessary [3]. This holds true also for the analysis of low microbial load communities by next generation sequencing [4]. Clearly, analysis at the limit of detection can pose serious problems to an accurate analysis as regards false positive results and wrong community structure by introduction of

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extraneous DNA. This can happen through carryover inoculation from laboratory surfaces and equipment, cross-contamination from one sample to another during extraction, aerosols from previous amplifications and air-borne environmental sources, inappropriate handling, and contaminated reagents and consumables [5]. There are procedures described for clinical, forensic, ancient DNA, and other applications to prevent sample contamination by DNA from exogenous sources [6–8] which will be not discussed here. This contribution focuses on the impact on the diagnosis of fungal pathogens of contaminated reagents and consumables employed in the sampling, extraction and amplification of target DNA from clinical samples. Solutions for in-house DNA decontamination procedures and commercially available DNA-free products will be presented with relevance for fungal diagnosis.

2 Sources of Material Contamination in the Course of Sample Collection, DNA Extraction, and Molecular Analysis

Reagents and consumables used in the molecular laboratory are generally not issued for the analysis of microbial sequences at very low concentration. Therefore, an important constraint of molecular analysis at the limit of detection is the potential presence of microbial DNA contaminating extraction chemicals, PCR reagents and consumables during the manufacturing process. Table 1 summarizes studies that indicated contamination of a variety of reagents and consumables by microbial DNA. The studies were conducted in the context of suitability of the material for sensitive molecular diagnosis. Contamination was found in the majority of reagents and consumables studied. DNA of fungal organisms including *Aspergillus* spp., *Pneumocystis* spp., *Saccharomyces cerevisiae*, *Candida* spp., and *Acremonium* spp. were detected in collection tubes for samples like blood, serum and urine, DNA and RNA extraction reagents as well as PCR buffer (Table 1, species in bold). The origin of the contamination could also be assigned to eubacteria, among them environmental organisms like *Alcaligenes* spp. and *Pseudomonas* spp., skin colonizers and opportunistic pathogens like *Propionibacterium* spp., *Serratia marcescens*, and *Sphingomonas* spp. as well as potential pathogens, including *Brucella* spp., *Coxiella burnetii*, *Escherichia coli*, and *Legionella* spp. The rate of contamination was found to be high in some materials (>50 % false positives), including forceps for tissue preparation, DNA extraction reagents and Taq polymerases (Table 1). Evidence for low and tolerable loads of contaminating microbial DNA (2 and 8 % false positive rate) was given for some Taq polymerases (Table 1, ref. [21]). The list of evidence of DNA contamination stresses the importance of taking care in the selection of materials and reagents specially

Table 1
DNA contamination of materials and reagents employed in molecular analysis of pathogens in clinical samples[a]

Process	% False positives (tests)	Origin	Reference
Sample collection			
Blood collection tubes	17 (185)	*Aspergillus* spp.	[9]
Blood serum tubes	10 (160)	*Aspergillus* spp.	[9]
	4 (50)	*Pneumocystis jirovecii*	[10]
Urine collection tubes	8 (25)	*Aspergillus* spp.	[9]
Forceps for tissue preparation	57 (23)	*Escherichia* spp., *Propionibacterium* spp., *Stenotrophomonas* spp., *Pseudomonas* spp.	[11]
Nucleic acid extraction and processing			
Zymolyase	n.d.	*Saccharomyces cerevisiae*	[12]
Lyticase	n.d.	Unspecified fungus	[12]
DNA extraction	100 (20)	*Burkholderia* spp., *Pseudomonas saccharophila*, *Ralstonia* spp., *Alcaligenes* spp.	[13]
	20 (20)	*Legionella* spp., *Aspergillus* spp.	[14, 15]
	n.d.	*Aspergillus* spp., *Candida* spp.	[16]
	n.d.	*Brucella* spp.	[17]
	<3 (36)	n.a.	[18]
Nucleic acid precipitation (glycogen)	22 (9)	*Acinetobacter lwoffii*	[19]
RNA stabilization reagent	5 (20)	*Aspergillus* spp.	[9]
PCR amplification			
Taq polymerase	100 (4)	Unspecified bacterium	[20]
	100 (4)	*Pseudomonas* spp.	[21]
	8 (24)	*Sphingomonas* spp., *Moraxella* spp.	[21]
	2 (41)	*Acinetobacter junii*	[21]
	n.d.	*Pseudomonas* spp., *Serratia marcescens*, *Escherichia coli*, *Salmonella* spp., *Shigella* spp.	[22]
	10–30 (n.m.)	*Coxiella burnetii*	[23]
Primers	0–100 (18–66)	*Delftia tsuruhatensis*, *Klebsiella* spp., *Paenibacillus* sp.	[24]
PCR buffer	n.d.	*Acremonium* spp.	[12]
Pipetting			
Pipette tips	18 (6/32)	Unspecified bacterium	[25]

[a]Samples of the same or different lots or samples from different manufacturers; signals were observed in negative PCR controls using DNA-free water; best match species were identified by sequencing of the amplicons and BLASTn search, *n.d.* not determined, *n.a.* not applicable, *n.m.* not mentioned

treated by elaborated decontamination procedures or, as far as available, low contamination commercial products manufactured under quality-controlled conditions for use for molecular microbial diagnosis.

3 Consumables and Reagents for Fungal DNA Analysis

The problem of false positive results in molecular analysis of fungal pathogens through contaminated consumables, buffers and reagents has been addressed by a number of approaches. Millar et al. [8] propose a risk assessment model detailing the manipulations, contamination hazards and risks, and corrective action involved in the broad-range 16S rRNA gene PCR diagnosis of bacterial blood stream pathogens which may serve as a guideline for fungal diagnostics by broad-range rRNA gene PCR and other assays. The model divides the analytical process into three parts, sample collection, DNA extraction and amplification, all of which demand careful handling and the availability of molecular grade, in particular DNA-free consumables and reagents. In the following sections ways of decontamination of materials and reagents and employment of commercially available DNA-free material are discussed as regards the setup of a reliable, highly sensitive system for the direct detection and identification of fungal organisms in clinical and other sample materials with low fungal loads.

3.1 Sample Collection

Consumables for the collection and handling of samples are in line with analytical processes other than molecular diagnosis of microbial DNA present at very low concentrations. Material for the collection and processing of blood to plasma and serum, stabilization of blood cells and preparation of tissue biopsies has been shown to be a potential source of exogenous DNA of fungal and bacterial organisms (Table 1). So far, material routinely tested from lot to lot for the absence of microbial DNA does not seem to be available from commercial sources. Millar et al. [8] proposed to prepare lots of sterile DNA-free collection tubes, EDTA solution and water for blood drawing. Reduction of amplifiable bacterial sequences and cells over up to 4 orders of magnitude to below the limit of detection was observed when surfaces of plastic ware were experimentally contaminated with DNA or microorganisms and treated with methanol radicals or ethylene oxide [26, 27]. Radical-based treatment was regarded superior to UV or gamma irradiation which tends to have a negative influence on the plastic consumables.

DNA decontamination of water and buffers can be achieved by UV or Gamma irradiation [7]. Water and buffers are also commercially available as molecular biology grade and certified human DNA-free products (e.g., Mo Bio, Carlsbad, CA, USA). Absence of microbial DNA, however, is not indicated and should be tested by the user by PCR negative control runs. Other sources of certified fungal (and bacterial) DNA-free water exist although available only as small volume products provided for PCR analysis (*see* Table 2, amplification reagents).

3.2 Consumables for Handling in Molecular Analysis

The selection of suitable plastic consumables employed for DNA extraction and molecular analysis, including pipette tips, sample tubes, centrifugation vials and PCR or Real-Time PCR tubes and plates is crucial for the avoidance of false positive results by contaminating DNA. Sterility and absence of nucleases as characters for molecular-grade articles are not a guarantee for the absence of exogenous DNA as evidenced by our own experience (Table 1). Therefore, as part of the setup of a system for low load fungal DNA analysis, testing of products from different suppliers for the absence of fungal DNA is recommended.

Radical gas treatment of plastics is a common procedure for the destruction of amplifiable sequences (see previous section). There are some suppliers of consumables which declare their products bacterial DNA-free (Table 2). Only one among the three suppliers listed in Table 2, however, files testing for bacterial and fungal DNA. Nevertheless, it seems that absence of bacterial DNA is a good indicator of the absence of fungal DNA as well from the point of view of our experience (see comment in Table 2). Nonetheless, to be sure as indicated above, consumables not explicitly labeled as tested for the absence of fungal DNA should be subjected to negative control run analysis in the laboratory.

3.3 DNA Extraction

As with other materials used for molecular biology, DNA extraction products are generally not designed for the purpose of ultra-sensitive detection of microorganisms at low loads in clinical and other specimens. In fact, they generally contain exogenous DNA of bacterial and fungal origin (Table 1). Systematic studies have been performed to eliminate contaminating microbial DNA from extraction buffers by binding the DNA to silica-based membrane columns in a procedure employing filtration washing [13]. Ethylene oxide treatment of plastic consumables, including mini spin columns, was successfully employed for the destruction of DNA contaminants [27].

Industry has reacted to the increasing demand for microbial DNA-free reagents and consumables by the supply of ultra-clean products for DNA extraction from clinical samples. Table 2 lists certified bacterial and fungal DNA-free products dedicated to the extraction of microbial DNA for the analysis of low loads of microorganisms. The products address the manual, semi-automated, and fully automated extraction of small and large sample volumes in the range 0.1 to 10 ml as well as tissue biopsies. Two products, MagNA Pure® (Roche) and easyMag® (bioMérieux), extract total nucleic acids, while all others aim at the preparation of microbial, including fungal DNA with low loads of human DNA. A variation of the standard easyMag® protocol was described by Wiesinger-Mayr et al. [28] by which bacterial DNA preparations were greatly depleted of human DNA. Excess host DNA can be a factor of false positive results and loss of assay

Table 2
Material and reagents for the analysis of low microbial loads

Component	Brand	Manufacturer	Absence of DNA tested[a]		Comment
			Bacterial	Fungal	
Consumables					
Filter tips, tubes, centrifuge vials	MGrade®	Greiner Bio-One (Kremsmünster, Austria)	+	+	
	Biopur®	Eppendorf (Hamburg, Germany)	+	–	
	Biosphere® Plus	Sarstedt (Nümbrecht, Germany)	+	–	Tested for absence of fungal DNA[b]
Extraction kits					
Manual protocols	UCP PurePathogen Blood	Qiagen (Hilden, Germany)	+	+	Manual DNA extraction; up to 8 ml blood
	MolYsis™	Molzym (Bremen, Germany)	+	+	Manual DNA extraction; up to 10 ml clinical samples
Automated systems	Blood Pathogen Kit™	Seegene (Seoul, South Korea)	+	+	Semi-automated; 1 ml blood; Seeprep12™ instrument (Seegene)
	SelectNA™ Blood Pathogen Kit	Molzym (Bremen, Germany)	+	+	Semi-automated; up to 10 ml clinical samples; Liaison® IXT instrument (Diasorin, Saluggia, Italy)
	MagNA Pure® LC Microbiology kit	Roche Diagnostics (Penzberg, Germany)	+	+	Automated DNA extraction; 0.1 ml samples; MagNA Pure LC 2.0 instrument (Roche)
	MolYsis SelectNA™*plus*	Molzym (Bremen, Germany)	+	+	Automated DNA extraction; 1 ml liquid samples, tissue biopsies; SelectNA™*plus* instrument (Molzym)
	EasyMag®	bioMerieux (Marcy-l'Étoile, France)	–	–	Modified automated protocol for 5 ml blood [29]; see text

Amplification reagents					
Taq DNA polymerase, master mix, water	Taq DNA Polymerase, DNA-free	Applichem (Darmstadt, Germany)	+	+	
	MolTaq 16S/18S	Molzym (Bremen, Germany)	+	+	
	innuTaq UltraPure DNA Polymerase	Analytic Jena (Jena, Germany)	+	–	
	MTP™ Taq DNA Polymerase	Sigma-Aldrich (St. Louis, MO, USA)	+	–	
	DFS-Taq DNA Polymerase	Bioron (Ludwigshafen, Germany)	+	–	
	Taq DNA Polymerase	Amresco (Solon, OH, USA)	+	–	
	DNA free-Taq DNA Polymerase	Xpress Bio (Frederick, MD, USA)	+	–	
	DF Taq Polymerase E (DNA-free)	Genaxxon (Ulm, Germany)	+	–	
	Mastermix 18S Basic	Molzym (Bremen, Germany)	+	+	Master mix for assays with custom primers
	Mastermix 18S Complete	Molzym (Bremen, Germany)	+	+	Broad-range 18S rRNA gene PCR assay for detection of fungal DNA
	Microbial DNA-free Water	Qiagen (Hilden, Germany)	+	+	
	DNA-free Water	Molzym (Bremen, Germany)	+	+	
	PCR Water, DNA-free	Applichem (Darmstadt, Germany)	+	+	

[a]+, information (homepage) on testing provided by manufacturer; –, no information available

[b]Own results, absence of fungal DNA shown at 40 cycles PCR employing Mastermix 18S Complete (Molzym)

sensitivity as a consequence of unspecific primer binding and amplification of non-target sequences [29].

Five of the 6 products are declared by the manufacturers to be routinely tested for the absence of bacterial and fungal DNA during the manufacturing process. As regards the product without information about testing for microbial DNA contamination, easyMag® (bioMérieux), there is evidence that also this system is suitable for sensitive analysis of pathogens, although demonstrated only for bacteria. By developing a modified easyMag® protocol for the extraction of bacterial DNA from 5 ml EDTA blood, Wiesinger-Mayr et al. [28] could detect a variety of Gram-positive and Gram-negative bacteria at low loads (10^1–10^2 cfu/ml) while extracts from negative extraction controls were negative over 40 cycles in the broad-range 16S rRNA gene assay employed which indicates that bacterial DNA contamination was below the detection level. The authors regarded the protocol as a promising system for bacterial DNA extraction. As indicated above, contaminating fungal DNA is likely to be absent, but should be proven before using the system for the development of a protocol for fungal DNA extraction.

3.4 Molecular Analysis

The record of references regarding contamination of amplification reagents comprises mainly bacterial DNA contamination (Table 1). As regards fungal DNA contamination, literature is scarce. Loeffler et al. [12] systematically studied contamination of reagents involved in the whole diagnostic process, including amplification. They found one component, the 10× PCR buffer of a certain lot of the product to be contaminated by fungal DNA which by sequence analysis of the amplicon could be assigned to *Acremonium* spp. This shows that care has to be taken in the selection of amplification reagents as was discussed before regarding sample collection and extraction.

Champlot et al. [7] conducted a systematic evaluation of various methods for the decontamination of PCR components. The authors provide protocols involving gamma-irradiation of water and short UV irradiation by which PCR buffers and other liquids can be decontaminated efficiently and rather easily from exogenous DNA. They point out that sensitive components like Taq DNA polymerase, primers and dNTPs are inactivated by UV and therefore need a different treatment. Employment of a protocol using a heat-labile endonuclease resulted in 99.5 % degradation of double-stranded DNA while the efficiency and sensitivity of the PCR assay was comparable to the control indicating that primers were not affected by the nucleolytic treatment. Although focused on the removal of bovine DNA contamination, the study provides valuable guidelines for the setup of a decontamination protocol for PCR components as regards fungal DNA.

When screening the information provided by manufacturers, several PCR components, including Taq DNA polymerases, master

mixes and molecular grade water are available which are certified bacterial and in many cases also fungal DNA-free (Table 2). The use of commercial products may be helpful in the reduction of fungal DNA contamination and the standardization of sensitive fungal target assays among laboratories. Importantly, when designing an analytical assay for the detection of minute amounts of fungal DNA, DNA-free products should also guarantee a high amplification activity [21].

Even though buffers and reagents of the amplification reaction are available DNA-free, either as single components or as master mixes (Table 2), primers designed for specific targets are generally produced under conditions which introduce considerable levels of microbial DNA into the primer preparation (Table 1). This is a frequently experienced problem which demands special care regarding decontamination (see above).

For routine practice, diagnostic assays for fungal DNA detection are available which have been thoroughly evaluated in clinical studies. Fungal assays and complete systems combining fungal DNA extraction and analysis are addressed in Chapters 1 and 2.

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Part II

Host Susceptibility and Defense

Chapter 7

Host-Derived Biomarkers for Risk Assessment of Invasive Fungal Diseases

Cristina Cunha, Samuel M. Gonçalves, and Agostinho Carvalho

Abstract

Invasive fungal diseases are major complications associated with the treatment of hematologic malignancies. The integration of host-derived biomarkers into clinical processes to predict the risk and progression of fungal disease is a promising approach in immunocompromised patients. Recent insights into human antifungal immunity have highlighted the remarkable influence of host genetics in modulating susceptibility to infection. In this chapter, we describe protocols to examine human genetic variation and to assess its functional consequences using the pattern recognition receptor PTX3 as an example.

Key words Invasive fungal disease, Single nucleotide polymorphism (SNP), Host biomarkers, Long pentraxin 3 (PTX3), Antifungal immunity, Immunocompromised patients, Risk stratification, Personalized medicine

1 Introduction

Invasive fungal diseases, particularly invasive aspergillosis (IA), are major complications associated with the treatment of hematologic malignancies [1]. Vaccines are not available, and despite noteworthy recent developments in diagnostic and therapeutic approaches, these diseases remain associated with unacceptable mortality rates [2, 3]. Concerns over excessive prescription of antifungal drugs and the remarkable burden these diseases convey to the healthcare systems are inspiring a shift from universal prophylaxis to risk stratification and preemptive approaches. Recent evidence continues to highlight the complexity of the multiple fungal-sensing immune systems and the remarkable influence of the host genetics in the ability to control infection risk and its progression. As a result of our improved understanding of the host–fungus interaction, several relevant target genes (and associated genetic variants) with the potential to be exploited in future personalized medical interventions in high-risk settings based on individual genetic risk have been identified.

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Chronic granulomatous disease and autosomal-dominant hyper-IgE syndrome (AD-HIES) are the most common examples of primary immunodeficiencies typically associated with susceptibility to IA [4]. These severe immune deficiencies with monogenic inheritance are usually limited to a very small number of individuals or families, but the identification of genetic defects is very informative on immune defense mechanisms. For most individuals however, genetic propensity to fungal disease has a polygenic inheritance, acting in concert with other clinical predisposing variants such as chemotherapy-induced neutropenia. Our increasing ability to analyze human variability at the DNA level has made possible the identification of several host genetic variants amenable to use in the categorization of patients with a high risk of infection and to target antifungal therapy (reviewed in [5, 6]). However, the clinical translation of this active field of research is still limited, mostly due to the heterogeneity of cohorts, sample size, case and control selection bias, and statistical misconceptions.

Among the most encouraging examples reported to date, a donor haplotype in Toll-like receptor 4 (*TLR4*) underlying a delayed T cell and natural killer T cell immune reconstitution [7] has been disclosed as an important risk factor for developing IA in recipients of allogeneic hematopoietic stem cell transplantation (HSCT) [8]. In addition, TLR4 variants have been previously linked with chronic aspergillosis in immunocompetent individuals [9] and fungal colonization in HSCT recipients [10]. However, and since the fungal ligand (or the host-derived molecule released in response to fungal infection) for TLR4 remains debated, uncovering the exact mechanism(s) through which TLR4 deficiency impacts antifungal immunity is required to strengthen the prognostic significance of its genetic variation.

It is noteworthy that, in addition to TLR4, genetic variation in other TLRs has also been proposed to influence the risk of IA. For example, a regulatory variant decreasing the expression of TLR3 in dendritic cells was found to impair the recognition of fungal nucleic acids and to compromise the efficient priming of protective memory CD8+ T cell responses, thereby rendering HSCT recipients more prone to develop IA [11]. Ultimately, the evaluation of regulatory variation impacting adaptive immunity might help to enhance the discriminatory potential of recent immunodiagnostic strategies based on the evaluation of fungal-specific adaptive immune responses [12]. Of interest, damage perception is coupled with pathogen-sensing pathways (especially intracellular TLRs) to restrain inflammation in experimental aspergillosis [13]. Therefore, it is not surprising that genetic variants determining a hyperactivation in danger signaling axes, and presumably underlying exuberant inflammatory responses, were also found to increase the risk for IA [14]. Finally, other examples include genetic variants in *TLR1* and *TLR6* [15], and *TLR5* [16], but further studies are warranted to definitely assure their predictive potential for fungal disease.

Dectin-1 deficiency has consistently been reported to contribute to susceptibility to IA [17–19]. In particular, a stop codon polymorphism in Dectin-1 (*CLEC7A*) compromising the surface expression of the receptor in myeloid cells and downstream cytokine production in response to fungal infection [20] significantly increased the risk of IA in HSCT recipients [18]. The fact that Dectin-1 deficiency in both transplant counterparts synergized towards risk of IA highlighted for the first time the pivotal contribution of non-hematopoietic Dectin-1 in antifungal immunity. The prominent biological plausibility of this association suggests Dectin-1 as an attractive candidate not only in risk stratification measures but also in immunotherapeutic strategies aiming at countering the defective Dectin-1 function.

Numerous positive associations between genetic variants in cytokine and chemokine genes and risk of IA have also been reported [21–26]. Most of these initial reports were however largely underpowered and performed in poorly characterized cohorts, thereby precluding definite conclusions about genetic variants affecting cytokine production in the context of IA. There are however some exceptions that assume particular relevance given the robust study design and functional validation [22, 27]. Among the exceptions, a haplotype in C-X-C motif ligand 10 *(CXCL10)* was mechanistically correlated to a deficient expression of this chemokine in dendritic cells and, accordingly, high levels of CXCL10 were also more frequently observed among patients surviving IA compared to unaffected controls [22]. More recently, variants in the genes encoding for IL-1β and beta-defensin 1 (DEFB1) were reported to increase the risk of IA in solid organ transplant recipients, supposedly by impairing fungal-induced proinflammatory cytokine secretion by mononuclear cells [27].

Next-generation sequencing technologies now provide exciting possibilities to pin down essential steps in the host-fungus interaction at a level of complexity previously unanticipated. The first genome-wide association studies (GWAS) exploring host susceptibility to IA are underway and are expected to provide unbiased insights into the genetic defects contributing to development of IA. The plausibility of these approaches has been recently demonstrated in invasive fungal diseases, in which functional genomics analyses have allowed the identification of new important players controlling susceptibility to candidemia [28, 29]. Finally, genetic analysis of gene expression represents another powerful approach enabling insights into the human genomic landscape by generating expression maps that might be revealed extremely useful for the functional interpretation of noncoding variants likely to arise from ongoing genome-wide initiatives [30].

A number of alternative strategies using mouse models of infection as a starting point have also been employed to define candidate genes involved in susceptibility to IA [31, 32]. Genetic mapping analysis of survival data of animals subjected to experi-

mental infection led to the identification of a non-synonymous polymorphism in human plasminogen (*PLG*), a regulatory molecule with opsonic properties, as an important modulator of susceptibility to IA in humans [32]. Genetic deficiency of additional molecules with opsonic activity—e.g., mannose-binding lectin (MBL) [33] and the long pentraxin 3 (PTX3) [34]—has also been disclosed as a major determinant of susceptibility to IA, pointing to a key contribution of the innate humoral arm to an adequate activation of protective antifungal immune responses. This is corroborated by the validation of the association of a *PTX3* haplotype and increased risk of IA in two independent, high-powered genetic association studies [34, 35]. The involved haplotype was found to compromise the alveolar availability of PTX3 and, at a cellular level, its expression during the developmental programming of neutrophil precursors in the bone marrow, leading to defective antifungal effector mechanisms of newly reconstituted neutrophils [34]. Importantly, this association was also recently replicated in recipients of lung transplant [36], highlighting a potential applicability of these markers in predicting infection across patients with intrinsically different predisposing conditions. Of interest, the alveolar levels of PTX3 have been demonstrated to discriminate microbiologically confirmed pneumonia in mechanically ventilated patients [37]. Given that these levels vary individually according to host genotypes [34], we can envisage the quantification of PTX3 in bronchoalveolar lavage fluids as a complementary surveillance measure in addition to the currently available diagnostic approaches. Finally, the fact that exogenous administration of PTX3 is able to revert the genetic defect *in vitro* [34] further highlights the potential of PTX3-based immunotherapies to treat (or prevent) IA [38].

Although the overall weight of the antifungal immune response is certainly driven by adding effects of single genetic factors and their complex interactions with clinical immune dysfunctions, PTX3 represents the most robust genetic marker identified to date. These consistent findings are expected to lay the foundations for well-designed prospective trials ultimately endorsing PTX3-based genetic testing in risk stratification approaches for IA. In this chapter, we will use PTX3 as an example to describe protocols to assess genetic variation in *PTX3* associated with susceptibility to IA, and to evaluate the associated functional consequences in circulating neutrophils from patients at-risk.

2 Materials

Prepare all solutions using ultrapure water and cell-culture grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Carefully follow all waste disposal regulations when disposing of human waste materials.

2.1 DNA Isolation

1. Quick-gDNA™ MiniPrep Kit (Zymo Research).

2.2 Single Nucleotide Polymorphism (SNP) Genotyping

1. KASP™ Assay Mix for the rs2305619, rs3816527, and rs1840680 SNPs in *PTX3* and KASP™ Master Mix (LGC Genomics).

2.3 Neutrophil Isolation

1. Histopaque 1077 (Sigma-Aldrich).
2. NaCl 0.2, 0.9, and 1.6%.
3. Dextran T-500 from Alfa Aesar (cat #: J63702) 3% in 0.9% NaCl (dextran/saline solution).
4. PBS: NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.5 mM, pH 7.2–7.4, 0.2 μm filtered.

2.4 Neutrophil Lysis

1. Neutrophil lysis buffer: Tris–HCl 10 mM, EDTA 5 mM, NP40 1%, and 1× protease inhibitors.

2.5 ELISA for PTX3

1. ELISA to detect human PTX3, for example the Pentraxin 3/TSG-14 DuoSet ELISA (R&D systems).
2. PBS (prepared as above).
3. Wash buffer: Tween 20 0.05% in PBS, pH 7.2–7.4.
4. Reagent diluent: bovine serum albumin (BSA) 1% in PBS, pH 7.2–7.4 (stored at 4 °C).
5. Substrate solution: 1:1 mixture of color reagent A (H_2O_2) and color reagent B (tetramethylbenzidine).
6. Stop solution: H_2SO_4 2 N.

2.6 Assessment of Neutrophil Effector Functions

1. Live conidia from *Aspergillus fumigatus*.
2. Fluorescein isothiocyanate (FITC) (Sigma-Aldrich).
3. RPMI Medium 1640 (Thermo Fisher Scientific).
4. Sabouraud Dextrose agar (BD Difco™).

3 Methods

For the purpose of this chapter, genetic variants in *PTX3* are given as examples of common variants that have been described to influence the risk of IA; however, these can be replaced with other known and novel genetic markers. The methods addressing the functional consequences of these variants and described below have been verified in human circulating neutrophils.

3.1 Isolation of Genomic DNA from Peripheral Blood

Reagents used to isolate genomic DNA are part of the Quick-gDNA™ MiniPrep Kit. By using the innovative Zymo-Spin™ Column technology, this kit yields highly purified RNA-free DNA, bypassing the need for RNase A treatment and excluding the use

of proteinase K and organic denaturants, thereby ensuring high quality for sensitive downstream applications, including SNP analysis. In addition, all reagents are compatible with commonly used anticoagulants (i.e., EDTA, heparin, citrate) in blood drawing procedures. Additional equipment required includes a microcentrifuge and a vortex.

1. Add 400 μL of lysis buffer to 100 μL of whole blood (4:1). Mix thoroughly by vortexing and allow the mixture to rest for 5–10 min at room temperature (*see* **Note 1**).
2. Transfer the mixture to a Zymo-Spin™ column in a collection tube and centrifuge at 10,000 × *g* for 1 min.
3. Wash the column with 200 μL of DNA pre-wash buffer and centrifuge as before.
4. Add 500 μL of DNA wash buffer to the column and centrifuge as before.
5. Elute the DNA in 50 μL of elution buffer and collect the purified DNA in a clean microcentrifuge tube (*see* **Note 2**).
6. Determine the concentration of DNA in each sample by light spectrophotometry (e.g., NanoDrop) using the elution buffer as blank.

3.2 Genotyping of PTX3 SNPs

The Kompetitive Allele-Specific PCR (KASPar) chemistry (LGC Genomics) is hereafter used as an example of a commercial on-demand assay to perform genotyping. This kit contains a KASP™ Assay Mix that is specific to the SNPs under analysis and consists of two competitive, allele-specific forward primers and one common reverse primer. Each forward primer incorporates an additional tail sequence that corresponds with one of two universal fluorescent resonance energy transfer (FRET) cassettes (FAM and HEX) present in the KASP™ Master Mix. This mix also includes the ROX™ passive reference dye, Taq polymerase, free nucleotides, and $MgCl_2$ in an optimized buffer solution.

1. Vortex thoroughly all the components of the KASPar assay prior to use.
2. Dispense 5 μL of each DNA sample onto a qPCR-compatible 96-well plate (*see* **Note 3**). Make sure to include at least three non-template controls (NTC) containing ultrapure water.
3. Prepare a master mix containing 5 μL of 2× reaction mix and 0.14 μL of the primer mix for each sample to be analyzed.
4. Add 5 μL of the master mix to each DNA and NTC well.
5. Seal the 96-well plate carefully and run the qPCR in a compatible instrument according to the following thermal profile (*see* **Notes 4** and **5**).

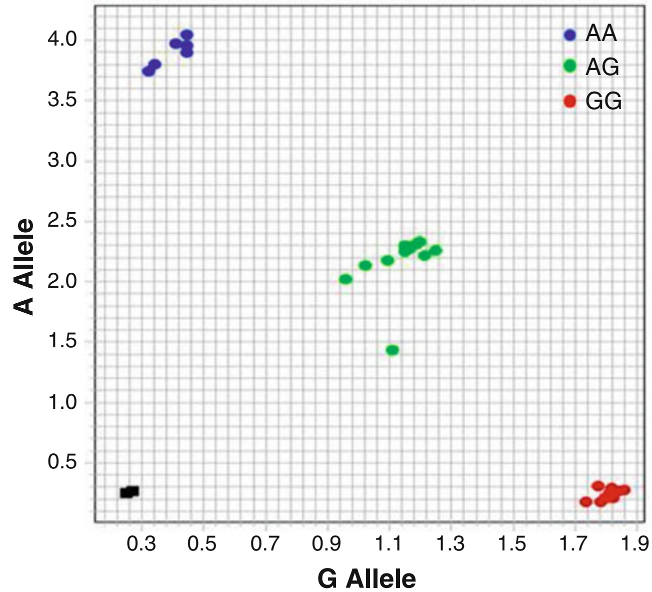

Fig. 1 Typical allelic discrimination plot obtained for the rs2305619 SNP in the *PTX3* gene. Genotyping was performed using KASPar chemistry in a 7500 Fast qPCR system (Applied Biosystems)

Holding stage	Cycling stage (10 cycles)		Cycling stage (32 cycles)		Post-PCR read (holding stage)
94 °C 15 min	94 °C 20 s	61 °C 60 s (−0.6 °C/cycle)	96 °C 20 s	55 °C 60 s	25 °C 60 s

6. Perform the allelic discrimination by reading fluorescence at 25 °C (Fig. 1).
7. In the event clear genotyping clusters are not observed, the plate should be thermally cycled further (three additional cycles of 20 s at 94 °C and 60 s at 57 °C) and read again at 25 °C.

3.3 Isolation of Circulating Neutrophils

1. Dilute the collected whole blood at a 1:4 proportion with 0.9% NaCl.
2. Layer 10 mL of Histopaque 1077 beneath the diluted whole blood using a pipette or a syringe (*see* **Note 6**).
3. Centrifuge at 400 × *g* for 40 min at 20 °C without brake (*see* **Note 7**).
4. Aspirate and discard the supernatant and resuspend the granulocyte/red blood cell (RBC) pellet in 20 mL of PBS.
5. Add an equal volume of dextran/saline solution, mix and incubate in the upright position for approximately 30 min at room temperature.

6. Aspirate and save the leukocyte-rich plasma (upper layer) using a pipette.
7. Pellet cells from the plasma by centrifuging at 250 × *g* for 10 min at 4 °C (*see* **Note 8**).
8. To remove residual RBC, subject cells to hypotonic lysis by resuspending the pellet in 20 mL of cold 0.2 % NaCl for exactly 30 s.
9. Restore isotonicity by adding 20 mL of ice-cold 1.6 % NaCl.
10. Centrifuge at 250 × *g* for 6 min at 4 °C and discard the supernatant. Repeat steps 8 and 9 until the cell pellet appears free of RBC.
11. Resuspend cells in ice-cold PBS and determine cell concentration by counting in a Neubauer chamber.

3.4 Preparation of Neutrophil Lysates

1. Transfer 10^6 neutrophils into an Eppendorf tube and remove the supernatant by centrifuging at 250 × *g* for 6 min at 4 °C.
2. Add 200 μL of lysis buffer and incubate 5 min on ice.
3. Collect the cell lysate by centrifugation at 12,000 × *g* for 15 min at 4 °C.
4. Store at −80 °C until the determination of intracellular PTX3 by ELISA.

3.5 Measurement of Intracellular PTX3 in Neutrophils by ELISA

All reagents from the ELISA kit for PTX3 should be brought to room temperature before use and the reconstituted components should be allowed to sit for at least 15 min with gentle agitation. Working dilutions should be prepared according to the manufacturer's instructions and used immediately.

1. Dilute the capture antibody to the working concentration using PBS and coat a 96-well microplate with 100 μL per well.
2. Seal the plate and incubate overnight at room temperature.
3. Wash each well three times with 400 μL of wash buffer (*see* **Note 9**).
4. Block the plate by adding 300 μL of reagent diluent to each well and incubate for 1 h at room temperature. Repeat the washing step as above.
5. Add 100 μL of sample (it may be necessary to dilute the samples prior use) or standards in reagent diluent per well. Cover the plate with an adhesive strip and incubate overnight at 4 °C.
6. Repeat the washing step as above.
7. Add 100 μL of the diluted detection antibody to each well and incubate for 2 h at room temperature.
8. Repeat the washing step as above.

9. Add 100 μL of the working dilution of streptavidin-horseradish peroxidase (HRP) to each well.
10. Seal the plate and incubate at room temperature for 20 min (*see* **Note 10**).
11. Repeat the washing step as above.
12. Add 100 μL of substrate solution to each well and incubate at room temperature for 20 min (*see* **Note 11**).
13. Add 50 μL of stop solution to each well and gently tap the plate to ensure thorough mixing.
14. Determine the optical density of each well immediately using a microplate reader set to 450 nm (*see* **Note 12**).

3.6 Measurement of Phagocytosis in Neutrophils

1. To estimate phagocytic activity, plate 5×10^5 freshly isolated neutrophils in 12 mm glass coverslips coated with serum proteins or purified fibrinogen in a 24-well plate.
2. Add live FITC-labeled conidia of *A. fumigatus* at an effector to fungal cell ratio of 1:5 in a final volume of 500 μL (*see* **Note 13**).
3. Incubate at 37 °C for 1 h in a humidified CO_2 culture chamber.
4. After incubation, remove the non-adherent conidia by washing carefully twice with PBS.
5. Add 300 μL of trypan blue (1 mg/mL in PBS) to each well for 15 min at 25 °C to quench the fluorescence of bound but uningested conidia.
6. Wash twice with PBS and fix the cells in 1 % paraformaldehyde for 15 min.
7. Mount coverslips with the cell side down onto microscope slides in 90 % glycerol in PBS, and seal with clear nail polish.
8. Quantify phagocytosis via phase contrast and fluorescence microscopy by counting at least 200 neutrophils per coverslip (*see* **Note 14**).

3.7 Evaluation of Neutrophil Fungicidal Activity

1. Incubate neutrophils with conidia of *A. fumigatus* at an effector to fungal cell ratio of 10:1 for 120 min in a final volume of 100 μL in 96-well flat-bottomed microtiter plates.
2. After incubation, freeze plates at −80 °C and rapidly thaw at 37 °C to lyse cells and harvest conidia.
3. Prepare serial dilutions (1:10) from each well in PBS (900 μL) and plate 100 μL onto Sabouraud dextrose agar plates.
4. Incubate plates at 28 °C for 48 h.
5. Count the number of colony-forming units (CFU) and calculate the percentage of CFU inhibition (referred to as the fungicidal activity) (*see* **Note 15**).

4 Notes

1. It is recommended to add β-mercaptoethanol to the genomic lysis buffer at a final dilution of 0.5 % (v/v) to improve lysis performance.
2. Ensure that the elution process occurs for at least 1 min to increase the DNA yield.
3. The use of at least 20 ng of DNA in each genotyping reaction is recommended. Use an identical DNA concentration for all samples in order to adequately normalize the fluorescence intensity.
4. The thermal profile presented is a typical example that has been specifically provided by the manufacturer with some minor modifications. Because of the underlying chemistry mechanisms of KASPar, the post-PCR read should always be performed below 40 °C.
5. An optimal cluster visualization is typically obtained using a 7500 Fast qPCR system (Applied Biosystems) and a post-PCR read at 25 °C.
6. Take care to preserve the interface between the Histopaque and the diluted blood solution, thereby avoiding contamination between both phases.
7. The temperature of 20 °C is crucial to achieve the best cell separation performance.
8. After separation, cells should be kept on ice at all times and handled gently to prevent damage or undesired activation of neutrophils.
9. Complete removal of liquid from the wells by inverting the plate and blotting it against clean paper towels is essential for an optimal performance of the ELISA test.
10. Avoid exposing the plate to light.
11. The length of incubation may vary depending on the concentration of PTX3. For that reason, check the plate every 5 min and stop the reaction earlier, if necessary.
12. If wavelength correction is available, set it to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm in order to correct for optical imperfections in the plate.
13. For the staining of live conidia of *A. fumigatus*, incubate 2×10^7 conidia in 2 mL of Na_2CO_3 50 mM buffer, pH 10.2 with FITC at a final concentration of 0.1 mg/mL (prepared in the same buffer) at 37 °C for 4 h. Wash the conidia thoroughly to eliminate residual FITC.

14. Enumerate the number of ingested conidia, or bound but not ingested. The data is typically presented as the percentage of neutrophils ingesting one or more conidia.
15. Calculate the inhibition of CFU—also known as fungicidal activity—using the following formula: % fungicidal activity = $(1-X/C) \times 100$ where X is the number of CFU obtained after infection of neutrophils, and C is the number of CFU in the absence of neutrophils. Perform three biological replicates for each condition and at least two technical replicates.

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Chapter 8

Assessment of Immune Responses to Fungal Infections: Identification and Characterization of Immune Cells in the Infected Tissue

Florian Sparber and Salomé LeibundGut-Landmann

Abstract

The immune system is important to protect the host from fungal infections. Diverse cell types belonging to the innate or adaptive branch of the immune system act in a tightly coordinated and tissue-specific manner. Experimental mouse models of fungal infections have proved essential for assessing the protective principles against different fungal pathogens. Besides pathological, histological, biochemical and molecular parameters, the analysis of phenotypic and functional aspects of immune cells in infected tissues is key for understanding the mechanisms of antifungal defense. In this chapter, we describe a method based on flow cytometry to assess innate and adaptive immune cells isolated from an *in vivo* context in a qualitative and quantitative manner.

Key words *Candida albicans*, Mouse models, Host defense, Innate and adaptive immunity, Mucosal tissue, Flow cytometry

1 Introduction

Fungi are abundant in the environment. They populate the soil, are found in the air we breathe, and they even live on our body surfaces. Some fungal species can cause diseases in humans and animals if they overgrow on body surfaces and/or gain access to the tissues where they usually do not reside. The mammalian immune system has evolved efficient defenses against fungi. Consistently, fungal diseases develop most frequently in immunocompromised individuals where these defenses are breached [1]. The host's armory against fungi comprises three main branches: epithelial barriers, innate immunity, and adaptive immunity. The epithelia covering all body surfaces including the skin, the gastrointestinal tract, the airways, and the female genital tract provide a tight physical barrier for invading microbes. In addition, they can sense the presence of potentially harmful pathogens and contribute actively to the induction of an inflammatory and antimicrobial

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response [2]. The innate immune system is composed of diverse populations of myeloid and lymphoid cells, which rapidly respond to an infectious insult. Neutrophils circulating in the blood stream enter the tissue and traffic to the site of infection to provide a critical first line of defense by phagocytosing and killing invading fungi, and by exerting extracellular fungistatic/fungicidal activities [3]. Mononuclear phagocytes have also emerged as important effector cells in innate antifungal immunity [4]. In addition, innate cells of the lymphoid lineage such as natural killer cells and innate lymphoid cells also contribute to host defense. The adaptive immune system is dedicated to provide long-term protection from fungi to which we are constantly exposed or to provide protection from reinfection. T helper cells secreting interferon-γ (IFN-γ) or Interleukin-17 (IL-17), so called Th1 and Th17 cells, play a critical role in antifungal immunity [5].

The mechanisms protecting from a particular fungal disease are determined by the fungal species involved as well as the tissue affected. Diverse barrier defects and/or immunodeficiencies predispose to different types of fungal infections. Defects in the epithelial barrier, which provide an entry port for fungi, are associated with an increased infection rate. Examples include the increased risk of fungal infections in burn patients [6] or the increased occurrence of *Malassezia* spp. infections in atopic individuals with barrier defects [7]. Neutropenia and defects in neutrophil function, such as those found in patients suffering from chronic granulomatous disease, are associated for example with systemic candidiasis, invasive aspergillosis, and other mould diseases [3]. Oropharyngeal candidiasis (OPC) as well as *Cryptococcus* and *Pneumocystis* airway infections on the other hand are a frequent consequence of T cell deficiencies such as those found in HIV$^+$ individuals/AIDS patients [8]. The recent identification of primary immunodeficiencies associated with isolated fungal infections contributed to the identification of novel and specific immune pathways critically involved in protection against certain types of fungal infections [9]. Such pathways are the IL-17 pathway associated with chronic mucocutaneous candidiasis (CMC) [10] or the CARD9-mediated signaling pathway associated with *Candida* infections of the central nervous system and deep-seated dermatophytosis [11, 12]. Studying these patients has greatly advanced the knowledge on host protection against different types of fungal diseases. In addition, animal models of local and systemic fungal infections that allow dissecting the cellular and molecular immune mechanisms *in vivo* proved instrumental for gaining a detailed understanding of tissue-specific host responses to fungi.

The successful defense of the mammalian host against pathogenic fungi is the result of a complex interplay between multiple different cell types that communicate via soluble molecules and cell contact-dependent interactions. Importantly, such cellular

interactions are tissue specific resulting in a high variability of antifungal immune mechanisms depending on the site of infection within the host. Experimental fungal infections in mice allow investigating the dynamics of immune responses and evaluating the outcome of fungus-mediated pathologies in the mammalian host. Tissues, molecules and cells can be analyzed with high resolution. Individual cells can be isolated from the infected tissues and analyzed on a per cell basis for their phenotypic and functional changes in response to the infectious insult.

In this chapter, we describe a procedure based on flow cytometry to analyze innate and adaptive immune cells isolated from the oral mucosa and draining lymph nodes of *Candida*-infected mice. Sublingual infection of mice with *C. albicans* is a model for mucocutaneous candidiasis in humans [13]. In this model, as in the human disease, IL-17 is critical for fungal control [10]. The epithelium as well as tissue-resident immune cells including dendritic cell (DC) subsets and cells of lymphoid origin coordinate the response [14–16]. Neutrophils and inflammatory monocytes rapidly infiltrate the infected tissue upon infection [14]. The inflammatory response peaks around day 1 post-infection [14]. The adaptive immune response is characterized by *C. albicans*-specific Th17 cells that are primed in cervical lymph nodes within 5–7 days post-infection through the action of DCs that migrate from the infected tissue to present fungal antigens to antigen-specific $CD4^+$ T cells in the lymph nodes [17].

2 Materials

2.1 Animals and Cells

1. C57BL/6 mice infected with *C. albicans* strain SC5314 [13] (optional). Of note, animals are not treated with immunosuppressive agents such as corticosteroids as they affect the trafficking and responsiveness of immune cells, which is not reconcilable with the aim of determining the natural immune response to *C. albicans* in the oral mucosa.
2. The DC line DC^{1940} [18] is grown in IMDM medium complete (see below).
3. Heat-killed (hk) *C. albicans* strain SC5314 for restimulation of T cells: Aliquot 10^8 *C. albicans* cells (from an overnight culture) in a 1.5 ml tube. Centrifuge for 1 min at $10{,}000 \times g$ to collect the yeast cells. Wash three times with 1× PBS. After the last wash, resuspend in 1 ml of 1× PBS. Incubate for 30–45 min in the boiling water bath. Let the suspension cool down, collect the cells by centrifugation, and resuspend in 1 ml of RPMI medium complete (see below). Adjust the concentration to 2.5×10^6 yeast cells/ml. Store aliquots at 4 °C.

4. Optional: *C. albicans*-derived antigenic peptides $pALS_{235-253}$ (sequence KGLNDWNYPVSSESFSYT) or $pADH1_{126-140}$ (sequence GSFEQYATADAVQAA) (e.g., from EMC microcollection). Prepare a stock of 500 ng/ml in RPMI medium complete. Store aliquots at −20 °C.

2.2 Anesthetics

1. Sublethal anesthetic mix: 9.34 mg/ml Ketamin 100 (Graeub), 2.17 mg/ml Rompun (Bayer), 280 μg/ml Prequillan (Agrovet AG) in 1× PBS.

2.3 Media, Buffers and Other Reagents

1. 1× PBS (e.g. BioConcept, Cat. 3-05F39-I).
2. Decomplemented fetal calf serum (FCS): Incubate FCS (e.g. PAA) at 56 °C for 20 min. Store aliquots at −20 °C.
3. Wash buffer: 2 mM EDTA (e.g. Ambion), 1% decomplemented FCS in 1× PBS. Store at 4 °C.
4. FACS buffer: 5 mM EDTA (e.g. Ambion), 1% decomplemented FCS, 0.05% Na_2N_2 (e.g. PAA) in 1× PBS. Store at 4 °C.
5. Percoll 40%: Prepare 90% Percoll/PBS by mixing 9 parts Percoll (e.g. Sigma-Aldrich) and 1 part 10× PBS (e.g. AppliChem). Dilute 90% Percoll/PBS to 40% Percoll/PBS solution using 1× PBS.
6. Digestion mix for tongue tissue: 4.8 mg/ml Collagenase IV (e.g. Thermo Fisher Scientific) and 200 μg/ml DNase I (e.g. Roche Diagnostics) in 1× PBS. Use 1 ml for each tongue sample.
7. Digestion mix for lymph nodes: 2.4 mg/ml Collagenase I (e.g. Thermo Fisher Scientific) and 200 μg/ml DNase I (e.g. Roche Diagnostics) in 1× PBS. Use 250 μl for each sample.
8. RPMI medium complete: RPMI1640 medium (e.g. PAA) supplemented with 10% decomplemented FCS, 100 IU/ml Penicillin, 100 μg/ml Streptomycin (e.g. Amimed), 2 mM L-Glutamine (e.g. PAA), 50 μM 2-Mercaptoethanol (e.g. Thermo Fisher Scientific). Store at 4 °C.
9. IMDM medium complete: IMDM Medium (e.g. Thermo Fisher Scientific) supplemented with 10% decomplemented FCS, 100 IU/ml Penicillin, 100 μg/ml Streptomycin, 2 mM l-Glutamine, 50 μM 2-Mercaptoethanol. Store at 4 °C.
10. 70% EtOH: prepare by mixing 7 parts EtOH abs. (e.g. Sigma-Aldrich) with 3 parts distilled water.

2.4 Reagents for Flow Cytometry

1. BD Cytofix/Cytoperm™ Fixation and Permeabilization Solution (BD Bioscience).
2. BD Perm/Wash™ buffer (BD Biosciences): dilute 1:10 in distilled H_2O prior to use.

3. Brefeldin A (e.g. Axon Lab AG). Prepare a stock solution of 5 mg/ml in EtOH abs. (Sigma-Aldrich). Store at −20 °C.
4. Fluorescence-labeled antibodies:
   Anti-mouse CD45 PE-Cy5 (clone 30-F11, e.g. BioLegend).
   Anti-mouse CD45.2 APC (clone 104, e.g. BioLegend).
   Anti-mouse CD11b PE-Cy7 (clone M1/70, e.g. BioLegend).
   Anti-mouse Ly6G Pacific Blue (clone 1A8, e.g. BioLegend).
   Anti-mouse Ly6C FITC (clone AL-21, BD Biosciences).
   Anti-mouse CCR2 PE (clone 475301, R&D Systems).
   Anti-mouse CD4 Pacific Blue (clone RM4-5, e.g. BioLegend).
   Anti-mouse CD90.2 Brilliant Violet 570 (clone 30H12, e.g. BioLegend).
   Anti-mouse IL-17A PE-Cy7 (clone TC11-18H10, e.g. BioLegend).
   Anti-mouse INFγ APC (clone XMG1.2, e.g. BioLegend).
   Anti-mouse CD32/CD16 (clone 2.4G2, BD Biosciences).
5. LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit (Molecular Probes).
6. BD CompBeads™ (BD Biosciences).
7. BD Calibrite Beads™ (BD Biosciences).

2.5 Supplies

1. Tabletop centrifuge with swinging-out rotor for 5 ml round-bottom tubes and for 15 ml and 50 ml conical tubes.
2. Heated water bath with temperature control.
3. 1 and 10 ml syringes with needles (23G, e.g. Braun).
4. Scissors and forceps for organ removal.
5. Conical polypropylene tubes (1.5, 15, and 50 ml, e.g., Trefflab; TPP).
6. 5 ml round-bottom polystyrene tubes (BD Falcon).
7. Round-bottom 96-well cell culture plates (e.g. , TPP).
8. Cell strainers 70 μm (BD Biosciences).
9. Flow cytometer and acquisition software (e.g., BD Instruments LSR II and BD FACSDIVA™).
10. Analysis software for flow cytometric data (e.g., FlowJo, Tristar).

3 Methods

Flow cytometry is a widely employed method for simultaneously obtaining qualitative and quantitative information on various cellular processes, including expression of surface markers, intracellular cytokines, and signaling proteins, or cell cycle analyses. It measures these characteristics on each cell individually in a high-throughput fashion and excels in characterizing heterogeneous cell populations. As flow cytometry is limited to analysis of cells in

suspension the preparation of the sample may require harsh enzymatic and physical treatments to isolate the cells from the tissue. For complex tissues, which are rich in extracellular matrix (ECM), isolating all leukocyte populations efficiently and with a good viability remains challenging.

This chapter describes the isolation and preparation of leukocytes from the infected tongue tissue and from the draining cervical lymph nodes for flow cytometric analysis. We focus on the separation and quantification of immune cells based on their expression of surface molecules and, in case of T cells on cytokine production. The proposed protocols can be extended to the analysis of intracellular signaling, effector molecules and cell proliferation. They can also be adapted for sorting cells from infected tissues for functional assay or molecular analyses. In this chapter, we also describe how flow cytometric data should be analyzed to limit false-positive events and artifacts, such as those caused by autofluorescent dead cells.

3.1 Isolation and Analysis of Leukocytes from the Infected Tissue (See Fig. 1)

This section describes the procedure to characterize immune cells, which were isolated from the oral mucosa of *C. albicans*-infected mice by means of flow cytometry. The analysis is focused on the tongue, which is easily accessible and contains a higher overall number of leukocytes compared to other parts of the oral mucosa. Of note, naïve mice harbor a relative low number of leukocytes in the tongue. Oral infection with *C. albicans* results in the recruitment and proliferation of immune cells and to a vast increase in the overall number of leukocytes in the mucosal tissue.

3.1.1 Perfusion and Tissue Removal

1. Anesthetize the animal by injection of 250 μl sublethal anesthetic mix intraperitoneally per 20–25 g weighed mouse. Adjust the injection volume for heavier mice accordingly. Check the reflexes to make sure the animal is fully anesthetized by pinching the rear foot with forceps before proceeding to the next step (*see* **Note 1**).
2. Douse the abdomen of the mouse with 70% EtOH. Remove the skin from the abdominal region. Open the thorax and remove a part of the rib cage to gain access to the heart without damaging any organ.
3. Cut the vena cava open. Hold the heart at the apex with a pair of straight forceps. With the other hand, slowly inject 10 ml of PBS into the right ventricle. The blood and PBS will flow out the open vena cava, while the lung will turn white and the liver pale brown. At the end, cut off the heart to avoid blood flowing back into the organs (*see* **Note 2**).
4. Excise the tongue using scissors and forceps and place it into a pre-labeled 1.5 ml tube filled with 0.5 ml of PBS. Keep the tube on ice until processing (*see* **Note 3**).

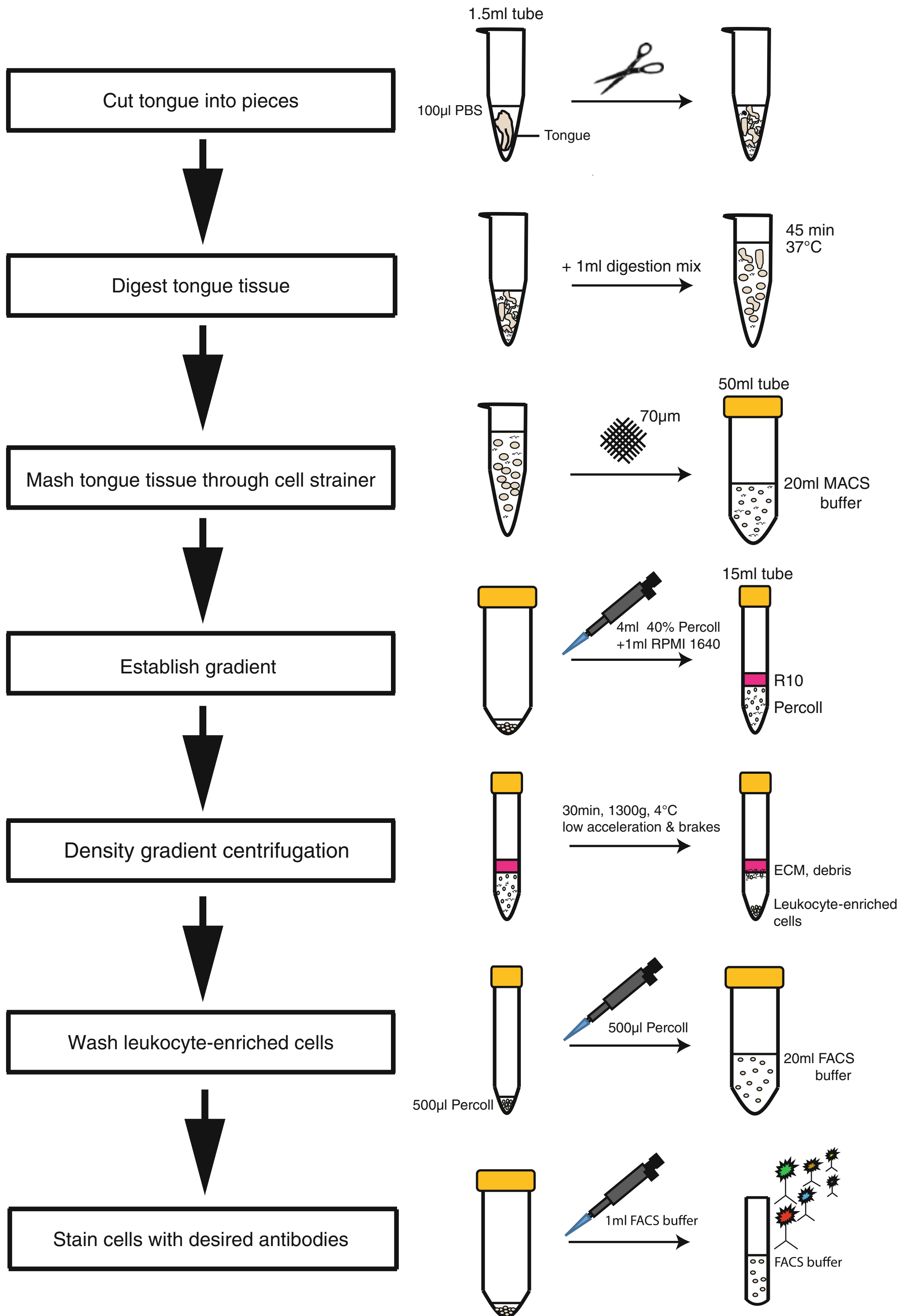

Fig. 1 Workflow for the isolation, enrichment, and analysis of leukocytes from infected tongue tissue

3.1.2 Preparation of a Cell Suspension

1. For processing the tongue, decant the PBS by pushing the tongue to the bottom of the 1.5 ml tube with the tip of a scissor and reversing the tube. A small volume of PBS (approx. 100 μl) will remain in the tube, together with the tissue.
2. Cut the tongue into small pieces using scissors (*see* **Note 4**).
3. Add 1 ml of digestion mix to the tube and mix the suspension well by inverting the tube several times (*see* **Note 5**).
4. Incubate for 45 min in the water bath at 37 °C. Invert the tube from time to time during the incubation period.
5. Pass the crude tissue suspension through a 70 μm cell strainer placed on a 50 ml tube, using the plunger of a 1 ml syringe. Rinse the strainer twice with 10 ml of wash buffer (*see* **Note 6**).
6. Centrifuge the 20 ml cell suspension for 10 min at 300 × *g*, 4 °C, to collect the cells (*see* **Note 7**).

3.1.3 Enrichment of Leukocytes Using Percoll Density Gradient Centrifugation

1. Decant the supernatant, resuspend the cell pellet in 4 ml of 40% Percoll/PBS solution, and transfer in a 15 ml conical tube. Carefully overlay with 1 ml of RPMI 1640 medium.
2. Centrifuge for 30 min at 1300 × *g*, 4 °C, with slow acceleration and no brakes (*see* **Note 8**).
3. Remove the upper phase using a 10 ml pipette leaving approximately 500 μl of Percoll with the cell pellet in the tube.
4. Resuspend the cell pellet in the 500 μl Percoll solution using a 1 ml pipette and transfer the entire suspension to a new 50 ml conical tube prefilled with 20 ml of cold FACS buffer.
5. Centrifuge the cell suspension for 10 min at 300 × *g*, 4 °C, to collect the cells.
6. Decant the supernatant, resuspend the cells in 1 ml of cold FACS buffer and transfer to a 5 ml round-bottom tube. Keep on ice.

3.1.4 Antibody Staining for Flow Cytometric Analysis

1. Centrifuge the cells for 5 min at 300 × *g*, 4 °C. Decant the supernatant.
2. Resuspend the cells in ≤1 μg anti-mouse CD32/CD16 in 50 μl of FACS buffer and incubate for 10 min at 4 °C.
3. Add 50 μl of antibody mix. The antibody mix contains all antibodies for cell surface staining (CD45.2, CD11b, Ly6C, Ly6G, CCR2, CD90.2; optimal amount is ~0.1–0.5 μg of each antibody/10^6 cells; the optimal concentration of each antibody should be determined experimentally) and the LIVE/DEAD® Fixable Near-IR Dead Cell Stain Dye (final dilution 1:1000) in FACS buffer.
4. Incubate for 30 min at 4 °C (*see* **Note 9**).

5. Wash off excess antibody by adding 4 ml of FACS buffer to each tube.
6. Centrifuge for 5 min at $300\times g$, 4 °C, decant supernatant.
7. Resuspend the cells in 200 μl FACS buffer.
8. Optional: Add a defined number of BD Calibrite Beads™ to each sample (ideally 2000–10,000 beads per sample).
9. Stain BD CompBeads™ with each antibody separately for compensation of fluorescence spectra (*see* **Note 10**).
10. Proceed to FACS data acquisition according to standard procedures.

3.1.5 FACS Data Analysis of Tongue-Derived Leukocytes (See Fig. 2)

Step 1. Plot the acquired events using FSC-Area (FSC-A) versus SSC-Area (SSC-A) and set a gate to exclude debris and ECM. Set a separate gate for the BD CompBeads™ (indicated with “*” in the figure).

Step 2. Exclude cell doublets using the FSC-A and FSC-Heigth (FSC-H) parameters (*see* **Note 11**).

Step 3. Gate on viable leukocytes based on their expression of CD45 and the exclusion of the LIVE/DEAD dye (*see* **Note 12**).

Step 4. Lymphocytes can be identified on the basis of CD90 expression (gate **P1**), myeloid cells are defined as CD11b$^+$ cells. A third population of CD90$^-$CD11b$^-$ cells can also be distinguished.

Step 5. CD11b$^+$ myeloid cells can be further divided into Ly6C^{high}Ly6G$^-$ inflammatory monocytes (**P2**) and Ly6C$^+$Ly6G$^+$ neutrophils (**P3**). The remaining population of Ly6C$^{low/-}$ cells comprises CD11b$^+$ DCs, macrophages and Ly6C^{low}NK cells.

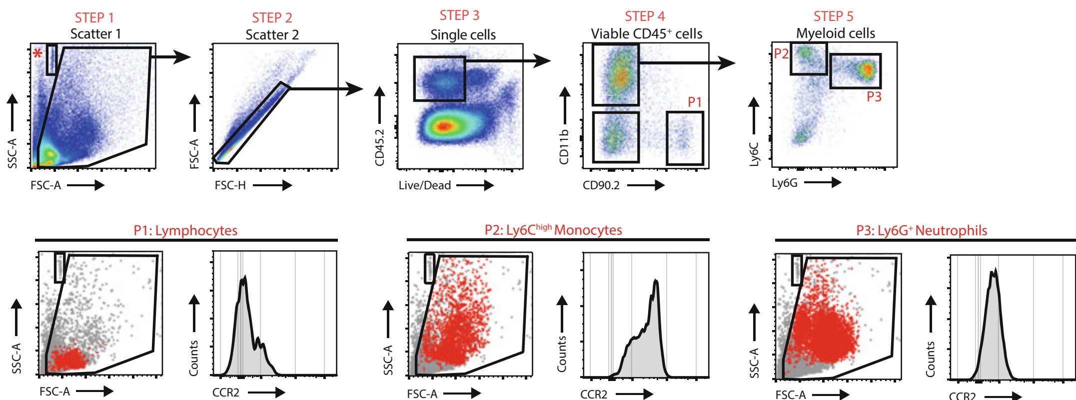

Fig. 2 FACS data analysis of leukocytes in the infected tongue tissue

Step 6. Overlaying populations **P1**, **P2**, or **P3** on the FSC-A/SSC-A profile confirms the high granularity of neutrophils and the smaller size and lower granularity of lymphoid cells, respectively. In addition, Ly6C^{high} inflammatory monocytes express high levels of the chemokine receptor CCR2, while neutrophils are low and the majority of lymphoid cells are negative for CCR2 expression.

Step 7. Absolute numbers of cells in any population of interest for a given sample can be calculated using the following formula: (number of events in the population of interest that were acquired and recorded for the sample) × (total number of BD CompBeads added to the sample)/(number of BD CompBeads that were acquired and recorded for the sample).

3.2 Analysis of *C. albicans*-Specific T Cell Responses During OPC

This section describes a method to analyze effector T cells in mice that were infected sublingually with *C. albicans*. It consists of the isolation of T cells from cervical lymph nodes and their restimulation with *C. albicans* to trigger cytokine secretion by activated fungus-specific T cells, which can then be examined by flow cytometry. Restimulation is done with hk *C. albicans* cells or with antigenic peptides to analyze the response of polyclonal or monoclonal T cell populations, respectively.

3.2.1 Preparation of Antigen Presenting Cells for T Cell Restimulation

1. The DC cell line DC^{1940} is maintained in IMDM medium complete at 10^5–10^6 cells/ml, 37 °C, 5 % CO_2. Passage the cells every 2–3 days and add fresh medium.
2. One day prior analysis of T cells from infected mice, seed 7×10^4 DC^{1940} cells per well in 100 μl IMDM medium complete in a round-bottom 96-well plate. Prepare 6 wells per sample: the restimulation of T cells with antigen-loaded and unpulsed DC^{1940} cells is carried out in triplicates (e.g. , for analyzing the T cell response of 3 infected and 3 naïve mice, prepare 36 wells of DC^{1940} cells).
3. Incubate cells overnight at 37 °C, 5 % CO_2 (*see* **Notes 13** and **14**).

3.2.2 Isolation of Cervical Lymph Node Cells from Infected and Naïve Control Mice

1. Euthanize mice by an approved protocol and spray the animal surface with 70 % ethanol (*see* **Note 15**).
2. Collect the cervical lymph nodes and transfer them in a 1.5 ml tube containing 250 μl wash buffer (1 tube per mouse).
3. Add 250 μl of digestion mix to the tube.
4. Incubate for 30 min at 37 °C (e.g. , in the water bath).
5. Pass the cell suspension through a 70 μm cell strainer placed on a 50 ml tube, using the plunger of a 1 ml syringe. Rinse the strainer once with 10 ml of wash buffer.

6. Centrifuge the cell suspension for 5 min at 300 × *g*, 4 °C, to collect the cells.
7. Count the number of viable cells (e.g. , by trypan blue dye exclusion) and resuspend them in RPMI medium complete at a concentration of 10^7 cells/ml.

3.2.3 T Cell Restimulation

1. Add 20 μl hk *C. albicans* (from Subheading 2.1, **step 3**) to half of the wells seeded with DC^{1940} cells (5×10^4 yeast cells/well). Add 20 μl RPMI medium complete to the remaining wells (unpulsed controls). Alternatively, DC^{1940} cells can be pulsed with antigenic peptides, e.g. , $pALS_{235\text{-}253}$ or $pADH1_{126\text{-}140}$ (100 ng/ml final).
2. Incubate for 2 h at 37 °C, 5 % CO_2.
3. Add 100 μl of cervical lymph node cells (from Subheading 3.2.2, **step 7**) to all the wells (*C. albicans*-pulsed and unpulsed DC^{1940} cells).
4. Incubate for 1.5 h at 37 °C, 5 % CO_2.
5. Dilute Brefeldin A stock solution in R10 complete medium to 110 μg/ml (1:45) and add 20 μl to each well (10 μg/ml final).
6. Incubate for 5 h at 37 °C, 5 % CO_2.
7. Harvest and transfer the cells from each well to a 5 ml round-bottom tube.
8. Add 1 ml of cold FACS buffer to each tube. Proceed to the next section immediately.

3.2.4 Antibody Staining for Flow Cytometric Analysis

1. Centrifuge the cells for 5 min at 300 × *g*, 4 °C. Decant the supernatant.
2. Resuspend the cells in ≤1 μg anti-mouse CD32/CD16 in 50 μl of FACS buffer and incubate for 10 min at 4 °C.
3. Add 50 μl of antibody mix. The antibody mix contains all antibodies for cell surface staining (CD45.2, CD4, CD90.2; optimal amount is ~0.1–0.5 μg of each antibody/10^6 cells; the optimal concentration of each antibody should be determined experimentally) and the LIVE/DEAD® Fixable Near-IR Dead Cell Stain Dye (final dilution 1:1000) in FACS buffer (*see* **Note 16**).
4. Incubate for 30 min at 4 °C (*see* **Note 9**).
5. Wash off excess antibody by adding 4 ml of FACS buffer to each tube.
6. Centrifuge for 5 min at 300 × *g*, 4 °C, decant supernatant.
7. Resuspend cells in 100 μl of BD Cytofix/Cytoperm solution and incubate cells for 20 min at 4 °C.
8. Wash cells by adding 1 ml of 1× BD Perm/Wash buffer.

9. Centrifuge cells for 5 min at 300 × *g*, 4 °C. Decant the supernatant.
10. Add 100 μl of an antibody mix containing IL-17 and IFN-γ antibodies (optimal amount is ~2 μg of each antibody/10^6 cells) in 1× BD Perm/Wash buffer to each sample. Incubate for 30 min at 4 °C.
11. Wash cells by adding 1 ml of 1× BD Perm/Wash buffer.
12. Centrifuge cells for 5 min at 300 × *g*, 4 °C and resuspend cells in 200 μl of FACS buffer.
13. Optional: Add a defined number of BD Calibrite Beads™ to each sample (ideally 2000–10,000 per sample).
14. Stain BD CompBeads with each antibody separately for compensation of fluorescence spectra (*see* **Note 10**).
15. Proceed to FACS data acquisition according to standard procedures.

3.2.5 Flow Cytometry Data Analysis of Candida-Specific T Lymphocytes (See Fig. 3)

Step 1. Plot the acquired events using FSC-A versus SSC-A and set a gate for lymphoid of cells (SSC-A low, FSC-A intermediate). Set a separate gate for the BD CompBeads (indicated with "*" in Fig. 3).

Step 2. Gate on single cells using FSC-A and FSC-H (*see* **Note 11**).

Step 3. Gate on viable leukocytes based on their expression of CD45 and the exclusion of the LIVE/DEAD marker (*see* **Note 12**).

Step 4. T helper cells can be identified on the basis of CD90 and CD4 expression.

Step 5. Display IL-17- and IFN-γ-positive events. Set the gates according to the unpulsed control sample.

Step 6. Absolute numbers of cells in any given population of interest for any sample can be calculated using the following formula: (number of events in the population of interest that were acquired and recorded for the sample) × (total number of BD CompBeads added to the sample)/(number of BD CompBeads™ that were acquired and recorded for the sample).

3.3 Further Options

- Identified cell populations may be further subdivided by assessing additional cell surface markers.
- Identified cell populations of interest may be isolated by cell sorting for functional studies.

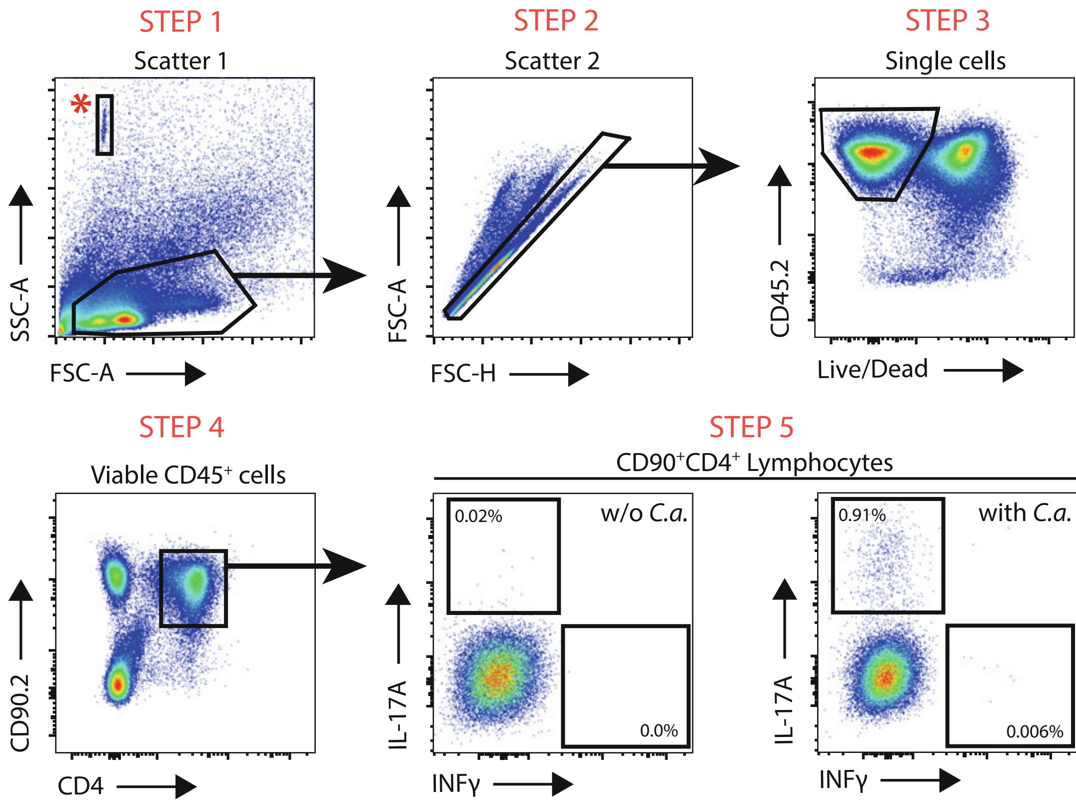

Fig. 3 Flow cytometry data analysis of cytokine-producing T lymphocytes

- Identified cell populations may be phenotypically and functionally characterized by including additional antibodies for surface molecules (e.g. , cell activation markers) and antibodies or reagents for visualizing intracellular molecules (e.g. , transcription factors, effector molecules, and posttranslational modifications of signaling molecules).
- The expression of a gene of interest may be tracked by using reporter mice expressing a reporter molecule (e.g. , green fluorescent protein) under the control of the promoter of the gene of interest.
- Comparative analyses may be performed with genetically modified mice (e.g. , gene knockouts).
- The analysis of immune cells in the mouse tongue described in Subheading 3.1 may be expanded to the buccal and sublingual mucosal tissues [19]. The cellular composition of these latter tissues slightly differ from the tongue. They are more difficult to prepare and yield a smaller number of leukocytes per mouse.

4 Notes

1. The duration to reach anesthesia may take 5–10 min, depending on the animal's body weight and the amount of anesthetic mix injected.
2. Perfusion of the animal is necessary for analyzing tissue-resident leukocytes. Without perfusion, the organ cell populations will be contaminated by blood cells that circulate through the organ's vasculature at the time point of analysis.
3. Collect as much of the organs as possible by cutting the tongue at its very posterior end, close to the pharyngeal region.
4. Cutting the tongue in very fine pieces will improve the efficiency of the enzymatic digestion.
5. The enzymatic digestion with collagenase and DNase is necessary to obtain a single cell suspension from organs rich in ECM. However, treatment with these enzymes can degrade cell surface antigens. It is thus recommended to check that the antibody epitopes for the cell surface antigens of interest are preserved. If necessary, the digestion protocol should be adjusted.
6. If several samples are handled in parallel, they should be kept on ice until they are processed one by one.
7. The supernatant may appear cloudy due to the high ECM content of the tongue.
8. The complex cellular composition of the tongue, which encompasses large numbers of muscle, epithelial and endothelial cells but comparably few leukocytes, even at the peak of the inflammatory response during OPC, necessitates an enrichment step to facilitate the identification of individual leukocyte populations. After density centrifugation with Percoll, leukocytes are enriched in the cell pellet, while ECM and the majority of the non-hematopoietic cells reside in the interface between the Percoll and the medium. The increased frequency of leukocytes after density centrifugation is accompanied by a loss of absolute leukocyte numbers per sample, hampering their absolute quantification.
9. Samples are generally kept at 4 °C during antibody staining to prevent unspecific binding of antibodies to cells. However, if particular antibodies are included in the staining panel, which require longer incubation periods and/or different incubation temperatures (e.g. , room temperature or 37 °C), separate staining steps for these antibodies should be included in the protocol.
10. The antibodies used to prepare the compensation controls using the BD CompBeads should be the very same ones as those used for staining the test samples of a given experiment to ensure the most accurate fluorescence compensation and calibration of the cytometer for each experiment.

11. Excluding cell doublets can also be achieved by plotting SSC-W versus SSC-H.
12. Exclusion of dead cells, which are enriched in tissue samples after enzymatic digestion and density centrifugation, is essential to avoid false positive signals due to unspecific antibody binding to dead cells.
13. Alternative sources of DCs such as bone marrow-derived DCs may be used instead of the DC line DC^{1940} for restimulating T cells.
14. The DC^{1940} cells may also be seeded on the day of the experiment at a concentration of 1×10^5 cells/well. The cells should rest at least 1 h after seeding before pulsing with hk *C. albicans* or *C. albicans*-derived peptides.
15. *Candida*-specific effector T cells can be found as early as day 4 post-infection in the cervical lymph nodes of infected animals. Frequencies increase until day 10 post-infection.
16. DC^{1940} cells express green fluorescent protein (GFP). FITC-conjugated antibodies should thus not be used when analyzing samples containing DC^{1940} cells.

Acknowledgments

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Part III

Screening Approaches to Fungal Pathogen Detection

Chapter 9

Histopathology

Renate Kain

Abstract

The detection of fungal elements and their characterization in patient specimens provides fundamental information. On histological sections fungi are most frequently seen on skin or mucosal surfaces or as mycotic thrombi or emboli that can occlude both arteries and veins in surgical specimen from immunocompromised patients or tissues obtained from autopsies. Microbial culture continues to be the central method for diagnosing fungal infection but is complemented by histomorphology using specific stains capable of identifying previously unsuspected fungal infections or for evaluating tissue invasion. These stains employ oxidizing reagents to create aldehyde binding sites on polysaccharides (1,2-glycol groups) of fungal cell walls for either Schiff's reagent or Silver ions. Gomori methenamine silver (GMS) and Periodic acid-Schiff (PAS) or their modifications are the most commonly used for tissue sections and in cytology specimens.

Key words Histochemistry, Schiffs' reaction, Silver stain, PAS stain

1 Introduction

On histological sections fungi are most frequently seen on skin or mucosal surfaces, especially in immunocompromised individuals. *Candida albicans* is part of the normal flora in the mouth. However, fungal invasion with ulceration and inflammation (Fig. 1a, b) can be provoked by disruption of the mucosal protective barrier or antibiotic treatment that suppresses the normal bacterial flora with resulting microbial dysbisosis [1]. Mycotic thrombi and subsequent emboli can occlude both arteries and veins (Fig. 2a–c), for example in the lung, and are often seen in surgical specimen or tissues obtained from autopsies of individuals with systemic fungal infections that are often fatal. Under these circumstances, fungi can be difficult to detect in the Hematoxilin and Eosin stain commonly used for routine histopathological examination (Figs. 1a and 2a) and can be confused with cellular debris or fibrin. Thus, special stains designed specifically to detect fungi should be performed when suspected morphologically or indeed on samples

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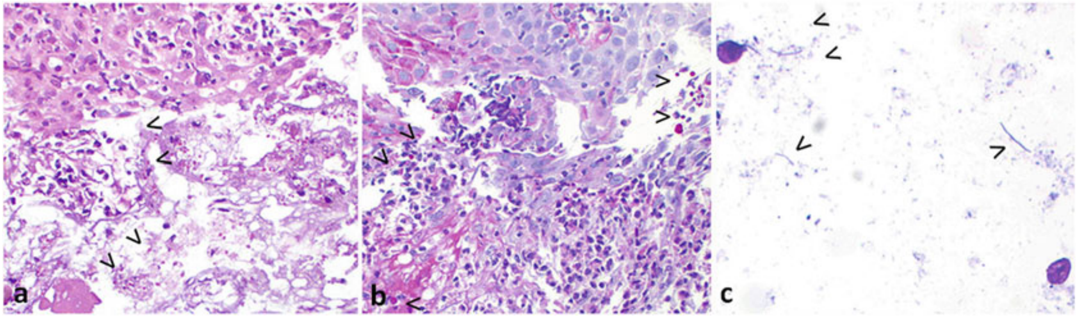

Fig. 1 Histology of an esophageal biopsy with marked granulocytic inflammation and necrosis of the epithelium (**a**). Fungal elements are suspected in H&E (*arrows*) and become clearly visible by PAS stain (**b**; *arrows*). Diagnosis is confirmed by brush cytology and Giemsa stain (**c**) that can also be used for microbial culture

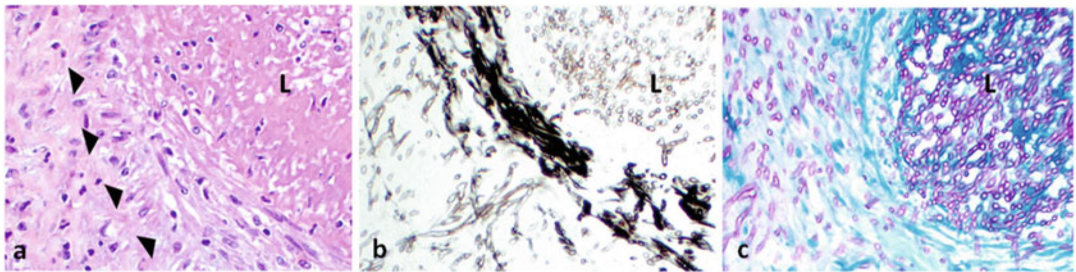

Fig. 2 Histology of a pulmonary artery on serial sections (**a**–**c**) in which the lumen (L) is filled with amorphous material that cannot be further specified by H&E staining alone (**a**). Fungal hyphae are clearly visible and destroy the vessel wall (*arrows*) by GMS (**b**, without counterstain) and PAS (**c**, Light Green counterstain). PCR amplification from paraffin sections confirmed presence of Aspergillus fumigatus

from immunocompromised patients in general (Figs. 1b and 2b, c). Fungi seen in histological sections are often fairly large and can be seen as hyphae, budding yeast, endosporulating spherules, or a combination of these forms. Their walls are rich in polysaccharides (1,2-glycol groups) which can be oxidized (by periodic acid or chromic acid) to create aldehyde binding sites for the Schiff or silver ions using Schiff's reagent or hexamine (methenamine) silver solutions [2].

The most frequently used special stain in routine histopathological examination is periodic acid-Schiff (PAS), originally described by Hotchkiss and McManus [3–5] (Fig. 1b). It is relatively simple, reliable and employs periodic acid to form dialdehydes which combine with Schiff's reagent to form an insoluble magenta compound. It stains most fungal organisms with the exception of *Histoplasma capsulatum* [6]. PAS is also used to detect neutral mucopolysaccharides, epithelial mucins, basement membranes and glycogen in tissue sections. For fungal staining PAS is often used after diastase (salivary amylase) pretreatment which removes glycogen and minimizes staining of host structures.

Another way to enhance visualization of fungi in section using PAS is a modification that utilizes Light Green instead of, or in addition to hematoxylin as nuclear counterstain (Fig. 2c).

In some protocols periodic acid is replaced by chromic acid which is routinely employed to oxidize the carbohydrates of fungal walls in the Grocott-Gömöri methenamine silver (GMS) stain to form aldehyde groups. These aldehydes will selectively react with silver nitrate in the hexamine-silver salt mixture (methenamine silver), reducing the silver ions to visible metallic silver (Fig. 2b). Light Green is commonly used; however, nuclear fast red can also be employed as a counterstain to demonstrate black-brown fungi against a pale green or light pink background [7, 8]. GMS is widely used in pathological practice however sometimes challenging, even in experienced hands, to avoid understaining or overstaining with formation of precipitates or high background. Therefore, both PAS and GMS are best used to demonstrate presence of fungi.

Brush biopsies taken from mucosal surfaces can be used for both, microbial culture and cytological examination. This method provides a fast confirmation of suspected fungal infection since the fungal hyphae can be detected in routinely used stains like Giemsa (Fig. 1c).

2 Materials

Unless otherwise specified prepare and store all solutions at room temperature using double distilled water (ddH_2O).

Generally, the hazard information mentioned below applies to reagents in concentrated form. In some instances, where concentrations are very low such as in buffers, good laboratory practice, such as avoiding spillages and splashes may be adequate. If in doubt refer to the Manufacturers' Safety Data Sheets. Follow appropriate waste disposal regulations when disposing waste material.

Glassware: use acid cleaned glassware (*see* **Note 1**)

2.1 Dewaxing and Dehydrating Tissue Sections

Solutions

1. *Xylene*. Available from commercial sources (*see* **Note 2**). As alternative, use commercially available nontoxic xylene substitutes (i.e., from Sigma-Aldrich).
2. *Absolute alcohol*. Available from commercial sources (*see* **Note 3**).
3. *Industrial Methylated Spirit*. Available from commercial sources (*see* **Note 4**).

2.2 Periodic Acid Schiff Stain (PAS)

1. *Periodic Acid*: Hazards—Corrosive to respiratory tract, skin, and eyes. Causes severe damage by ingestion. Wear gloves and visor.

   0.5% aqueous Periodic Acid (to make 500 ml):

Measure out 500.0 ml ddH_2O into conical flask. Weigh out 2.5 g periodic acid, put into conical flask, stir until dissolved. Filter into clean plastic storage bottle and label appropriately (*see* **Note 5**).

2. *Schiff's Reagent:* Hazards—Causes irritation by inhalation, ingestion and contact with skin and eyes.

   Good quality Schiff's reagent can be obtained commercially (i.e., Merck, Chroma). The reagent, however, is easily self-made following the formulation of FEULGEN and ROSSENBECK [9]: Work in an extraction fume hood. Heat 200.0 ml ddH_2O to boil in a conical flask. Take from the heat, add 1.0 g Pararosaniline (C.I. 42500) and shake well until the dye is dissolved (*see* **Note 6**). Cool down to 50 °C and add 20.0 ml 1 M HCl, mix well. Cool to 25 °C and add 10.0 ml 10% sodium metabisulfite, mix well. Close flask with a stopper, allow to stand in the dark for 24 h at room temperature. Add 2.0 g activated (animal) charcoal, shake vigorously for 1 min, then filter the solution (i.e., Whatman Nr. 2 filter paper) into a dark (brown) bottle with a screw cap. Store at 4 °C (*see* **Note 7**).

3. *Harris' Hematoxylin*—nuclear counterstain:

   Recommended are commercially available, ready to use hematoxylin solutions (Ehrlich's hematoxylin, Harris's hematoxylin, and Mayer's hematoxylin).

4. *Scott's Solution* (Blueing agent for hematoxylin; tap water substitute) (to make 1 l): Weigh out 3.5 g sodium bicarbonate and 20.0 g of magnesium sulfate, dissolve in 1 l of ddH_2O (*see* **Note 8**).

5. *1% Light Green in 1% Acetic Acid*—collagen stain (to make 500 ml):

   Weigh out 5.0 g Light Green SF yellowish, place in conical flask and add 500.0 ml ddH_2O. Add carefully 5.0 ml glacial acetic acid to the conical flask, swirl it to dissolve the dye. Filter into a clean plastic stock bottle and label appropriately (*see* **Note 9**).

6. *Light Green Working Solution.*

   1% Light Green in 1% acetic acid—1 ml.

   1% acetic acid—9 ml.

7. *Amylase (if diastase treatment to remove glycogens is desired).*

   Weigh out 0.06 g (60.0 mg) amylase and stored in plastic universal bottles in the fridge. Add 10 ml of ddH_2O immediately prior to use and filter solution onto slide (*see* **Note 10**).

2.3 Grocott-Gomori Methenamine Silver Stain (GMS)

1. *5% Aqueous ChromiumTrioxide (chromic acid)* (to make 500 ml):

   Measure out 500.0 ml ddH_2O into conical flask. Weigh out 25.0 g chromium trioxide. Add slowly to conical flask of ddH_2O to reduce heat buildup and completely dissolve. Filter into clean plastic bottle and label appropriately.

2. *1% Aqueous Sodium Metabisulfite* (to make 500 ml):
   Measure out 500.0 ml ddH_2O into a conical flask. Weigh out 5.0 g sodium metabisulfite and place in flask with water. Stir and when dissolved filter into a clean plastic bottle and label appropriately.
3. *3% Aqueous Methenamine (Hexamine)* (to make 500 ml):
   Weigh 15.0 g Hexamine, place in a conical flask. Measure 500 ml ddH_2O and add to Hexamine. Dissolve and mix. Place in a clean plastic bottle and label appropriately. Store at 4 °C.
4. *5% Aqueous Silver Nitrate* (*see* **Note 11**) (to make 200 ml):
   Weigh out 10.0 g Silver Nitrate and dissolve in 200.0 ml Distilled Water. Filter in to a clean stock bottle label appropriately and store in cupboard in the dark.
5. *5% Aqueous Sodium Tetraborate (Borax)* (to make 500 ml):
   Measure out 500.0 ml ddH_2O and put in a conical Flask. Weigh out 25.0 g of Sodium Tetraborate and dissolve in the ddH_2O. Pour the solution into a clean 500 ml plastic bottle. Label appropriately.
6. 5% *Aqueous Sodium Thiosulfate* (Hypo, *see* **Note 12**) (to make 1 l):
   Measure out 1 l ddH_2O into conical flask. Weigh out 50.0 g sodium thiosulfate and add to conical flask. Dissolve thoroughly and pour into clean plastic stock bottle. Label appropriately.
7. *0.2% Aqueous Gold Chloride (Sodium Chloroaurate)* (to make 500 ml):
   Measure 500.0 ml ddH_2O and place in a clean 500 ml stock bottle. Carefully pour 1.0 g Sodium Chloroaurate into the clean stock bottle. Dissolve and label appropriately.
8. *1% Light Green in 1% Acetic Acid*—collagen stain (to make 500 ml):
   Weigh out 5.0 g Light Green SF yellowish, place in conical flask and add 500.0 ml ddH_2O. Add carefully 5.0 ml Glacial Acetic Acid to the conical flask, swirl it to dissolve the dye. Filter into a clean plastic stock bottle and label appropriately.
9. *Stock Hexamine Silver Solution.*
   3% aq. Methenamine (Hexamine) 400 ml.
   5% aq. Silver Nitrate 20 ml.

   Add the silver nitrate solution to the methenamine solution. A white precipitate forms but clears on shaking. Solution keeps well at 4 °C.
10. *Silver Incubating Solution:*
    Borax solution—5 ml.
    Distilled water—25 ml.
    Methanamine silver—25 ml.

Filter into a Coplin jar and place in water bath preheated to 60 °C.

11. *1 % Acetic Acid* (to make 500 ml):

    Measure 495.0 ml ddH_2O into a conical flask. Measure 5.0 ml Glacial Acetic Acid and carefully add to distilled water. Place in plastic stock bottle and label appropriately (*see* **Note 13**).

12. *Light Green Working Solution*.

    1 % Light Green in 1 % acetic acid—1 ml.

    1 % Acetic Acid—9 ml.

13. *Nuclear Fast Red solution* (to make 200 ml):

    Weigh out 0.2 g nuclear fast red and 10.0 g aluminum sulfate, dissolve in 200.0 ml ddH_2O and heat with stirring to dissolve. Leave overnight to cool. Filter into clean plastic stock bottle. Label appropriately. The solution keeps for 1 year. It needs to be filtered before use if precipitates form are visible.

    Comparable solutions are commercially available, ready to use.

3 Methods (*See* Note 14)

3.1 Dewaxing of Tissue Sections (See Note 15)

1. Dewax sections by placing in xylene 2 × 10 min.
2. Immerse slides in absolute alcohol and agitate 2 × 1 min.
3. Immerse slides in fresh 96 % industrial methylated spirit, agitate 1 × 1 min.
4. Immerse slides in fresh 80 % industrial methylated spirit and agitate 1 × 1 min.
5. Wash slides in running tap water 1 × 5 min.

3.2 Dehydrating of Tissue Sections (See Note 16)

1. Rinse section with 80 % alcohol and agitate 1 × 30 s.
2. Rinse section with 95 % alcohol and agitate 1 × 30 s.
3. Rinse section with absolute alcohol and agitate 2 × 30 s.
4. Rinse section with xylene and agitate 1 × 30 s.
5. Rinse section with xylene and place in a container of xylene 1 × 1 min.
6. Prepare coverslip with drop of mounting medium (*see* **Note 15**).
7. Dry excess xylene from around section and mount onto coverslip.

3.3 Periodic Acid Schiff (PAS) Stain (See Note 17)

Steps 2 and **3** for PAS with Diastase treatment only.

1. Take sections to water (*see* Subheading 3.1).
2. Treat section with amylase for 15 min at room temperature.
3. Wash well in tap water for 5 min.

4. Treat section with 0.5 % periodic acid for 10 min.
5. Wash 3× in tap water, 1× in ddH_2O (*see* **Note 18**).
6. Place silde into a cuvette with Schiff's Reagent, stain for 20 min at 65 °C (*see* **Note 19**).
7. Wash well in (25 °C warm) running water for 5 min.
8. Stain nuclei with hematoxylin for 20 s.
9. Blue in Scott's solution (2–5 min).
10. Rinse in running water (2 min).
11. Dehydrate, clear, and mount (*see* Subheading 3.2).

    Alternative Protocol with Light Green counterstain.
    Follow **steps 1**–**7** of PAS staining above.

12. Place slide into a cuvette with Light Green for 2 min.
13. Dehydrate, clear, and mount (*see* Subheading 3.2).

3.4 Grocott-Gömöri Modification of Methenamine Silver Stain (GMS)

1. Take sections to water (*see* Subheading 3.1).
2. Oxidize in 5 % chromic acid for 20 min.
3. Wash 3× in tap water.
4. Bleach in 1 % sodium metabisulfite (*see* **Note 20**).
5. Rinse in several changes of ddH_2O.
6. Place in hexamine (methenamine)-silver solution at 65 °C in the dark for 20 min (up to 60 min or until fungi are blackened and background is clear, *see* **Note 21**).
7. Wash well in many changes of ddH_2O.
8. Tone in 0.2 % gold chloride for 1 min or until grey.
9. Rinse in ddH_2O.
10. Fix in 5 % sodium thiosulfate for 10 s.
11. Wash in running tap water.
12. Counterstain in Light Green working solution for 20 s (*see* **Note 22**).
13. Rinse in ddH_2O.
14. Dehydrate, clear, and mount (*see* Subheading 3.2).

4 Notes

1. Some color reagents, like silver nitrate, precipitate when glassware has not been properly cleaned or contains residues of reagents. Insure proper washing, rinsing with copious amounts of RV water. In some instances, autoclaving glassware containing ddH_2O which is discarded before glassware is used, may be recommended.

2. Vapor irritates respiratory tract and eyes, may cause dermatitis to skin and burns to eyes. Ingestion causes irritation of the mouth, throat and gastrointestinal tract and may prove fatal. Work in fume exhaust cabinet. Wear chemical resistant gloves. Flammable, keep away from sources of ignition.
3. May be harmful by inhalation, if swallowed, or absorbed by the skin, causing irritation. Mists or vapors are irritating to the eyes, mucous membranes, and respiratory tract. Has been linked to birth defects and cancer in humans. Handle and maintain in a fume extraction hood. Wear chemical resistant gloves. Highly flammable, keep away from sources of ignition.
4. Harmful through inhalation, in contact with skin and if swallowed. Handle and maintain in a fume extraction hood. Wear chemical resistant gloves. Highly flammable, keep away from sources of ignition.
5. Store at room temperature. Make fresh and change weekly.
6. Be careful—Pararosaniline (C.I. 42500) foams.
7. Solution in stock bottle is stable for 6 months. Change working solution after 2 weeks.
8. The use of Scott's solution can be substituted by blueing in running hot tap water. Alternatively, 1:10 stock solutions of blueing agents are commercially available from several companies.
9. Alternatively use commercially available Goldner's staining III solution.
10. Sensitizer. May cause allergic respiratory reaction. May be harmful if inhaled. Toxicology not fully investigated.
11. Causes internal damage by ingestion. Corrosive to eyes and skin. Inhalation may cause irritation. Wear gloves and eye protection.
12. Harmful by ingestion and inhalation. Irritating to eyes. No hazard when used with good laboratory practice.
13. Burns eyes. Irritating to respiratory system. May be absorbed by skin. Will cause severe damage if swallowed. Wear gloves and eye protection.
14. Times indicated have been established at the author's histology laboratory using 2 mm thick paraffin sections of tissue fixed in 7.5% buffered formaldehyde and may need to be empirically determined and optimized in each laboratory individually.
15. Work in an extraction fume hood.
16. Usually xylene soluble nonaqueous mounting medium is used however there are several mounting media commercially available that do not contain dangerous organic solvents however are compatible with xylene based protocols.

17. The Schiff reagent is prepared by decolorizing basic fuchsin with hydrochloric acid and anhydrous metabisulfite. This forms a colorless fuchsin sulfurous acid which will combine with aldehydes. When this combination takes place, the reagent is recolorized by the aldehyde and renders a pink-to-red-to-purple, relatively insoluble product. Accordingly, the quality of Schiff's reagent can be tested by adding a drop of formaldehyde (7% or 10% as used for fixation)—there should be an instantaneous change of color to purple.
18. Proper washing ensures that any remaining periodates formed during oxidation are removed and cannot react with Schiff's reagent to generate background.
19. Preheating the staining cuvette speeds up the staining procedure.
20. Sodium metabisulfite removes excess chromic acid however this step can be ommited.
21. Proper determination of staining endpoint with a microscope, in many instances, is essential to a successful outcome. Tissue should be the color of a brown paper bag. If the fungi are not turning black, check and make the following solutions fresh: chromic acid, borax, sodium metabisulfite. If the working Methenamine-Silver solution turns cloudy, discard and make fresh.
22. Light Green can be replaced by incubating in 0.1% nuclear fast red solution for 5 min if red nuclear counterstain is preferred.

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Chapter 10

Culture-Based Techniques

Birgit Willinger

Abstract

The detection of fungal elements and their characterization in patient specimens provides fundamental information. Culture-based methods, though often slow, may yield the specific etiological agent, and may allow susceptibility testing to be performed. Proper collection and transportation of the specimen is essential. Particularly, sterile materials are important for diagnosis of invasive fungal infections.

Therefore, culture and direct microscopy should be performed on all suitable clinical specimens when fungal disease is suspected. Numerous different media for culturing and identifying fungi are available, and those important for diagnosing mycoses as well as the most important staining methods for direct microcopy are described.

Key words Media, Specimen processing, Preanalytic aspects, Microscopy, Staining methods

1 Introduction

The detection of fungal infections by histopathology and fungal culture remains the cornerstone of the diagnosis in fungal infections [1]. Culture, though often slow, sometimes insensitive and sometimes confusing with respect to contamination, can identify the specific etiological agent, and may allow susceptibility testing to be performed. The proper collection and transportation of the specimen is essential since sterile materials are critical for accurate diagnosis of invasive fungal infections. However, culture of skin, nails, hair, and mucous membranes is also important for superficial infections.

Whenever a fungal infection is suspected, culture should always be attempted and the use of specific mycological media is advised [2]. Fungi isolated from sterile sites should be identified to the species level. Fungi have longer generation times than those of most bacteria, which results in slower recovery of the organisms from specimens. [3]. Thus, everyone performing cultures has to be aware that it may take long time (e.g., several weeks) before fungi grow in culture.

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Histopathology plays an essential role in diagnosing and identifying fungal pathogens. However, appropriate tissue sample cannot always be obtained from critically ill patients. In addition, it is often difficult to identify the specific pathogen based solely on morphological characteristics, because of the similarities in histopathological characteristics of different organisms: for example, *Fusarium* spp., and other filamentous fungi are indistinguishable from *Aspergillus* in tissue biopsies [3]. As *Aspergillus* is far more commonly encountered than the other pathogens mentioned, a pathologist often may describe an organism as *Aspergillus* or *Aspergillus*-like based upon morphological features alone. This can hinder diagnosis and may lead to inappropriate treatment [4]. This is especially true for invasive infections as clinical symptoms are rarely characteristic. Accordingly, the combination of histology with culture is the most successful approach to proving infection.

Culture remains one of the key methods for diagnosing fungal infection, even though it is often slow, sometimes insensitive and can be confusing because of contamination. Cultures can reveal the specific etiological agent and may allow susceptibility testing to be performed. The proper collection and transportation of the specimen are essential. In particular, sterile materials are important for diagnosis of invasive fungal infections. However, direct microscopy of any specimen without fixative is most useful in the diagnosis of superficial, subcutaneous and invasive fungal infections and, in those settings, should always be performed together with culture. Recognition of fungal elements can provide a reliable and rapid indication of the mycosis involved. Various methods for direct microscopy can be used: KOH-preparation, India Ink preparation, Calcofluorwhite and others.

Culture is an important basis for identification of fungi. Yeasts are identified by their assimilation pattern and their microscopic morphology while moulds are recognized by their macroscopic and microscopic morphology [5]. Chromogenic media and test kits facilitate the rapid identification of *Candida* spp. and certain common yeasts can be identified immediately once visible growth is observed (Fig. 1).

Identification of filamentous fungi can be much more cumbersome and generally requires information from macroscopic and microscopic morphology [6].

Proteomic profiling by mass spectral analysis has recently been introduced for the use in species differentiation of a variety of microorganisms as an alternative to the conventional identification schemes. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) generates characteristic mass spectral fingerprints with unique signatures for each microorganism. These are ideal for accurate microbial identification to the genus and species levels and have the potential to be used for strain

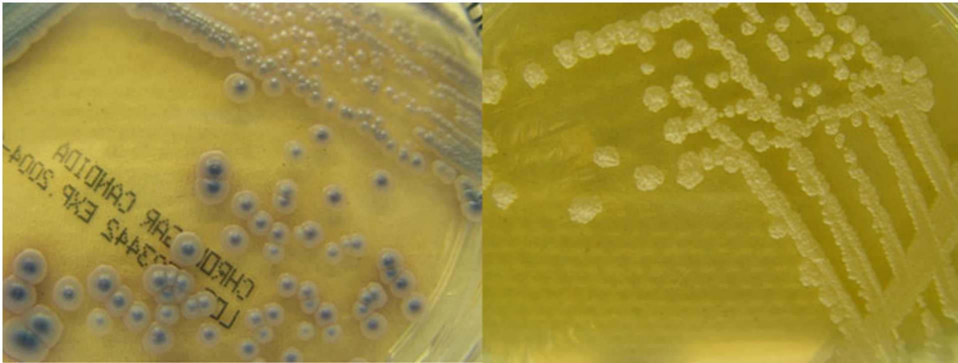

Fig. 1 *C. tropicalis* on CHROMagar Candida® (Becton Dickinson) and Sabouraud dextrose agar

typing and identification [5]. Each of the methods discussed here should be performed exactly as described by the various manufacturers.

2 Materials

Specimens sent for the detection of fungi have to be inoculated onto media that in combination will ensure the growth of all media fungi that might be clinically significant [7]. Although most fungi grow on the standard culture media, such as blood agar and chocolate agar, media specific for fungal growth must also be used. Specific media for fungi are for instance Sabouraud glucose agar with cycloheximide, potato dextrose agar, malt extract dextrose agar, cornmeal agar, and brain heart infusion agar [7, 8]. Cycloheximide is added to media whenever rapidly growing saprophytic fungi have to be detained from overgrowing slow-growing primary pathogens. The use of selective media such as Sabouraud agar containing antibiotics with chloramphenicol and gentamicin is recommended, whenever bacteria being possibly present shall be prevented from growth.

Some fungi require highly specialized medium supplements. Enriched media should be used whenever fastidious thermally dimorphic fungi are suspected.

The optimal growth temperature for most clinically important fungi is 30 °C. Simultaneous incubation at 30 °C and 35–37 °C should be reserved for when thermally dimorphic organisms may be present [7]. The duration of incubation varies and is dependent from the organisms expected to grow. Most yeasts grow in 2–3 days, while filamentous fungi need a prolonged incubation time. The dimorphic fungi may take as long as 3–6 weeks.

For clinical specimens it is recommended to use a combination of media with and without cycloheximide. A combination of media

lacking cycloheximide with media containing cycloheximide is recommended in order to detect fungi that are commonly considered saprobes or contaminants, but can also act as opportunistic pathogens [8].

2.1 Media Without Cycloheximide

1. Sabouraud 2 % (or 4 %) dextrose agar (SDA): Chloramphenicol (40 mg/L), penicillin, streptomycin, and gentamicin are commonly used as antibacterial agents.
2. Inhibitory mould agar (IMA): This is an enriched medium and contains chloramphenicol (125 mg/L), but gentamicin (50 mg/L) and/or chloramphenicol or ciprofloxacin can also be added instead. It has been shown to be superior to SDA as a primary medium [9].
3. Malt Extract Agar [10]: This medium is recommended as an alternative to SDA. It can also be used in order to stimulate sporulation in a wide range of filamentous fungi.

2.2 Media with Cycloheximide

Cycloheximide prevents rapidly growing saprobic fungi from masking the presence of slower growing pathogenic fungi including dermatophytes and certain dimorphic, endemic fungi (e.g., *Histoplasma* spp., *Blastomyces dermatitidis*, *Coccidioides* spp., and *Paracoccidioides brasiliensis*). Be aware that cycloheximide inhibits the growth of some pathogenic or potentially pathogenic fungi such as *Cryptococcus neoformans*, *Cryptococcus gattii*, *Aspergillus* spp., *Scedosporium prolificans*, *Neoscytalidium dimidiatum*, many *Candida* spp., and most mucoraceous fungi.

1. Mycosel Agar® (BD, Franklin Lakes, NJ, USA) including cycloheximide (0.4 g/L) and chloramphenicol (0.05 g/L).
2. Sabouraud Agar with Chloramphenicol and Cycloheximide® (BD, Franklin Lakes, NJ, USA).

2.3 Enriched Media

Enriched media are used for the improved recovery of dimorphic or other fastidious fungi. It should be considered that blood enrichment may promote the growth of dimorphic fungi, but inhibits the conidiation in the mould form.

1. BHI (Brain Heart Infusion) agar (with or without 5–10 % sheep blood) with chloramphenicol (50 mg/L) and gentamicin (50 mg/L).
2. SABHI agar with chloramphenicol (50 mg/L) and gentamicin (50 mg/L). SABHI has some of the ingredients of both SDA and BHI-agar.

2.4 Specialized Media

1. Chromogenic agar: Differential media for isolation and presumptive identification of some *Candida* spp. (*see* **Note 1**).
2. Media for recovery of lipid-requiring *Malassezia* spp. [8]:

(a) SDA with olive oil overlay: isolation of lipid-requiring *Malassezia* spp. However, only *M. furfur*, *M. pachydermatis*, and *Malassezia yamatoensis* grow well on these media.

(b) Dixon's medium 7 (Malt Extract 36 g Desiccated Oxbile 20 g Tween 40 10 ml Peptone 6 g Glycerol 2 ml Oleic Acid, 2 ml DI Water 1000 ml).

3. Potato Flake Agar (PFA, Rinaldi 1981 JCM) or potato dextrose agar (PDA) [11]: This is an effective isolation/identification medium because it promotes conidiation of moulds that can be important for more rapid treatment decisions in patients with life-threatening filamentous fungal infections (e.g., bone marrow transplant patients). These media are often recommended for slide cultures in order to stimulate conidiation and pigmentation.
4. Czapek-Dox-Agar for the routine cultivation of fungi and identification of *Aspergillus* spp. *and Penicillium* spp.
5. Dermatophyte Test Medium (DTM): This medium is meant for the growth of dermatophytes. These fungi change the color of the medium from yellow to red within 14 days.
6. Trichophyton Agars Nos. 2–7: for the differentiation of *Trichophyton* species.
7. Vitamin Free Agar (Trichophyton Agar No 1): for the differentiation of *Trichophyton* species.
8. Urea Agar with 0.5 % Glucose for detection of urease production in certain fungi. It is the same as used in bacteriology and is commercially available in prepared or dehydrated form. It is mainly used for the differentiation of yeasts and yeast-like fungi.
9. Bird Seed agar (Staib Agar) with Guizota abyssinica seed (available in most stores that sell bird feed). Only *C. neoformans* and *C. gattii* produce phenol oxidase, which breaks down the substrate and results in the production of melanin. Thus these species grow as dark brown to black colonies. All other yeasts show unpigmented colonies.

2.5 Blood Culture Systems

The use of specialized mycological blood culture bottles being used by some blood culture systems can shorten the time to detection relative to standard bacterial blood culture bottles [8, 12, 13]. Mainly commercial blood culture systems such as Bactec (BD, Franklin Lakes, NJ, USA), BacTAlert (bioMérieux, l'Etoile, France), or the Lysis centrifugation isolator system (Wampole Laboratories, Cranbury, NJ, USA) are used.

1. Lysis-centrifugation system: dimorphic moulds can be reliably and rapidly recovered in the lysis-centrifugation system. For optimal recovery, the concentrate can be inoculated to a choice of media with and without cycloheximide. Chocolate agar can be added for the recovery of fungi [8, 14].

2. Standard bacterial blood culture bottles are most useful for the recovery of *Candida* spp. and some moulds.

2.6 Media Used for Antifungal Susceptibility Testing

RPMI-1640: RPMI 1640 (with L-glutamine and a pH indicator but without bicarbonate) supplemented with glucose to a final concentration of 2% (RPMI 2% G) is recommended by the European Committee of Antimicrobial Susceptibility Testing [15, 16]. The use of 2% rather than the standard 0.2% concentration of glucose has been shown to result in better growth and facilitate the determination of endpoints. Antifungal susceptibility testing should be conducted as described by EUCAST [15] or CLSI [17–19] without any changes or adaptations.

2.7 Media Selection

The simultaneous use of two or three media is recommended for the recovery of fungi from a majority of clinical specimens. For normally sterile and nonsterile, non-dermatological sources select one or two media each from category A and B (*see* Table 1). For dermatological specimens (skin, hair, nails) select one medium each from category B and C. For specimens from throat, urine, and genital tract one medium from category C is sufficient.

Table 1
Media for primary isolation of fungi

Category	Type of medium	Examples
A	Without cycloheximide	SDA PDA, PFA BHI agar SABHI agar
B	With antibacterial agents	SDA, BHI or SABHI IMA
C	With antibacterial and antifungal agents	Mycosel agar
D	For dermatophytes	DTM
E	Selective and differential media for yeasts	Chromogenic media

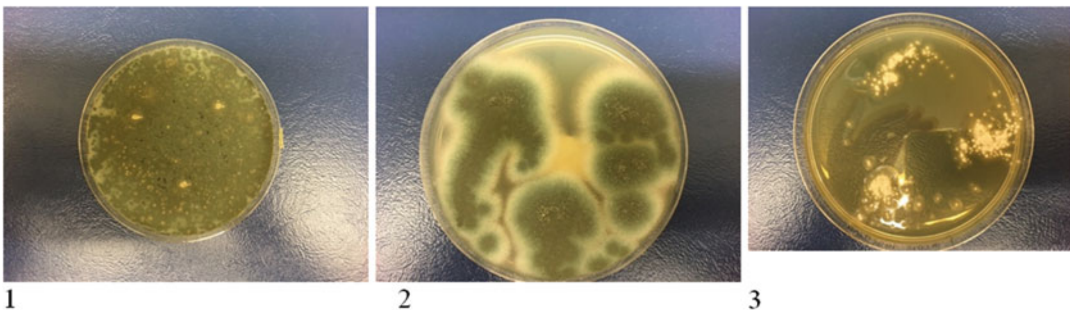

Fig. 2 *Aspergillus fumigatus* on three different media: (1) Sabouraud dextrose agar, (2) Potato dextrose agar, and (3) Mycosel agar

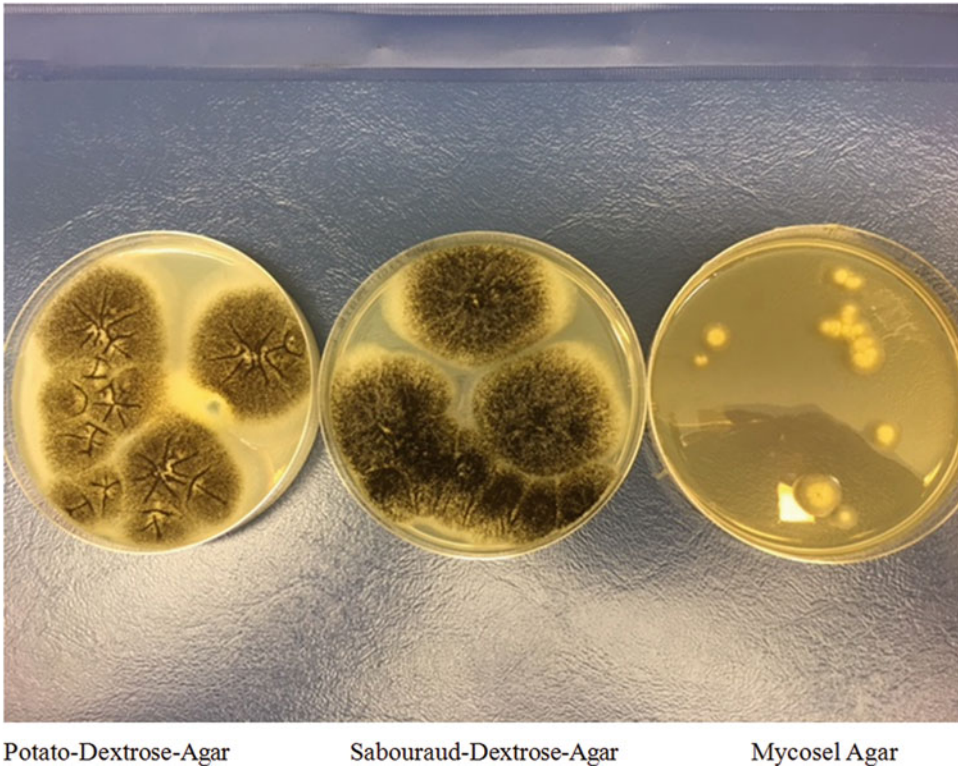

Fig. 3 *Aspergillus niger* on three different media

Category E media can be used as stand-alone media for primary isolation and/or presumptive identification.

The appearance of fungi on different media is shown in Figs. 2 and 3.

3 Methods

3.1 Staining Methods

Potassium hydroxide (KOH) preparation ([6]; *See* **Note 1**)

1. Make a 15% KOH solution.
2. Place a portion of the specimen on a labeled slide.
3. Add 2–3 drops of the KOH solution to the slide.
4. Put a cover slip over the preparation.
5. Heat gently over the flame. Do not boil!
6. Dab the excessive fluid gently with a small piece of filter paper.
7. Examine the slide carefully for hyphal elements, spores, or conidia.

India Ink Preparation [6]

1. Place a small drop of India ink on a glass slide.
2. Mix with an equal amount of centrifuged spinal fluid sediment or a suspension of isolated yeast (3500 × *g* for 10 min). Sputum or similar specimens can be cleared with KOH and then mixed with India ink.
3. Put a coverslip on top and examine the slide for encapsulated yeast cells.
4. Check for a well-demarcated halo around yeast cells.

3.2 Processing of Specimens

3.2.1 Skin Scrapings, Nail Scrapings and Epilated Hairs When Tinea Is Suspected (See Notes 2–4)

1. Remove any ointments or other local applications in patients with suspected tinea or ringworm with an alcohol wipe.
2. Use a blunt scalpel, tweezers, or a bone curette, firmly scrape the lesion, particularly at the advancing border.
3. For babies, young children and awkward sites such as interdigital spaces use a bone curette.
4. Make a wet mount preparation in KOH for direct microscopy.
5. Inoculate specimen onto two different media with and without cycloheximide also containing chloramphenicol and gentamicin.
6. Incubate cultures at 26 °C.
7. Maintain cultures for 4 weeks.

3.2.2 Skin Scrapings When Candidosis Is Suspected (See Notes 5–7)

1. Make a wet mount preparation in KOH for direct microscopy.
2. Inoculate specimens onto Sabouraud's dextrose agar slopes containing chloramphenicol and gentamicin.
3. Incubate at 35 °C.
4. Maintain cultures for 4 weeks.

3.2.3 Skin Swabs When Candidosis Is Suspected

1. Smear swab onto heat sterilized glass slide for Gram stain.
2. Inoculate specimens onto Sabouraud's dextrose agar slopes containing chloramphenicol and gentamicin.
3. Incubate at 35 °C.
4. Maintain cultures for 4 weeks.
5. Whenever a dermatophyte is suspected, include a Sabouraud's agar slope containing cycloheximide or Mycosel® and incubate at 26 °C.

3.2.4 Skin Scrapings from Patients with Suspected Pityriasis Versicolor

1. Make a wet mount preparation in KOH for direct microscopy.
2. Inoculate specimens onto DIXON's agar slopes for isolation of Malassezia furfur.
3. Incubate at 26 °C.
4. Maintain cultures for 4 weeks.

3.2.5 Urine (See ***Notes 8–13****)*

1. Collect urine first thing in the morning after overnight incubation in the bladder. A midstream clean catch or catheterized specimen is best, as this minimizes the presence of genital flora.
2. Centrifuge the urine for 10–15 min at 2000 rpm. Decant the supernatant and pool the sediment (approximately 0.5 ml) if necessary.
3. Prepare a direct smear of the sediment in KOH for direct microscopy.
4. Inoculate 0.05–0.1 ml of the sediment onto Sabouraud's agar with gentamicin and chloramphenicol and incubate duplicate cultures at 26 and 35 °C. Alternatively, use a calibrated loop for quantification.
5. Maintain cultures for 4 weeks.

3.2.6 Cerebrospinal Fluid, CSF (See ***Notes 14*** *and* ***15****)*

1. Note the volume of CSF, if lesser volumes than the requested 3–5 mL are received.
2. Centrifuge 3–5 mL of CSF at 3500 × *g* for 10 min.
3. Keep the supernatant of CSF for cryptococcal antigen testing.
4. For direct microscopy use 1 drop of the sediment to make an India ink mount for detection of *C. neoformans.*
5. Resuspend the remaining sediment in 1–2 ml of CSF.
6. Inoculate onto Sabouraud's dextrose agar with chloramphenicol and gentamicin and a chromogenic medium and incubate duplicate cultures at 26 and 35 °C.
7. Inoculate onto brain heart infusion agar (BHIA) supplemented with 5 % sheep blood and incubate at 35 °C.
8. Inoculate onto Bird Seed Agar if *C. neoformans* is suspected.
9. Maintain cultures for at least 4 weeks.

3.2.7 Sputum, Bronchial Washings and Throat Swabs, Bronchoalveolar Lavages (BAL), (See ***Notes 16–18****)*

1. Bronchial washings and sputa should be collected upon rising in the morning as overnight incubation and growth of fungi in the lungs will increase the likelihood of isolating pathogenic fungi.
2. Patients should not eat before specimen collection.
3. All specimens must be sent to the laboratory and processed as soon as possible, a delay of longer than two hours at room temperature may impede the detection of some fungi. Store at 4 °C if short delays in processing are anticipated.
4. Any bits of blood, pus or necrotic material should be plated directly onto media.
5. Make wet mount preparations in KOH (1 drop) and Gram stained smears (1 drop) of all suspicious areas.
6. Inoculate sample onto Sabouraud's dextrose agar with chloramphenicol and gentamicin and a chromogenic medium and incubate duplicate cultures at 26 and 35 °C.

7. Inoculate sample onto Brain heart infusion agar (BHIA) supplemented with 5 % sheep blood and incubate at 35 °C.
8. Maintain cultures for 4 weeks.

*3.2.8 Tissue Biopsies from Visceral Organs (See **Notes 19–21**)*

1. Tissue samples should be kept moist with saline or BHI broth and be transported to the laboratory as soon as possible.
2. Tease tissue specimens aseptically apart using a sterile petri dish.
3. In case areas of pus or necrosis are present, inoculate these directly onto the isolation media
4. Prepare a slide for direct microscopic examination.
5. Mince the tissue sample into pieces as small as possible with a sterile scalpel blade
6. Inoculate the minced or homogenized material onto
   - Sabouraud's dextrose agar with chloramphenicol and gentamicin and a chromogenic medium and incubate duplicate cultures at 26 °C.
   - Brain heart infusion agar (BHIA) supplemented with 5 % sheep blood and incubate at 35 °C.
7. Maintain cultures for 4 weeks.

3.2.9 Blood and Bone Marrow

Mainly commercial blood culture systems such as Bactec (BD, Heidelberg, Germany), BacTAlert (bioMérieux, Marcyl'Etoile, France) or the Lysis Centrifugation Isolator System (Wampole Laboratories, Cranbury, NJ, USA) are used. When using these systems the processing of the samples should be done as described by the respective manufacturer.

4 Notes

1. Chromagar Candida (Becton Dickinson, Franklin Lakes, NJ, USA) and Candida ID Agar (bioMérieux, Marcy l'Etoile, France) have been shown to allow easier differentiation of *Candida* species in mixed yeast populations than the traditional Sabouraud Glucose agar. Moreover, more rapid identification of *Candida* species can be achieved on these media than with conventional test kits. These media reduce the need for subculture and further biochemical tests and considerably simplify and shorten the identification procedure mainly for *C. albicans*, *C. tropicalis* and *C. krusei* [5]. *C. glabrata*, the second most common *Candida* sp. cannot be identified reliably on chromogenic media.
2. Potassium Hydroxide (KOH) preparation: if it is not possible to examine the specimen directly after preparation, the slide may be put in a wet chamber for several hours. In this case the

preparation not only does not dry but also the fungal elements may be detected more easily.

3. If multiple lesions are present, choose the most recent for scrapings as old loose scale is often not satisfactory.
4. Remove the tops of any fresh vesicles as the fungus is often plentiful in the roof of the vesicle.
5. When using DTM Agar one has to be aware that some of the non-dermatophyte fungi may cause false positive changes in color. DTM should therefore be only used as an adjunct to other media [7].
6. In patients with suspected candidiasis the young "satellite" lesions which have not undergone exfoliation are more likely to yield positive results if they are present. Otherwise the advancing scaly border should be scraped. When lesions in the flexures are moist and very inflamed it is more satisfactory and less painful to roll a moistened swab firmly over the surface.
7. In patients with suspected cutaneous manifestations of systemic pathogens scrap the lesions with a bone curette or blunt scalpel as for tinea. A biopsy may be required in some cases.
8. Do not use urine from a collection bag. Also, 24 h urine samples are unacceptable.
9. Identify and report yeasts recovered from routine urine bacteriology cultures of catheterized urine or urine obtained by sterile procedure regardless of colony count. However, the isolation of yeasts from clean catch specimens must be interpreted with caution and is only significant with additional support from other clinical and laboratory investigations.
10. Colony counts have not proved to be diagnostically useful. In order to distinguish contamination from colonization or infection obtain a new urine sample to verify funguria. Also, performing a Gram stain and proving the presence of fungi by microscopy will give an important clue that a fungal infection is present [20].
11. Be aware of *C. glabrata*. Although this species grows well on blood agar, the organism grows slowly, and colonies may not appear for 48 h or more. Thus, inoculate Sabouraud agar slants and keep them at least for 7 days if specific urine cultures for fungi are requested [21].
12. Seal negative bacteriological cultures from patients with clinical evidence of an infection with tape and maintain these cultures at 26 °C for 4 weeks to exclude the presence of a slow growing fungus.
13. Urine samples must be processed as soon as possible, a delay of longer than two hours at room temperature may impede the

detection of some fungi. Store at 4 °C if short delays in processing are anticipated.

14. If there is a delay in processing the CSF, it rather should be kept at room temperature or incubated at 30 °C.
15. Cultures from patients undergoing treatment for cryptococcal meningitis should be maintained for 3 months, so that dormant viable cells, which do not start to grow until after a 1 month period, will not be missed [10].
16. Twenty-four hour samples from the respiratory tract are unacceptable because they become overgrown with bacteria and fungal contaminants.
17. Bronchial washings and sputa will usually be contaminated with throat flora. For this reason interpretation of results may be difficult from poor quality specimens.
18. Perform mucolytic pretreatment of viscose respiratory samples using Sputasol (Oxoid Microbiological Product, ThermoScientific Fisher, Loughborough, UK), unless the samples are already sufficiently fluid. This leads to an improved homogenization of samples and is associated with a more equal distribution of pathogens in the sample.
19. It is essential that histopathology also receive a portion of the tissue in formalin for rapid frozen sectioning with staining.
20. Be aware that Mucorales will not survive the chopping up or tissue grinding process. If on clinical and/or radiological evidence mucormycosis is suspected then gently tease the tissue apart and inoculate it directly onto the isolation media. If you are not sure, maintain the specimen in saline or BHI broth until the results of the frozen sections are known.
21. Negative bacteriological cultures from patients with clinical evidence of an infection should be sealed with tape and maintained at 26 °C for 4 weeks to exclude the presence of a slow-growing fungus.

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Chapter 11

Serological Approaches

Barbora Weinbergerova, Iva Kocmanova, Zdenek Racil, and Jiri Mayer

Abstract

The diagnosis of invasive fungal diseases (IFD) based on clinical, radiological, and conventional microbiological findings is not reliable and is often delayed. Non-culture-based methods with higher sensitivity and specificity may reduce diagnostic time and result in decreased IFD morbidity and mortality. These methods are now increasingly used to manage patients at risk of IFD. Among available biomarkers, fungal antigens have been investigated as an aid to early diagnosis and are predominantly used as screening tests to prompt antifungal treatment mainly in patients with hematological malignancies. The revised version of the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) consensus definitions includes some of these biological markers (galactomannan, 1,3-beta-D-glucan, cryptococcus antigen).

Key words Galactomannan, The Platelia *Aspergillus* enzyme immunoassay, Latex agglutination, *Aspergillus* lateral-flow device, 1,3-beta-D-glucan, Fungitell assay

1 Galactomannan Detection

1.1 Introduction

Galactomannan (GM) is a critical microbiological criterion for diagnosis of invasive aspergillosis (IA). GM represents a fungal cell wall component of *Aspergillus* along with chitin, 1,3- and 1,4-beta-D-glucan [1]. Detection of this carbohydrate molecule was recorded in 1992 by Stynen, using a monoclonal antibody (MoAb) EBA-2 [2] which specifically binds to four galactofuranosyl residues. Circulating GM can be detected in serum or plasma, bronchoalveolar lavage fluid (BALF), cerebrospinal fluid (CSF), and other body fluids.

The release of GM is thought only to occur during cell wall lysis when nutrients are limited in necrotic oxygen deprived tissue [3]. An "*in vitro*" model demonstrated that GM is not released into circulation during infection until the fungus invades the endothelial compartment [4]. This suggests that circulating GM cannot be detected in the serum until angioinvasion by the fungus occurs. Furthermore, it has been observed that serum GM (S-GM) is almost always readily detected in neutropenic hematological and

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human stem cell transplant (HSCT) patients as a result of poor immune response where angioinvasion is observed in characteristic "halo" signs in high resolution computer tomography (HRCT) [5]. On the other hand, with non-neutropenic patients, such as hematological patients after bone marrow recovery or non-hematological patients treated with long-term corticosteroids and other immunosuppressive regimens, a significant local inflammation limiting the angio-invasive process occurs, and patients could be S-GM negative [6].

1.1.1 Latex Agglutination

The original GM assay was a latex agglutination test with a detection limit of 15 ng/mL [7]. This assay was superseded by an enzyme immunoassay (EIA) able to detect less than 1 ng/mL [2], and most reports on assay now relate to the EIA assay.

1.1.2 Aspergillus Lateral-Flow Device

A recent test developed and marketed for *Aspergillus* spp. detection is an antigen test identifying an extracellular glycoprotein antigen only produced during active growth of the fungus. This test uses a lateral flow device (LFD) format [8]. The assay is specific for *Aspergillus* spp. and reacted positively to sera from patients diagnosed with IA by GM and 1,3-beta-D-glucan (BDG) assay results [8, 9]. A meta-analysis of Pan et al. assessed the diagnostic accuracy of the LFD test in patients with IA according to EORTC/MSG definitions from seven published studies [10]. The pooled sensitivity, specificity, and diagnostic odds ratio (DOR) were 0.86 (95% CI, 0.76–0.93), 0.93 (95% CI, 0.89–0.96), and 65.94 (95% CI, 27.21–159.81) in the LFD test using BALF, and 0.68 (95% CI, 0.52–0.81), 0.87 (95% CI, 0.80–0.92), and 11.90 (95% CI, 3.54–39.96) in the LFD test using serum (*see* Table 1). The authors concluded that the *Aspergillus* LFD had a positive diagnostic value in immunocompromised patients at risk of IA. The LFD using BALF might have a better performance than the serum LFD test. The *Aspergillus* LFD test showed promising results for IPA diagnosis. Furthermore, the LFD test can be performed easily and provides rapid results. Therefore, it may be a reliable alternative for IPA diagnosis in centers where GM results are not rapidly available and in centers with few samples.

Table 1
Diagnostic accuracy of *Aspergillus* lateral-flow device (LFD) test for IA diagnosis

	Meta-analysis	SEN (%)	SPE (%)	DOR
Serum	Pan et al. [10]	68	87	11.90
BALF	Pan et al. [10]	86	93	65.94

SEN pooled sensitivity, *SPE* pooled specificity, *DOR* diagnostic odds ratio

1.1.3 Platelia™ Aspergillus Enzyme Immunoassay

The Platelia™ *Aspergillus* EIA, a single commercial assay (Bio-Rad Laboratories, Marne La-Coquette, France), is the most commonly used technique for GM detection and for this reason, we will focus exclusively on this method. It is a one-stage immunoenzymatic sandwich microplate assay that detects GM in biological samples.

This assay uses rat EBA-2 MoAb, which are directed against *Aspergillus* GM. The MoAb are used to coat the microplate wells, bind the antigen, and then detect the antigen bound to the sensitized microplate (conjugate reagent: peroxidase-linked MoAb).

Cutoff is calculated by dividing the optical density (OD) of the patient's sample by that of the threshold control, which contains 1 ng/L of GM. GM concentration is expressed as an index, and with serum (and plasma) a cutoff GM index for 0.5 positivity (IP) is recommended.

Regular monitoring for S-GM is recommended with neutropenic patients who have a relatively high a priori probability (>5–10%) of developing IA—including patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) undergoing intensive chemotherapy and patients receiving allogeneic HSCT in the early engraftment phase [11–13].

In addition to blood, GM can be detected in CSF or BALF [14, 15]. Experience of GM detection in pleural fluid, sputum, urine, and other specimens is insufficient to formulate specific recommendations. BALF and serum are currently the only specimens with manufacturer approval for EIA analysis.

The EIA performance of S-GM and BALF-GM testing has been thoroughly reviewed and reassessed in recent meta-analyses [16, 17]. The reported pooled sensitivity and specificity in hematological patients is 62 and 95% consecutive positivity of S-GM, and for BALF-GM: 82 and 92% (*see* Table 2). The heterogeneity of sensitivity may be related to several factors that can influence results, including patient characteristics [18], variability in the definitions of IPA [19, 20], different cutoff of OD values of GM used in BALF [15, 21, 22], administration of mould-active antifungal drugs [23–25], presence of neutropenia [24, 26, 27], and finally standardization of BAL procedures [28]. GM EIA specificity may

Table 2
Diagnostic accuracy of galactomannan (Platelia Aspergillus EIA) test for IA diagnosis

	Meta-analysis	SEN (%)	SPE (%)	NLR	PLR
Serum	Arvanitis et al. [17]	62	95	0.40	12.1
BALF	Heng et al. [16]	82	92	0.19	10.9

SEN pooled sensitivity, *SPE* pooled specificity, *NLR* negative likelihood ratio, *PLR* positive likelihood ratio

Table 3
Possible causes of galactomannan test false positivity (Platelia *Aspergillus* EIA)

Treatment with beta-lactam antibiotics (e.g., piperacillin–tazobactam)
Plasma-Lyte solution administration
Patients with graft versus host disease
Bifidobacterial lipoglycan (present in human gut, especially in neonates)
Gastrointestinal tract mucositis
Patients with multiple myeloma
Contamination of sample during processing

be limited by the false positivity caused by numerous factors, including cross-reactivity with certain beta-lactam antibiotics and a Plasma-Lyte solution [29, 30]. These factors are listed in Table 3.

In the commercial kit, there is a pretreatment step with an ethylenediaminetetraacetic acid (EDTA) solution which is heated in order to precipitate proteins and break up immune complexes. This may reduce the activity of the GM acid labile furanosyl side chains, and some galactofuranosyl moieties of glycoproteins may also be lost in this step [3]. It has been suggested that a microfiltration concentration step can be used to increase analytical and clinical sensitivity [31]. Certain authors have described problems with the reproducibility of the assay [32].

1.2 Materials

All procedures strictly correspond to the manufacturer‘s instructions [33].

1. Microplate (12 strips of 8 wells each) coated with antiGM MoAb.
2. Concentrated washing solution (Tris–NaCl buffer, 1 % Tween® 20, 0.01 % thimerosal).
3. Positive, cutoff and negative control serum (freeze-dried human serum).
4. Conjugate (antiGM MoAb/peroxidase labeled with 0.01 % thimerosal).
5. Sample treatment solution (EDTA acid solution).
6. Tetramethylbenzidine (TMB) substrate buffer (citric acid and sodium acetate, 0.009 % hydrogen peroxide, 4 % dimethylsulfoxide).
7. Chromogen TMB Solution (90 % dimethylsulfoxide solution with 0.6 % TMB).
8. Stopping solution (1.5 N sulphuric acid).

1.3 Methods

1.3.1 Specimen Collection

The test is performed on serum and BALF (*see* **Note 9**). Collect samples according to standard laboratory procedures. Unopened samples can be stored at 2–8 °C for up to 5 days (for serum) or 1 day (for BALF) prior to testing. For longer storage, keep the serum at −70 °C and the BALF at −20 °C or less. Serum samples and BALF can be subjected to a maximum of four freezing–thawing cycles.

1.3.2 Treatment of the Sera/BALF

All control sera must be processed at the same time as samples.

1. Pipette 300 μL of each test material and control into individual 1.5 mL polypropylene tubes.
2. Add 100 μL of treatment solution to each tube.
3. Mix tubes thoroughly with vigorous mixing or vortexing to blend thoroughly. Tightly close the tube to prevent opening during heating; use a cap lock for snap cap tubes.
4. Heating of tubes
   (a) Heat block option: heat tubes for 6 min in a heat block at 120 °C (recommended are Grant block). Tubes must only be placed in the block when the prescribed temperature is reached.
   (b) Water bath option: heat tubes for 3 min at 100 °C.
5. Carefully remove hot tubes from the heat block or the boiling water bath and place in a centrifuge. Centrifuge tubes at 10,000 × *g* for 10 min.
6. The supernatant is used for GM antigen detection.

1.3.3 Enzyme Immunoassay

1. Prepare washing solution and controls.
2. Prepare a chart for identification of test samples and controls.
3. Add 50 μL of conjugate to each well. Next, add 50 μL of supernatant to each well as designated above.
4. Cover plate with plate sealer or other means to prevent evaporation, ensuring that the entire surface is covered and watertight.
5. Incubate microplate in a dry microplate incubator for 90 ± 5 min at 37 °C (±1 °C).
6. Remove the plate sealer. Aspirate the contents of all wells into a waste container (containing sodium hypochlorite). Wash the plate five times using a minimum of 800 μL of washing solution. After the fifth wash, invert the microplate and gently tap on absorbent paper to remove remaining liquid.
7. Rapidly add 200 μL of chromogen TMB solution to each well, avoiding exposure to bright light.
8. Incubate microplate in the dark at room temperature (18–25 °C) for 30 ± 5 min.

9. Add 100 μL of stopping solution to each well, utilizing the same order for addition of chromogen TMB solution. Mix well.
10. Read the optical density of each well at 450 nm (reference filter of 620/630 nm). Microplates must be read within 30 min after adding stopping solution.

1.3.4 Interpretation of Results

1. Calculate a validity criteria and then an index for each test sample.
2. IP = OD sample/mean cutoff control OD.
3. Samples with an IP < 0.50 are considered to be negative for GM antigen.
4. Samples with an IP ≥ 0.50 are considered to be positive for GM antigen, but for the BALF this point is debatable (*see* **Notes 3–6**, **8** and **11**) [15, 21, 22].

1.4 Notes

1. Strictly comply with proposed protocol.
2. According to manufacturer recommendations, a positive result for S-GM and BALF-GM is ≥0.50 IP.
3. For clinical practice use there are both ECIL (European Conference on Infections in Leukemia) recommendations for S-GM positivity: A single positive GM of ≥ 0.7 IP or two consecutive samples of ≥0.5 IP and for BALF-GM positivity: 1 sample of ≥0.5 IP and EORTC/MSG recommendations for S-GM positivity: Two consecutive samples of ≥0.5 IP and for BALF-GM positivity: 1 sample of ≥0.5 IP [13, 20].
4. In case of isolated S-GM positivity, mainly in patients without risk factors for IA (neutropenia, corticotherapy, etc.), it is necessary to consider possible false positivity (*see* Table 3).
5. A lower clinical utility and false negative S-GM test is possible among patients treated with antifungal prophylaxis affecting *Aspergillus* spp. (posaconazole, voriconazole or echinocandins). In this case, a BALF-GM examination may be advantageous.
6. A negative test cannot rule out an IA diagnosis. Serum samples from patients at risk for IA should be tested twice a week.
7. The performance of the Platelia™ *Aspergillus* EIA has not been evaluated with neonatal samples.
8. The Platelia™ *Aspergillus* EIA may be affected by reduced detection of GM in patients with chronic granulomatous disease (CGD) and Job's syndrome.
9. The Platelia™ *Aspergillus* EIA has not been evaluated for use with plasma or other sample types such as urine or CSF.
10. Other genera of fungi such as *Penicillium*, *Alternaria*, *Paecilomyces*, *Geotrichum*, and *Histoplasma* have shown

reactivity with rat, EBA-2 MoAb used in the assay for Aspergillus GM detection. Histoplasmosis should be considered in endemic areas including parts of the United States.

11. The BALF sample results from non-immunocompromised patient should be interpreted with caution.

2 1,3-Beta-D-Glucan Detection

2.1 Introduction

The glucose polymer 1,3-beta-D-glucan (BDG), a major cell wall component of most fungal species (with exception of zygomycetes and *Cryptococcus* spp.), is released in blood and tissues in the course of IFD. Measurement of BDG levels is based on activation by BDG of factor G of the coagulation cascade in the amoebocyte lysate from the horseshoe crab, which leads to quantifiable transformation of a chromogenic substrate [34]. Four commercial BDG antigenemia assays are available (Fungitell, Fungitec-G, Wako, and Maruha) with similar accuracy for the diagnosis of IFD in hemato-oncological patients [35]. The kits differ in the source of the amoebocyte lysate from the horseshoe crab, *Limulus polyphemus* (North-America) or *Tachypleus tridentatus* (Japan). This results in different reactivities for BDG detection and cutoffs for defining positivity of tests: Fungitell 60–80 pg/mL, Fungitec-G 20 pg/mL, Wako and Marhua 11 pg/mL. The Fungitell assay has been approved by the Food and Drug Administration (FDA) and is available in the United States and Europe; the other three tests are available only in Japan. The screening performance of serum BDG in patients with IFD has been evaluated in the recent meta-analysis of prospective cohorts studies with the pooled sensitivity, specificity, positive likelihood ratio, negative likelihood ratio, and diagnostic odds ratio (DOR): 75 %, 87 %, 5.85, 0.30, and 19.53, respectively (*see* Table 4) [36]. In another recent meta-analysis, the BDG assay diagnostic performance in proven/probable IFD was better with two consecutive than one single positive test result (DOR: 111.8

Table 4
Diagnostic accuracy of 1,3-beta-D-glucan test (Fungitell assay) for IFD diagnosis

	Study	**SEN**	**SPE**	**NLR**	**PLR**
Serum	Hou et al. [36]	75 %	87 %	0.30	5.85
	Study	**SEN**	**SPE**	**NPV**	**PPV**
BALF	Mutschlechner et al. [39]	79 %	39 %	86 %	28 %

SEN pooled sensitivity, *SPE* pooled specificity, *NLR* negative likelihood ratio, *PLR* positive likelihood ratio, *NPV* negative predictive value, *PPV* positive predictive value

vs 16.3) [35]. For 2 consecutive tests, sensitivity and specificity were 49.6 and 98.9%, respectively. Estimated positive and negative predictive values for an IFD prevalence of 10% were 83.5 and 94.6%, respectively. Because sensitivity is low, the test needs to be combined with clinical, radiological, and microbiological findings.

Many issues remain open and need to be addressed in further studies (e.g., BDG monitoring in high-risk patients, diagnostic yield in allogeneic HSCT recipients, detection in biological fluids other than blood, utility in follow-up of IFD). Attention should be paid to the potential causes of false-negative results (ongoing antifungal prophylaxis or therapy, pathogenic fungi not detected by the BDG assay, such as zygomycetes and *Cryptococcus*, non/semi-invasive fungal disease), and false-positive results (*see* Table 5).

Serum BDG appears to be a good marker for *Pneumocystis* pneumonia (PCP). The recent meta-analysis showed high diagnostic accuracy of the BDG assay for PCP with the pooled sensitivity 96%, specificity 84%, and DOR 102.3 [37]. Because the sensitivity for PCP is particularly high, the BDG assay can be used as a screening tool for PCP.

Rose et al. retrospectively studied and evaluated the utility of BALF-BDG for the diagnosis of IFD in a large unselected population of high-risk patients [38]. BALF-BDG was 100% sensitive for Pneumocystis. For all IFD, BALF-BDG had a sensitivity of 53% and specificity of 68%. A prospective multicenter analysis of the Fungitell assay was performed on BALF samples obtained from solid-organ transplantation patients suffering from probable, proven, or no IFD [39]. Based on a 100 pg/mL cutoff per test, sensitivity, specificity, positive, and negative predictive values were 79.2, 38.5, 27.6, and 86.3% (*see* Table 4). Although the high negative predictive value of the Fungitell assay in BALFs may support exclusion of pulmonary IFD, the low positive predictive value

Table 5
Possible causes of 1,3-beta-D-glucan test false positivity

Thrombocyte infusion with leukocyte-removing filters
Human blood products (immunoglobulins or albumins)
Hemodialysis/hemofiltration with cellulose membranes
Beta-lactam antibiotics (e.g., piperacillin–tazobactam, amoxicillin–clavulanate)
Severe mucositis
Cellulose dressings
Contamination of specimens by organic dust or waste at bedside or during preanalytical or analytical processing
Bacterial infections

limits its utility as a screening tool for early IFD diagnosis. Identification of the BAL-specific factors that may interfere with the performance of the assay could improve the clinical usefulness of BALF-BDG testing.

2.1.1 Fungitell Assay

The Fungitell assay is a protease zymogen-based colorimetric assay for the qualitative detection of BDG in serum. The assay is based upon a modification of the Limulus Amoebocyte Lysate (LAL) pathway. BDG activates Factor G, a serine protease zymogen. The activated Factor G converts the inactive proclotting enzyme to an active clotting enzyme, which in turn cleaves pNA from the chromogenic peptide substrate, Boc-Leu-Gly-Arg-pNA, creating a chromophore that absorbs at 405 nm. The Fungitell kinetic assay, described below, is based upon the determination of the OD rate increase produced by a sample. This rate is interpreted against a standard curve to produce estimates of BDG concentration in the sample.

2.2 Materials

All procedures strictly correspond to manufacturer instructions [40].

1. 96-well, uncoated microplates, with lids.
2. Lyophilized (1, 3)-β-D-glucan specific LAL.
3. 1.2 M KCl.
4. 0.2 M Tris–HCl pH 7.4.
5. 0.25 M KOH.
6. Lyophilized pachyman.
7. Reagent water.

All laboratory materials must be glucan-free (e.g., pipette tips, test tubes, container).

2.3 Methods

2.3.1 Specimen Collection

Serum samples should be collected in sterile tubes and allowed to clot. The serum is then separated from the clot and decanted to a container. Serum samples can be stored at 2–8 °C before assay or frozen at −20 °C or colder. Testing should be conducted promptly to avoid the possibility of sample degradation.

2.3.2 Preparation of Glucan Standard

1. Dissolve the glucan standard with the volume of reagent water and prepare five standards (concentration 100; 50; 25; 12.5; and 6.25 pg/mL) (*see* **Note 6**).
2. When plotting the standard curve, multiply the concentration of the standards by five so that the range is from 500 to 31 pg/mL (the volume of standard in the assay is 25 μL per well or five times the volume of the serum).

2.3.3 Serum Pretreatment Reagent

1. The alkaline serum pretreatment reagent converts triple-helix glucans into single-stranded glucans, which are more reactive

in the assay. The high pH also inactivates the serine proteases and serine-protease inhibitors in a serum that can give a false positive and a false negative result, respectively.

2. Prepare the serum pretreatment reagent by combining equal volumes of 0.25 M KOH and 1.2 M KCl, and vortexing well.

2.3.4 Procedure

1. Transfer 5 μL of identical serum sample to at least two of its designated wells at least twice. Repeat this parallel testing for each serum sample (*see* **Notes 1–4**).
2. Add 20 μL of the serum pretreatment reagent to each well containing serum.
3. Agitate the plate for 5–10 s to mix the well contents (the reader's plate agitation function may be used), then incubate for 10 min at 37 °C (*see* **Note 5**).
4. Reconstitute one vial of Fungitell reagent by adding 2.8 mL of reagent water and then adding 2.8 mL of pyrosol buffer. Swirl the vial gently to dissolve completely—do not vortex.
5. At the end of serum pretreatment incubation, remove the plate from the incubating plate reader and add the standards and negative controls to the plate (25 μL at least twice for parallel testing).
6. Add 100 μL of Fungitell reagent to each well (containing negative controls, standards, and samples) using the stepper pipettor.
7. Insert the plate into the microplate reader (equilibrated to 37 °C) and shake for 5–10 s. Read the plate at 405 nm minus 490 nm, for 40 min at 37 °C.
8. Collect the data and analyze as follows: Examine OD plots of test samples, and check for kinetic trace patterns other than a smooth increase comparable to those of standards. Invalidate plots indicating optical interference. Calculate the mean rate of OD change (milli-absorbance units per minute) for all points between 0 and 40 min.

2.3.5 Interpretation of Results

1. The results are expressed in pg/mL of serum and range from non-detectable (<31 pg/mL) to >500 pg/mL and are printed out by the software or read from the standard curve. Accurate values above 500 pg/mL require that the sample be diluted in reagent water and retested.
2. BDG values < 60 pg/mL are interpreted as negative results.
3. Values ≥80 pg/mL are interpreted as positive. A positive result means that BDG was detected. A positive result does not define the presence of disease and should be used in conjunction with other clinical findings to establish a diagnosis (*see* **Notes 7** and **8**).

4. Values from 60 to 79 pg/mL suggest a possible fungal infection. Additional sampling and testing of sera is recommended. Frequent sampling and testing improves the utility for diagnosis.
5. The laboratory performing the test should inform the ordering physician that the Fungitell test does not detect certain fungal species such as the genus *Cryptococcus*, which produces very low levels of BDG. The test also does not detect Zygomycetes such as *Absidia*, *Mucor*, and *Rhizopus*, which are not known to produce BDG.

2.3.6 Procedural Limitations

1. The tissue locations of fungal infection, encapsulation, and the amount of BDG produced by certain fungi may affect the serum concentration of this analyte.
2. Some individuals have elevated levels of BDG that fall into the indeterminate zone. In such cases, additional testing is recommended.
3. The frequency of patient testing will depend upon the relative risk of fungal infection.
4. Positive results have been found in hemodialysis patients, subjects treated with certain fractionated blood products such as serum albumin and immunoglobulins, and in specimens or subjects exposed to glucan-containing gauze (*see* **Note 9**).
5. Test levels were established with adult subjects. Infant and pediatric normal levels approach those of adults.

2.4 Notes

1. Work on lab table after disinfection with alcohol. Do not use flow box.
2. Cover plates and do not tip over or turn upside down in order to avoid contamination of biological material.
3. Avoid unnecessary movement of covered plates, and avoid moving unnecessarily over plates, as dust particles, e.g., from our clothes or hair, can affect test.
4. Pipette sample carefully into the well, and leave a sample drop in the well‘s upper left corner. Since pipette tip can touch the wall, insure that all sample residue in the tip is transferred into the well.
5. Handle only the side of the plate; do not touch the bottom when carrying plates.
6. Glucan standard is viscous. Pipette slowly with care, and do not leave residues in the tip. Begin with the lowest glucan standard concentration, and change pipette tip with each concentration used.
7. With positive results, it is necessary to provide a differential diagnosis of IA and invasive candidiasis (GM, mannan, and antimannan).

8. With extremely high levels and typical clinical and radiological (pulmonary X-ray/HRCT) findings, it is necessary to consider Pneumocystis pneumonia.
9. With an isolated positive value of serum BDG in patients with low risk of IFD, it is necessary to consider possible false positivity (*see* Table 5).

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Chapter 12

Isolation of Nucleic Acids for Fungal Diagnosis

P. Lewis White and Rosemary A. Barnes

Abstract

PCR can aid in the diagnosis of invasive fungal disease (IFD). While the large number of "in-house" methodologies drives technological diversity, providing robustness, they make it difficult to identify optimal strategies, limiting standardization, and widespread acceptance. No matter how efficient, PCR utility will be limited by the quality of extracted nucleic acid. This chapter highlights benefits and limitations affecting the nucleic acid extraction process, before focusing on recent recommendations that through multicenter evaluation have provided optimal and standardized methodology.

Key words Sample type, DNA extraction, Fungal infection

1 Introduction

1.1 Background Information

Conventional mycological methods (microscopy and culture) for the diagnosis of invasive fungal disease (IFD) lack sensitivity. Development of a variety of non-culture techniques can improve patient management by providing earlier diagnosis, or by excluding IFD (primarily invasive aspergillosis (IA)) and preventing unnecessary empiric treatment [1–3].

Non-culture approaches have focused on the detection of fungal biomarkers, in particular galactomannan enzyme linked immunosorbent assay (GM-EIA) for IA [4, 5], *Candida* antibodies and mannan antigen for invasive candidosis (IC) [6], crytpococcal antigen (CRAG) [7], direct immunofluorescence for *Pneumocystis* pneumonia (DIF-PCP) [8], and β-D-Glucan (BDG) for broad range fungal detection [9]. CRAG has proven invaluable for diagnosis of cryptococcal meningitis, but the performance of other biomarkers (GM-EIA, BDG, DIF-PCP) is more variable, [4, 5, 7–9]. Thus the opportunity to improve IFD diagnosis remains and combining antigen testing with molecular based tests has been shown to be beneficial for IA [1–3, 10].

Publications on the application of molecular methods to aid in the diagnosis of IFD are numerous although performance varies

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greatly [11, 12]. Behind this diversity lies the lack of an agreed consensus method. Differing etiologies and manifestations may prevent a single method and many "in-house" assays have been developed [11, 12]. Standardized methodology is needed for incorporation of molecular methods into IFD consensus definitions [13–16].

Given the range of fungi capable of causing IFD, of the non-culture assays only molecular based methods have the capacity to be truly pan-fungal. However, pan-fungal approaches can be associated with poor specificity [17], unless multiplexed with a range of probes specific for particular fungal pathogens [18]. PCR can also be designed to be species specific providing epidemiological evidence comparable to culture, with the benefits of rapidity and enhanced sensitivity [19].

The range of specimens (blood, CSF, tissue biopsy, respiratory and other fluids), different extraction methods and PCR assays has resulted in a wide variety of published methods. The performance of PCR is dependent on the quality of the nucleic acid (NA), which is dependent on the efficiency of the NA extraction technique. This should be optimized to the sample tested, which in turn is dependent on the disease manifestation of the suspected infective agent and clinical condition of the patient. This chapter focuses on the intricacies of NA extraction; limiting and influencing factors for each step of the molecular process are be highlighted together with recommended protocols.

1.2 Specimen Type: Factors for Consideration

A summary of factors influencing the choice of sample and consequently NA extraction methodology/PCR efficiency is shown in Fig. 1.

1.2.1 Patient Cohort and Likely IFD

The choice of specimen is the first experimental variable. When selecting a sample various factors should be considered to avoid inappropriate results [20]. The patient's underlying condition will provide information indicating the most likely cause of IFD in a particular patient cohort (e.g., *Candida* in ICU patients, *Aspergillus* in hematology patients and *Pneumocystis* or *Cryptococcus* in HIV patients). It will also influence the likely site of infection, indicating the optimal specimen. However, this may be influenced by whether a whether a patient is suitable for invasive procedures necessary to obtain bronchoalveolar lavage (BAL) fluid, cerebrospinal fluid (CSF), or tissue biopsies.

Most fungi are capable of causing a wide range of manifestation and clinical symptoms will determine the specimen for testing. Less invasive or localized disease (e.g., Aspergilloma or intra-abdominal candidal abscesses) release less fungal target and samples to specific to the area of infection may be required to avoid reporting potentially false negative PCR results. Yamakami et al. reported that the degree of tissue invasion was important and less invasive

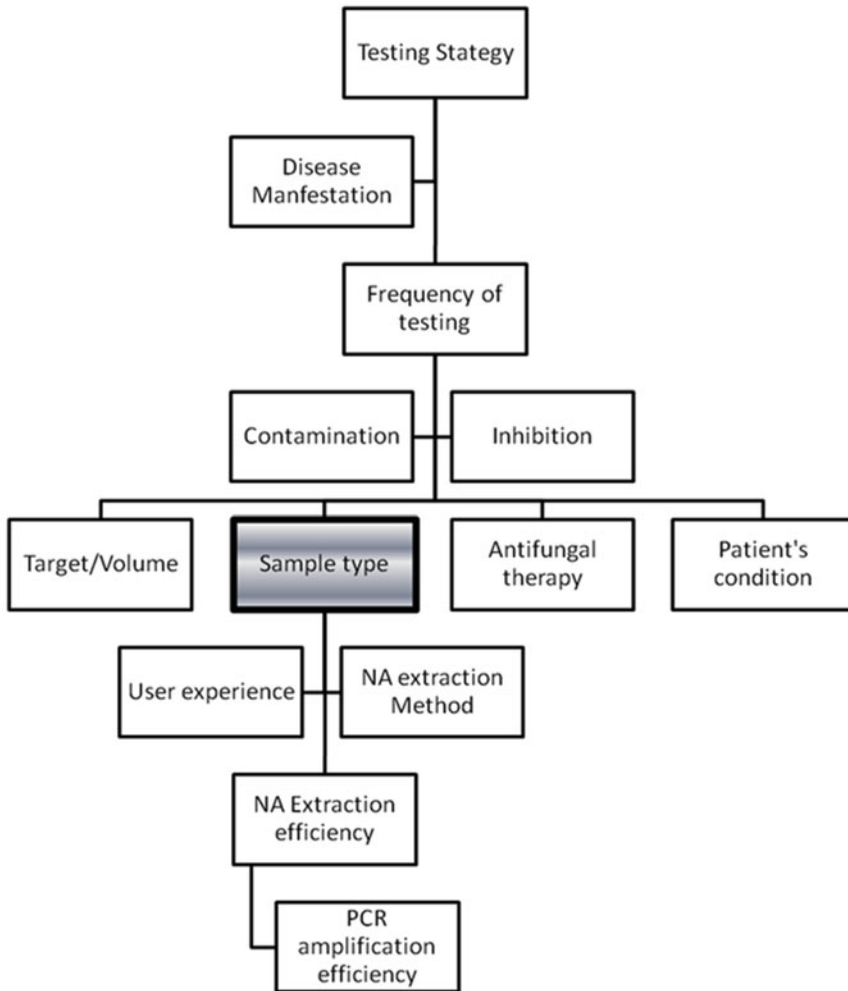

Fig. 1 Factors that could influence PCR performance

infections such as aspergilloma had a lower frequency of positive PCR results compared to proven/presumptive cases of invasive pulmonary aspergillosis [21].

1.2.2 Testing Strategies

The testing strategy will also dictate the specimen choice, and is also influenced by the incidence of IFD. Given that the incidence of most common IFD in developed countries (IA and IC) are relatively low, even in high risk populations, screening strategies to exclude disease are likely to be more useful. The basis of these tests is a high sensitivity/negative predictive value, where negative results can be used confidently to rule out disease and unnecessary antifungal therapy. To achieve this level of sensitivity frequent testing is required excluding the use of invasive samples. Easily obtainable samples (e.g., blood) will be required. Low positivity rates may represent of the transient nature of the circulating target.

Most fungal pathogens are commensal or environmental organisms and infection can begin at any time requiring frequent (≥two times per week) large volume (≥4 ml) sampling to address this problem. The relationship between sample volume and positive PCR result has been noted [22].

To provide diagnostic confirmation of clinical suspicion of IFD, targeted testing from the area of infection (BAL, CSF or tissue biopsies) is required. Presence of target in circulatory samples may be transient and *Aspergillus* DNA is detected in only 11–12 % of blood samples from patients with proven/probable IA. Testing a single blood sample may result in false negativity [23, 24]. PCR testing of tissue biopsies may also be useful in confirming histological evidence of IFD, and providing species identification when culture is negative.

Antifungal therapy has the potential to lower fungal burden to below detectable levels for PCR and lead to negative results [23–25]. It is unwise to perform screening PCR on patients receiving antifungal therapy, including prophylaxis with posaconazole, unless there is evidence of progression or breakthrough IFD. Theoretically, the actions of antifungal therapy on the fungal cell walls/membranes will cause cell lysis and release free DNA, requiring protocols to be modified accordingly. Sample types and DNA extraction techniques should reflect this possibility.

1.2.3 Sample Contamination and Inhibitors

Frequent testing and large sample volumes increase the opportunity for false positive reactions and PCR inhibition. Up to 10 ml of blood have been used for DNA extractions prior to performing *Aspergillus* PCR [25, 26]. False positive PCR results with BAL and sputum samples can be due to inhalation of airborne *Aspergillus* conidia or *Candida* colonization of the mucosal membranes [27–29]. Contamination of vacutainers used to take specimens has been reported [30, 31]. In two studies a high proportion of citrate vacutainers and to a lesser extent EDTA vacutainers were contaminated with *Aspergillus*, [30, 31]. Further environmental fungal contamination of molecular biology reagents has been noted, including general molecular biology reagents (proteinase K/spin columns/lysis buffers) and enzymes specifically used for fungal NA extraction processes (Zymolase/Lyticase). This particularly affects pan-fungal PCR performance, although lysis buffer contamination can cause *Aspergillus* PCR false positivity [31–35].

Extraction is responsible for their removal of potential inhibitors of PCR and failure to do this will impact on PCR amplification. Inhibition of *Aspergillus* PCR due to the presence of anticoagulants has been demonstrated [36]. Using DNA spiked in plasma (0.15 mg/ml) and a nested PCR (by design highly sensitive and often hampered by false positive results) Garcia et al. reported that use of heparin and citrate inhibited PCR whereas EDTA did not [36]. PCR inhibition due to the presence of anticoagulants could

also result from patients receiving heparin for clinical purposes (e.g., after heart by-pass surgery or for treatment of thrombosis). The use of an internal control (IC) PCR identifies potential false negative results and is essential in a diagnostic assay as documented in the European Aspergillus PCR initiative (EAPCRI) recommendations [37, 38].

1.2.4 Source of Fungal DNA

The fungal element (Intracellular fungal DNA within hyphae/conidia/spore (whether viable/nonviable/phagocytosed), or free DNA (DNAemia)) to be targeted by NA extraction will vary with sample type and IFD. For IC, fungemia will occur in approximately two-thirds of cases [39] and yeast cells are certainly present within the circulation making these are logical target for PCR testing. Yet in studies comparing *Candida* PCR testing of whole blood with plasma/serum, where yeast cells have been removed by blood fractionation, showed improved sensitivity with the cell free fractions, indicating a greater abundance of DNAemia [40–42]. The source of free DNA is postulated to be a result of the host immune response or antifungal therapy on the integrity of the yeast cell structure.

For IA, inhalation of airborne conidia, airway colonization, and initiation of the infective process infer that when testing respiratory samples DNA extraction protocols should target intracellular DNA. Yet the effect of alveolar macrophages may also provide a free DNA source requiring different DNA extraction methods; although a combination approach should be feasible (see below). As IA progresses and disseminates through tissue, viable hyphae invade the circulation. PCR positivity in blood could be dependent on the extent of angioinvasion. In clinical practice blood cultures are rarely positive even in disseminated *Aspergillus*, despite the fact that blood culture bottles inoculated with 1–10 cfu of *Aspergillus* conidia becoming positive within 24 h [43–45]. Nonviable fungal fragments resulting from phagocytosis and platelet attachment post angioninvasion could provide an alternative source of DNA [28]. With macrophages thought to be mainly responsible for ingestion of conidia, and neutrophils targeting hyphae [28], the available target in high risk neutropenic patients with invasive disease could be limited. Another circulatory source of phagocytosed fungi may result from translocation of particle-laden alveolar macrophages from the lungs via the bloodstream to extrapulmonary organs [46]. In an animal model, both serum and WB PCR assays were positive one hour after inoculation with *Aspergillus* conidia, excluding disease and angioinvasion, as germination and hyphal formation takes between 6 and 24 h to develop, possibly associated with this translocation mechanism [47, 48].

A wide range of *Aspergillus* PCR manuscripts publications successfully used serum/plasma and WB [11, 12, 49], establishing that both DNA targets are available in blood. Although

standardized methods exist for both sample types it is possible to target both DNAemia and intracellular DNA sources in a single extraction (see below).

1.3 Extraction Method: Factors for Consideration

The specimen type influences the complexity and efficiency of the extraction method. Sample volume and type limit the available target which can be further compromised by the method used. Complex methods are associated with more individual steps increasing the opportunity for DNA losses and contamination. Greater intricacy can affect extraction efficiency, particularly if inexperienced technicians perform the test.

Methods targeting fungal cells need to lyse the cell wall/ membrane. The use of zymolase has been abandoned due to its contamination with fungal DNA [32, 33] and was initially replaced, at great expense, with recombinant lyticase [50, 51]. The use of enzymatic processing not only increases cost but prolongs the procedure. Van burik et al. demonstrated that bead-beating was highly efficient at extracting DNA from both hyphae and conidia [52]. It has been widely used to replace enzymatic disruption of fungal cells in whole blood and BAL, reducing both costs and turnaround time [53–63]. The use of bead-beating instead of enzymatic lysis is recommended by the EAPCRI [37].

The use of automated DNA extractors has reduced hands-on time, minimized crossover contamination and improved interlaboratory comparison when compared to manual methods [64]. A comparison of automated extractors for the extraction of DNA from *Aspergillus fumigatus* in WB showed three extractors Qiagen EZ1 advance XL, Roche MagNA Pure LC, and BioMerieux EasyMag were all capable of detecting down to 10^1 conidia ml^{-1} of EDTA-WB [34]. While the EasyMag generated a superior quality of DNA it was also associated with *Aspergillus* contamination.

The extraction of fungal DNA from WB has been shown to be beneficial [58, 65, 66], but requires additional steps to concentrate and lyse the fungi, and specialist training was required. Automated extraction of serum and plasma allows methods to be in line with routine molecular methods [66–68], an important factor if fungal PCR is to attain widespread use.

The use of manual and automated extraction processes to detect fungal DNA in other samples (CSF, tissue biopsies or other sterile fluids) is less well validated. No standardized methods are currently available and specific DNA sources have not been clarified. To provide optimal performance it would be logical to attempt to extract both free and intracellular DNA from fluids, whereas from biopsies it is essential that the tissue is sufficiently digested to gain access to the invading fungal elements.

Specific protocols for the main sample types discussed will be described below.

2 Materials

2.1 Extraction from EDTA Whole Blood

Blood should be collected using **EDTA** as an anticoagulant and a minimum of 3 ml whole blood should be used as the specimen input. EDTA whole blood may be stored at −80 °C until testing and this process will aid in the lysis of erythrocytes.

Reagents:

1. Red Cell Lysis buffer (RCLB): 10 mM Tris, Adjust pH to 7.6, 5 mM $MgCl_2$, 10 mM NaCl.
2. White Cell Lysis buffer (WCLB): 10 mM Tris, 50 mM NaCl, 0.2% SDS, 10 mM EDTA, Adjust pH to 8.0, proteinase K (200 μg/ml, stored separately at −20 °C and added to buffer immediately prior to use).
3. Alternative Commercial Red cell lysis buffer (RCLB): Cell lysis buffer (Promega).
4. 50 mM NaOH.
5. Ceramic MagNA lyser Green beads (Roche).
6. Proteinase K (200 mg) Molecular grade water.
7. Tween 20 (Polyethylene glycol sorbitan monolaurate).
8. EZ1 Advance XL Tissue kit (Qiagen).

Once prepared all in-house reagents should be filter sterilized using a clean cabinet into single use aliquots and stored at room temperature (unless otherwise indicated).

2.2 Extraction from Serum or Plasma

A minimum of 0.5 ml serum or plasma should be used as the specimen input. Serum and plasma may be stored at −80 °C until testing. Serum and plasma should be removed from WB prior to freezing, as this will cause haemolysis that could inhibit PCR amplification.

Reagents:

1. EZ1 Advance XL DSP Virus kit (Qiagen).
2. 1× Molecular grade Tris-EDTA (TE) buffer.

2.3 Extraction from Deep Respiratory Samples

A minimum of 1.0 ml of bronchoalveolar or bronchial fluid is required. Samples may be stored at −80 °C until testing.

Reagents:

1. Ceramic MagNA lyser Green beads (Roche).
2. Sample process buffer (10% Sodium Dodecyl Sulfate (SDS) and Proteinase K (200 mg)).
3. Sputazol (0.1% dithiothreitol in phosphate buffer; Oxoid Unipath, Basingstoke, UK).
4. EZ1 Advance XL Tissue kit (Qiagen).

3 Methods

Gloves and a laboratory coat should be worn at all times and sample preparation should be performed in a category II safety cabinet.

All DNA extraction described here utilizes the Qiagen EZ1 Advance XL automated extractor. This system can be replaced with any other commercial NA extraction method provided the EAPCRI recommendations on sample volume (≥3 ml EDTA WB, ≥0.5 ml plasma/serum), elution volume (≤100 μl), and mechanical fungal lysis are followed [37, 38, 69]. When testing WB the following commercial assays have been used successfully in place of the Qiagen EZ1 advance XL: Roche High Pure Template DNA kit, Roche MagNA Pure LC, BioMerieux EasyMag, BioMerieux MiniMag [34, 58]. (For a full list of platforms/kits that have been successfully applied for the testing of serum and WB *see* refs. [38, 39]). For BAL, CSF and tissue samples no methodological recommendations currently exist.

Given that the burdens, particularly in circulatory samples, are likely to be at the limit of real-time PCR detection every eluate should tested in duplicate, as a minimum requirement, and an IC PCR should be performed to avoid the reporting of false negative results [37, 38].

3.1 DNA Extraction from EDTA Whole Blood

3.1.1 Extraction Controls

To prepare extraction controls for *Candida* and *Aspergillus* PCR:

1. In mycology laboratory culture *C. albicans* and *A. fumigatus* on SABC agarose plates by incubating at 30 °C for 2 days for *C. albicans* or until *A. fumigatus* sporulates. After inoculating the *Aspergillus* plates seal with tape to stop contamination of laboratory.
2. For *Candida* scrape a 2–3 colonies with a sterile loop and resuspend in 10 ml sterile distilled water.
3. For *Aspergillus* transfer the sporulating culture to a Class I laminar flow hood and remove seal.
4. Using good aseptic technique transfer spores with a dry cotton swab to 10 ml of sterile distilled water containing a drop of Tween 20 to prevent clumping.
5. Transfer suspensions back to the mycology laboratory and perform 1/10 dilutions using sterile water. Usually a 1/1000 dilution provides a number that is quantifiable using a counting chamber.
6. Count the number or *Candida* blastospores and *Aspergillus* spores using a Fuchs-Rosenthal counting chamber. Record the number of cells/spores per μl
7. In Class 2 laminar flow cabinet divide EDTA blood in to 3 ml aliquots.

8. Depending on the counts spike the positive control with 10 blastopsores of *Candida* and 10 conidia of *Aspergillus*.
9. Transfer controls back to DNA extraction Laboratory for immediate use or storage at −80 °C.

This process can be easily replicated for alternative yeast species. However, for mould species that do not regularly sporulate and for species, such as *Pneumocystis jirovecii* that are difficult to culture quantification of organism will be difficult and using quantified DNA for a positive control material may be preferential.

3.1.2 Sample Preparation

In a Class II safety cabinet a minimum of 3 ml of the EDTA blood samples should be transferred to a sterile 15 ml labeled tube for immediate testing or storage at −80 °C until testing.

3.1.3 WB Sample Extraction (See Fig. 2)

Before performing any Fungal DNA extractions all work surfaces and pipettes should be decontaminated using Microsol and DNAzap.

1. Bring all samples and controls to room temperature. Ensure samples and controls are **defrosted thoroughly**.
2. Mix 3–4 ml of EDTA blood with 10 ml of red cell lysis buffer.
3. Centrifuge at 2000 ×*g* for 15 min in a sealed rotor centrifuge and discard the supernatant.
4. Repeat **steps 2** and **3**.
5. Resuspend the pellet in 1 ml white cell lysis buffer with proteinase K, transfer to labeled sterile 1.5 ml screw-cap tubes, and incubate at 65 °C for 45 min.
6. Centrifuge specimens in white cell lysis buffer at 10,000 ×*g* in a sealed microcentrifuge for 10 min. While this is proceeding dispense 20–30 μl of ceramic MagNA lyser green beads into 1.5 ml sterile tubes.
7. Discard the supernatant, if minimal white cells remain go to **step 10**.
8. If a significant white cell pellet remains add 200 μl of 50 mM NaOH. Incubate at 95 °C for 5 min or until pellet is no longer visible.
9. Centrifuge at 10,000 ×*g* in a sealed microcentrifuge for 10 min and discard the supernatant.
10. Add the equivalent of 20–30 μl of the predispensed (to avoid contamination of batch) ceramic beads and bead-beat vigorously for 30 s.
11. Add 190 μl of EZ1 G2 buffer to each sample and agitate the ceramic beads at least ten times by multiple depressions of the pipette to wash material off the beads.

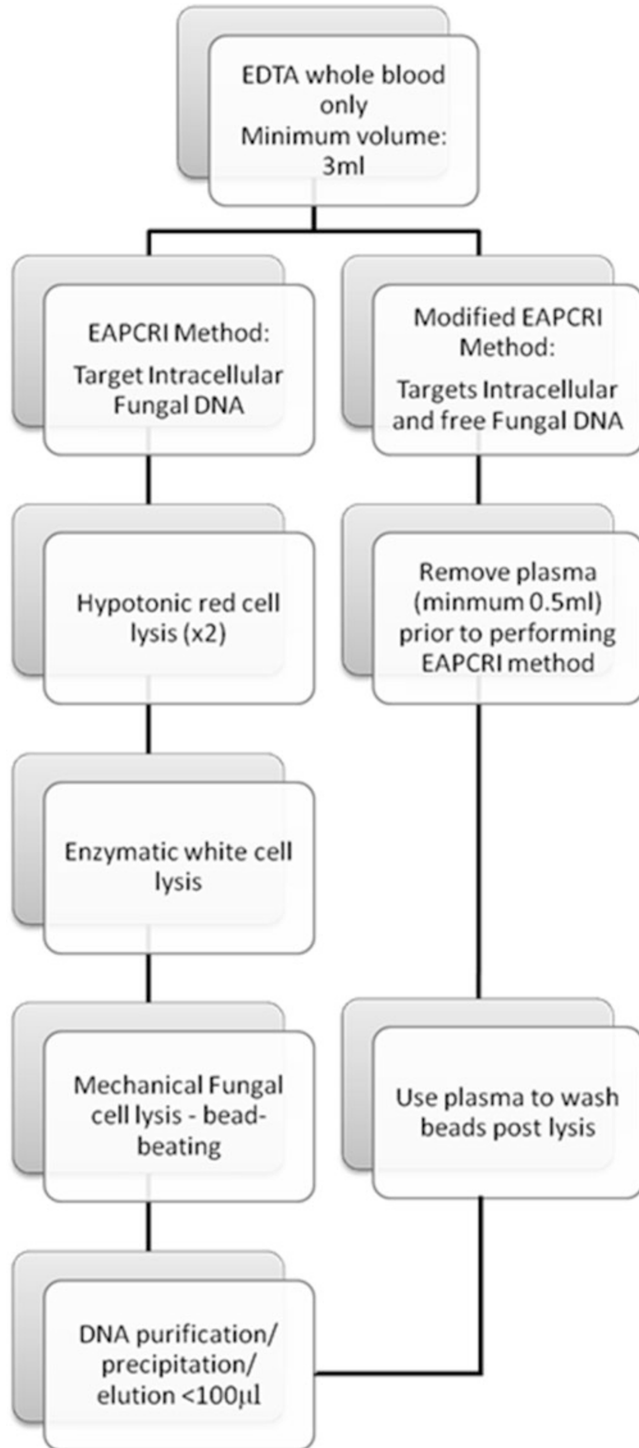

Fig. 2 Nucleic acid extraction from whole blood

12. Transfer washings to fresh labeled tubes containing 10 μl of EZ1 proteinase K and incubate at 56 °C for 30 min.
13. Meanwhile, set up the EZ1 advanced XL extractor by inserting the correct Tissue DNA kit card and the turning on the machine using the toggle switch at the back left. **Do not turn on the machine without a card inserted!**
14. Press start and follow the on screen instructions for data tracking.
15. Choose an elution volume of 60 μl.
16. Invert the correct number of reagent cartridges, open the instrument door and remove the specimen processing rack and reagent cartridge rack.
17. Insert the correct number of reagent cartridges and put the reagent cartridge rack back into the machine.
18. Take the specimen processing rack to a category II laminar flow cabinet and insert the correct number of 1.5 ml sterile elution tubes into row 1 (**Do not remove the caps**), sterile tips and tip holders into row 2.
19. On completion of the incubation (**step 12**) place the specimens into row 4.
20. Transfer the sample processing rack and insert it into the instrument. **Remove the caps from both the sample and elution tubes**.
21. Close the instrument door and press start.
22. The entire process takes approximately 15 min to complete.
23. On completion remove elution tubes and incubate the tubes at 70 °C for 10 min prior to capping. Discard all waste and UV irradiate the instrument for 30 min.
24. Extracts can be stored at 4 °C for up to 24 h or at −80 °C for longer periods prior to amplification. To prevent NA degradation avoid repeated freeze/thawing procedures.

3.2 DNA Extraction from Serum or Plasma

3.2.1 Extraction Controls

To creating a DNA source for controls:

1. Culture the desired fungi using Sabouraud media at 30–37 °C for 48 h for yeasts or until filamentous fungi have sporulated.
2. For *Candida* or yeasts scrape a large number of colonies with a sterile loop and resuspend in 1 ml sterile distilled water.
3. For *Aspergillus* and other filamentous moulds transfer the sporulating culture to a Class I laminar flow hood. Using good aseptic technique transfer a heavy inoculums of spores with a dry cotton swab to 3 ml of sterile distilled water containing a drop of Tween 20.

4. Take 1 ml of the desired suspension and centrifuge at $10,000 \times g$ for 5 min, decant the supernatant and discard.
5. Mechanically disrupt the fungal cells by bead-beating for 30 s with the equivalent to 20–30 μl of ceramic MagNA lyser green beads.
6. Pulse centrifuge and then extract the DNA using the EZ1 Advance XL tissue kit as described in Subheading 3.1.3 (**step 11** onwards) or using any commercial DNA extraction kit.
7. Quantify the NA using a nanophotometer. The extract can be stored at −80 °C until required or diluted to the necessary concentration with TE buffer prior to storage.
8. Ideally calculate the concentration of the extract in genomes per μl:

   Concentration of the extract (g/μl)/weight of the fungal genome (g).
   Weight of the Fungal genome (g) = Genome weight (Daltons) × |1.661 × 10^{-24}, where base pair = 660 Da.

   Example:

   *A. fumi*gatus genome = 29.3 Mb.

   Weight in Daltons = $29.3 \times 10^{6} \times 660 = 1.9338 \times 10^{10}$ Da.

   Weight in Grams = $1.9338 \times 10^{10} \times 1.661 \times 10^{-24} = 3.21 \times 10^{-14}$ g.

   If the DNA extract concentration = 15 ng/μl = 1.5×10^{-8} g/μl.

   Then concentration in genomes = 1.5×10^{-8} g/μl/3.21×10^{-14} g = 4.7×10^{5} genomes/μl.

To prepare control material for testing serum/plasma:

9. Remove the quantified genomic fungal DNA extract (usually *A. fumigatus* or *C. albicans*) from the −80 °C freezer. If not already diluted, use TE buffer to dilute the DNA to the desired a concentration of 1 genome/μl and aliquot in to 20 μl volumes.
10. Store excess diluted DNA aliquots at −80 °C until required.
11. In Class 2 cabinet (Room 137) divide serum/plasma in to 0.5 ml aliquots.
12. Store excess serum aliquots at −80 °C until required.
13. For each extraction a positive and negative control are required.
14. For the positive control spike 0.5 ml of serum with 10 μl of diluted DNA extract.
15. For the negative control use 0.5 ml of unspiked serum.
16. Store control samples at −80 °C until required. To avoid degradation do not expose the positive control samples to more than one freeze–thaw cycle.

3.2.2 Sample Preparation

In a Class II safety cabinet 0.5 ml of serum/plasma should be transferred to a sterile labeled 1.5 ml Eppendorff tube for immediate testing or to be stored at −80 °C before testing.

3.2.3 Plasma/Serum Sample Extraction

Before performing any fungal DNA extractions all work surfaces and pipettes should be decontaminated using Microsol and DNAzap.

1. Bring all samples and controls to room temperature.
2. Set up the EZ1 advanced XL extractor by inserting the DSP Virus kit card and the turning on the machine using the toggle switch at the back left. **Do not turn on the machine without a card inserted!**
3. Press start and follow the on screen instructions for data tracking.
4. Choose a sample volume of 400 μl and elution volume of 60 μl.
5. Invert the correct number of reagent cartridges, open the instrument door and remove the specimen processing rack and reagent cartridge rack.
6. Insert the correct number of reagent cartridges and put the reagent cartridge rack back into the machine. Load the correct number of non-skirted 2 ml tubes into the heating block position.
7. Take the specimen processing rack insert the correct number of labeled 1.5 ml sterile elution tubes into row 1 (**Do not remove the caps**), sterile tips and tip holders into row 2. 1.5 ml tubes containing 60 μl of internal carrier RNA into row 3 (**Do not remove the caps**), and the specimens into row 4 (**Do not remove the caps**). Transfer the sample processing rack and insert it into the instrument. **Remove the caps from all tubes**.
8. Close the instrument door and press start.
9. The entire process takes approximately 45 min to complete.
10. On completion remove elution tubes and incubate the tubes at 70 °C for 10 min prior to capping. Discard all waste and UV irradiate the instrument for 30 min.
11. Store extracts at 2–8 °C for up to 24 h or at −80 °C for longer periods. To prevent NA degradation avoid repeated freeze/thawing procedures.

3.3 DNA Extraction from Bronchoalveolar Lavage and Bronchial Fluids

3.3.1 Extraction Controls

To prepare control material for respiratory sample testing:

1. Remove the quantified genomic fungal DNA extract (usually *A. fumigatus*) from the −80 °C freezer as described in Subheading 3.2.1.
2. Attain a suspension of fungal spores as described in Subheading 3.1.1.

3. Store excess diluted DNA aliquots at −80 °C until required.
4. In Class 2 cabinet (Room 137) divide BAL fluids in to 1.0 ml aliquots.
5. Store excess BAL aliquots at −80 °C until required.
6. For each extraction a positive and negative control are required.
7. For the positive control spike 1.0 ml of BAL with 10 μl of diluted 1 genome/μl DNA extract and 10 spores (e.g., *A. fumigatus* conidia).
8. Store the spiked BAL samples at −80 °C until required. To avoid degradation do not expose the positive control samples to more than one freeze–thaw cycle.
9. For the negative control use 1.0 ml of unspiked BAL.

To produce control material containing just free DNA target, exclude fungal spores and for intracellular DNA exclude the free DNA.

3.3.2 Sample Preparation

Due to the risk of category III pathogens (*Mycobacterium tuberculosis*) in deep respiratory samples all BAL/NBL fluid require inactivation in a level III containment laboratory using a class I safety cabinet. Heat the sample to be extracted (minimum volume 1 ml) at 120 °C for 30 min after which the sample is safe to be processed in level II containment laboratories for immediate testing or storage at −80 °C. An alternative approach dependent on the extraction method, is to utilize the lysis buffer provided to inactivate any potential pathogens (e.g., a minimum of 10 min in Nuclisens Lysis buffer (BioMerieux)).

3.3.3 Sample Extraction (See Fig. 3)

N.B. Before performing any Fungal DNA extractions all work surfaces and pipettes should be decontaminated using Microsol and DNAzap.

1. Bring all samples and controls to room temperature. Ensure samples and controls are **defrosted thoroughly** and vortex.
2. If the sample is particularly mucoid mix 1:1 (v/v) with 0.2% (final concentration) SDS and proteinase K (200 μg/ml) and incubate for 30 min at 56 °C. Alternatively use and equal volume of Sputazol as per manufacturer's instructions.
3. Centrifuge a minimum volume of 1.0 ml of BAL fluid at 10,000 ×*g* for 10 min.
4. Transfer the supernatant to a new sterile 1.5 ml microfuge tube.
5. Bead-beat the remaining pellet for 30 s using Roche MagNA LYser green
6. beads.

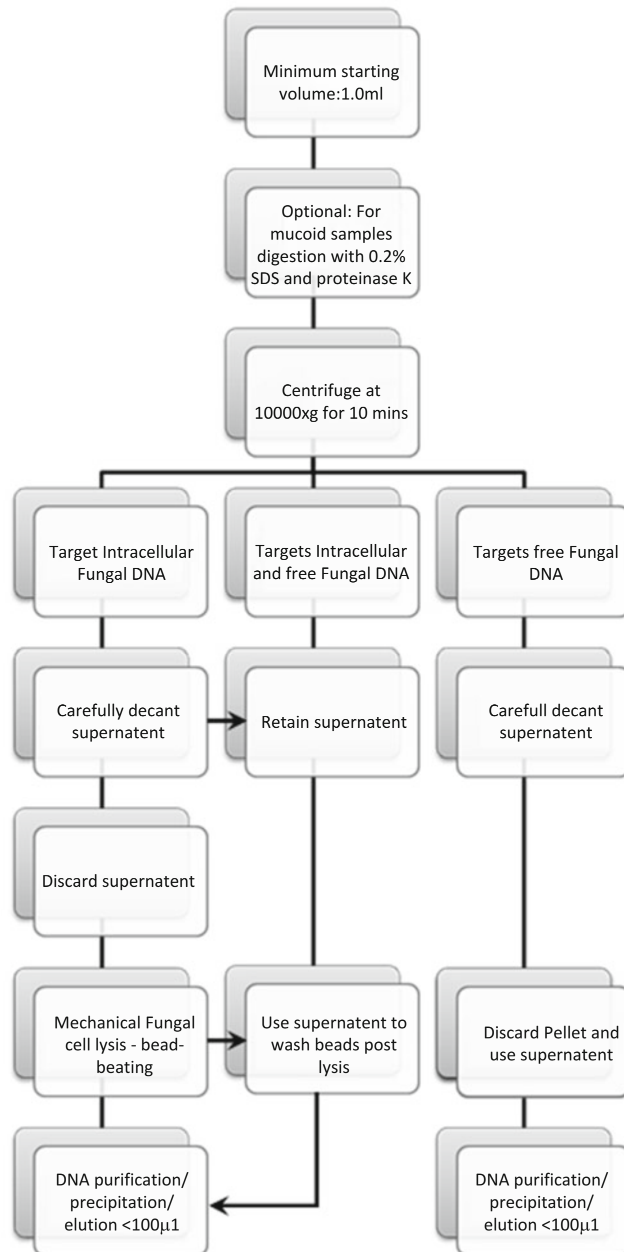

Fig. 3 Nucleic acid extraction from deep respiratory samples

7. Pulse centrifuge, and wash the pellet/beads with 200 µl of supernatant and transfer the combined washings to a tube containing 190 µl of EZ1 G2 buffer and 10 µl of EZ1 proteinase K and incubate at 56 °C for 30 min.
8. At this point set up the EZ1 advanced XL extractor by inserting the correct Tissue DNA kit card and the turning on the

machine using the toggle switch at the back left. **Do not turn on the machine without a card inserted!**

9. Press start and follow the on screen instructions for data tracking.
10. Choose an elution volume of 60 μl.
11. Invert the correct number of reagent cartridges, open the instrument door and remove the specimen processing rack and reagent cartridge rack.
12. Insert the correct number of reagent cartridges and put the reagent cartridge rack back into the machine.
13. Take the specimen processing rack to a category II laminar flow cabinet and insert the correct number of 1.5 ml sterile elution tubes into row 1 (**Do not remove the caps**), sterile tips and tip holders into row 2.
14. On completion of the incubation highlighted in **step 12** place the specimens into row 4.
15. Transfer the sample processing rack and insert it into the instrument. **Remove the caps from both the sample and elution tubes**.
16. Close the instrument door and press start.
17. The entire process takes approximately 15 min to complete.
18. On completion remove elution tubes and incubate the tubes at 70 °C for 10 min prior to capping. Discard all waste and UV irradiate the instrument for 30 min.
19. Extracts can be stored at 4 °C for up to 24 h or at −80 °C for longer periods prior to amplification. To prevent NA degradation avoid repeated freeze/thawing procedures.

4 Notes

4.1 Extractions in General

To avoid wasting clinical samples it is essential that all control material and any kits/reagents be screened for fungal contamination prior to use, and this should be performed on a batch basis.

For each extraction procedure one positive and one negative control must be included, and when testing larger batches of samples ($n = > 20$) it is advisable to include more control samples. The positive material used should represent the suspected DNA source in that particular sample (e.g., for WB intracellular DNA in the form of fungal organism, or for serum/plasma simply genomic Fungal DNA). Positive controls should represent burdens typically encountered in the clinical scenario, but at levels providing 100% reproducible PCR detection to prevent high rates of unnecessary extraction run failures (approximately 5–20 genomes). Extraction controls for fungal PCR can be prepared up to one month prior to

extraction and stored at −80 °C until required. Prior to extraction a positive control and negative control should be thawed along with any stored clinical samples and all processed in an identical manner.When lysing fungal cells mechanical disruption is cheaper, quicker; reduces the risk of contamination; and is more efficient than enzymatic lysis. It is not necessary when extracting specimens (serum/plasma) targeting free DNA, in fact it may be detrimental to the yield of free DNA extracted. If a bead-beater is not available then vortexing in the presence of ceramic/glass beads for 30–60 s is sufficient, although if possible it is wise to use a vortex-adaptor to hold several tubes in place during a single process.

The use of an IC is essential for any diagnostic PCR assay. The IC target should be present at a concentration that is similar to that for the expected pathogen, for IFD this can be very low (Cq: >35 cycles). To avoid over reporting of inhibition the IC should be at a concentration that achieves 100% reproducible detection with consistent quantification cycles (Cq: <38 cycles), so expected IC crossing points should be in the range of 33–37 cycles. Ideally, the IC target will be spiked into the sample prior to extraction and in doing so limits the need for positive extraction controls, as the efficiency of extraction for each sample will be individually monitored. The IC target should not be concomitantly extracted human DNA. The concentration of human DNA will differ between specimens resulting in varying Cq values and identifying inhibition across samples becomes difficult. The IC target should not be another fungal pathogen that could interfere with pathogen of interest through competitive PCR amplification. Using assays for non-fungal pathogens already at use in the laboratory for a fungal IC is feasible, although prolonged amplification of these targets could result in contamination issues, and the use of nonspecific (junk) DNA targets or assays not used in the lab maybe preferable. For the testing of serum and plasma the IC target should be a DNA source that can be spiked directly into the sample prior to extraction, monitoring for individual extraction efficiency and inhibition. While for WB and respiratory samples the IC target can be DNA or organism (e.g., *Bacillus* spores) depending on the method used, although if DNA is used for these sample types it should not be introduced prior to bead-beating, as this can fragment and bind DNA. If the IC cannot be incorporated prior to extraction then using a PCR master-mix spiked with IC DNA can be utilized, but only provides information regarding PCR inhibition. It cannot be used to monitor DNA extraction efficiency and a positive extraction control must be used.

In summary, efficient extraction of nucleic acid is critical to successful PCR amplification. This is particularly relevant for the detection of IFD where circulating DNA sources may be close to the limit of detection by molecular methods (≤1 genome/ml). Losses associated with DNA extraction are accentuated by multiple

steps and simplifying procedures is beneficial [70]. The use of automated platforms (described above) reduce work burden, minimize crossover contamination, and improve inter-laboratory reproducibility compared to manual methods [64]. However, they do require the purchase of relatively expensive equipment. The development of new and/or improved technologies, particularly specific to the detection of IFD (T2MR *Candida*) could enhance performance and robustness further [71]. The availability of commercial PCR assays for the detection of IFD when combined with standardized DNA extraction protocols using commercially sourced kits will allow molecular testing to gain widespread use outside specialist reference settings.

4.2 Extractions from WB

Control material for WB extractions consists of EDTA blood taken from a healthy donor (including commercially sourced animal donor material) and should be divided into 3 ml aliquots and stored at −80 °C. Freezing WB samples prior to testing assists in the lysis of erythrocytes. The positive material used for WB samples is intracellular DNA in the form of fungal organism. For invasive yeast infections spiking blood with blastospores represents infection, although for invasive mould disease compromise may be necessary. For invasive mould disease hyphal invasion of tissue and blood vessels occurs, and non-phagocytosed spores are unlikely to be found in the circulation, but spiking WB with hyphae is inaccurate due to difficulties in quantifying this fungal morphology that is also multinucleate. An alternative is to use recently germinated spores (germlings) that can be quantified but also exhibit a hyphal element.

The WB extraction described (Fig. 2 and Subheading 3.1.3) remains time consuming, taking approximately 3–4 h to complete, but the necessity of each step has been highlighted [72]. While removing one of the erythrocyte lysis steps saves time (30 min) and does not particularly affect PCR performance, especially if blood has been frozen prior to testing, it can result in a heavily blood stained pellet potentially resulting in inhibition. This is issue is accentuated if both erythrocyte lysis steps are excluded and was shown to reduce the reproducibility of detection [72]. Leukocyte lysis is critical to performance and exclusion causes a 60% reduction in reproducibility and significant inhibition. Sample volumes <3 ml and elution volumes >100 μl reduce reproducibility by ≥50%. Fungal cell lysis, preferably through bead-beating, is also critical [72].

The WB extraction technique described (*see* Subheading 3.1.3) only targets any intracellular fungal DNA sources, with any free DNA decanted in **steps 2** and **3**. It can be modified to target both DNA sources available in blood (Fig. 2). To do this decant 0.5 ml of plasma from the EDTA WB on sample receipt (prior to freezing),

proceed with **steps 1–10**. Post bead-beating (**step 10**) replace 190 μl of EZ1 G2 buffer with the 0.5 ml plasma and extract these washings using a commercial DNA extraction platform as described for serum and plasma [73].

4.3 Extractions from Serum/ Plasma

The control material is serum (separated from clotted blood) or plasma (separated from EDTA blood) provided by a healthy donor (including commercially sourced animal donor material) and is divided into 0.5 ml aliquots that are stored at −80 °C. All material must be screened for fungal contamination prior to use. Positive controls should represent the suspected DNA source, in this case free DNA (DNAemia).With extraction from serum/plasma targeting DNAemia the use of commercially available kits is recommended, and provides quality controlled manufacture achieving temporal and inter-laboratory consistency. In the EAPCRI study investigating the performance of *Aspergillus* PCR 96.6% of the commercial protocols evaluated were able to reach the designated threshold of detection on at least one occasion, and permits fungal PCR to be performed in generic molecular diagnostic laboratories alongside bacterial and viral assays [38]. A minimum volume of 0.5 ml sample should be used, with larger volumes up to 1 ml potentially improving performance, but limiting the extraction options available and DNA should be eluted in ≤100 μl [38]. Several automated platforms are available but if manual extraction is performed spin columns (Qiagen Qiamp DNA kits or Roche High Pure template DNA kits) should be used. Larger volumes of serum/plasma can be processed by loading multiple volumes to the same column or through the purchase of the large volume kits. To improve DNA recovery it may also be beneficial after adding the elution buffer to incubate the spin column at room temperature for 2–5 min prior to centrifugation. These recommendations can also be applied to manual extractions with spin columns of specimens other than serum/plasma. The use of carrier RNA/DNA may be beneficial for increasing the efficiency of extraction of low concentrations of target DNA.

While there are no major technical differences when extracting fungal DNA from serum and plasma the formation of clot material potentially reduces available DNA in serum and the clot has been shown to contain significant amounts of target DNA [69, 74]. Unfortunately, processing the clot in routine practice is difficult. Avoiding the formation of the clot may provide greater amounts of free DNA in the cell free fraction leading to earlier crossing points, which is important as circulatory fungal DNA concentrations are regularly at the limit of real-time PCR detection [69]. During disease *Aspergillus* DNA burdens in blood are often at the limits of PCR performance. Using plasma could improve performance while maintaining methodological simplicity of serum testing

4.4 Extraction from Respiratory Samples

There are three issues when preparing controls for extractions of deep respiratory samples. Firstly, obtaining a representative specimen control matrix can be difficult as volunteers for bronchial or bronchoalveolar washing are infrequent. Secondly, yeasts are commensal organisms of mucosal membranes and airborne environmental moulds are regularly inhaled so low grade contamination is difficult to avoid. Finally, two DNA sources (fungal cells and free DNA) are likely to be present and the decision of which target, possibly both, should be used to generate the positive control sample.

The DNA source is unlikely to significantly affect Ct values at higher burdens (≥50 genomes per sample), with Ct values similar whether the targeted DNA be sourced from fungal cells or free DNA. At lower burdens there can be a marked difference in detecting free and intracellular DNA, consequently effecting PCR efficiency (Fig. 4). More complicated procedures targeting the organism will be less efficient resulting in later Ct values that will increase if the method is not optimized for one of the sources (Fig. 4). Given that the extraction protocol described targets both DNA sources the positive extraction controls described also contain both targets (Fig. 3). While human DNA should not be used as an internal control to monitor for inhibition, when testing respiratory samples (e.g., BAL) determining the amount of human DNA is indicative of the amount of cellular material in the sample, and the quality of the sampling process. Testing for the presence of human DNA besides an internal control PCR prevents the reporting

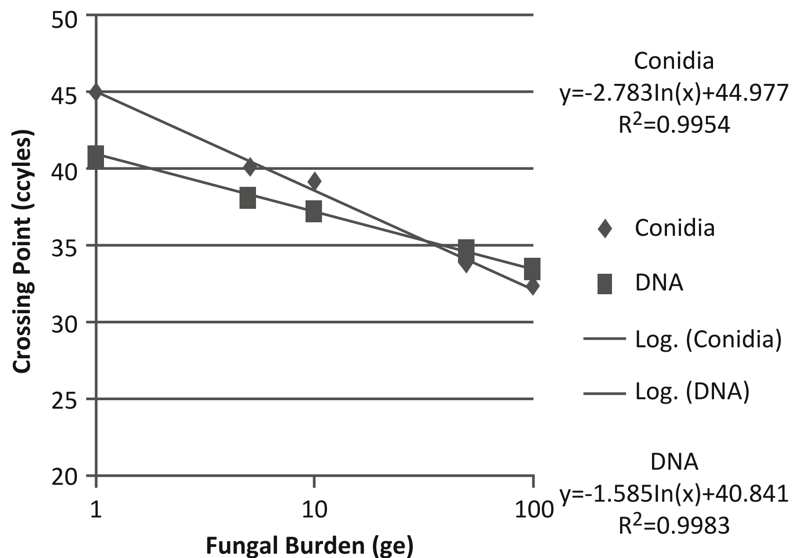

Fig. 4 *Aspergillus* PCR efficiency when detecting free DNA and conidial based DNA sources in bronchoalveolar lavage fluid

of false negatives due to inadequate sampling. In addition, for infections such as PCP the immune/inflammatory response is related to disease severity, and for non-HIV susceptible individuals this may be great despite low fungal burdens. In this scenario targeting human DNA may be helpful for determining the significance of infections associated with low burdens.

The above protocol targets both free and intracellular fungal DNA. To only target intracellular DNA, post centrifugation **step 3** remove the supernatant and discard (Fig. 3). To only target free DNA, the supernatant obtained after centrifugation **step 4** can be extracted directly. Alternatively the naive BAL sample can be extracted without the bead-beating stage. Bead-beating a naive BAL sample and continuing with the extraction may appear to provide a mechanism for extracting both free and intracellular DNA but is optimal for neither. Bead-beating a fluid, rather than a pellet, provides a degree of buffering, results in the formation of froth and is a potential mechanism for fragmenting free DNA.

4.5 Extraction from Other Sample Types (Cerebrospinal Fluid or Tissue)

Fungal infection in tissue proceeds through hyphal invasion and it is difficult to generate representative positive control material. Surplus clinical or animal-model material from confirmed IFD will be in limited supply and has ethical implications. As this sample type is used for diagnostic confirmation, and not to exclude IFD assay, specificity, and ruling out contamination is the key requirement. It is acceptable to perform these processes using only a negative process control, such as molecular grade water. This monitors for any contamination that may have entered during procedure. Currently, there are no recommendations for the PCR testing of tissue biopsies, with most studies following local protocols. Consequently, no specific method will be recommended, but a few steps essential for success will be considered. Fresh rather than formalin-fixed paraffin-embedded (FFPE) specimens are preferable as the extraction protocol is less complicated. However, if microscopic fungal elements have been seen in histological specimens, retrospective molecular identification using the fixed specimen, maybe useful if no fresh specimen is available. If paraffin-embedded tissue is used this can be removed by washing twice with xylene. It is essential to thoroughly digest the tissue to gain access to the invading fungi and this can be improved by finely slicing or homogenizing the tissue prior to tissues digestion using proteinase K/SDS or liquid nitrogen. Bead-beating of the digest is an efficient and cost effective method of lysing fungal cells, after which DNA should be purified using commercially available methods. In a study comparing the efficiencies of five commercially available tissue DNA extraction methods for detecting fungal DNA in FFPE tissues the TaKaRa Dexpat (Takara Bio Inc, Japan) and the QIAmp DNA FFPE Tissue Kit (Qiagen, Germany) provided optimal performance, particularly for moulds [75]. This study provides a good source for methods for

fungal DNA extraction from FFPE, although the use of recombinant lyticase is not necessarily required.

For CSF samples access to material is also a problem, as is identifying the precise DNA source in this specimen type. Unlike BAL testing, environmental contamination and colonization are not issues, like tissue testing, diagnostic confirmation is most important. Consequently, the use of only a negative process control is acceptable. If excess CSF material is available, positive controls could be developed in line with BAL testing (Subheading 3.3.1). When extracting DNA from CSF samples a minimum of 0.5 ml CSF should be used and DNA can be extracted in line with BAL methods (Subheading 3.3.3), excluding **step 2** and similarly can be modified to target free or intracellular DNA or both (Fig. 3).

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Chapter 13

Prerequisites for Control of Contamination in Fungal Diagnosis

Stefan Czurda and Thomas Lion

Abstract

Nucleic acid amplification methods facilitate rapid and sensitive detection of clinically relevant fungal pathogens, and can be employed using a variety of patient specimens. However, contamination from various exogenous sources constitutes a serious threat to the validity of amplification-based fungal assays. In this chapter, common origins of fungal contaminants that compromise molecular fungal testing are described, and measures for preventing contamination are proposed. Detailed guidelines for sample handling, reagent selection, contamination screening, and decontamination procedures are provided.

Key words PCR reagent contamination, Contamination prevention, DNase-mediated decontamination, Invasive fungal infection, Molecular fungal diagnostics

1 Introduction

Nucleic acid amplification techniques (NAAT) have great potential to overcome limitations of current gold standard methods in fungal diagnostics which offer limited sensitivity or are inherently slow in providing diagnostic information [1–4]. Major advantages of NAAT methods include speed and high sensitivity enabling the detection of minute amounts of fungal DNA. Conversely, since only a few target molecules are required for sufficient amplification, even traces of contaminating DNA may become a major problem by leading to false positive results [5]. Hence, the major benefit of amplification-based testing, the ability to detect minimal amounts of target DNA, is a double-edged sword. Generally, fungal amplification techniques are amenable to contamination from various sources. Panfungal or broad-spectrum detection methods are much more vulnerable to trace amounts of nucleic acid contamination than species-specific assays, due to their ability to detect contaminating DNA from various fungal sources [1]. Several reports describing potential sources of fungal contamination in NAAT testing have been published. Airborne fungal spores and conidia are ubiquitously present in

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indoor environments, and constitute serious contaminants during various working steps including DNA extraction, master mix preparation, and sample amplification setup [6–8]. Moreover, the human skin is colonized by various fungal species [9], and therefore represents an additional important source of sample contamination. Further, there is considerable risk that traces of fungal DNA are present in reagents required for fungal cell lysis, DNA extraction, and nucleic acid amplification [6, 10–14]. It was shown that commercially available enzymes required for digestion of the fungal cell wall can contain traces of fungal nucleic acids [6, 13]. Fungal DNA was also detected in components of commercial kits for nucleic acid extraction [11], and even in sterile tubes for the collection of clinical specimens [15]. Recently, it was reported that traces of contaminating DNA may be present in oligonucleotide and master mix solutions for real-time PCR analysis [10]. Together, these observations indicate that virtually any step during a NAAT workflow may be at risk of contamination. Here we provide practical guidelines for effective contamination control. Their implementation greatly reduces the risk of false positive results, and can therefore improve the validity of fungal amplification-based assays.

2 Materials

1. Spatially separated laminar air flow (LAF) workbenches equipped with UV light for DNA decontamination.
2. Lab coat.
3. Nitrile gloves.
4. 1.5 % hypochlorite solution (*see* **Note 1**).
5. One-way cleaning cloths.
6. Sterile disposable arm sleeves.
7. DNA-free safe lock tubes (Biopure™, Eppendorf).
8. DNA-free pipette tips (Biosphere™, Sarstedt).
9. DNA-free water (Molzym, Germany).
10. Nucleic acid extraction kit including DNA-free reagents (MolYsis™ Complete 5 DNA extraction kit, Molzym).
11. Prescreened PCR oligonucleotides free of fungal nucleic acids (*see* Subheading 3.4).
12. Prescreened PCR master mix reagents free of fungal nucleic acids (*see* Subheading 3.4).
13. DNA-free 96-well reaction plate (Life Technologies).
14. Tris buffer (20 mM Tris–HCl, 5 mM $MgCl_2$, pH 8) free of fungal nucleic acids.
15. Double-strand specific DNase (ArcticZymes).

3 Methods

3.1 General Handling Measures to Prevent Fungal Contamination

1. Handling of patient samples used for testing by NAAT should occur exclusively in an LAF workbench to avoid contamination by airborne fungal spores or conidia.
2. Lab coat, fresh nitrile gloves, and sterile arm sleeves should always be worn and changed frequently. Arm sleeves should completely cover the skin.
3. Handling of PCR reagents (preparation of primer/probe and master mix aliquots, master mix pipetting procedures) should be performed in an LAF workbench placed in a location spatially separate (different room) from the workplaces where DNA extraction, PCR setup and amplification are performed (*see* **Note 2**).
4. The surface of all materials and reagents transferred into the LAF workbench should be wiped with hypochlorite solution using a one-way cleaning cloth.
5. LAF workbench surfaces, pipettes, and other equipment should be regularly cleaned with hypochlorite solution.
6. Laboratory waste should be collected in solid, liquid-proof bags and discarded immediately after completion of all working steps. Sterile bags should be used if they are placed within the LAF workbench.
7. UV-decontamination of the LAF workbench should be performed on a daily basis, after completion of all working steps (*see* **Notes 3** and **4**).
8. Frequently used equipment such as tabletop centrifuge, heating block, vortex machine, and pipettes should be placed permanently in the LAF workbench, if possible, to prevent exogenous contamination during sample handling.
9. Materials not certified as DNA-free need to be pretested for possible fungal contaminants (*see* Subheading 3.4).

3.2 Contamination-Free DNA Extraction

1. Fungal DNA contamination was reported in reagents used for DNA extraction. To exclude contamination with traces of fungal DNA, use extraction kits that include certified DNA-free reagents and consumables.
2. Use certified DNA-free pipette tips, reaction tubes and water during isolation of fungal nucleic acids.
3. Perform DNA extraction exclusively in an LAF workbench to avoid contamination with airborne spores or conidia.
4. If vortexing of samples during DNA extraction is required, briefly centrifuge tubes afterwards to remove liquid from the lid. This measure avoids aerosol formation when opening the

tubes which could lead to cross-contamination between different patient samples processed in parallel.

5. Fungal cell walls are difficult to lyse thus requiring additional materials such as glass beads for bead-beating or enzymes for cell wall degradation. These materials may not be included in commercial extraction kits. To prevent contamination from materials not certified as DNA-free, perform regular screening using an appropriate number of negative control reactions.

3.3 Contamination-Free DNA Amplification

1. Master mix preparations should be performed in an LAF workbench placed in an area physically separated from the space of patient sample handling.
2. Use certified DNA-free pipette tips, water, and consumables during the amplification procedure.
3. Use master mix solutions containing uracil-*N*-glycosylase (UNG), and perform a digestion step prior to amplification to reduce the risk of carryover contamination by PCR amplicons.
4. Customized DNA oligonucleotides (primers, probes) and master mix solutions are at considerable risk of containing traces of fungal nucleic acids. Perform regular screening of these reagents as described in Subheading 3.4.
5. If traces of fungal nucleic acids are detected during screening, perform decontamination as described in Subheading 3.5.

3.4 Screening of Reagents at Risk of Containing Fungal Contamination

Whenever possible, use certified DNA-free reagents and consumables for fungal DNA extraction and amplification procedures. Other materials should be screened for potential contamination by traces of fungal material (*see* **Note 5**). A guideline for contamination screening is outlined below.

1. When subjecting materials or reagents to contamination screening, ensure that all other materials included in the workflow are free of fungal traces to prevent difficulties in assigning contamination to a specific component.
2. Use DNA-free water as negative control during the screening procedure.
3. Perform an adequate number of replicates during each screening run to allow statistical evaluation of contamination rates with appropriate significance (*see* **Note 6**).
4. Calculate the rate for quantitative assessment of reagent contamination: contamination rate (%) = number of false positive test results/number of all test results × 100.
5. Screening should be performed whenever a new batch of a materials not certified as DNA-free is intended to be used.
6. Contamination rates of a particular reagent may differ from batch to batch. Monitoring of each batch is therefore very important.

7. Pretesting of different small batches of a particular reagent may facilitate the identification of an uncontaminated batch which then can be purchased in larger quantities.
8. If contamination is repeatedly observed in oligonucleotides or PCR master mixes, it may be helpful to switch to a different provider (*see* **Notes 7** and **8**). If this is not possible, perform the PCR procedure for reagent decontamination, as described in Subheading 3.5.
9. If complete decontamination of a reagent is not achievable, and traces of fungal DNA persist, it may be necessary to account for the occurrence of a low percentage of contaminated reactions. In such instances, repeated testing with multiple negative controls should be performed and evaluated only if there is no indication of contamination in a particular test run. However, the occurrence of stochastic events may not entirely preclude the observation of false positive test results.

3.5 DNase-Mediated PCR Reagent Decontamination

If fungal DNA contamination in PCR reagents is observed, a decontamination method based on the enzymatic activity of double-strand specific DNase (dsDNase) [16] is a rapid and easy way to remove traces of fungal nucleic acids [10]. Due to its double-strand specific activity, single-stranded DNA molecules such as primers and TaqMan probes remain undigested as long as no double-stranded structures (e.g., internally formed hairpins or primer dimers) are formed. To reduce the risk of primer dimer formation during enzymatic treatment, we do not recommend decontaminating mixtures of different oligonucleotides in a single reaction. Rather, each oligonucleotide should be decontaminated in a separate reaction. Adjustments of incubation temperature and time during DNase-mediated decontamination may be necessary for optimal results [10]. It is necessary to consider, however, that dsDNase treatment may affect the efficiency of the ensuing PCR amplification. This may become an issue if detection of very low fungal burden is required. Hence, after decontamination of individual reagents, the parameters of PCR efficiency should be tested and compared with assays using untreated reagents to control the effect on PCR performance.

3.5.1 Oligonucleotide Decontamination

1. Reconstitute lyophilized primers and probes in DNA-free water at a concentration of 100 μM.
2. For each oligonucleotide, prepare a separate reaction mix. Dilute the 100 μM stock using DNA-free Tris-buffer (20 mM Tris–HCl, 5 mM $MgCl_2$, pH 8) to obtain the desired working stock concentration (e.g., 10 μM for primers and 2.5 μM for probes).
3. Preheat each reaction at 40 °C for 5 min to allow partial denaturation of double-stranded DNA structures.

4. Subsequently, add 2.5 μl dsDNase (5 U/μl), 2.5 μl 1 mM DTT to the diluted working stock to obtain a final volume of 100 μl per reaction. Perform DNase-mediated digestion at 40 °C for 30 min followed by an enzyme deactivation step at 65 °C for 15 min.
5. Store decontaminated primer/probe solutions at −20 °C in small aliquots to avoid repeated freeze-thawing.

3.5.2 Master Mix Decontamination

1. Add 2.5 μl dsDNase (5 U/μl) and 2.5 μl of 1 mM DTT per 100 μl of master mix solution.
2. Perform dsDNase treatment at 37 °C for 20 min followed by an enzyme deactivation step at 60 °C for 20 min.
3. Use decontaminated master mix immediately because freezing may affect subsequent performance.

4 Notes

1. Hypochlorite solution can cause irritations of the eyes or the respiratory tract, and may be substituted by other suitable DNA decontamination reagents (e.g., DNA away, Molecular BioProducts).
2. Repeated opening of reaction tubes may increase the risk of contamination by airborne spores. It is advisable therefore to prepare and store all reagents in small aliquots.
3. The light intensity of UV lamps and thus the efficacy of decontamination in biological safety cabinets are greatly affected by accumulation of dust and dirt. Regular cleaning of the UV lamps is therefore required.
4. The UV light intensity decreases with time, and necessitates regular replacement according to the manufacturer's recommendation.
5. If no fungal DNA contamination is detected in tested materials, sufficient amounts should be stored to facilitate adequate screening of future material batches.
6. To monitor contamination-free handling, multiple negative control reactions covering every step of the assay should be included.
7. If contamination is observed, identification of the biological source of contaminating DNA should be attempted. This may provide information on how the contamination had occurred and serve as a basis for eliminating the source. Amplification of the internal transcribed spacer region (ITS2) [17], which is highly variable among different fungal species, followed by DNA sequencing, may be instrumental for precise taxonomic assignment of the contaminating DNA.

8. Manufacturers should be informed about the presence of contaminated products to provide a stronger impetus for future availability of certified DNA-free materials.

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Chapter 14

Broad-Spectrum Molecular Detection of Fungal Nucleic Acids by PCR-Based Amplification Techniques

Stefan Czurda and Thomas Lion

Abstract

Over the past decade, the incidence of life-threatening invasive fungal infections has dramatically increased. Infections caused by hitherto rare and emerging fungal pathogens are associated with significant morbidity and mortality among immunocompromised patients. These observations render the coverage of a broad range of clinically relevant fungal pathogens highly important. The so-called panfungal or, perhaps more correctly, broad-range nucleic acid amplification techniques do not only facilitate sensitive detection of all clinically relevant fungal species but are also rapid and can be applied to analyses of any patient specimens. They have therefore become valuable diagnostic tools for sensitive screening of patients at risk of invasive fungal infections. This chapter summarizes the currently available molecular technologies employed in testing of a wide range of fungal pathogens, and provides a detailed workflow for patient screening by broad-spectrum nucleic acid amplification techniques.

Key words Invasive fungal infections, Emerging fungal pathogens, Panfungal PCR, Molecular fungal diagnostics, Broad-range fungal diagnostics

1 Introduction

Invasive fungal infection (IFI) is a leading cause of morbidity and mortality in high-risk patients including allogeneic stem cell recipients, intensive care patients, individuals undergoing high-dose chemotherapy, and patients with acquired or innate immune deficiencies [1, 2]. Although *Aspergillus fumigatus* and *Candida albicans* are still considered the main agents of IFIs, the spectrum of fungal pathogens has dramatically expanded well beyond these two species over the past decade [3]. Serious life-threatening IFIs are not only caused by non-*albicans* species of *Candida* (*Candida glabrata*, *Candida tropicalis*, *Candida krusei*, and *Candida parapsilosis*) and non-*fumigatus* representatives of the genus *Aspergillus* (*Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus ustus*), but reportedly include also a variety of novel and taxonomically diverse opportunistic pathogens

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such as various yeast-like organisms (*Trichosporon*, *Malassezia*, *Rhodotorula*, and *Hansenula* spp.) and moulds (*Fusarium*, *Acremonium*, *Scedosporium* spp., and Mucormycetes) [3, 4]. Today it is clear that virtually any fungal species may cause a potentially lethal infection in immunocompromised individuals [5]. Application of broad antifungal prophylaxis in-high risk patients has conceivably increased the occurrence of resistant species and has facilitated the emergence of hitherto uncommon fungal pathogens [6]. This development should be taken into account in the selection of suitable diagnostic tools. Rapid and sensitive diagnostic methods that cover a broad range of fungal species are mandatory for efficient screening and early diagnosis in febrile immunocompromised patients [7, 8]. A variety of broad-spectrum (panfungal) assays based on nucleic acid amplification techniques (NAAT) have been described including endpoint PCR [9–11], real-time PCR [7, 12–15], and RNA-based detection systems [16]. However, to date these techniques have not been included in the consensus definitions for diagnosis of invasive fungal diseases by the European Organization for Research and Development of Cancer and the Mycoses Study Group (EORTC/MSG) due to the current lack of standardization [17]. Nevertheless, these assays have the potential to become an important alternative or supplement to conventional diagnostic methods such as culture techniques, microscopic examination, and histopathology which are inherently slow and suffer from low sensitivity [1, 5, 18].

The majority of NAAT screening assays target the ribosomal DNA (rDNA) genes (18S, 28S, and 5.8S) which carry highly conserved sequences for broad-range detection, and are present in multiple copies facilitating highly sensitive analysis [9, 19, 20]. A published comprehensive selection of primers targeting the fungal ribosomal genes can serve as a basis for the development of broad-spectrum detection assays [21]. Various types of clinical specimens such as bronchoalveolar lavage [22], tracheal secretions [15], fresh or paraffin-embedded tissues from primarily sterile body sites [11, 23], plasma [12], serum [15], and whole blood [7, 15] can be used for molecular testing [24]. The fungal load in peripheral blood specimens in patients with IFI may be very low [25], thus requiring highly sensitive detection methods. Viable *Aspergillus* hyphae are rarely found in peripheral blood because the tight architecture of the fungal filaments prevents cell shedding. However, detachment of small fragments may occasionally occur and drive the systemic spread of infection via the bloodstream, which is particularly evident in severely immunocompromised patients [26, 27]. Little information is available on the amount of fungal elements circulating in peripheral blood (PB) in this setting. Small test series in patients with hematological malignancies as well as blood samples from mice and rabbits experimentally infected with *A.fumigatus* showed fungal loads in a range of 10–100 colony

forming units (CFU) per ml [24, 25, 28]. In line with these data, the European *Aspergillus* PCR Initiative has defined the requested minimum detection limit of *Aspergillus*-specific PCR assays at 50 *A. fumigatus* conidia eluted in 100 μl or 27 rRNA gene copies per μl eluate (based on a presumptive mean copy number of 54 per *A. fumigatus* genome) [29]. The estimated levels of *Candida* pathogens required for reliable DNA-based PCR detection in PB during invasive infection are 5–10 CFU/ml. However, the presence of a lower initial fungal burden during early stages of candidemia is conceivable, indicating that the achievement of higher sensitivity levels in PCR-based assays may be beneficial [30]. The broad-spectrum/panfungal assay presented here is based on our experience in molecular diagnostics in the hemato-oncological and allogeneic stem cell transplantation setting, and facilitates screening in patients at risk of IFI. The TaqMan® probe-based system includes two separate PCR reactions [12]. Reaction I covers primarily moulds, and reaction II yeasts and Mucormycetes. Both PCR reactions target highly conserved regions within the 28S rRNA multicopy gene. Locked nucleic acids are incorporated into primers and probes facilitating increased binding efficacy and specificity of DNA amplification [31]. The screening assay permits rapid and sensitive detection of more than 80 different fungal pathogens, and has been adapted for use in clinical samples containing low amounts of fungal material such as PB [12]. The hands-on time required for DNA extraction and PCR analysis is approximately 6 h. The following workflow describes the application of the assay in the screening of patients at risk of IFI using PB specimens, but can be readily adapted to the analysis of other clinical materials.

2 Materials

2.1 Blood Sample Processing

1. 5 ml Biopure™ DNA-free safe lock tubes (Eppendorf).
2. Biosphere™ DNA-free pipette tips (Sarstedt).
3. Lab coat.
4. Nitrile gloves.
5. Hypochlorite solution (Bleach).
6. Disposable sterile arm sleeves.

2.2 DNA Extraction

1. MolYsis™ Complete 5 DNA extraction kit (Molzym, Germany).
2. 2 ml Biopure™ DNA-free safe lock tubes (Eppendorf).
3. Acid-washed glass beads (Sigma-Aldrich).
4. Disrupter Genie device (Scientific Industries) for bead beating.

5. PhHV (phocine herpes virus) cell culture supernatant: quantified virus spiked into the primary sample is used to control DNA extraction and occurrence of PCR inhibition.

2.3 PCR Primers and Probes: Panfungal Assay

Primers and TaqMan® probes (Eurogentec):

1. Reaction I forward primer I: 5′-TAA AGC TAA ATA YYG GCC RGA GA-3′.
2. Reaction I reverse primer I: 5′-CT[T] TYC AAA GTG CTT TTC A[T]C-3′.
3. Reaction I reverse primer II: 5′-CTC T[T]T TCA AAG TTC TTT TCA [T]C-3′.
4. Reaction I TaqMan® probe: 5′-6-FAM-ACT [T]GT [G]CG [C]TA [T]CG-BHQ-1-3′.
5. Reaction II forward primer I: 5′-GGG TGG TRA RYT CCW TCT AAR GCT AA-3′.
6. Reaction II forward primer II: 5′-GGG WGG TAA ATC YCW CCT AAA GCT AA-3′.
7. Reaction II reverse primer I: 5′-CTC T[T]T YCA AAG TKC TTT TCA[T]C-3′.
8. Reaction II TaqMan® probe: 5′-6-FAM-A[C]TT[G]T [T]C[G] [C]TA [T]CG-BHQ-1-3′.

Nucleotides in brackets carry LNA modifications (*see* **Notes 1** and **2**).

2.4 PCR Primers and Probe: PhHVcontrol Reaction

1. PhHV forward primer: 5′-GGG CGA ATC ACA GAT TGA ATC-3′.
2. PhHV reverse primer: 5′-GCG GTT CCA AAC GTA CCA A-3′.
3. PhHV TaqMan® probe: 5′-6-FAM-TTT TTA TGT GTC CGC CAC CAT CTG GAT C-TAMRA-3′.

2.5 PCR Equipment, Reagents, and Consumables

1. TaqMan® Gene Expression Master Mix (Thermo Fisher Scientific) containing uracil-DNA glycosylase (UNG) and nucleotides including dUTP instead of dTTP to permit elimination of potential contamination by carryover of amplicons.
2. 96-Well Reaction Plate (Thermo Fisher Scientific).
3. Optical Adhesive Film (Thermo Fisher Scientific).
4. DNA-free water (Molzym).
5. 7500 Real-Time PCR System (Thermo Fisher Scientific).
6. 1.5 ml Biopure™ DNA-free safe lock tubes (Eppendorf).

3 Methods

3.1 Blood Sampling and Processing

1. Detailed procedures to avoid contamination during the complete workflow are compiled in the Chapter "Prerequisites for control of contamination in fungal diagnosis."
2. At least 3 ml of EDTA-anticoagulated peripheral blood (*see* **Note 3**) from patients at risk of IFI are required.
3. Gently invert EDTA-blood tube several times and store the sample in a DNA-free 5 ml tube (*see* **Note 4**) at −80 °C.

3.2 DNA Extraction

1. DNA extraction is performed using the MolYsis™ Complete 5 DNA extraction kit, according the recommendations of the manufacturer, with minor modifications outlined herein. All buffers and enzymes indicated below are derived from the MolYsis™ Kit.
2. Use DNA-free pipette tips for all steps during extraction procedure.
3. Thaw frozen blood at room temperature, and add 250 μl CM buffer per 1 ml of sample, vortex for 15 s, and incubate at room temperature for 5 min.
4. Briefly centrifuge to clear lid (*see* **Note 4**).
5. Add 250 μl buffer DB1 per 1 ml of sample and 10 μl of MolDNase B, vortex for 15 s and incubate at room temperature for 15 min.
6. Centrifuge for 10 min at 12,000 × *g* (*see* **Note 4**).
7. Remove and discard the supernatant.
8. Resuspend pellet in 1 ml RS buffer and transfer sample into a DNA-free 2 ml tube.
9. Centrifuge for 5 min at 12,000 × *g*.
10. Remove and discard the supernatant.
11. Add quantified PhHV standard diluted in DNA-free water to the sample.
12. Resuspend pellet in 80 μl RS buffer, add 20 μl BugLysis solution and 1.4 μl beta-mercaptoethanol, vortex for 15 s.
13. Incubate reaction for 60 min at 45 °C on a thermoblock shaker at maximum vibration.
14. Briefly centrifuge (pulse spin) to remove liquid from the lid.
15. Add glass beads (*see* **Note 5**) and perform bead beating for 2 min using the Disruptor Genie device.
16. Add 150 μl RP buffer and 20 μl Proteinase K, vortex for 15 s.
17. Incubate reaction for 10 min at 56 °C on a thermoblock shaker at maximum vibration.

18. Preheat deionized water on a thermoblock at 70 °C.
19. Briefly centrifuge to remove liquid from the lid.
20. Add 250 μl CS buffer, vortex for 15 s.
21. Briefly centrifuge to remove liquid from the lid.
22. Add 250 μl AB buffer, vortex for 15 s.
23. Briefly centrifuge to remove liquid from the lid.
24. Place column in a 2 ml collection tube and transfer lysate onto column.
25. Centrifuge for 45 s at 12,000 × *g*.
26. Place column in a fresh 2 ml collection tube, add 400 μl WB buffer.
27. Centrifuge for 45 s at 12,000 × *g*.
28. Place column in a fresh 2 ml collection tube, add 400 μl of 70 % ethanol.
29. Centrifuge for 3 min at 12,000 × *g*.
30. Place column in a fresh 1.5 ml collection tube, and add 100 μl preheated deionized water (at 70 °C) to the column.
31. Incubate for 1 min at room temperature, and centrifuge for 1 min at 12,000 × *g*.
32. Store eluate in DNA-free tubes at −20 °C for subsequent PCR analysis (*see* **Note 6**).

3.3 Real-Time PCR Reactions

1. Use DNA-free pipette tips during the entire procedure.
2. Set up PCR reaction I in a total volume of 25 μl including 12.5 μl TaqMan® Gene Expression Master Mix, 400 nM of each primer, 40 nM TaqMan® probe, and 5 μl eluted patient sample DNA (*see* **Note 7**).
3. Set up PCR reaction II in a total volume of 25 μl including 12.5 μl TaqMan® Gene Expression Master Mix, 400 nM of each primer, 50 nM TaqMan® probe, and 5 μl eluted patient sample DNA.
4. Set up PhHV control PCR reaction in a total volume of 25 μl including, 16.4 μl TaqMan® Gene Expression Master Mix, 900 nM of each primer, 200 nM TaqMan® probe, and 5 μl eluted patient sample DNA.
5. Perform amplification using the following cycling profile: 2 min at 50 °C (uracil N-glycosylase-mediated degradation of potentially present contaminating amplicons containing dUTP), 10 min 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C.
6. Run reactions I and II for each patient sample in triplicates.
7. Run one PhHV control reaction for each patient sample.

8. On each plate, include at least three no-template control (NTC) reactions (5 μl DNA-free water), and a positive control for reaction I (PCR with 100 fg *A. fumigatus* DNA as template) and for reaction II (PCR with 100 fg *C. albicans* DNA) (*see* **Note 8**).
9. Include an NTC (5 μl DNA-free water) for the PhHV control reaction on the plate.

3.4 Result Interpretation

For PCR-based test systems employed in clinical diagnosis, extensive validation is required to establish criteria for negative and positive test results. The screening assay presented permits detection of more than 80 different fungal pathogens, but the assay performance in individual fungal species can display some differences. During validation of the assay, the limit of detection (LOD) was defined for all clinically relevant fungal pathogens. This was accomplished by the establishment of cutoff C_T values representing the LOD for reactions I and II. In view of potential inter-laboratory differences in assay performance, it may be necessary to adapt the cutoff values accordingly. General recommendations for result interpretation are listed below:

1. Cutoff values at our center for reaction I and II are 36.5 and 37.5, respectively. Adjustment to local assay performance may be required.
2. In clinical testing, results of both PCR reactions are evaluated independently. At least two of three replicates per reaction have to display C_T values at or above the established LOD for a positive test result. Two consecutive positive results within a febrile episode are required to support the diagnosis of IFI [7].
3. NTCs for reactions I and II have to be negative or display C_T values beyond the respective LOD for a valid test result.
4. Spiked PhHV control reactions in patient samples have to display values within a predefined range to ensure adequate assay performance (DNA extraction efficiency, absence of PCR inhibition).
5. NTC for the PhHV control reaction must be negative for a valid test result.
6. Positive controls for reactions I and II have to display values within a predefined range for a valid test result.
7. Testing has to be repeated if results do not meet the indicated validity criteria.

4 Notes

1. Oligonucleotides for real-time PCR containing locked-nucleic acids are available at licensed companies, and are more expensive than conventional primers and probes without modifications.

2. Most primers used in the assay presented contain degenerated bases (wobble positions). Based on our experience, we recommend purification with desalting of primers with degenerated bases to facilitate equal distribution of the primer variants.
3. Testing of at least 3 ml EDTA-PB as starting material is recommended by White et al. [29]. It is necessary to bear in mind, however, that it may not be possible to collect 3 ml blood samples in all instances (e.g., in young pediatric patients).
4. Handling, storage, and centrifugation of 5 ml tubes requires compatible laboratory equipment.
5. Glass beads can be added by using an inverted 1 ml DNA-free pipetting tip. The glass beads should represent approximately one-third of the total sample volume.
6. The DNA extracted from patient material should be stored in aliquots to avoid multiple freezing and thawing steps in case repeated testing is required.
7. For long-term storage, aliquots of master mix solutions and primer/probe stocks should be prepared and stored at −20 °C. More than two freezing and thawing steps should be avoided to prevent a negative impact on assay performance.
8. Genomic DNA for positive control reactions was isolated from cultures of *Aspergillus fumigatus* and *Candida albicans*. Nucleic acid extraction was performed essentially as described (*see* Subheading 3.2), but extraction was started at **step 9**, and addition of the PhHV control was omitted. DNA was quantified using Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific, USA). Aliquots of quantified DNA were stored at −80 °C.

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Chapter 15

Genus- and Species-Specific PCR Detection Methods

Jan Springer and Jürgen Löffler

Abstract

PCR-based detection of fungal pathogens offers a sensitive and specific tool for the diagnosis of invasive fungal infections. A large variety of different clinical specimen types can be used as original material. However, certain precautions, in addition to the published MIQE guidelines [1], need to be taken to prevent contaminations from airborne fungal spores and PCR reagents. In addition, the European *Aspergillus* PCR Initiative (EAPCRI) recently defined standards for *Aspergillus* PCR [2, 3], following these recommendations leads to superior sensitivity. The combination of fungal PCR with the galactomannan ELISA assay increases the sensitivity for the detection of *Aspergillus* DNA from blood, compared to a single assay only [4, 5].

Key words DNA, Detection, Fungi, PCR, Amplification, Broad-range PCR, Species-specific method

1 Introduction

PCR-based detection of fungal pathogens offers a sensitive and specific tool for the diagnosis of invasive fungal infections. A large variety of different clinical specimen types can be used as original material. However, certain precautions, in addition to the published MIQE guidelines [1], need to be taken to prevent contaminations from airborne fungal spores and PCR reagents. In addition, the European *Aspergillus* PCR Initiative (EAPCRI) recently defined standards for *Aspergillus* PCR [2, 3], following these recommendations leads to superior sensitivity. The combination of fungal PCR with the galactomannan ELISA assay increases the sensitivity for the detection of *Aspergillus* DNA from blood, compared to a single assay only [4, 5].

Mortality due to invasive fungal infections (IFI) remains unacceptably high, particularly in hematological patients [6]. In these heavily immunocompromised patients, the outcome from IFI strongly depends upon the extent of infection, but also whether specific diagnosis is made and if treatment with an effective drug is begun early enough [7]. However, the low sensitivity of commonly

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used fungal detection methods leads either to a delayed start or to a broad but frequently unjustified use of expensive and/or toxic antifungal agents [8].

A step towards a more targeted and economic use of antifungal agents was made by the introduction of molecular detection methods for fungal DNA in clinical samples.

In this chapter, we focus on the peculiarities of such molecular detection methods for mould infections, especially on molecular assays for the most frequent and thus most devastating mould infection, invasive aspergillosis (IA).

The highest incidence (10–20 %) and mortality rates (60–90 %, in CNS aspergillosis up to 100 %) of IA have been reported following allogeneic HSCT and heart or lung transplantation. The reason for such patients developing IA is that the underlying disease and its treatment with chemotherapy induce bone marrow failure resulting in a lack of functioning polymorphonuclear granulocytes and impaired T cell-mediated immunity [7]. Such losses of function especially of the innate immunity rapidly lead to impaired local defenses, including of the oral cavity, and of the gastrointestinal and respiratory tracts. In the latter, fungal spores can germinate, infect lung tissue and progress to angioinvasion, which usually leads to further dissemination to other organs [9]. Nevertheless, direct demonstration of *Aspergillus* infection is rare by culture of sterile body fluids, and obtaining tissue by lung biopsy is seldom possible, which again hampers proper diagnosis.

Due to the low and inconstant frequency of fungal pathogens in sterile body fluids, successful recovery requires highly sensitive and specific detection procedures, such as polymerase chain reaction (PCR) based assays. Furthermore, relatively large sample volumes (usually of whole blood, plasma, serum, or bronchoalveolar lavage) need to be processed, which is demanding and requires specific protocols for DNA extraction. Additionally, due to the omnipresent environmental exposure to airborne fungal spores, specific and rigid preventative measures need to be taken for successful PCR-based diagnosis of fungi. This includes among others the use of (1) fungal DNA-free reagents and consumables, (2) an adequate number of positive and negative controls, (3) laminar air flow hoods for all working steps, and of course, (4) the guidelines described in the MIQE (minimum information for publication of quantitative real-time PCR experiments, [1]).

Finally, investigators need to consider that highly sensitive tests such as PCR assays usually deliver a high negative predictive value for the disease. In consequence, this means that IFI normally can be ruled out if a PCR assay used as a screening test shows a negative result. On the other hand, PCR positive results of such assays can be used for confirmation of IA.

In bivariate analyses, the EAPCRI group observed significant positive correlations between PCR sensitivity and the volume of

plasma used for DNA extraction, the ratio between the volume of plasma used for extraction and the subsequent elution volume, the reaction mixture volume used for PCR, the analysis of ≥2 replicates, and the use of an internal control [10].

In the following chapters, we address these points and describe in detail laboratory methods and protocols for the sensitive and specific detection of *A. fumigatus* and other medically relevant moulds isolated from clinical specimens. This includes PCR primer and probe sequences, PCR protocols for broad-range and genus-specific PCR tests as well as adequate control assays.

2 Materials

2.1 Broad-Range Fungal PCR

Broad-range PCR assays can be of enormous relevance as screening tools, such as for prospective screening of blood samples during neutropenic episodes in patients with acute leukemia or after allogeneic stem cell transplantation and in other high-risk patient cohorts. However, due to large homologies of primers and probes with many different fungal species and genera and due to the omnipresence of fungal spores in the air, the risk of contamination is high. In consequence, strict controlling of these PCR assays is mandatory.

As a broad-range fungal approach we describe a modified version of the assay published by Einsele et al. [11]. The primers amplify a region of the 18S ribosomal gene cluster, a consensus sequence for fungal pathogens. Depending on the detected fungal pathogen the amplicon size varies between 482 and 503 bp. The described probes, specific for various *Aspergillus* and *Candida* species were revised, now using a broad-range hydrolysis probe facilitating the detection on a real-time PCR platform, such as the StepOnePlus machine (Applied Biosystems). Alternatively, the primers can be used in a conventional PCR assay. Then, amplicon analysis should be performed by gel electrophoresis followed by DNA staining with ethidium bromide or SYBR green. Species identification is possible by amplicon analysis via sequencing.

1. 50 μM forward primer: 5′-ATTGGAGGGCAAGTCTGGTG-3′.
2. 50 μM reverse primer: 5′-CCGATCCCTAGTCGGCATAG-3′.
3. 5 μM probe: cgg[+T]aa[+T]tc[+C]ag[+C]tc[+C]aaWagcgt labeled with FAM and BHQ1; five nucleotides are modified (locked nucleic acid, LNA, [+nt]), one is degenerated whereas W replaces A or T (unpublished own data).
4. 2× TaqMan GenEx master mix (Applied Biosystems; GEX).
5. Molecular grade water.
6. 96-well PCR plate and cover or strips, suitable for real-time PCR.

2.2 Aspergillus-Specific PCR

As the incidence of *Candida* infections, especially in hematological patients is reduced due to routine azole prophylaxis, *Aspergillus* is main the pathogen in this patient cohort. Broad-range fungal PCR has the potential to detect *Aspergillus* but could fail if other fungi are present. Especially in bronchoalveolar lavage (BAL), broad-range fungal PCR might detect colonizing *Candida* spp. as commensals. Then, detection of a second fungus is unlikely due to preferred amplification of the more frequent pathogen. *Aspergillus* spp. or other fungal pathogens, present in an expected lower amount than *Candida* will therefore be missed. This obstacle can be bypassed by using pathogen-specific PCR assays. We established a real-time PCR for the LightCycler machine and adapted this assay for the StepOnePlus machine by exchanging the FRET (fluorescence resonance energy transfer) probes by a hydrolysis probe [12]. *Aspergillus*-specific oligonucleotide primers and probes were designed to target the multi-copy ribosomal operon within the internal transcribed spacer 1 (ITS1) and the 5.8S region. In the LightCycler assay different *Aspergillus* species can be differentiated by their specific melting temperatures. Three pathogen groups are described. Due to the short amplicon size, sequencing cannot further elucidate the exact species of *Aspergilli*. Using the hydrolysis-based assay, detection on an *Aspergillus* spp. level is possible.

1. 5 or 50 μM Asp fum F (5′-GCAGTCTGAGTTGATTATCG TAATC-3′).
2. 5 or 50 μM Fungi5.8_R (5′-CAGGGGGCGCAATGTGC-3′).
3. 3 μM Fungi5.8_FL (5′-AATGCGA TAAGTAATGTGA ATTGCAGA-FL-3′, where FL is fluorescein).
4. 3 μM Fungi5.8_LC640 (5′-LC Red640-TCAGTGAATCATC GAGTCTTTGAACGC-PH-3′, where PH is phosphate).
5. Dimethyl sulfoxide (DMSO).
6. 25 mM $MgCl_2$.
7. LightCycler FastStart DNA Master HybProbe (Roche).
8. Glass capillaries.
9. Hydrolysis probe: 24 μM ITS-PF (5′-FAM-CAGCGAAA TGCGATAAGTAATGTGAATTGCA-TAMRA-3′, where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine).
10. TaqMan GenEx master mix (Applied Biosystems).

2.3 Mucorales-Specific PCR

Improved diagnostics as well as effective anti-*Aspergillus* prophylaxis and treatment has recently led to a decrease of infections caused by *Candida* and *Aspergillus* and to a rise of the so-called “rare fungal pathogens” including members of the order of Mucorales, *Fusarium* spp., and others. Invasive mucormycosis is a fast progressing and often fatal fungal infection and difficult to

diagnose. Standard techniques are insensitive, whereas molecular assays provide a tool for fast species identification. A semi-nested conventional assay specific for Mucorales, targeting the 18S rRNA was described by Bialek et al. [13]. Further identification to at least genus level is possible by amplicon sequencing. Others modified this assay by using EvaGreen and high resolution melting curve analysis (HRM) to identify species without sequencing in a real-time PCR format [14]. The melting temperatures (*Tm*) for each tested species were as follows: for *Rhizopus microsporus*, 76.46 °C; for *Rhizopus oryzae*, 76.59 °C; for *Mucor racemosus*, 76.78 °C; for *Mucor circinelloides*, 76.98 °C; for *Rhizomucor pusillus*, 77.87 °C; and for *Lichtheimia corymbifera*, 78.56 °C. All HRM results were confirmed by sequencing.

1. Outer primers ZM1 (5′-ATT ACC ATG AGC AAA TCA GA-3′).
2. ZM2 (5′-TCCGTC AAT TCC TTT AAG TTT C-3′).
3. Inner primer ZM3 (5′-CAA TCC AAG AAT TTCACC TCT AG-3′).
4. 10 mM Tris–HCl (pH 8.3).
5. 50 mM KCl.
6. 2.5 mM $MgCl_2$.
7. AmpliTaq DNA polymerase (Roche).
8. 100 mM of each dNTP.

2.4 *Fusarium*-Specific PCR

Invasive fusariosis is a usually disseminated fungal infection with a radiologic picture similar to IA; however, different from aspergillosis, patients have frequently disseminated nodular and papular skin lesions and positive blood cultures. Their outcome is usually poor, and largely dependent on the recovery of the immune status of the host, particularly neutropenia [15]. A *Fusarium*-specific conventional PCR was established by Hue et al. [16]. The oligonucleotides P28SL and P58SL amplify a fragment of the ribosomal genes (rRNAs) spanning the ITS2 and a part of the 5.8S and 28S gene region. The amplicon has a length of 329 bp.

1. P58SL.5′-AGT ATT CTG GCG GGC ATG CCT GT-3′.
2. P28SL.5′-ACA AAT TAC AAC TCG GGC CCG AGA-3′.
3. Agarose.
4. Tris–acetate–EDTA buffer (TAE).
5. 100-bp DNA Ladder.
6. Ethidium bromide/SYBR Green.

2.5 Control PCR Assays (IC/EC)

In each PCR, numerous negative controls for all extraction and detection steps (pretested material, e.g., human serum from healthy donors and PCR-grade water), extraction controls (EC)

spiked with control material, e.g., *Aspergillus* conidia or *Bacillus subtilis* DNA, positive PCR controls with low copy numbers of target DNA and PCR inhibition controls (IC) need to be included.

The IC is a second independent PCR assay to monitor samples for PCR inhibition. The amount of IC spiked in every sample should result in a stable signal (crossing point/Cq value). Reduction of the expected real-time value indicates PCR inhibition which is extrapolated to affect also the PCR performance of the pathogen-specific assay. The same template amount and PCR condition should preferably be used. Ideally, the IC is performed on the same plate as the fungal assay. In principle, every target except the specific pathogen can be used. The amount of IC should be in the range of the expected pathogen load. As this is quite low for *Aspergillus* in blood, the lowest stable signal should be aimed.

1. 16S S 5′-ggTCTT gAC ATC CTC TgA cAA tCC TA-3′.
2. 16S A 5′-AAC TgA ATg CTg gCA ACT AAg ATC A-3′.
3. Hydrolysis probe 16S TM 5′-JOE-AgAgTgACAgg TggTgCATggTTg TC-BHQ1-3′.
4. *Bacillus subtilis* DNA (as template to spike into PCR master mix).
5. TaqMan GenEx master mix (Applied Biosystems).

3 Methods

In general, reagents and templates should be stored at 4 °C. By using "hot-start" Taq polymerase all procedures can be carried out at room temperature and in DNA-free UV cabinets. To avoid lab-based cross-over contaminations, gloves are mandatory and should always be exchanged if things outside of the cabinet are touched (*see* **Notes 1** and **2**).

3.1 Broad-Range PCR

1. Amplification reactions are performed in a 20 μl volume containing 12.5 pmol of forward and reverse primers (being equal to 0.25 μl of a 50 μM solution), 1.9 pmol of probe, and 10 μl of TaqMan GenEx master mix (Applied Biosystems; *see* **Notes 3** and **4**).
2. With adjustment of the amount of primers and probe to 0.88 and 5 μl of template (*see* **Note 5**), 4.13 μl water has to be added to every reaction.
3. Prepare a PCR master mix and aliquot 15 μl in every well.
4. Use a support base to avoid contact of wells to any surface (*see* **Note 6**).
5. Add the negative controls first, then the different templates, and at the end the positive controls to the dedicated wells. If possible separate the positive controls spatially from the rest to avoid cross-contaminations.

6. Seal the plate and spin it for 1 min at $900\times g$ to remove bubbles.
7. Amplification is carried out as follows: uracil-DNA glycosylase (UNG; *see* **Note 3**) activation at 50 °C for 2 min, initial *Taq* polymerase activation at 95 °C for 10 min, followed by 50 cycles (*see* **Note 7**) of 95 °C for 15 s, 55 °C for 10 s, and 72 °C for 25 s.
8. In each run, negative and positive controls must be included. As the fungal load is often close to the limit of detection, eluates should be analyzed at least in duplicates.

3.2 *Aspergillus*-Specific PCR

1. Real-time PCR (qPCR) amplification is performed in 20 μl using a LightCycler PCR machine (model 1.5; Roche). Each 20 μl reaction mixture contains 2 μl of LightCycler FastStart DNA Master HybProbe, 62.5 nM Asp fum F, 125 nM Fungi5.8_R (*see* **Note 8**), 150 nM (each) probe Fungi5.8_FL and Fungi5.8_LC640, 1.25 μl of DMSO, 4 μl of $MgCl_2$ (25 mM), and 10 μl of extracted DNA (*see* **Note 5**).
2. Target DNA amplification is carried out as follows: initial *Taq* polymerase activation was at 95 °C for 9 min, followed by 55 cycles (*see* **Note 7**) of 95 °C for 10 s, 54 °C for 30 s, and 72 °C for 25 s, followed by melting curve analysis.
3. In each run, negative and positive controls are included. *Aspergillus*-specific amplification yields DNA amplicons of 153 bp in length.
4. Different *Aspergillus* species can be distinguished by their specific melting temperatures: 64 °C for DNA of *A. fumigatus*, *A. versicolor*, *A. lentulus*, and *A. nidulans*, 58 °C for *A. terreus* and *A. niger*, and 54 °C for *A. flavus* (Fig. 1).

This LightCycler assay was converted into a hydrolysis-based probe assay to be run on a different PCR cycler and to increase sample capacity. The LightCycler is able to process 32 samples including controls in parallel, which is too low for high throughput assays with specimens analyzed in duplicates or triplicates. Here, a 96-well system is preferable. Real-time PCR amplification is performed in a 21 μl mixture using a StepOnePlus machine (*see* **Note 9**). Each reaction mixture contains 10 μl TaqMan GenEx master mix, 300 nM Asp fum F, 600 nM Fungi5.8_R (*see* **Notes 3** and **8**), 143 nM probe ITS-PF, and 10 μl of extracted DNA (*see* **Note 5**). Amplification is carried out as follows: uracil-DNA glycosylase (UNG) activation is at 50 °C for 2 min, and the initial *Taq* polymerase activation is at 95 °C for 10 min, followed by 55 cycles of 95 °C for 15 s, 54 °C for 30 s, and 72 °C for 30 s (*see* **Note 7**). *Aspergillus*-specific amplification yields DNA amplicons of 153 bp in length.

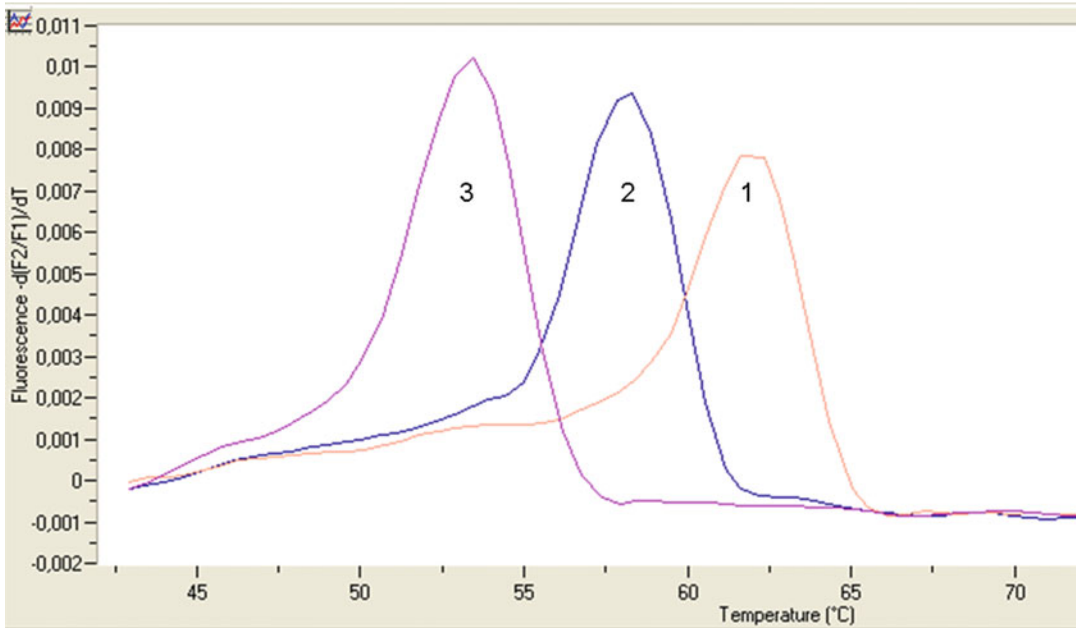

Fig. 1 Melting curves of amplified DNA from three different *Aspergillus spp.* groups (1: *A. fumigatus*, *A. versicolor*, *A. lentulus*, *A. nidulans*, 2: *A. terreus, A. niger*, 3: *A. flavus* DNA) in the LC assay

3.3 Mucorales-Specific PCR

This assay is a semi-nested PCR reaction meaning that the PCR product of the first round will be re-amplified with one primer of the first round in a second PCR cycling round. The outer primers ZM1 and ZM2 amplify a 386 bp amplicon in *Rhizopus arrhizus*, which will be re-amplified in the second reaction using primers ZM1 and ZM3. This results in a PCR product of 175–177 bp length, depending on the Mucorales species.

1. The reaction mixture of the first PCR consists of 10 μl template DNA (*see* **Note 5**) in a total volume of 50 μl, with final concentrations of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, and 2.5 mM $MgCl_2$, 1 mM of each primer of the outer primer sets (ZM1/ZM2), 1.5 U of AmpliTaq DNA polymerase, and 100 mM of each dNTP.
2. Identical reaction mixture is used for the nested PCR except that 1 μl of the first reaction (template), 50 mM of each dNTP, and 1 mM of each inner primer (ZM1/ZM3) is used.
3. All reaction mixtures are thermally cycled once at 94 °C for 5 min, 35 times at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, and then once at 72 °C for 5 min.
4. As all PCR reaction tubes of the first PCR have to been opened, special care for carry-over contaminations must be applied (*see* **Note 2**).

3.4 Fusarium-Specific PCR

1. This PCR assay is performed with the following temperatures and PCR cycles: initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 68 °C for 1 min, and extension at 72 °C for 1 min. The thermal cycles are terminated by a final extension of 10 min at 72 °C.
2. 5 μl of the PCR products are analyzed on a 2% agarose gel after electrophoresis in Tris–acetate–EDTA buffer.
3. A 100-bp DNA Ladder is used as a molecular size marker. Gels are stained with ethidium bromide (1 mg/ml) and visualized under UV light.

3.5 Internal Control PCR (IC)

1. The reaction mixtures (21 μl in total, *see* **Note 9**) contain 0.12 μM primer 16S S and 0.12 μM 16S A, 0.07 μM hydrolysis probe 16S TM, 1000 plasmid copies of *Bacillus* DNA, 10 μl of TaqMan GenEx master mix (*see* **Note 3**), and 10 μl of template DNA.
2. Amplification should use the same cycling protocol as the pathogen specific assay (see above).
3. Also the same amount of template as in the pathogen specific assay should be used. Amplification of *Bacillus subtilis* DNA is quite stable under different cycling protocols but should be tested for any other new fungal PCR condition.

Duplexing both PCR reactions is an option by combining both targets in one reaction well. This monitors exactly the PCR conditions in each well, respectively. However, primer limited conditions should be established to enable the full efficiency of the pathogen detection reaction without any loss of sensitivity. This has to be optimized for each system and should result in stable signals in both assays. For the tested duplex combination targeting *Aspergillus/Bacillus subtilis* DNA in parallel, we found reduced sensitivity for detecting *Aspergillus* DNA when detection of *Bacillus* DNA was performed in parallel. Therefore, in this case, both targets are analyzed in monoplex reactions. As both assays are performed in different reaction wells, PCR conditions in the first well have to be extrapolated for the other.

If cell-free unbound DNA is extracted from serum samples, quality control (external control, EC) for DNA extraction consists of one negative control (fungal DNA-free human serum) and one *Bacillus*-positive serum specimen (spiked with 10,000 plasmid copies of *Bacillus subtilis* DNA). Besides the pathogen PCR detection, *Bacillus* DNA is amplified in a second reaction. This control reaction is used independently of the pathogen target (monoplex), but within the same PCR run (same PCR cycling condition).

4 Notes

1. Wear a second pair of **gloves** simultaneously over the first. For some brands this is possible with the same type. With others you have to use two different types of glove. The upper glove is used for work outside of the UV cabinet and can be easily discarded when working in the inside. The inner pair of gloves should exclusively be used in the DNA-free environment.
2. As fungal spores are omnipresent in the air, **contaminations** might be present in kits, reagents, and consumables. They can affect DNA extraction and PCR detection methods in the laboratory as well as the clinical samples themselves (contaminated blood collection tubes). Intensive monitoring is mandatory. Small aliquots of reagents, especially water aliquots, should be prepared and reused only 1–2 times. Samples should be handled under laminar flow, whereas pipetting of PCR steps should be done in DNA-free work places (UV cabinet). The workflow from DNA extraction to detection should be unidirectional and take place in separated rooms.
3. The **commercial PCR master mix** contains all necessary reagents for amplification. The producer guarantees stability and consistency of the product. Nevertheless, variability of the quality can occur in different batches. This should be tested especially if high sensitivity is important. The best is to use a single batch for a whole set of experiments. As mixes are stable for several months, sufficient reagents should be ordered for a whole study period.

   Some PCR amplification mixes contain uracil-DNA glycosylase (UNG). This enzyme degrades DNA containing uracil after activation at 50 °C, which can be helpful to prevent carryover contaminations if amplicons have incorporated uracil instead of thymidine.

   Some PCR mixes use a "hot-start" Taq polymerase which has to be activated prior to use (10 min at 95 °C). This enzyme allows pipetting of PCR reactions at room temperature. No unspecific amplification occurs during PCR handling.
4. For homogenous distribution, all PCR components (except the template) should be mixed together in a **master mix**. For volume calculation, take the number of samples in your individual run and add 10% (e.g., for 10 samples use calculation of 11-fold). An automated calculation can be done in an Excel sheet (*see* Table 1). Sample number can be adjusted by changing cell C2.
5. **Amount of template**: As a variety of different clinical sample types exists to diagnose fungal infections, the proportion of the original clinical sample volume to the volume of sample

Table 1
Excel calculation of master mix (broad-range assay)

1	A	B	C	Excel calculation
2		1×	11	
3	2× GEX	10	110	=C2×B3
4	50 µM primer_for	0.25	2.75	=C2×B4
5	50 µM primer_rev	0.25	2.75	=C2×B5
6	5 µM probe	0.38	4.125	=C2×B6
7	h20	4.13	45.375	=C2×B7

analyzed in the PCR reaction has to be considered. This proportion is not only dependent on the volume tested in the PCR reaction but also on the specimen volume used for DNA extraction and on the elution volume. Whole blood and tissue samples contain higher amounts of human DNA than cell-free materials, such as serum and plasma. Therefore, tissue biopsies and whole blood samples are more likely to inhibit PCR reactions by an excess of human DNA.

In general, only EDTA-stabilized blood specimens should be used for DNA extraction. Citrate and heparin inhibit PCR reactions.

Many fungal PCR detection systems, especially from blood, work close to the limit of detection (LOD). High sensitivity is therefore needed. Besides high PCR efficiency, low elution volume, high input of template DNA and sample replication can help increasing the chances to detect even small amounts of target DNA. This concentration effect might not only affect the target DNA but also other parameters, e.g., human DNA. Thus, increasing the template amount can be counterproductive. This can be monitored by an IC assay or by using two different template amounts. As an example, if 2.5 and 5 µl of a positive sample are used for PCR amplification, one would expect 2 different Cq values. Testing of 5 µl should result in a decrease of Cq = 1, as the double amount of target is present and detected. One Cq value is equal to a twofold change in target concentration.

Exceptions are expected in inhibited samples or in samples with such a low fungal load that they are not reproducible (Cq >40). An optimization for tissue sample eluates is shown by Babouee et al. [17]. They tested different amounts (0.5–10 µl) of formalin-fixed, paraffin-embedded (FFPE) tissue sample eluates in two different PCR assays. There, 0.5 and 2 µl PCR

input showed the best results. In contrast, higher input volume is possible from extracts of cell-free blood materials as serum or plasma due to lower risk of PCR inhibition (own data).

6. By lifting the plate, you can **monitor complete filling** of all dedicated wells. For beginners it may be helpful to use colored reagents changing the color when template is added.
7. **High cycle number** can lead to unspecific amplification but can also compensate PCR inhibition partly. Amplification of only a few target copies should result in approximately Cq = 40 depending on the PCR efficiency.
8. **Asymmetric PCR** assays can enhance and stabilize real-time PCR signals. The DNA strand to which the probe binds is preferably amplified by applying twofold excess of the appropriated primer.
9. Using a commercial **twofold concentrated PCR mix** allows taking the same amount for assay components and PCR template. In a total volume of 20 μl, this means 10 μl. If 10 μl of an eluate are tested, the ratio of PCR mix to eluate is slightly diminished. Using 10 μl of twofold concentrated PCR mix in a total volume of **21 μl** reduces the end concentration from onefold to 0.95-fold. The PCR reaction still works fine and for this approach primers and probe have to be included in 1 μl.
10. To ensure **homogenous concentrations** of IC template in every test sample, spiking should be done into the PCR master mix. As an example, 10 test samples require a total of 11,000 plasmid copies. IC template should be hold in small volumes (e.g., by adding 0.1 μl per PCR reaction [10,000 copies/μl] resulting in adding 1.1 μl to the master mix).

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Chapter 16

Identification of Fungal Pathogens in Tissue Samples from Patients with Proven Invasive Infection by Fluorescence *In Situ* Hybridization

Ilka McCormick Smith and Volker Rickerts

Abstract

Identification of fungal pathogens in clinical samples by hybridization with short oligonucleotide probes is increasingly used in the diagnosis of invasive fungal infections. Rapid and specific fungal identification has been documented in different diagnostic settings allowing for specific patient management. Identification of fungal pathogens in formalin-fixed, paraffin-embedded tissue samples appears to be rewarding as these materials are stored in pathology archives offering an insight into the etiology of deep fungal infections that is often not achieved by non-molecular tests. In contrast to PCR based methods, amplification of target sequences is unnecessary limiting the potential for contamination and localization within infected tissue is possible helping to distinguish between colonization and infection.

Key words FISH, Invasive fungal infection, Formalin-fixed, Paraffin-embedded tissue, Candidiasis, Aspergillosis

1 Introduction

Fluorescence *in situ* hybridization (FISH) has been successfully used in environmental microbiology for the identification, localization, and quantification of phylogenetically defined microbes in divers samples [1]. In diagnostic mycology, FISH has predominantly been reported in three settings. First, yeasts including *Candida albicans*, *Cryptococcus neoformans*, and *Histoplasma capsulatum* have been accurately identified by specific FISH probes from precultivated blood culture bottles [2–4]. Commercially available FISH assays have been evaluated in single and multicenter trials for the identification of common agents of candidemia from blood culture bottles, demonstrating the applicability of FISH in diagnostic microbiology laboratories [5]. Second, the fungal pathogens *Cryptococcus neoformans* and *Cryptococcus gattii* were directly visualized in cerebrospinal fluid specimens from patients with cryptococcosis, demonstrating that FISH targeting fungi is

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feasible directly from uncultivated clinical samples [4, 6]. Third, formalin-fixed, paraffin-embedded (FFPE) tissue samples from patients with proven invasive fungal infection have been studied using *in situ* hybridization (ISH), which uses non fluorescent detection for pathogen visualization and less common FISH [7, 8]. As FFPE samples from patients with invasive fungal infections are stored in pathology archives, they represent a useful resource to define the etiology of invasive fungal infections by molecular methods. However, the use of FISH on FFPE specimens appears to be associated with challenges, especially in mould infections. These include first, a potential for low fluorescence signals probably due to low rRNA numbers, second, an uneven distribution of the signal in the fungal mycelium and a potential for autofluorescence [9]. Nevertheless, FISH used in combination with PCR and sequencing are powerful tools to characterize the etiology of invasive fungal infections including difficult to diagnose cases such as those caused by uncultivated pathogens or mixed fungi [10, 11].

The basic steps in hybridization experiments include fixation of samples containing fungi with formaldehyde or ethanol in order to preserve the integrity of fungal cells and allow the uptake of labeled oligonucleotides to reach their intracellular targets [1]. FISH applications in diagnostic mycology target ribosomal RNA (rRNA) with oligonucleotide probes. The availability of ribosomal RNA sequences of many fungi allows the targeted *in silico* design of oligonucleotide probes. Depending on which parts are targeted, probes hybridizing with single species or phylogenetically defined groups may de designed. After hybridization, unbound probes are *washed away* from samples. The hybridization signal is detected using Epifluorescence-, or confocal laser scanning microscopy (FISH), or microscopy (ISH).

2 Materials

2.1 Probe Design

1. The Silva-homepage is a comprehensive online resource for FISH targeting ribosomal RNA (http://www.arb-silva.de/fish-probes/).
2. Determine the fungal target species that should be detected by FISH. Check if probes have already been developed. The website probebase (http://131.130.66.201/probebase/) lists established rRNA-probes including some fungal probes.
3. Obtain good quality 18S- and/or 28S rRNA sequences of target fungi that should be identified by hybridization and non-target fungi. Choose a sequence (15–30 bp) with a complete match for target fungi with more than one mismatch with non-target fungi. The mismatches should be in central positions of a probe, as this is associated with better differentiation than

mismatches at the 3′ or 5′ end of the oligonucleotide. The website probecheck (http://131.130.66.200/cgi-bin/probecheck/probecheck.pl) allows to query a potential probe sequence against a database containing quality checked 18S-, or 28S rRNA. It also offers links to outside tools to determine probe characteristics such as melting temperature or potential hairpins (Oligo Calc) and BLAST® to query GenBank.

4. The accessibility of rRNA molecules differs among different localizations. Maps grading the accessibility are a useful resource to identify promising target regions [12, 13]. The homepage Mathfish (http://mathfish.cee.wisc.edu/probeaff.html) predicts the hybridization efficiency of a given probe with a target RNA sequence, an important determinant of the signal that may be obtained with the probe in a hybridization experiment.
5. Select fungi (target and non-target) for empirical probe testing. Choose non-target fungi with 1 and more mismatches.
6. Order DNA probes labeled with Cy3 or Cy5 at the 5′ end purified by HPLC procedure for experimental evaluation.

2.2 Experimental Evaluation of Probes

1. Fungi: Grow yeasts to *log* phase in shaking culture. For moulds, we use germlings grown without shaking for the initial experimental evaluation of probes. Depending on the growth rate of a mould, incubation between 6 and 24 h may be adequate for many species. Fungi can be fixed in ethanol (50 %) or buffered formalin (37 %, 1:2 in PBS) overnight, washed in PBS three times, and stored in 50 % ethanol.
2. We compare the performance of newly designed probes with target and non-target fungi in comparison to a positive control probe producing bright fluorescence with fungi (EUK 516) and a nonsense probe that does not hybridize with fungal rRNA using identical fluorophores and microscopy settings (non-EUB338) [9] (Fig. 1).

   Several controls may be processed in parallel. Hybridization buffer without probes may be used to determine if autofluorescence is an issue with the target organisms. Hybridization after treatment of samples with RNAse has been used to document that detected signals are due to interaction of labeled probes with the rRNA-target [14].
3. For testing FFPE specimens from patients with invasive fungal infections, we cut 5 μm sections from tissue blocks. The cuts are placed on microscopy slides. Prior to hybridization, we deparaffinize them by dipping into octane for 10 s.

   To produce clear signals in FFPE tissue samples, in our experience, probes with hybridization efficiency comparable to EUK 516 are necessary. If monolabeled DNA probes fail to

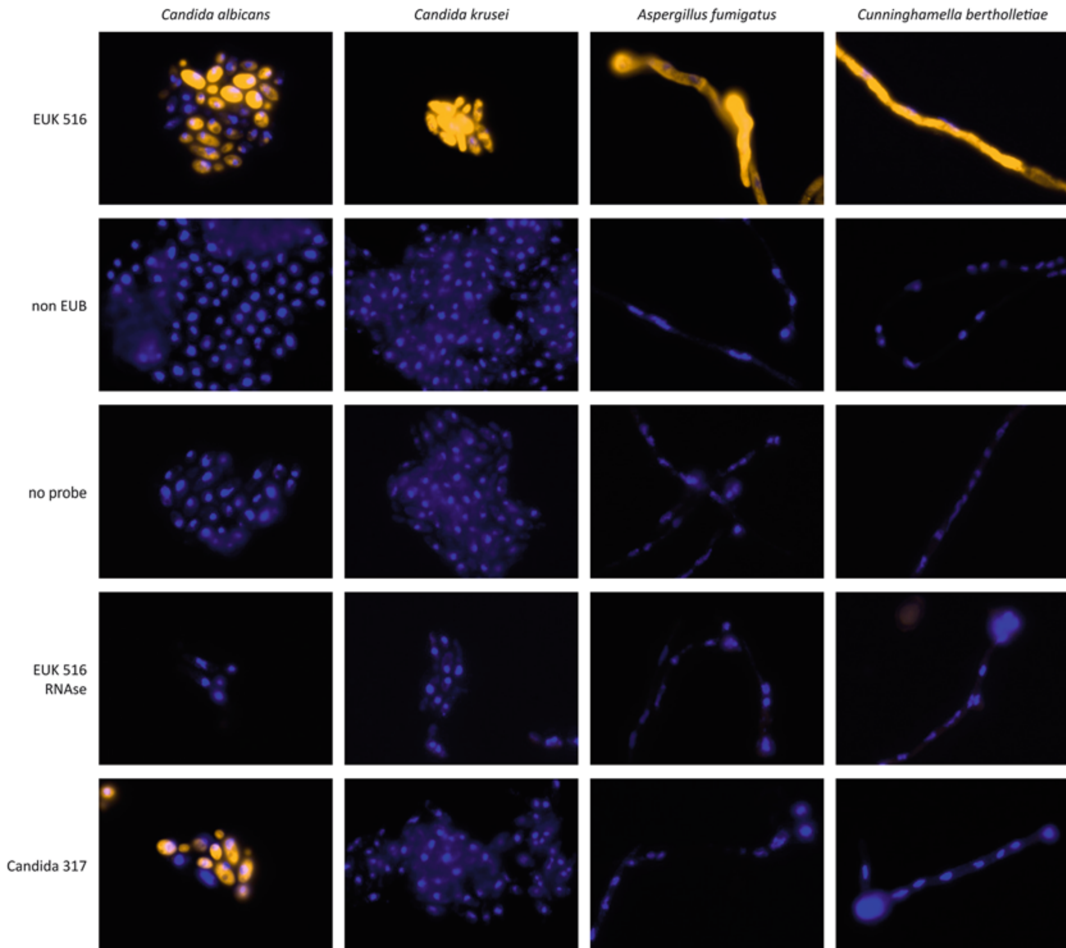

Fig. 1 Evaluation of FISH probes using cultivated fungi. Formalin-fixed fungi were incubated with the unspecific probe EUK516 (first row), the nonsense probe nonEUB338 (second row), without any probe (row 3), with EUK516 after treatment with RNAse (row 4), and with the specific probe *Candida* 317 targeting *Candida albicans* and close relatives (row 5). All probes labeled with Cy3, coded as *orange*. Counterstaining with DAPI (*blue*). Note difference in signal intensity of individual yeasts cells and uneven signal distribution in mould hyphae. The presence of a signal with the unspecific probe for all fungi documents the correct hybridization procedure. The absence of a signal in rows 2–3 excludes unspecific binding or autofluorescence. The absence of a signal in row 4 suggests probe binding to RNA determined the signal. Comparison between target fungi (*Candida albicans*) and non-target fungi documents the specific hybridization of probe *Candida* 317 (row 5)

produce sufficient signals, labeling with multiple fluorophore molecules, use of PNA probes or CARD-FISH may prove useful to augment signal [1].

DAPI is often used in parallel to stain double stranded DNA allowing the localization of fungi within samples and nuclei within fungal elements (Fig. 2).

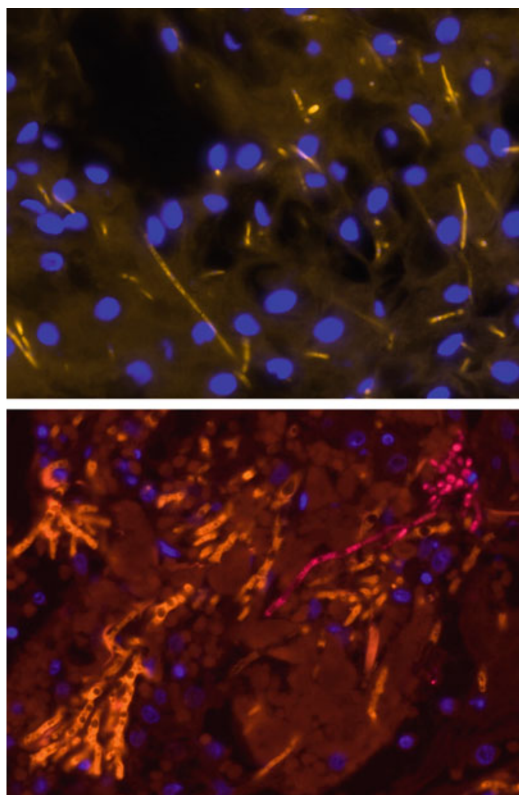

Fig. 2 Hybridization of formalin-fixed, paraffin-embedded tissue samples. *Upper*: patient with esophageal candidiasis. Yeast and pseudohyphae were visualized by hybridization with probe *Candida* 317 (labeled with Cy3, coded as *orange*), suggesting *C. albicans* as etiologic agent as confirmed by broad-range PCR and sequencing. *Lower*: mixed deep fungal infection. Fungal elements were visualized by hybridization with probe *Candida* 317 (labeled with Cy5, coded as *red*) and *Aspergillus* F (labeled with Cy3, coded as *orange*). Broad-range PCR and sequencing confirmed presence of DNA from *C. tropicalis* and *A. fumigatus* in the sample

2.3 Hybridization Procedure

Prepare all solutions using deionized sterile water.

1. SET-buffer (25×).
   NaCl 3.75 M, EDTA 25 mMol, Tris 0.5 M (pH 7.8) (all from Sigma). This buffer is used as ingredient for hybridization buffer (pure), to remove coverslips after hybridization (5× at 4 °C) and for washing away unbound probes after hybridization [0.2× at 50 °C (depending on probes used)].
2. Bovine serum albumin 2 % (Sigma).
3. Dextran 2.5 g in 5 ml (Fluka).
4. Poly A 25 mg in 2.5 ml (Sigma).
5. Salmon DNA pure (Sigma).

6. SDS 0.02% (Sigma).
7. DAPI (2500 ng/μl stock).
8. FISH probes stocks are prepared at 500 ng/μl and stored at −20 °C in the dark. Working solution for use in hybridization buffer is at 50 ng/μl (*see* **Note 1**).
9. Microscope slides partitioned into 6–10 wells (Diagnostika; Menzel-Gläser).
10. Coverslips.
11. Hybridization chamber (Lock and lock food container).
12. Kimwipes.
13. Vectashield mounting fluid (Vector Labs).
14. Nail varnish to fix coverslips after application of mounting fluid.

3 Methods

Defrost ingredients for hybridization buffer, DAPI, and probe stocks.

1. Prepare fresh Hybridization buffer for each experiment (5× SET, dextran 10%, BSA 0.2%, polyadenosine 0.1 mg/ml, salmon testes DNA 20 μg/ml, and SDS 0.02% (*see* Subheading 2.3, **item** 7).
2. Mix hybridization buffer with probes (50 ng/μl) and DAPI (25 ng/μl) 2 μl each in 100 μl hybridization buffer.
3. Apply fungal elements on slides in different partitions, let dry, use one slide per probe or probe combination to prevent diffusion of probes between partitions (*see* **Note 2**).
4. Apply hybridization buffer with probes and DAPI on fungal material (approximately 10 μl per well, or 50–80 per FFPE embedded tissue section).
5. Apply coverslip, preventing formation of bubbles
6. Bring slides into hybridization chamber on Kimwipes soaked with water and close chamber
7. Place hybridization chamber in hybridization oven at 50° (depending on probe melt temp). Incubate for 4–24 h.
8. Remove hybridization chamber from oven.
9. Remove coverslips by dipping in cold 1:5 SET buffer (*see* **Note 3**).
10. Wash three times (10 min) in SET 1:100 at 47 °C (depending on probe Tm).
11. Let dry in the absence of light.
12. Apply mounting solution.

13. Apply new coverslip.
14. Fix coverslip with Nail varnish.
15. Use epifluorescence or confocal laser scanning microscope, filters, and lasers matching excitation and emission wavelength of fluorophores (*see* **Notes 4** and **5**).

4 Notes

1. Probes may lose signal intensity by repeated freeze–thaw cycles. Prepare small portions of working solutions.
2. Use separate slides for individual probes or probe combinations as probes may diffuse between wells.
3. Mycelia may detach from wells during hybridization or post hybridization washes. Use of yeast cells and small germlings is favorable.
4. In tissue sections, fungal elements can be limited to small areas. Scanning a conventionally stained section may help localize fungal cells in the tissue.
5. Fluorescence signal within fungal cells may be limited to small foci often adjacent to non-necrotic host tissue.

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Chapter 17

Nuclear Magnetic Resonance Spectroscopy-Based Identification of Yeast

Uwe Himmelreich, Tania C. Sorrell, and Heide-Marie Daniel

Abstract

Rapid and robust high-throughput identification of environmental, industrial, or clinical yeast isolates is important whenever relatively large numbers of samples need to be processed in a cost-efficient way. Nuclear magnetic resonance (NMR) spectroscopy generates complex data based on metabolite profiles, chemical composition and possibly on medium consumption, which can not only be used for the assessment of metabolic pathways but also for accurate identification of yeast down to the subspecies level. Initial results on NMR based yeast identification where comparable with conventional and DNA-based identification. Potential advantages of NMR spectroscopy in mycological laboratories include not only accurate identification but also the potential of automated sample delivery, automated analysis using computer-based methods, rapid turnaround time, high throughput, and low running costs.

We describe here the sample preparation, data acquisition and analysis for NMR-based yeast identification. In addition, a roadmap for the development of classification strategies is given that will result in the acquisition of a database and analysis algorithms for yeast identification in different environments.

Key words NMR spectroscopy, Yeast, Identification, Classification, Nuclear magnetic resonance

1 Introduction

Identification of fungi relied traditionally on phenotypic information from microscopy, culture, and/or a limited array of biochemical tests. For fungi like yeasts, in which phenotypic differences are sparse, molecular identification methods are essential. Drawbacks of molecular identification methods are relatively high running costs and relatively long turnaround times. In particular in a clinical environment, rapid and objective high-throughput identification methods are essential to avoid delays for using the most efficient therapy. Novel technologies like nuclear magnetic resonance (NMR), infrared and Raman spectroscopy, and mass spectrometry generate complex data based on metabolite profiles of microorganisms (metabolome) [1–6]. Metabolomic methods can detect genotypic and phenotypic differences in closely related yeast

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species and genetically modified yeast strains [5, 7–9]. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry has already entered clinical microscopy laboratories as a routine method for the identification of yeast isolates [10–12]. Metabolomic analysis of spectroscopic data provides better discrimination between microbes than transcriptomics and proteomics [8, 13].

While MALDI-TOF mass spectrometry based identification systems have been established for routine analysis in microbiological laboratories, NMR spectroscopy provides similar accuracy but adds some advantages. The ease of sample preparation for NMR spectroscopy makes automation and thereby high-throughput data acquisition possible [14]. The acquisition and automated statistical analysis of NMR spectra is rapid (5–10 min), shows limited variability after repeated data acquisition, and is noninvasive. In particular the noninvasive nature of NMR spectroscopy has important bearings on *in vivo* diagnosis of infective lesions, in particular in the brain for an etiological identification of abscess causing microorganisms [15–17]. Mass spectrometry and NMR spectroscopy are to some extent complementary methods. While MALDI-TOF mass spectrometry of biological samples mainly identifies proteins, NMR spectroscopy identifies small molecules and metabolites. Both methods are attractive in biology and biomedicine because they are capable of simultaneous recognition of hundreds of cellular compounds. While cells are destroyed during the acquisition of MALDI-TOF mass spectra, NMR spectra of living cells can be acquired under physiological or other conditions. For diagnostic purposes, NMR spectroscopy can be applied to isolated compounds, cell and tissue extracts, biological fluids like blood plasma, urine, or cerebrospinal fluid, cell suspensions, tissue biopsy specimens, and *in vivo*. NMR spectroscopy of yeast was not only able to identify isolates to the level of species and subspecies based on isolated cultures [1, 4, 9, 16] but also to assess susceptibility to antifungal drugs [18], distinguish between different causes of meningitis based on cerebrospinal fluid [19, 20], and follow up the success of antifungal treatment based on biofluids from patients [20]. While running costs for NMR spectroscopy are low, a disadvantage is its high costs for the instrumentation.

Conventional approaches of using NMR spectroscopy have the aim to identify metabolites or metabolic pathways that are of importance in biochemistry, bioengineering, industrial processes (for example food industry) but also in diagnosing diseases, assess metabolic pathways involved in pathogenesis and potential therapy [5, 21]. For the purpose of unambiguous assignment of NMR signals to particular metabolites, time-consuming multidimensional correlation NMR spectra are required. For the purpose of rapid identification, automated analysis approaches on NMR

spectroscopic features (signals) are used that do not require the assignment of these features to particular chemicals.

NMR spectra of biological samples are characterized by high dimensionality (several thousand individual data points) and scarcity of available samples (in our case, the number of genetically independent yeast isolates that belong to particular taxa). A statistically meaningful analysis of a limited number of high-dimensional data points presents a challenge. Therefore, several automatic feature extraction methods (for example [22–24]) that explore statistical properties such as the spectral variability, similarity, or dissimilarity within groups can be used to develop classifiers for the identification of new, unknown yeast isolates. As NMR spectroscopic features are highly redundant (data do not span the entire high-dimensional space) and neighboring spectral features of NMR spectra are highly correlated, these highly correlated characteristics of neighboring spectral features can be exploited for feature extraction as successfully shown for closely related yeast species [25]. Efficient feature extraction methods should identify most discriminatory regions in the spectrum, which are then analyzed by classification methods like linear discriminate analysis or artificial neural networks [26].

Considering that at least 5–10 samples (isolates) per feature (for example spectral region) are required to achieve a robust and reliable classification and subsequent identification, a large number of independent yeast isolates is needed for the training process of those classifiers [26]. Classifier development would involve more than hundred strains per taxa. Classifiers developed on relatively small data sets can result in overly optimistic classification with attendant poor generalizability, so that new, independent samples will not be classified correctly [4, 27]. The larger the data base for classifier training, the more likely it is that randomly selected isolates of a particular class (sub-species, species, genus) are truly representative of the data space (phenotypic range) occupied by all members of that class. The selection of a cohort of yeast strains for classifier training should include a wide range of isolates that is not only representative for a particular application, environment or location but should include diverse strains. Hereby, type strains and commonly used reference strains allow comparison with reference methods and laboratories, but additional well-identified and documented strains improve the generalization potential of a classifier. Such quality-assured strains are available from accredited public microbial culture collections [28, 29].

In this article, we first focus on the description of protocols for the acquisition and processing of NMR spectra from environmental, industrial or clinical yeast isolates for rapid and high-throughput identification. As classifiers used for the identification of yeasts based on their NMR spectra are not yet readily available, we further explain a strategy to develop such classifiers.

2 Materials

2.1 Microorganisms

Yeast strains can be of environmental, industrial, veterinarian, or clinical origin. They need to be viable and pure cultures. Culture conditions (including media) should be to some extent standardized (*see* **Note 1**). Filamentous fungi are less suitable for NMR based identification.

2.2 Yeast Culture

Culture of yeast cells should be performed using either static cultures on plates or liquid medium. Here, we focus on standard procedures for most common yeast species.

1. DYPA plates (2 % dextrose, 0.5 % yeast extract, 1 % peptone, 2 % agar).
2. Incubator operating at 27 °C or a similar temperature.
3. Bunsen burner.
4. Inoculation loops.

2.3 NMR Spectroscopy

1. Economy, high-throughput NMR tubes of 5 mm diameter.
2. Phosphate buffer saline (PBS, consisting of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4) made up with sterile water and at least 25 % deuterated water, containing 0.05 % 3(trimethylsilyl)propionic-2,2,3,3-d4 acid (TSP).
3. 1 ml plastic tubes.
4. Vortexer.
5. Pipettes (0.2–1 ml volume).
6. Inoculation loops.
7. NMR spectrometer (*see* Fig. 1b, c) equipped with a 5 mm ^1H probe (also other probes like ^1H/broad band or $^1H/^{13}C$ inverse-detection probes can be used).
8. Software for data acquisition and processing.

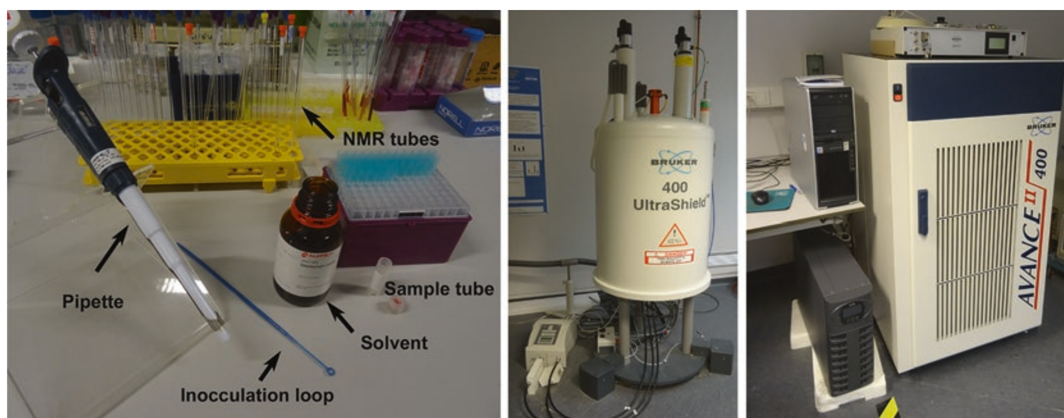


Fig. 1 Equipment required for the preparation (**a**) and acquisition of NMR spectra from yeast isolates (**b**—magnet of an 400 MHz NMR spectrometer, equipped with a 5 mm ^1H, ^{13}C probe head and **c**—console and processing computer of the NMR spectrometer)

2.4 Data Analysis

1. Data base containing NMR spectra of the yeast species that need to be identified. The data base should contain at least 30 strains per species. Each strain that is part of this reference data base needs to have a confirmed identity (*see* **Note 2**).
2. Classification software.

3 Methods

3.1 Yeast Culture

1. Pure yeasts are plated out on DYPA plates using sterile inoculation loops in an aseptic environment near the flame of an Bunsen burner.
2. DYPA plates are incubated for 48 h in an incubator operating at a temperature of 27 °C.
3. Culture plates are transported to the NMR spectrometer. Plates can be kept at room temperature for up to 5 h.

3.2 Sample Preparation for NMR Spectroscopy

Suspensions of yeast cells in PBS (containing at least 25% D_2O, PBS/D_2O) are prepared immediately before the acquisition of NMR data.

1. Cells are gently removed from the DYPA plates, without removing any agar, using an inoculation loop (at least 10^7 and up to 10^9 colony forming units, cfu; Fig. 2a).
2. The cell mass is suspended to homogeneity in a 1.5 ml plastic tube containing 0.5 ml PBS/D_2O. Hereby, cells are removed from the inoculation loop (Fig. 2b) and the suspension is briefly vortexed.
3. A volume of at least 0.5 ml of the homogeneous yeast suspension is transferred to an 5 mm NMR tube using a pipette. Make sure that no air bubbles are trapped in the NMR tube (Fig. 2c, d).

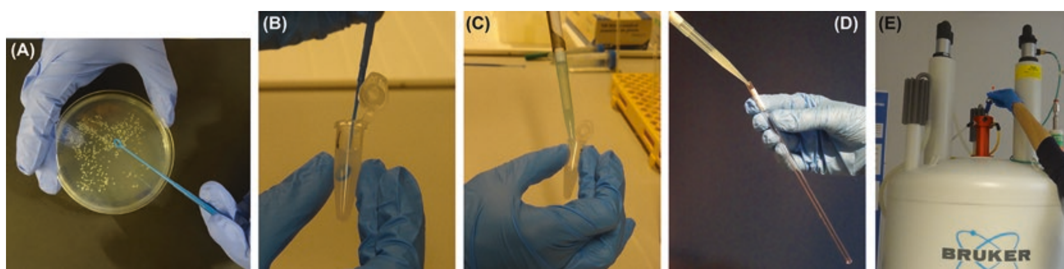


Fig. 2 Steps required for sample preparation to acquire an NMR spectrum of yeast suspensions. (**a**) Scrap 10^6–10^8 yeast cells from the culture plate using an inoculation loop, (**b**) suspend the yeast cells in 0.5 ml PBS/D_2O (containing TSP), (**c**, **d**) transfer the yeast suspension to an NMR tube, (**e**) put the NMR tube in a rotor and transfer it to the magnet of the NMR spectrometer

3.3 Acquisition of NMR Spectra

NMR spectra should be acquired as quickly as possible after sample preparation to avoid sedimentation of the yeast cells at the bottom of the NMR tube. Make sure that the NMR probe is tuned and matched to the ^{1}H frequency (*see* **Note 3**). NMR spectra have to be acquired at a defined temperature (27 °C). The most frequently used protocol for the acquisition of 1D ^{1}H NMR spectra is the 1D Nuclear Overhauser Enhancement Spectroscopy (NOESY) with water suppression, averaging over typically 32 scans, repetition times of 5 s, a spectral width of 12 ppm, acquiring 8 or 16k data points and spinning the sample at 20Hz to avoid sedimentation of the yeast cells during data acquisition (*see* **Note 4**).

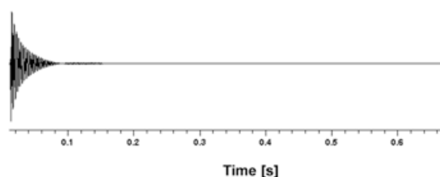
1. Put NMR tube in rotor and load sample into the magnet of the NMR spectrometer (*see* Fig. 2e).
2. Generate a data file for the acquisition of a ^{1}H NMR spectrum based on a previously saved protocol (*see* **Note 5**).
3. Start spinning the sample.
4. Lock the sample (D_2O) and shim (*see* **Note 6**).
5. Adjust the receiver gain.
6. Start acquisition of the NMR spectrum.

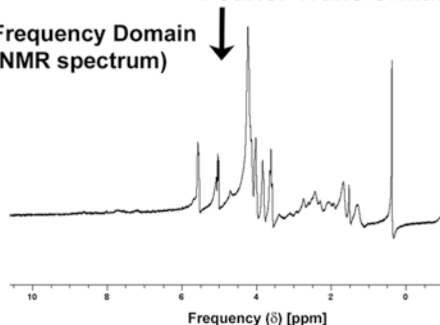
3.4 Data Processing

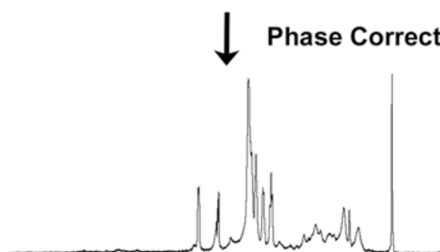
There are several possible approaches to use NMR spectra of microbes for their identification. Either the free induction decay (FID, time domain) or the NMR spectrum in the frequency domain is used for further analysis. In the first case, no additional data processing is required. However, NMR spectra are more commonly used in biological and biomedical applications. Here, we discuss in detail the steps required for preparing NMR spectra (frequency domain) for further analysis (*see* Fig. 3 and **Note 7**).

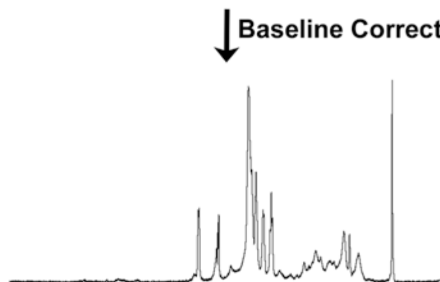
1. Perform Fourier transformation using the manufacturer's (NMR spectrometer) or third party software.
2. Perform phase and baseline correction according to documentation of the respective software.
3. Align/calibrate the frequency using the TSP signal (chemical shift (δ) = 0 ppm).
4. Generate features for further analysis. The simplest and most commonly used features are the full range of data points of the normalized spectrum or integrals of spectral regions (binning, using even or variable width bucketing). For some specific applications, further transformation like the use of first derivatives of the spectrum or rank-orders of the original features provided better classification results (*see* **Note 7**). Spectral regions containing signals from "artifacts" like solvents (water) or calibration standards (TSP) need to be excluded.
5. Export the spectral features to the classification software.

Typical examples of closely and distantly related clinical and industrial yeast isolates are shown in Fig. 4.









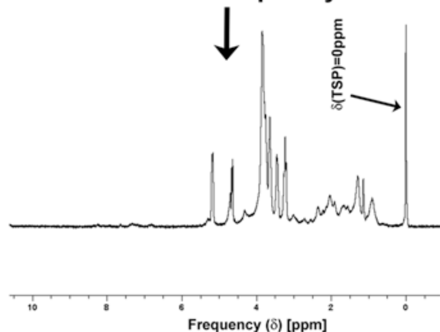


Fig. 3 Steps required for the processing of an NMR spectrum. All steps can be routinely performed using the standard software of the NMR spectrometer or NMR software from other providers. The acquired data are originally in the time domain (free induction decay, FID). An NMR spectrum (frequency domain = chemical shift, δ) is generated by Fourier transformation. Phase errors are adjusted by phase correction. For accurate quantification (for example by integration), baseline correction is essential. In order to be able to align all NMR spectra, the frequency (chemical shift) is corrected using the TSP signal (far right, δ(TSP) = 0 ppm)

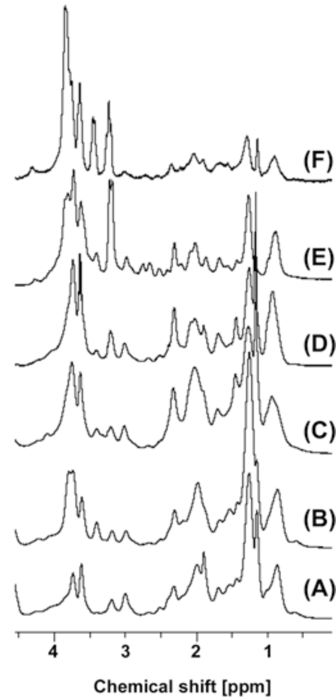


Fig. 4 NMR spectra of suspensions of yeast isolates. Closely and distantly related isolates are displayed and include (**a**) *Saccharomyces cerevisiae*, (**b**) *Candida albicans*, (**c**) *Saccharomyces bayanus*, (**d**) *Saccharomyces pastorianus*, (**e**) *Metschnikowia pulcherrima*, and (**f**) *Cryptococcus gattii*

3.4.1 Quality Control

To avoid misidentification due to poor spectral quality, minimum standards are required (*see* also Fig. 5). The most important parameters are the signal-to-noise ratio (SNR) and the spectral resolution (line width of reference peak). For both, manufacturers of NMR spectrometer provide routines for rapid assessment. For automated assessment of SNR, it is important to define a signal region of the spectrum that contains signals for all microbial isolates (typically chemical shift regions between 0.9–1.5 ppm and 3.0–3.8 ppm) and avoid signals from solvents (typically the region of remaining water signal between 4.5 and 4.8 ppm). For NMR spectra of yeast, noise can usually be defined in the chemical shift region 9–10 ppm. From our experiences, an SNR > 20 is essential. The line width of the TSP signal should not exceed 10 Hz (*see* **Note 8**).

3.5 Data Analysis

Identification of unknown yeast strains based on their NMR spectra is done automatically by classification of the exported NMR data using a validated classifier. As only limited classifiers are publicly available and classification/identification questions may arise in different contexts (yeast species relevant for agro-industrial applications, environmental or clinical samples), we describe the

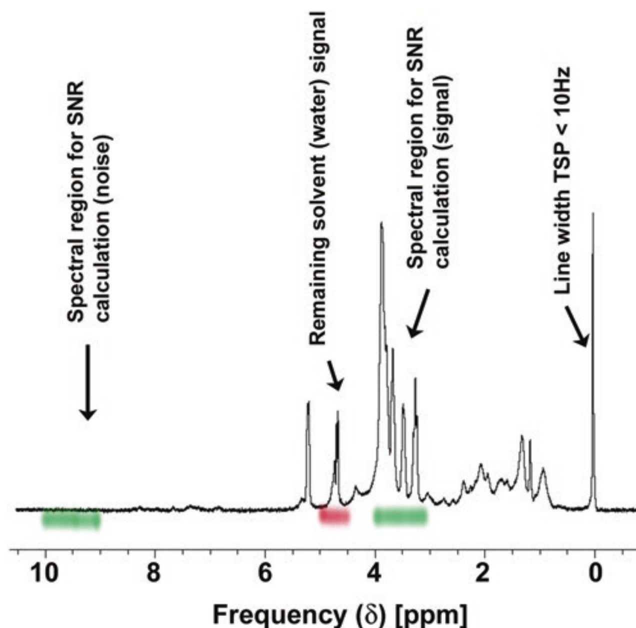


Fig. 5 Quality assessment of NMR spectra acquired from yeast isolates. Typical parameters to assess the quality of the NMR spectrometer can be determined using the manufacturer's software. For the identification of yeast isolates based on their NMR spectrum, the line width of a reference peak (TSP) should be smaller than 10 Hz. The signal-to-noise ratio (SNR) can be determined by defining a noise region (without signals) in the chemical shift region 9–10 ppm and a signal region in the chemical shift range 3.0–3.8 ppm. The SNR should be at least 20. For classification/identification, chemical shift regions that contain artifacts (for example water or TSP) should be removed

generation and validation of classifiers below. The development of accurate and robust classifiers for the identification of microbes consists of three processes as illustrated in Fig. 6.

3.5.1 Feature Reduction

One intrinsic problem of using spectroscopic data for identification and diagnostic applications is that they consists of several thousand data points (high-dimensional feature space) but only very few samples (for example, yeast strains) per class (for example, yeast (sub) species). In order to avoid overtraining of classifiers and to arrive at a required sample to feature ratio of 5–10, features need to be reduced to the most discriminatory, removing non-discriminatory variance [26, 27, 30]. A data set of NMR spectra that was generated from independent yeast strains should be based on at least 5–10 times the number of selected features per species. For example, if the final classifier for identifying species A from species B is based on three spectral regions at least 15–30 independent strains of species A and B should be part of the data base (*see* **Note 9**). A variety of successful feature reduction and feature selection procedures are available of which only few can be mentioned:

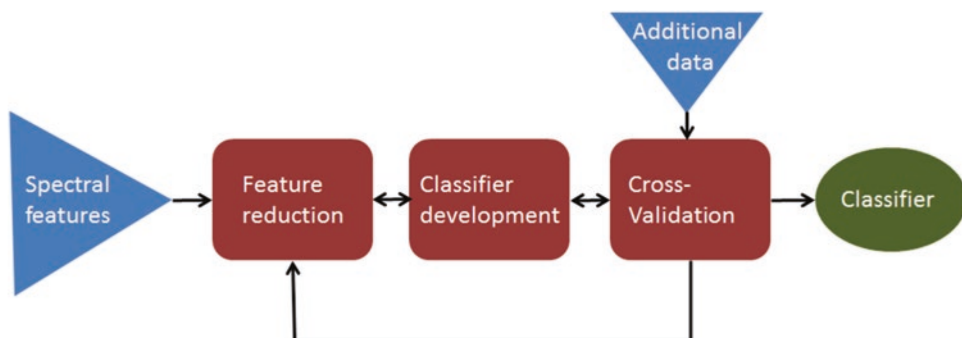


Fig. 6 Flow chart for the development of classifiers for the identification of yeast isolates based on the NMR spectra. Spectral features like all data points of the normalized NMR spectra or normalized integral regions (buckets) are used as input. The feature reduction step reduces the original features to a few, most discriminatory features. These reduced features are then used to develop classifiers. After cross-validation by randomly splitting the original data set into training and tests sets, validation using a data set of new, independent isolates is necessary. If the validation of the independent data set results in insufficient specificity and/or sensitivity, the new data set is added to the original training set and all training steps are repeated. Otherwise, the resulting classifier can be used for the identification of new yeast isolates

1. Prior-knowledge driven selection: Based on knowledge of spectral regions in the NMR spectra (representing particular compounds) that contain mainly noise, artifacts (for example solvents) or signals from metabolites that can be used as potential markers for the identification of a particular microbial species, parts of the NMR spectrum can be excluded (noise, artifacts) or exclusively used as feature. This method is highly subjective, introduces potential bias, and is not suitable for (sub)species that are (metabolically) closely related.
2. Filtering methods rank features according to their discriminative power (and not according to the variance). A potential disadvantage of ranking methods is that they require prior knowledge of the classes (species, subspecies) present in the data (*see* **Note 10**). If the biochemical origin of a successful classification is of interest (for example, identification of biomarkers), extracted features from ranking methods have the advantage that they preserve their spectral identity (*see* **Note 11**). Several ranking models have been used to identify most relevant features like *t* test [31], Kruskal–Wallis test [32], or Fisher discriminant [33].
3. Projection or wrapper methods transform the data into a feature space and estimate the relevance of those features to the overall learning task. They are often used in combination with the eventual classifier to guide the selection method. Commonly used methods are principal component analysis [34, 35] and principal coordinate analysis [35]. Disadvantages of these methods are that, due to the transformation of the original

data into principal components, extraction of spectral features (metabolites) is not directly possible (*see* **Note 12**) and, due to ordering principal components according to maximum variance, the first principal components are not necessarily most discriminatory [27].

4. To overcome some of the limitations of the above mentioned standard methods, a variety of approaches have been taken. Examples for the use of NMR spectra for classification purposes include feature selection by using the Gini importance of random forests [23] or a statistical classification strategy that uses a genetic algorithm based region selection, which retains the spectral identity of selected features [22].

In our experience, different feature extraction methods result frequently in a similar classification, which is an indirect indication for the robustness of the classification process.

3.5.2 Development of Classifiers

Classifiers are developed using the lowest number of selected and most discriminatory features that would result in correct and robust classification of an independent test set of yeast isolates. For classifier development, a large training data sets of NMR spectra from independent yeast isolates is required. Ideally, a training set for classifier development contains ten times more samples (per class) than features used in the final classifier, which reduces the risk of overtraining (*see* **Note 13**). Small training sets would result in overly optimistic classification with poor generalizability, so that new independent isolates are more frequently incorrectly identified [4, 9]. Various types of classifiers have been used for the analysis of spectroscopic data. They include linear discriminant analysis, k nearest neighbors, simple correlation methods, decision trees, neural networks and support vector machines. Experience has shown that the simpler classification approaches performed often more robust on independent test data than more complex and sophisticated approaches [26, 27].

3.5.3 Cross-Validation

In particular for small data sets, splitting in an independent training and validation set is not always advisable due to the risk of overtraining. Cross-validation strategies aim to overcome the bias introduced by using the whole data for the training/development process of a classifier and at the same time for the assessment of its performance. The simplest cross-validation approach is the leave-one-out method, which uses all but one spectrum for the classifier development and then tests its performance using the left out spectrum. This process is then repeated N-times (N = number of spectra in the data set). Another approach is the random splitting of the data set in a training and a test set. A bootstrap-based cross-validation method [36] was used for the classification of different Candida species [4]. Hereby, random resampling with replacement

of samples for the training and validation process of classifiers was typically repeated 1000 times. The ultimate classifier consisted of the weighted output of the 1000 different bootstrap classifier coefficient sets. These ultimate classifiers are then again tested against new isolates, which are then again added to the training process until convergence of the accuracy for training and independent test set is achieved [4].

Each classifier yielded probabilities of class assignment for the individual spectra. For each sample x, the a posteriori probabilities $pm(x)$ for all classes (taxa) m _ 1, 2, …, K is calculated according to

$$p_m(x) = \left(1 + \sum_{n=1, n \neq m}^{K} \left(\frac{1}{p_{mn}(x)} - 1\right)\right)^{-1}$$

where $p_{mn}(x)$ is the class probability for x when submitted to the individual pair classifier C_{mn}. The individual probabilities of belonging to one of the predefined taxa are normalized by dividing them by the sum of all individual probabilities (e.g., $p_1[x] + p_2[x] + \ldots + p_K[x]$). The identification of a new isolate to one of the classes (taxa) with confidence is achieved if the probability of belonging to one class is larger than the average between 1 and even probabilities ($(1 + K^{-1})/2$). For example, for a classifier containing five different species, identification with confidence is achieved if the NMR spectrum of this isolate is assigned to one of the species with a probability larger than 60% ($(1 + 1/5)/2 = 0.6$). For classification probabilities that are smaller than ($(1 + K^{-1})/2$), the assignment of a spectrum to any species group is indeterminate.

4 Notes

1. Culture conditions (time, temperature, medium) should allow optimal growth of the respective yeasts. They should be kept as constant as possible. Previous experiments have shown that minor variations in terms of duration of culture, temperature, additional storage have little or no effect on identification results [4]. Culture conditions should represent the natural environment of the yeast. For example, while the growth temperature for environmental and industrial strains is usually 25 or 27 °C, it is conventionally 30 °C for clinical isolates. It is important to keep culture conditions for the to-be-identified yeast identical to the conditions used for the acquisition of a training data set.
2. A data base of NMR spectra from the yeast species that need to be identified is necessary to train classification (identification) software. The data base should contain all species, subspecies, and varieties that are of relevance. Each entity, to which identification is attempted, such as species, subspecies or variety,

should be represented by the largest feasible genetic diversity of strains. If such a data base is not available it can be acquired using the acquisition protocol described above. This data base can then be used to train classifiers using commercial or purposely developed classification software. For further reading see also refs. [4, 9, 37].

3. Tuning and matching is usually performed for each sample. However, we have noticed that no or only marginal adaptations to the tuning and matching of the probe have to be made if similar samples (same type of NMR tube filled with the same volume of suspended yeast cells in the same buffer solution) are measured. Therefore, we only tune and match the probe for the first measurement of the day.
4. Once an acquisition protocol is selected, it is important to avoid unnecessary modifications to the protocol. The repetition time should be approximately five times the T_1 relaxation time of the metabolites. For much diluted samples (low cell numbers of 10^5–10^6), the number of scans should be increased from 32 to 128 or even 512, which will increase the total acquisition time of the NMR spectrum. We assume that power levels for radiofrequency pulses have been adjusted ones for the acquisition protocol. In our experience, it is also not necessary to adjust parameters for water suppression for each individual sample.
5. A template for the optimized acquisition protocol should be saved and protected from modifications. Depending on the manufacturer of the NMR spectrometer and the respective software version, fully automated acquisition of a previously optimized protocol is possible and advised.
6. Fully automated locking and shimming is advised. Starting from a base shim set, the shimming protocol should be optimized for speed of acquisition rather than highest quality of shimming as (1) the samples are very similar and (2) cell suspensions will result in relatively large line widths anyhow.
7. Many purpose developed software packages but also commercially available software (for example, from manufacturers of NMR hardware) provide routines for automated processing and extraction of spectral parameters.
8. Cell suspensions are more inhomogeneous than chemicals dissolved in an NMR solvent. Therefore, shimming will always be of lower quality and subsequently, line widths of signals in the NMR spectra of yeast suspensions will be broader than in high resolution NMR spectra of liquids. A line width of 10 Hz is sufficient for successful classification of suspensions of intact cells (bacteria, yeast) [4, 9, 23, 25, 37].
9. It is advisable to use commonly utilized reference strains (for example type strains and frequently used strains from culture

collections) as well as strains that are relevant for the respective application (for example, clinical isolates, environmental isolates, agro-industrial isolates). The identity of isolates should be confirmed by generally accepted biochemical or molecular identification methods.

10. For the training of classifiers used for identification purposes, one relies on well characterized microbial strains. In this case, prior knowledge of to-be-identified species/subspecies (classes) is essential. However, this approach will assign isolates that belong to a species/subspecies that was not part of the training set (outlier) to the (biochemically) closest class but not to the correct class.
11. Extracted features that directly correspond to regions of the NMR spectrum can be interpreted in terms of compounds (metabolites) that generate the respective signals. For identification purposes, this is of limited interest. However, if it is of relevance to know what compounds have contributed to a successful discrimination between different microbes, preservation of the spectral identity is beneficial.
12. Threshold-based methods like loading or score plots can determine which features have mainly contributed to particular principal components.
13. If the number of features is relatively small when compared to the number of independent NMR spectra (from different yeast strains), classification results are overly optimistic. This becomes apparent when such a classifier is retested on an independent data set of newly acquired isolates. It is therefore important to add new isolates and to retrain already existing classifiers until the performance of the classification under training conditions is similar to the performance on independent test sets [4].

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Chapter 18

T2 Magnetic Resonance for Fungal Diagnosis

Fainareti N. Zervou, Ioannis M. Zacharioudakis, Jaclynn Kurpewski, and Eleftherios Mylonakis

Abstract

Rapid diagnostic methods for fungal infections are long awaited and are expected to improve outcomes through early initiation of targeted antifungal therapy. T2Candida panel is a novel qualitative diagnostic platform that was recently approved by the US Food and Drug Administration (FDA) for diagnosis of candidemia with a mean time to species identification of less than 5 h. T2Candida panel is performed on the fully automated T2Dx instrument in whole blood K_2EDTA specimens and is able to detect 5 *Candida* spp., namely *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, and *Candida glabrata*. By combining magnetic resonance with molecular diagnostics, T2*Candida* panel amplifies DNA and detects the amplified product by amplicon-induced agglomeration of supermagnetic particles and T2 Magnetic Resonance (T2MR) measurement. Here we describe the materials and methods needed to diagnose candidemia with the T2Candida panel.

Key words T2*Candida* panel, T2 magnetic resonance, T2MR, *Candida*

1 Introduction

Parallel to the increase of immunocompromised patients, an increase in the incidence of invasive fungal infections has been documented [1], and *Candida* spp. infections currently represent 6% of all hospital-acquired infections (HAIs) in the USA [2]. Evidence from a meta-analysis of randomized controlled trials has indicated that invasive candidiasis is associated with a high and unreduced 30-day mortality rate that surpasses 30% [3].

Early initiation of treatment and specifically treatment initiation within the day of symptom onset and blood culture draw is related with a significantly reduced mortality down to 15% [4, 5]. However, automated blood culture methodologies, which are routinely used for the diagnosis of candidemia, take up to 2–5 days to grow and lead to a crucial delay in treatment initiation. Specifically, it has been shown that in 89% of candidemic patients empiric antifungal therapy is not started on the day of blood culture draw [6].

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Therefore, a highly sensitive, specific, and rapid diagnostic method is expected to allow early initiation of antifungal therapy and subsequently improve outcomes [7, 8]. Moreover, a rapid diagnostic method has the potential to reduce the administration of empiric antifungal treatment to patients who test negative, reducing this way the adverse effects of antifungal therapy [9], as well as the antimicrobial pressure that is associated with development of resistance to antifungal agents [10–12].

Technological advances in the field of nanotechnology coupled with the proven applications of magnetic resonance have recently presented a rapid, fully automated, qualitative, sensitive, and specific diagnostic platform, the "T2Candida panel" [13]. T2Candida panel combines nuclear magnetic resonance and PCR molecular assays to directly detect and identify *Candida* spp. from whole blood samples [13].

T2Candida panel initially lyses the red blood cells and concentrates the pathogen cells and cellular debris. Thereafter, *Candida* spp. cells are lysed with mechanical bead beating and DNA is amplified. The amplified DNA is directly detected in whole blood samples by amplicon-induced agglomeration of Supermagnetic Particles and T2 Magnetic Resonance (T2MR) measurement. An internal control, that is a synthetic DNA target, monitors T2*Candida* panel performance in each reaction. The T2*Candida* panel can identify five *Candida* spp. and the following three results can be reported:

1. *Candida albicans/Candida tropicalis.*
2. *Candida parapsilosis.*
3. *Candida glabrata/Candida krusei.*

Initial studies on the performance of the T2*Candida* panel have been promising [14]. The analysis of artificially supplemented blood samples with both the BACTEC 9050 automated blood culture system (Becton Dickson) and T2Candida panel (T2Biosystems, Inc, Lexington, MA, USA) showed a significant change in time to diagnosis and an advantage of T2Candida panel in identification of *C. glabrata* when the arbitrary 5 day duration (which is used in most laboratories before the samples are discarded) was used.

The sensitivity and specificity of the T2*Candida* panel has also been validated in a clinical trial that included analysis of both prospectively collected blood samples of more than 1,800 patients with symptoms of bloodstream infection and blood samples technically spiked with known clinically relevant concentrations of the 5 *Candida* spp. [15]. Different *Candida* spp. clinical isolates were used for each specimen. The overall sensitivity per assay of the T2*Candida* panel was found to be 91.1 % and the overall specificity 99.4 %, with a mean time to species identification of 4.4 ± 1.0 h. Further, using quantified spiked samples, the limit of detection

(LOD) of T2*Candida* panel was estimated to be 1 CFU/mL for *C. tropicalis* and *C. krusei*, 2 CFU/mL for *C. albicans* and *C. glabrata*, and 3 CFU/mL for *C. parapsilosis.*

The 5 detectable *Candida* spp. are responsible for more than 95% of the total *Candida* infections [16]. It should be noted that the T2Candida panel does not estimate the resistance profile of the isolated *Candida* spp. Although different species have generally distinct antifungal resistance patterns [17], the increasing prevalence of resistant *Candida* spp. outlines the importance of identification of the resistance pattern of the isolating strains [12, 18]. For those reasons, this new diagnostic approach will most likely be incorporated in diagnostic algorithms that will also include blood culture methodologies.

The introduction of this technology in diagnostic algorithms will increase the cost per patient tested, but it is expected to provide an economically self-supporting policy if savings from shorter hospital stays and termination of excess empiric antifungal treatment are taken into account [19, 20]. Expected benefits in terms of morbidity, mortality and costs remain to be confirmed in clinical practice. However, these preliminary results are highly promising and indicate that this new technology may represent a paradigm shift in the field of infectious diseases diagnostics.

Below, we summarize the materials needed and the methods used to diagnose candidemia using T2*Candida* panel that is currently approved by the US Food and Drug Administration (FDA).

2 Materials

1. T2Dx instrument (T2 Biosystems), a fully automated sample-to-result system, which completes all steps in T2*Candida* panel after specimen loading.
2. Barcode Reader.
3. 4 mL plastic Vacutainer K_2EDTA venous blood collection Tubes 13 mm (*see* **Notes 1** and **2**).
4. T2*Candida* Sample Inlet, where the K_2EDTA whole blood specimen is loaded (*see* **Note 3**) (*see* Fig. 1).
5. T2*Candida* Base that contains calcium hypochlorite and a Lysis Reagent comprising a detergent mix and 0.09% sodium azide in an aqueous buffer solution used for the T2Candida panel (*see* **Note 3**) (*see* Fig. 2).
6. T2*Candida* Reagent Pack that contains the refrigerated reagents used for the T2*Candida* panel (*see* **Notes 4** and **5**) (*see* Fig. 3).
7. *Candida albicans, C. parapsilosis,* and *C. glabrata* (APG) External Positive Control.

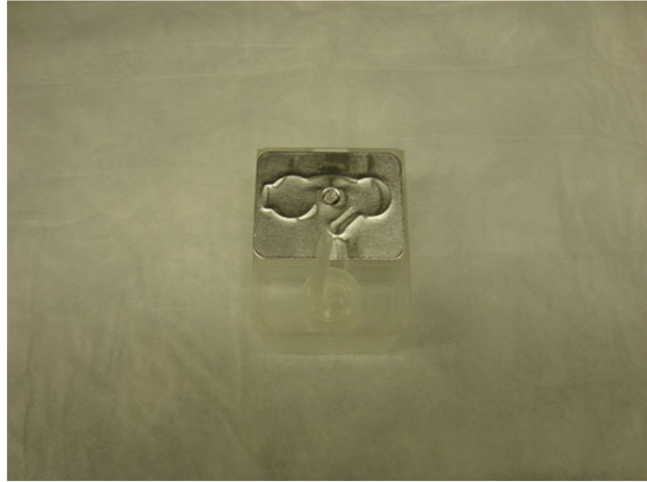

Fig. 1 T2*Candida* sample inlet

Fig. 2 T2*Candida* base

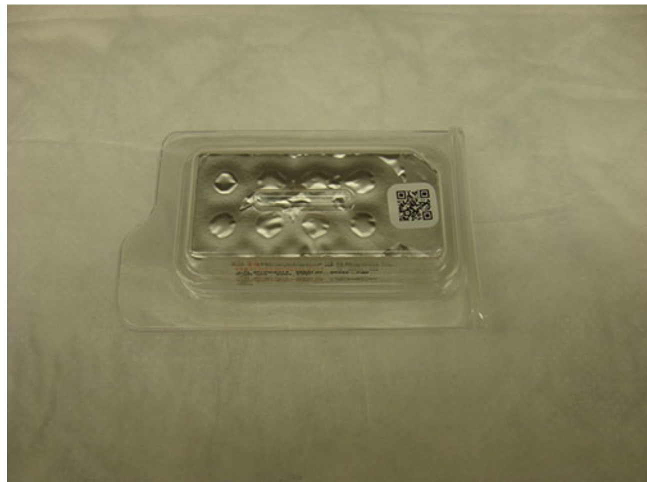

Fig. 3 T2*Candida* reagent pack

8. *Candida tropicalis, C. parapsilosis,* and *C. krusei* (TPK) External Positive Control.
9. Negative external control.
10. Powderless disposable gloves.
11. Eye protection.
12. Lab coat.
13. Bleach-Rite (Current Technologies Inc.) or household bleach at 5% sodium hypochlorite diluted 1:10.
14. 70% Isopropyl Alcohol.
15. Lint-free wipes.
16. Absorbent pads.
17. Distilled or deionized water.
18. Biohazard waste bags.

3 Methods

3.1 Blood Specimen Collection and Storage

1. Blood specimens should be collected with the same aseptic procedure as blood cultures (*see* **Note 6**).
2. After blood collection, the K_2EDTA whole blood specimen must be inverted 8–10 times to thoroughly mix the blood with the K_2EDTA anticoagulant.
3. K_2EDTA whole blood specimen should be stored at 15–25 °C for up to 12 h before analysis or at 2–8 °C for up to 2 days. If stored at 2–8 °C the specimen should remain at room temperature (15–25 °C) until temperature equilibration is achieved (approximately 20 min) before analysis.

3.2 Work Area Disinfection (See Note 7)

1. Wear fresh gloves, spray Bleach-Rite®, or equivalent, onto a new lint-free wipe, wipe the preparation area benchtop in a unidirectional motion and discard the wipe (*see* **Notes 8** and **9**).
2. Using a new lint-free wipe each time, repeat the same procedure to clean the touchscreen, barcode scanner, benchtop area where the T2Dx instrument is located and drawer panel of T2Dx instrument (*see* **Notes 8** and **9**).
3. Change the pair of gloves and repeat the cleaning procedure using 70% isopropyl alcohol to wipe all work surfaces (*see* **Note 9**).
4. Place a disposable absorbent pad on the work surface.

3.3 Setup

1. After work surface disinfection, confirm that the T2Dx instrument is operational.
2. Wear a new pair of gloves, remove the specimen from storage and confirm that the blood volume is at least 3 mL and that the

barcode is undamaged. If there is any blood on the exterior of the vacutainer, clean with standard lab procedures.

3. Allow blood sample to equilibrate to room temperature as previously described.
4. Wear fresh gloves and remove the required number of T2*Candida* Reagent Packs, T2*Candida* Bases, and T2*Candida* Sample Inlets from storage (*see* **Note 10**).
5. Wear fresh gloves, take out one T2*Candida* Sample Inlet, one T2*Candida* Base, and one T2*Candida* Reagent Pack and put them on the clean disposable absorbent pad that you have placed on the work surface (*see* **Note 11**).
6. Check the barcodes for integrity.
7. Briefly mix the Reagent Pack by agitating in a horizontal motion for 3–5 s (*see* **Notes 12** and **13**).
8. Insert the T2*Candida* Reagent Pack onto the T2Candida Base using the orientation notch on the T2*Candida* Reagent Pack for appropriate alignment.
9. Push down the T2*Candida* Reagent Pack touching only the site of the barcode and not the foil until an audible snap-in sound is heard (*see* **Note 14**).

3.4 Sample Loading

1. Resuspend the patient sample in the capped blood collection tube by inverting the tube a minimum of 8–10 times (*see* **Note 15**).
2. Uncap the blood collection tube using standard procedures and dispose the cap in the biohazard bag.
3. Invert the T2*Candida* Sample Inlet and use it to recap the blood collection tube with a push and twist notion (*see* **Notes 16** and **17**).
4. Invert the T2*Candida* Sample Inlet assembly and the blood collection tube and assure that the blood level in the blood collection tube drops as the blood is flowing into the T2*Candida* Sample Inlet (*see* **Note 18**).
5. Place the T2*Candida* Base on a flat surface and snap the T2*Candida* Sample Inlet onto it by pushing it down until an audible snap-in sound is heard indicating effective loading (*see* Fig. 4).

3.5 Loading the T2Candida Panel on the T2Dx Instrument

1. Wear fresh gloves and press the "Load" button on the touch screen of the T2Dx instrument.
2. Scan the K_2EDTA blood collection tube, the T2*Candida* Base and the T2*Candida* Reagent Pack following the instrument instructions. If the barcode reader does not work, put the information in manually using the grey button next to the field, it will open a keyboard on the touchscreen.

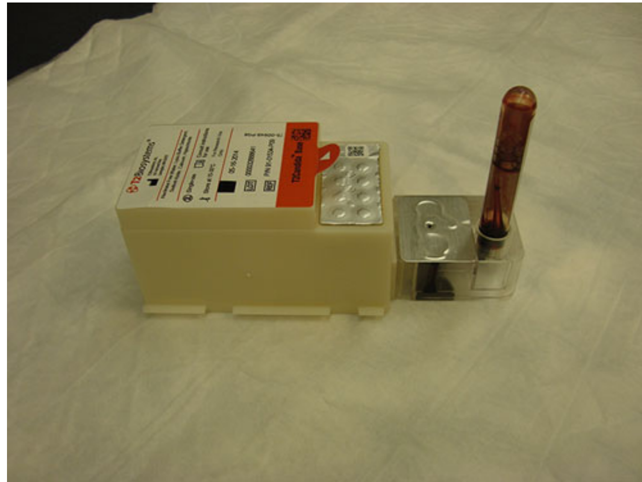

Fig. 4 Place the T2*Candida* sample inlet onto the T2*Candida* base

3. The system will open an available drawer and will instruct the user to load the fully assembled T2*Candida* panel (*see* **Notes 19** and **20**).
4. Press "Next" and when indicated by the instrument remove the top seal from the T2*Candida* Cartridge by pulling on the tab (*see* **Note 21**).
5. Ensure that all components are loaded and press "Confirm" on the touchscreen of the T2Dx instrument. The locking mechanism will engage to hold the T2Candida panel in place and the drawer will close (*see* **Note 22**).
6. The T2Dx instrument automatically selects the next available drawer for loading another sample.
7. Wear fresh gloves and repeat steps C-E for each sample until there are available drawers.

3.6 Instrument Unloading

1. Once the analysis is finalized, the "Run Complete" indicator will appear on the display screen.
2. All the used and unused disposables and waste are contained in the T2*Candida* Cartridge. To remove the used T2*Candida* Cartridge from the T2Dx instrument, wear fresh gloves and press "Unload" on the touch screen (*see* **Note 23**).
3. Follow the T2Dx instructions displayed on the screen to open the drawer.
4. Remove the used T2*Candida* Cartridge using a biohazard waste bag (*see* **Note 24**).
5. Tie up the biohazard bag and then discard the bag in accordance with local waste disposal regulations.

6. Dispose the gloves into the biohazard waste.
7. Wear fresh gloves, and press "Next" twice on the display screen and drawer will close.
8. Repeat for all the drawers until a "Run Complete" indication appears.

3.7 Quality Control

1. The T2*Candida* panel is designed to perform a quality control during each specimen analysis. Specifically, as described above, an Internal Control, which is a synthetic DNA target, is automatically introduced into each specimen. If the Internal Control is invalid a "Negative" result is reported and "Invalid" is displayed as the "Internal Control" result. This means that the T2Candida panel result cannot be interpreted since the sample might contain inhibitors that interfered with T2Candida panel. In that case, a different sample from that patient must be run.
2. Positive and negative external controls are also provided by T2Biosystems for further quality control. A positive (APG or TPK) and a negative external control are suggested to be run at least once a month with alteration between the multiplex blends APG and TPK positive external controls with each quality control check. Also, a quality control check with External Controls is suggested after a new reagent lot is received into the lab or when a significant maintenance to the T2Dx instrument is performed.

3.8 Data Interpretation

1. Each valid T2*Candida* panel returns 3 total results for *C. albicans/C. tropicalis* (A/T), *C. parapsilosis* (P), and *C. glabrata/C. krusei* (G/K) (Table 1).

Table 1
Interpretation of T2*Candida* panel results

T2Dx result	Result interpretation
A/T: positive; IC: valid	*C. albicans* and/or *C. tropicalis* were detected
A/T: negative; IC: valid	Neither *C. albicans* nor *C. tropicalis* were detected
P: positive; IC: valid	*C. parapsilosis* was detected
P: negative; IC: valid	*C. parapsilosis* was not detected
K/G: positive; IC: valid	*C. krusei* and/or *C. glabrata* were detected
K/G: negative; IC: valid	*C. krusei* and/or *C. glabrata* were not detected
IC: invalid	The internal control was invalid; all results are negative and cannot be interpreted

A/T: *C. albicans/C. tropicalis*, P: *C. parapsilosis*, K/G: *C. krusei/C. glabrata*, *IC* internal control

4 Notes

1. T2*Candida* panel can be used only for human whole blood specimens collected with K_2EDTA as anticoagulant.
2. Each K_2EDTA whole blood specimen should contain at least 3 mL of blood for optimal panel performance.
3. T2*Candida* Cartridge, consisting of T2*Candida* Sample Inlet and T2*Candida* Base, should be stored at 15–30 °C. Expiration date should be noted.
4. The T2*Candida* Reagent Pack contains the Internal Control DNA and probe-coupled Supermagnetic particles that hybridize to the Internal Control sequence amplicon, the Reagent consisting of dNTPs and *Candida* Primers, a thermostable polymerase (T2Biosystems, Inc.), *C. albicans/C. tropicalis* particles (probe-coupled Supermagnetic Particles that hybridize to either *C. albicans* or *C. tropicalis* amplicons), *C. parapsilosis* particles (probe-coupled Supermagnetic Particles that hybridize to *C. parapsilosis*), and *C. krusei/C. glabrata* particles (probe-coupled Supermagnetic Particles that hybridize to either *C. krusei* or *C. glabrata*).
5. T2*Candida* Reagent Pack should be stored at 2–8 °C. Expiration date should again be noted.
6. Specimens should not be drawn through a central line or port from which antifungal therapy is administered.
7. Due to the low LOD of the T2*Candida* panel thorough disinfection of the work area should take place to avoid contamination.
8. Allow the bleach solution for at least 3 min to achieve successful disinfection.
9. Should gloves become soiled, replace them with a clean pair following standard lab procedures.
10. Do not touch the inner contents. The outer packaging is considered contaminated and should not be removed at this time.
11. Be careful not to touch the outside of the packaging or the foil on top of the T2*Candida* Reagent Pack.
12. Visually inspect the contents of the Reagent Pack to ensure all solutions are homogeneous. If settled particles are present at the bottom of the well(s) repeat **step 7** for up to three times until homogenous. If it is not discard and replace with a new Reagent Pack. Once homogeneous, gently tap on the benchtop to displace any trapped air bubbles, and visually confirm that air bubbles removal.

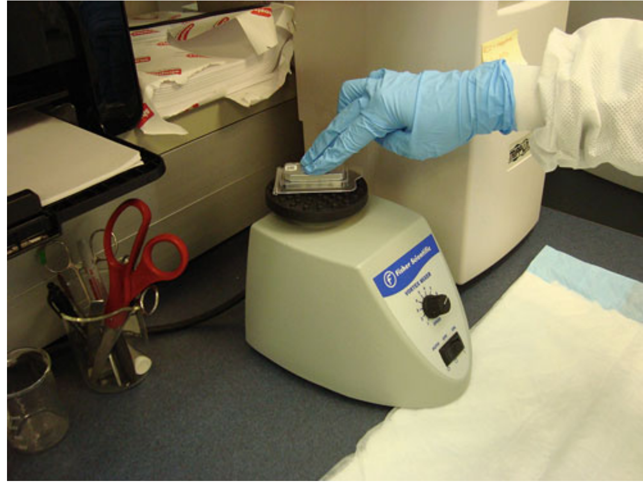

Fig. 5 Using a vortex mixer with a rubber head and cover to mix the reagent pack

13. A vortex mixer with a rubber head and cover can be used to mix the reagent pack instead of manually shaking it. The dial should be set at about 8, on a scale of 1–10. Place the reagent pack (still in the plastic case) on the vortex mixer. Place two fingers on the top of the plastic case; gently push down to start the mixing process. Hold for a count of 5. Check the bottom of the reagent pack to check that there are no particles in the bottom of the wells, without tipping it over. If there are particles present, repeat the process up to three times. If there are still particles, discard the reagent pack and warm up a new one, retry (*see* Fig. 5)
14. The left side of the reagent tray needs to go into the cartridge first, wiggle it a bit to set it in place and then push down on the side with the barcode. It may take a little adjustment. If there is no audible snap heard, discard the cartridge and prepare again. The reagent tray may not have been able to be locked securely in place.
15. Check for visible clots when inverting the blood tube. Clots will slow down or stop the flow of blood into the inlet module. If multiple or large clots are seen, discard the blood and request a new sample. If a small clot is seen, try attaching the blood tube to the inlet. If the blood fills the inlet, it will save a patient redraw.
16. Center the barcode label with the front of the inlet module using a twisting motion so that the barcode can be easily read (*see* Fig. 6).
17. Make sure that a good seal has been formed between the blood collection tube and the T2Candida Sample Inlet before

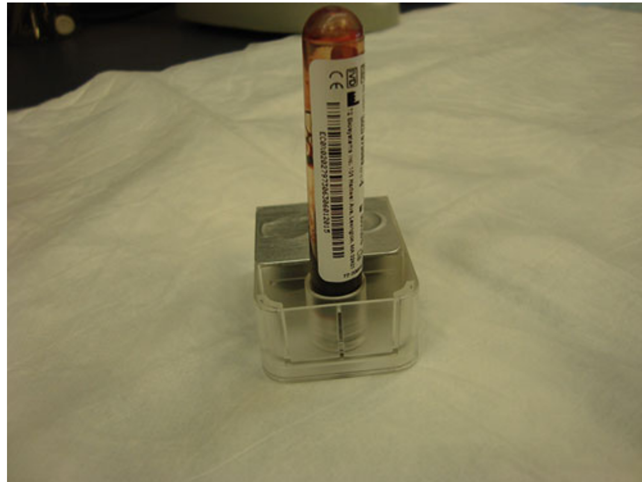

Fig. 6 Center the barcode label with the front of the sample inlet module so that the barcode can be easily read

inverting the T2*Candida* Sample Inlet assembly and the blood collection tube.

18. If the blood is not moving into the inlet module reservoirs from the blood tube, gently tilt the tube side to side. Push the blood tube down gently and twist a few times, then gently tap the inlet module on the counter. If there is no blood flowing into the reservoirs after this, discard the sample and inlet module and request a new specimen.
19. The T2*Candida* panel should be level when placed in the drawer in contact with the metal rails and the location pins.
20. Pick the assembled T2*Candida* Panel up by placing left hand over the top of the cartridge body and grabbing both sides of it or grab both ends of the cartridge body and place the little finger under it for stability. Load the T2*Candida* Panel and without letting go, gently wiggle it in place to make sure it is seated properly and the posts are aligned (*see* Figs. 7 and 8).
21. Hold the T2*Candida* Panel in place after loading and gently pull off label. Place it on the counter under the drawer. This serves as visual confirmation that the label has been removed and proper loading has taken place. Ensure all components under the label are visible (10 pipet tips, 7 small tubes, and 1 large tube). Stop loading if something is missing (*see* Fig. 9).
22. When pressing Confirm, watch the locking mechanism to confirm that it engages for proper attachment. If it does not, stop loading. If the Cartridge is not seated correctly, loading cannot proceed. Press "Cancel" and repeat the loading steps.

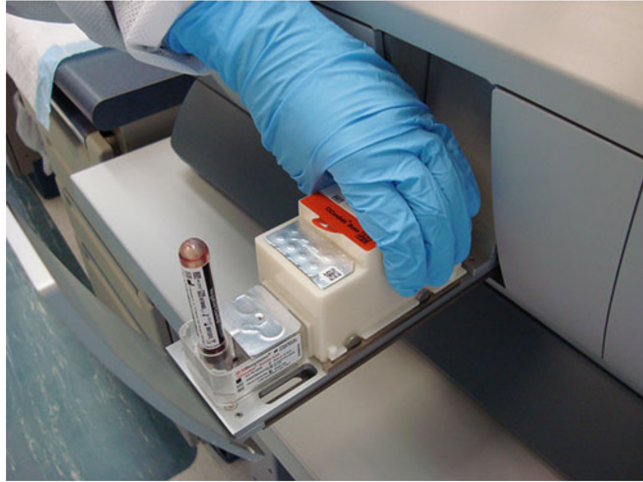

Fig. 7 Pick the assembled T2*Candida* panel up by placing left hand over the top of the cartridge body and grabbing both sides of it or grab both ends of the cartridge body and place the little finger under it for stability

Fig. 8 Load the T2*Candida* panel and without letting go, gently wiggle it in place to make sure it is seated properly and the posts are aligned

23. The used T2*Candida* Cartridge contains DNA amplification products and should be handled as contaminated. Standard lab procedures should be followed to avoid lab contamination. It should be noted that T2*Candida* Base and Reagent Pack contain sodium azide as preservative and should not be disposed in the plumbing system to avoid accumulation of deposits in metal piping where conditions for explosive conditions could develop.

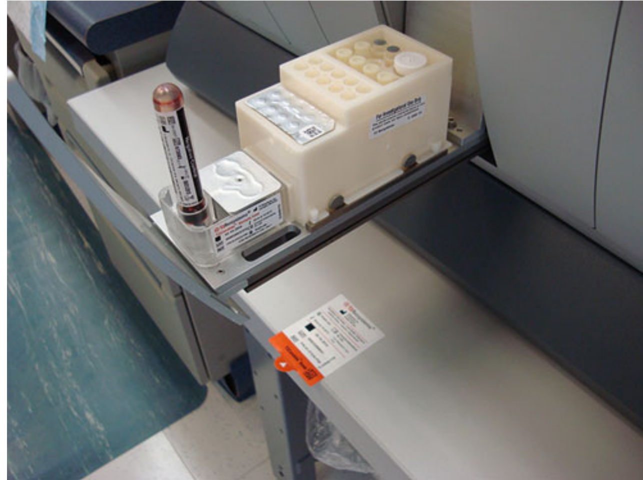

Fig. 9 Hold the T2*Candida* panel in place after loading and gently pull off label. Place it on the counter under the drawer

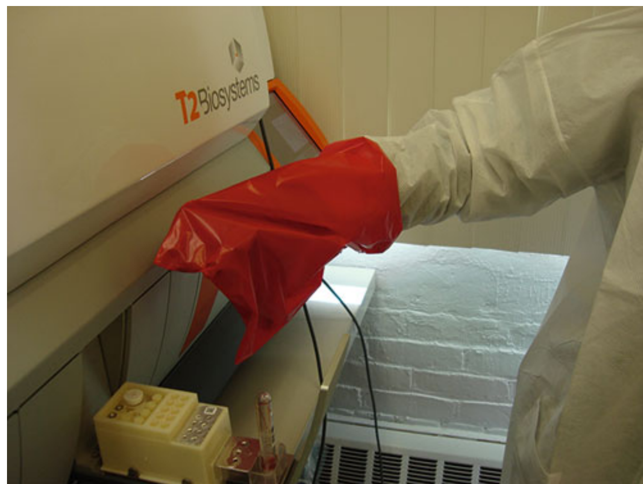

Fig. 10 Place gloved hand all the way to bottom of the bag

24. Removing the T2*Candida* Panel with the biohazard bag as a second glove is awkward, but doable. Place gloved hand all the way to bottom of the bag and grab the back end of the T2*Candida* Panel with that hand. Pull bag over the hand holding the T2*Candida* Panel and the T2*Candida* Panel itself with other gloved hand; pull from the top, then bottom. Keep going until the T2*Candida* Panel is inside the bag. Grab top of the bag, let go of the cartridge and close bag with a twist tie (*see* Figs. 10, 11, and 12).

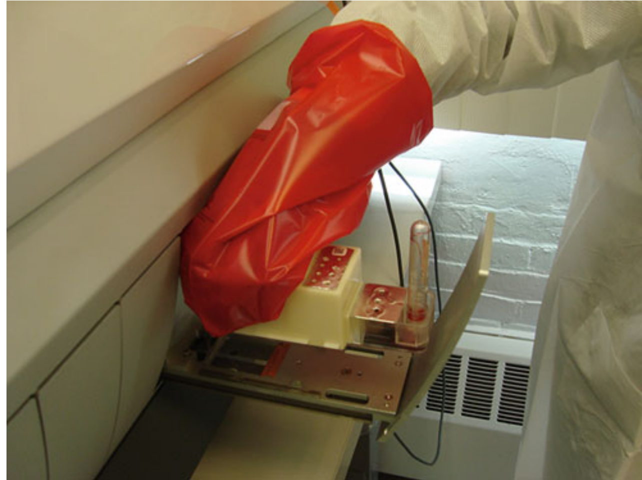

Fig. 11 Grab the back end of the T2*Candida* panel with the gloved hand and the biohazard bag as a second glove

Fig. 12 Grab top of the bag, let go of the cartridge, and close bag with a twist tie

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Part IV

Identification of Fungal Species

Chapter 19

Fungal Species Identification by MALDI-ToF Mass Spectrometry

Oliver Bader

Abstract

MALDI-TOF MS has become the standard method for routine identification of most microbial organisms in clinical laboratories and has largely replaced biochemical assays. Classification relies on extensive well curated databases, ideally covering the full spectrum of microorganisms encountered in the specimens at hands. The protocols for harvesting cells and procuring material suitable for downstream MALDI-TOF MS analyses vary in specific details between the different groups of organisms, e.g., gram-positive or -negative bacteria, mycobacteria, or fungi. With respect to fungi, methods further vary between yeasts and moulds; and even among different mould genera if they do not lyse in a similar fashion. Purification of microbial materials from clinical specimen allows the direct identification of bacteria; however this is not yet fully adapted to fungi. In this chapter, I look into the differences between the underlying methods for yeast and moulds, and for production of samples suitable for MALDI-TOF MS species identification from cultures and different clinical materials.

Key words MALDI-ToF, MALDI Biotyper, VITEK MS, Saramis, Andromas

1 Introduction

In most large diagnostic laboratories routine identification of microbial species has been taken over by the purely biophysical and bioinformatics-driven Matrix-Assisted Laser Desorption/Ionization time-of-flight mass spectrometry (MALDI-TOF MS) approach over the past decade. This presents the major workflow change since Robert Koch's time in the way species are determined in microbiology.

The idea of the method dates back to the 1970–1990s [1, 2] and was first described with yeasts in 2001 [3]. Chemically ionized cellular contents serve as the analytes, which are desorbed from the target plate by laser fire and accelerated in an electric field along an evacuated flight tube. Time-resolved impact on a detector yields a characteristic biomarker mass spectrum, which can be used to search a database of reference entries. It is not necessary to know

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the identity of the biomarker masses; it is only important that the observed masses are highly reproducible within isolates of the same species, and sufficiently different between highly related species to allow a precise species distinction. Samples are prepared directly from cells harvested from cultures or isolated from patient material, either by directly depositing and lysing on the target plate, or by a quick protein extraction protocol.

It is generally accepted that this methodology shows higher precision than biochemical and microscopic methods at significantly reduced turnaround times, where databases are properly evaluated. At the time of writing there were four commercial curated systems largely evaluated in the literature: the MALDI Biotyper (Bruker Daltonics, Bremen, Germany), the AXIMA@SARAMIS database (AnagnosTec, Potsdam, Germany and Shimadzu, Duisburg, Germany), its successor VITEK MS (bioMérieux, Marcy l'Etoile, France), and the Andromas system (Andromas, Paris, France). The algorithms underlying three of these systems are described in detail in Welker et al. [4]. Several independent studies have looked at the individual systems and their performances regarding fungi, to each other as well as to other standard methods such as biochemistry or sequencing of the rDNA/ITS loci. For a summary of studies until 2012 the reader is referred to Bader et al. [5]. It was the general observation that false classifications are only rarely observed. Spectra of species not represented in the databases are rather interpreted as "unknown." Previously unrepresented species become identifiable after addition of reference spectra to the databases. All fungal species commonly encountered in clinical routine practice are now included in the databases. Even closely related yeast species which cannot be discriminated with common biochemical methods such as those of the *Candida ortho-/meta-/parapsilosis* complex can be resolved without difficulty by MALDI-TOF MS.

Phylogenetic relationships among moulds are closer than among yeasts [6] and here species-boundaries are not drawn as easily. Therefore, while commercial systems will readily identify moulds frequently occurring in clinical samples, they are neither equipped to resolve larger clusters of closely related moulds nor do they provide deeper phylogenetic insights in a routine fashion. Instead, individual databases for specific taxonomic problems of interest have been created by several research groups. However, to date there is no central repository for such databases and they must be obtained from the authors of the respective publication, and evaluated locally.

2 Materials

2.1 Laboratory Equipment

1. A work environment equipped for fungal culture, including space free of air movement for handling of cultures producing conidia.

2. Pipettes, pipette tips.
3. A table top centrifuge for reaction tubes capable of reaching ~15,000 × *g*.
4. A centrifuge for 15 mL tubes, capable of reaching ~4000 × *g*.
5. MALDI-TOF mass spectrometer and software for microbial species identification.
6. Clean MALDI target plates.

2.2 Basic Consumables and Chemicals

1. Media for subculture from clinical specimen (Sabouraud's agar and Sabouraud's broth).
2. Sterile cotton swabs.
3. Inoculation loops.
4. Deionized water, MALDI grade.
5. High grade ethanol, without denaturant.
6. Formic acid, MALDI grade.
7. Acetonitrile, MALDI grade.
8. α-hydroxy cinnamic acid (HCCA).
9. Trifluoroacetic acid.
10. Unused toothpicks.
11. High quality 1.5 mL reaction tubes.
12. High quality 15 mL centrifuge tubes.

2.3 Additional Consumables and Chemicals

1. Sodium dodecyl sulfate (SDS) solution as detergent.
2. Saponin as detergent.
3. Tween 80 as detergent.
4. Brij-97 as detergent).
5. CAPS as buffer substance.
6. Sterile filters (0.2 μm pore size).
7. 25 mm filter membrane (0.45 μm pore size).
8. Na_2PO_4 as buffer substance.
9. $NaHCO_3$ as buffer substance.
10. Sinapinic acid as alternative matrix substance.
11. Chocolate agar.
12. Pasteur pipettes.

3 Methods

3.1 Preanalytical Procedures

Direct identification of microorganisms from clinical materials is highly desirable, especially where there is an increased need for short turnaround times. This applies to materials that can be

considered "growth media" themselves and contain a high microbial burden. In a broad sense, these are positive blood cultures, liquid cultures or native urine, and possibly cerebrospinal fluid. With all of these, analyses are hampered by highly abundant interfering substances such as cations other than H^+ disturbing the ionization process or altering observed masses. Also, small proteins and peptide breakdown products present in the sample may give rise to mass peaks that partially overlap with spectra from fungi [7]. This necessitates a proper purification procedure before analysis. Here, additional washing steps with deionized water [7–9], low concentrations of detergents such as SDS [7], saponin [10], or Tween 80 [9] may increase sample purity. Also, in contrast to pure colonies picked from agars, multiple species may be present and their combined mass patterns may, if at all, result in low identification scores or ambiguous results [9, 11].

3.1.1 Harvesting from Blood Cultures Bottles

Several approaches to purification of bacterial or fungal cells from positive blood cultures prior to sample preparation have been developed [12]. However, fungus-positive blood cultures of clinical origin are low in numbers and represent only minor fractions of total samples in larger studies. Only two studies [9, 13] have used a larger number of clinical samples to properly evaluate the process with respect to yeasts.

Therefore, to date, the entire process is not standardized. Each of these approaches needs to be validated locally. There are four different technical pathways to microbial enrichment and contaminant removal from such samples: differential centrifugation [8], differential lysis [9, 10], filtration [14], and the use of gel matrices [15, 16]. While suitable for bacteria, none of these approaches served to full satisfaction for yeasts. However, steps from all protocols can be used in different combinations, leading to several different options that may serve as starting points for further development. At least two kits, namely the Sepsityper [17] from Bruker Daltonics and the Vitek MS blood culture kit from bioMérieux, are partially based on these protocols and are commercially available.

Note: all of these protocols necessitate the use of blood culture bottles containing no charcoal as starting material.

Differential centrifugation (adapted from Ferreira et al. [8]).

1. Transfer 4 mL of the positive blood culture into a centrifuge tube.
2. Centrifuge at 2000 × *g* for 30 s and transfer supernatant to new 1.5 mL tube to separate microorganisms from host cells.
3. Centrifuge at 15,500 × *g* for 5 min and discard supernatant to collect microorganisms.
4. Wash pellet once with deionized water.
5. Proceed with full extraction (Subheading 3.2.4).

Differential lysis (adapted from Spanu et al. [9]).

1. Transfer 8 mL of the positive blood culture to a centrifuge tube.
2. Spin at 10,000 rpm for 2 min at room temperature and discard supernatant.
3. Wash pellet twice with 1 mL deionized water.
4. Recentrifuge, remove residual liquid, and resuspend cells in 1 mL 0.1 % Tween 80.
5. Incubate for 2 min, spin down, and discard supernatant.
6. Wash pellet twice in pure water (1 mL).
7. Proceed with full extraction (Subheading 3.2.4).

Differential filtration (adapted from Fothergill et al. [18]).

1. Transfer 2 mL of blood culture broth into a new tube.
2. Add 1 mL lysis buffer (0.6 % Brij-97 in 0.4 M CAPS pH 11.7, filtered at 0.2 μm).
3. Vortex briefly and incubate for 2–4 min at room temperature.
4. Pass the resulting lysate in a constant stream through a 25 mm filter with 0.45 μm pore size.
5. Ensure the liquid does not back up and keep the sample application area to roughly 1 cm^2.
6. Wash membrane first three times with wash buffer (0.05 % Brij-97, 0.45 % NaCl, 20 mM Na_2PO_4 pH 7.2, filtered at 0.2 μm), then three times with distilled water, each time completely covering the membrane.
7. Recover the cells from the membrane by firm scraping with a swab.
8. Directly apply to MALDI target and proceed with **step 3** of on-target-lysis (Subheading 3.2.3).

3.1.2 Harvesting from Urine

Yeasts have also been directly identified from urine samples with high microbial burden [19].

Differential centrifugation (adapted from Ferreira et al. [20], modified by Kim et al. [19]).

1. Transfer 3 mL native urine into a fresh tube.
2. Centrifuge at 2000 × *g* for 30 s and transfer supernatant to fresh tube to separate from host cells.
3. Centrifuge at 14,100 × *g* for 10 min and discard supernatant to collect microorganisms.
4. Wash cells once with deionized water.
5. Resuspend pellet in 1.5 mL wash solution (0.1 % SDS, 0.015 M $NaHCO_3$) and incubate at 37 °C for 10 min.

6. Centrifuge at 14,100 × *g* for 10 min and discard supernatant to collect microorganisms.
7. Wash pellet once with deionized water.
8. Proceed with on-target-lysis (Subheading 3.2.3) or full extraction (Subheading 3.2.4).

3.1.3 Yeast Culture

For yeasts, cells growing on agar plates serve as samples. The agars of choice are Sabouraud's or Columbia blood, but also the analysis from Chromagar plates is possible [21, 22].

3.1.4 Mould Culture

Moulds are more difficult to analyze than yeasts. In addition to the more complex phylogeny, this is also due to their more complicated morphology and stronger cell walls preventing proper lysis during the extraction step. Spectra made from different morphologies may vary [23] significantly. Therefore, for example, spectra obtained from conidia will not give rise to good identifications in a library prepared from liquid culture mycelium. It is therefore eminent that the culture and sample preparation methods used match the ones used for making of the reference library. Taxonomical resolution and species identification reliability increase if more masses can be measured reproducibly. Informative spectra are achieved either using mycelial cells scraped from agar plates [10, 24, 25] or harvested from liquid cultures. For clinically relevant moulds where identification with cells from agar plates was not successful, we found this culture protocol, followed by a full extraction procedure (Subheading 3.4), particularly useful:

(Adapted from the Fungi Library, Bruker Daltonics)

1. From an agar plate, inoculate a moderate number of conidia or mycelial fragments into 5 mL Sabouraud's broth in a tightly sealing screw cap vessel using a (wet) cotton swab.
2. Incubate overnight at 30 °C, or appropriate temperature, on a turning wheel.
3. This should yield a dense turbid culture of conidial germlings or small mycelial aggregates (Fig. 1).
4. Proceed with full extraction (Subheading 3.2.4).

3.1.5 Harvesting from Short Cultures

Another possibility to analyze materials that need fast processing is to shorten incubation times. For specimen where only a single species can be expected, e.g., blood cultures, a sufficient amount of cells for subsequent MALDI-TOF analysis by direct deposit can be harvested. This procedure works in 50–80 % of the cases with bacteria, but in the literature it currently fails to identify yeasts [26–28]. However, with prolonged incubation times, it may have some worth for fungi.

(Adapted from Verroken et al. [27] and Zabbe et al. [28]).

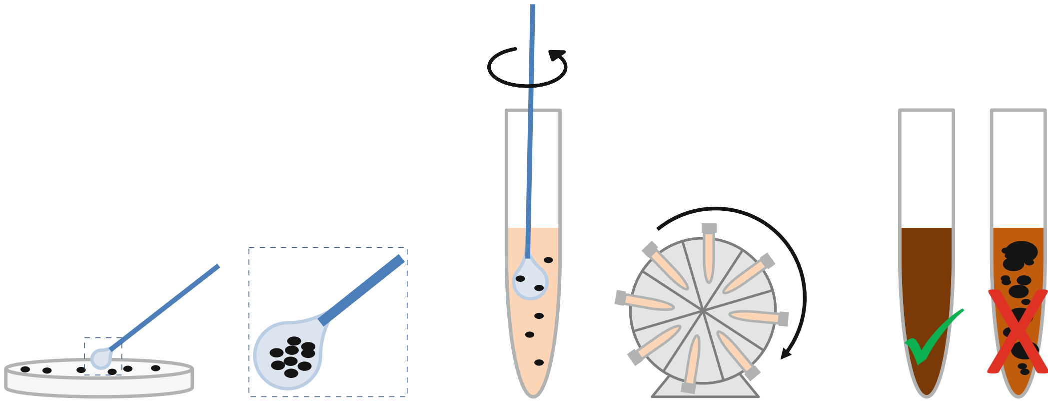

Fig. 1 Mould culture using liquid media. A high inoculum on a turning wheel yields a turbid culture of small mycelial aggregates, rather than large single flocks

1. Plate the blood culture suspected positive for yeast growth (e.g., by Gram or Calcofluor White stain) on chocolate agar.
2. Incubate at 37 °C for at least 5 h.
3. Using a 10 μL inoculation loop, carefully harvest cells by "swabbing" the microbial film growing on the plate surface.
4. Proceed with **step 2** of on-target-lysis (Subheading 3.2.3).

3.2 Sample Preparation Procedures

3.2.1 Preparation and Handling of Matrix Solution

Several different matrix substances have been used for diagnostic MALDI-TOF procedures. These are most commonly, but are not restricted to, sinapinic acid (SA) or alpha-hydroxycinnamic acid (HCCA). Commercial applications usually restrict protocols to HCCA, with which reference spectra have been generated.

1. Prepare solvent (50% acetonitrile, 2.5% trifluoroacetic acid, 47.5% water). Use only high-quality (MALDI-grade) chemicals and only plastic ware free of chemical softening agents, as such compounds may inhibit ionization.
2. Add 10 mg/mL matrix substance into the solvent tube and let dissolve until solution is clear.
3. Alternatively, prepare a saturated solution by adding crystals to the solvent until some substance remains undissolved at the bottom. Make sure the solution is at least 10 mg/mL.
4. Aliquot and store at ambient temperature (~2 weeks shelf life).

3.2.2 Direct Deposit

In this most basic sample preparation procedure (also called "smear preparation" or "direct transfer"), a small amount of unprocessed cells harvested from a culture, usually an agar plate, is directly deposited onto the MALDI target plate. Derived from this procedure, the entire process is also called "intact cell mass spectrometry" ("ICMS"). The spot is then overlaid with a small amount of matrix solution also lysing the cells. Evaporation of the solvent

embeds the released cell components into a crystalline matrix. This protocol is suitable for most bacteria, however, as under these conditions most fungal cells will not lyse sufficiently, it only rarely results in mass spectra sufficient for fungal species identification.

1. Pick a single colony with a toothpick.
2. Carefully spread the cells onto the MALDI target plate.
3. Overlay the spot with 0.8–1 μL matrix solution and let crystallize.

3.2.3 On-Target Lysis

This procedure (also called "short extraction," or "extended direct transfer, eDT") follows the same steps as the direct deposit to which a lysis step with 25% (VITEK MS) to 70% (MALDI Biotyper) formic acid is added. In the case of the MALDI Biotyper, modified cutoff values or supplemented databases have been applied when using this procedure (Subheading 4.7).

Yeast Protocol

1. Pick a single colony with a toothpick.
2. Carefully spread the cells onto the MALDI target plate.
3. Overlay the cells on the target plate in ~1 μL 70% formic acid (v/v) and let dry.
4. Overlay the spot with 0.8–1 μL matrix solution and let crystalize.

Mould Protocol

1. Harvest a small amount of mycelium from liquid culture using an inoculation loop, a cut-off pipette tip, or a glass Pasteur pipette. From an agar plate, scrape a small amount of cells.
2. Carefully spread the cells onto the MALDI target plate, remove excess liquid with a pipette.
3. Mix the cells on the target plate in ~1 μL 70% formic acid (v/v) and let dry.
4. Overlay the spot with 0.8–1 μL matrix solution and let crystalize.

3.2.4 Full Extraction

This procedure extends the above protocols and improves the protein extraction by use of larger cell amounts, protein precipitation with ethanol, an extended formic acid lysis step in a liquid environment, and finally by protein solubilization with acetonitrile. Conveniently, ethanolization of cell material also inactivates most pathogenic organisms. Both, formic acid and acetonitrile are standard chaotropic protein solvents used in many proteomic applications.

Yeasts Protocol

1. Pick several colonies using an inoculation loop.
2. Resuspend cells well in 300 μL deionized water in a 1.5 mL reaction tube. There should be no aggregates left; if resuspension with the loop is insufficient, resuspend cells by pipetting.
3. Add pure ethanol to ~75 % (900 μL), mix by vortexing.
4. Place in table top centrifuge and harvest cells for ~2 min at maximum speed.
5. Remove ethanol thoroughly and leave pellet to dry at ambient temperature.
6. Thoroughly resuspend the pellet in an appropriate volume 70 % formic acid (Fig. 1).
7. Usually pipetting up-and-down works better then swirling.
8. Incubate ~5 min at ambient temperature.
9. Add an equal volume acetonitrile to solubilize small proteins and mix.
10. Remove debris by centrifugation in a table top centrifuge at maximum speed for 2 min.
11. Place 1 μL of supernatant on MALDI target plate.
12. Overlay the spot with 0.8–1 μL matrix solution and let crystalize.

Mould protocol (adapted from the Fungi library, Bruker Daltonics).

1. Harvest mycelium from liquid culture using a cut-off pipette tip or Pasteur pipette into a 1.5 mL reaction tube. Generally, small mycelial flakes are handled easier than large mycelial balls or mycelial structures growing at the culture surface. Remove medium as thoroughly as possible by repetitive centrifugation and aspiration.
2. Resuspend cells well in a total of 300 μL deionized water (including potential residual liquid from culture). There should be no larger aggregates left; if resuspension by vortexing is insufficient, resuspend cells by pipetting up-and-down with a cut-off pipette tip.
   - Optionally, also material scraped from an agar plate can be used for extraction in analogy to the yeast protocol, and placed directly into the 300 μL water aliquot.
3. Add pure ethanol to ~75 % (900 μL), mix by vortexing.
4. Centrifuge for ~2 min at maximum speed in a table top centrifuge to harvest cells.

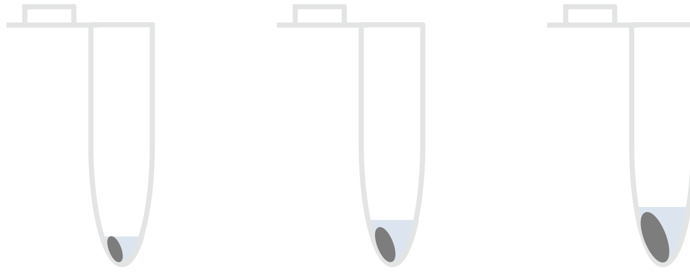

Fig. 2 Pellet size. During full extraction, the volume of formic acid and acetonitrile added must be increased accordingly with increasing pellet size; otherwise the formic acid lysis step will not work efficiently

5. Remove ethanol thoroughly and leave pellet to dry at ambient temperature. A dry pellet is characterized by flaking off the tube wall.
6. Thoroughly resuspend the pellet in an appropriate volume 70% formic acid (Fig. 2).
7. Usually pipetting up-and-down works better then swirling for small volumes. Macerating the pellet with an inoculation loop or pipette tip may also aid in resuspension.
8. Incubate ~15 min at ambient temperature. Prolonged incubation may resolve insufficient lysis, however extended incubation in formic acid may lead to formylation of proteins and thus change masses of biomarkers.
9. Add an equal volume acetonitrile to further solubilize small proteins and mix.
10. Centrifuge in a table top centrifuge at maximum speed for 2 min to remove debris.
11. Place 1 μL of supernatant on MALDI target plate.
12. Overlay the spot with 0.8–1 μL matrix solution and let crystalize.

4 Notes

1. Since acetonitrile is more volatile than water it will evaporate earlier from the tube than water, limiting the shelf-life of matrix solutions. Matrix from solution with too few acetonitrile will not crystalize properly, and not properly lyse cells during direct deposit or on-target-lysis.
   - Prepare fresh matrix frequently.
   - Keep matrix in tightly sealing reaction tubes, e.g., with a rubber seal.

- Store matrix at ambient temperature. Acetonitrile will not solidify at −20 °C; in freezers there will be phase separation in the matrix tubes and increased acetonitrile evaporation over time. This leads to precipitation of matrix substance and reduction in acetonitrile content.

2. Repeated analyses from the same sample may be required for some approaches. During full extraction, the optimal time point to store cells for longer times is after ethanolization. Due to the volatile character of acetonitrile (*see* **Note 1**) and the reactive nature of formic acid, an extract itself has only very limited shelf-life.
   - Prepare a sufficiently large and dense but still well homogenized batch of ethanolized cells (Subheading 3.2.4, **steps 1**–**3**) and store at −20 °C. To continue, transfer an aliquot only and proceed with **step 4** of the respective protocol.
3. Use of too high cell numbers in relation to matrix and/or extraction reagents lead to uninformative spectra.
   - For on-target lysis, use only a thin layer of cell material.
   - For the full extraction procedure, increase first the amount of formic acid and subsequently of acetonitrile if the pellet exceeds the usual size (Fig. 2)
   - Note, that adding more formic acid once acetonitrile has been added does not improve extraction efficiency.
4. Some moulds may be difficult to harvest from medium by centrifugation, as their density is similar to that of medium/water.
   - Step-wise replace medium by 75 % ethanol. Due to density differences of medium and ethanol cells may be easier harvested from ethanol.
   - Insufficient drying of the ethanolized pellet during extraction can reduce the concentration of formic acid to inefficient levels and mislead during judging the biomass (Fig. 2).
   - If in doubt, completely dry the pellet before proceeding to lysis; a fully dry pellet is characterized by flaking of the tube wall.
   - Spin down residual liquid in a centrifuge a second time, aspirate collected supernatant.
   - Prolong air drying.
   - Use a vacuum centrifuge to assist drying, but do not prolong.
5. Some fungi have stronger cell walls than others and may not lyse efficiently with the given procedures.
   - During extraction, prolong the formic acid incubation step.

- Resuspend again during the incubation step.
- During extraction, additional lysis steps may be performed, e.g., mechanical disruption using a bead-beater or glass beads [24, 29, 30].

6. Strong pigmentation can inhibit ionization of the analyte [29, 31, 32].
   - For moulds, use liquid cultures suppressing pigment formation [31] or include additional preanalytical washing steps [32].
7. The MALDI Biotyper database may yield log scores below the threshold for species identification (2.000) when using the on-target-lysis protocol, since yeast reference spectra have been created using the full extraction protocol.
   - Matches with lower log-scores starting from 1.700 [21, 33–36] have been shown to be acceptable as species-specific for yeasts if additional criteria are met: the list of identifications must encompass a certain number of database hits ($n = 2$ [21] or $n = 3$ [16]) of a single species at the top, have no other species intermingled [21], and a certain difference in the log(score) to the next species (e.g., 0.200 [37]) must be evident.
   - The MALDI Biotyper database can be supplemented with reference MSPs created with the on-target lysis method [38, 39], however this is not applicable to the IVD-certified version, and must be quality-controlled locally.
   - Similar procedures for moulds are not yet established.
8. After prolonged use accumulated dirt in the ion source will require use of higher laser power.
   - Manually increase laser power.
   - Clean the ion source.
   - Contact service of the manufacturer.
9. If qualitatively good spectra are obtained, but they do not give rise to matches in the database:
   - Make sure you are using the sample preparation method properly recommended by the manufacturer for the database used.
   - If this is a more general problem, i.e., not just in a single, but many isolates normally identifiable without problems, calibrate the instrument and remeasure. If the problem cannot be solved call for service by the manufacturer.
   - Identify the species using molecular methods, e.g., by sequencing of the ITS region, tubulin and/or calmodulin genes. If the species is not included in the database a new

reference entry can be generated (not applicable to closed IVD systems) or the specific isolate made available to the manufacturer for database inclusion.

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Chapter 20

Immunological Identification of Fungal Species

Filomena Nogueira*, Fabian Istel*, Leonel Pereira, Michael Tscherner, and Karl Kuchler

Abstract

Immunodetection is described in this chapter as a technique for producing specific antibodies for antigen detection of the major human fungal pathogens. In the case of *Candida* spp., heat-killed cells are used to immunize mice over a couple of weeks and then splenocytes are isolated and further fused with myelomas to easily propagate the antibodies produced in the mice. The resulting antibodies follow a purification process where antibody levels and concentrations are determined. Fungal cells are also lysed to obtain whole cell extracts as a prior step for identification of antigens using immunoprecipitation. Finally, this method permits the production of specific antibodies against fungi and the identification of the respective antigens in an *in vivo* model.

Key words Immunodetection, Antibodies, Antigens, *Candida*, *Aspergillus*, Zygomycetes, *Mucor*, *Rhizopus*, *Fusarium*, *Cryptococcus*

1 Introduction

Immunodetection comprises valuable tools for the identification of human fungal pathogens and, in combination with other in-use diagnostic tests, gives complementary and more specific information. Early diagnostics by using immunological assays attempt to detect circulating antigens by the use of specific antibodies. Several studies were initially based on raising rabbit or goat antisera against antigens present on the surface of fungi such as spores or hyphal filaments [1]. Attempts were made to differentiate fungal pathogens by raising polyclonal antibodies (pAbs), but were regarded as unsuccessful as pAbs demonstrated poor specificity [2, 3]. Cross-reactivities were a major issue resulting in many false positive results identifying non-targeted species rather than facilitating differentiation from specific species of interest [3–6]. To overcome these issues, Richardson and colleagues started developing highly specific

*These authors contributed equally to this work.

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monoclonal antibodies (mAbs), once the hybridoma technology in the mid-1970s [7] changed the perspective on the use of antibodies [8]. Monoclonal antibodies have monovalent affinity facilitating the identification of specific markers of fungal species or genera. Immunization of model animals such as rabbits, goats, or mice with a fungal antigen (e.g., heat-killed fungal cells) triggers an immune response that results in antibody production by B lymphocytes. Hybridomas are hybrid cell lines produced through the fusion of a B cell with a myeloma cell (B cell cancer). This process immortalizes cells permitting the production of hybrid cells with infinite growth while keeping the characteristics of the B cell for antibody production. Antibodies produced by the hybridoma display single epitope specificity able to differentiate fungi at the species, genus, and isolate levels [9–11].

In this chapter, we give an extended overview on the main human fungal pathogens most commonly occurring in the clinical setting and requiring accurate diagnostics. Detailed information will be provided on techniques for immunodetection of *Candida* spp., *Aspergillus* spp., *Cryptococcus* spp. and also for emerging fungi such as Zygomycetes and *Fusarium* spp.

1.1 *Candida* spp.

The genus *Candida* comprises more than 150 species of which only a minority is known to cause infections in the human host. About 65 % of all *Candida* species are not able to grow at 37 °C and therefore obviously unsuited for the role of a pathogen [12]. However, several *Candida* strains adapted to the human host and pose a severe problem in the clinical setting. Among the most common *Candida* strains causing infections are *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* [13–16]. These strains are known to cause superficial infections of skin and mucosa, but also invasive infections that can be life-threatening. Recent studies estimate that 250,000 people suffer from invasive candidiasis every year [13, 14]. These infections are characterized by a high mortality of up to 50 % depending on the setting of the study [13, 14, 16].

Importantly, *Candida* spp. can become resistant to most antifungals that are currently used in the clinics. In a recent study, 7 % of all *Candida* isolates were resistant to fluconazole and 2 % to echinocandins. Among the *C. glabrata* isolates, even 10 % were resistant to fluconazole and 4 % to echinocandins [14]. This is consistent with other studies that observed a higher incidence of multidrug resistant *C. glabrata* strains compared to other *Candida* strains [13, 15].

Another significant problem is the diagnosis of invasive *Candida* infections. Blood cultures of patients suffering from invasive Candidiasis are often negative, especially in the case of deep-seated candidiasis [17]. This highlights the need for diagnostics other than culture based methods. Among these non-culture based methods, there are immunological methods, such as β-D-glucan or

mannan assays. However, there are many other putative targets that might be suitable for immunological methods.

During invasive fungal infections, a variety of fungal antigens are released in the host. In the past, several assays have been developed based on the detection of fungal cell wall components or the detection of antibodies against these cell wall antigens. Commercially available kits are based on the detection of fungal cell wall polysaccharides. While several of those assays show promising results [18], there have been reports on false-negative results due to antifungal treatment [19, 20]. Likewise, there are also reports on false-positive results due to contact with cotton gauze or bacteremia [20]. These reports suggest that there is a need to improve the diagnostic approaches by implementing assays with higher sensitivity and specificity to reduce false positive findings.

In the past years, several advances have been made to identify other antigens released by the fungus upon infection. *C. albicans* is known to grow in hyphal form in order to invade tissue or to escape the host's immune response. Yeast-like transition to hyphal form causes significant changes both in the morphology of the cell wall and in the proteome of *C. albicans*. Therefore, presented antigens are apparently specific for hyphal growth. These antigens are commonly designated as *C. albicans* germ-tube-specific antigens (CAGTA) [21].

Many of the antigens are enzymes involved in glycolysis or glucose fermentation such as Fba1, Pgk1, Eno1, and Adh1 [22, 23]. Interestingly, these four proteins have also been reported to bind human plasminogen, thereby creating plasmin. This proteolytic enzyme might enhance the ability of *C. albicans* to invade tissues [24, 25]. Met6 is an essential methyltransferase involved in methionine biosynthesis. However, it might also play a role in reducing the amount of intracellular homocysteine conferring toxicity to *C. albicans* [26]. Ino1 is the first step in the *de novo* synthesis of inositol-containing compounds. While inositol can also be imported by the transporter protein Itr1, *de novo* synthesis might be important for virulence [27]. Bmh1 is the only 14-3-3 family protein in *C. albicans* being responsible for proteome control at the posttranscriptional level, protein and DNA binding, and regulation of exocytosis and vesicle transport. It has been shown to be necessary for growth as well as for hyphal formation [28] but is apparently downregulated during biofilm formation [29].

The most relevant antigens detected by CAGTA antibodies are Met6, Ino1, Eno1, Adh1, Pgk1, Bmh1, Egd2, and Sap2 [21, 30–32].

1.2 *Aspergillus* spp.

Aspergillus infections are a leading cause of morbidity and mortality in the immunocompromised setting that manifest as chronic, allergic or invasive pulmonary aspergillosis (IPA) [9]. Chronic pulmonary aspergillosis (CPA) is the most common form, often

occurring in patients diagnosed with tuberculosis [30–33]. Chronic and allergic forms of aspergillosis are more common than invasive disease [34–36]. Cutaneous aspergillosis is a rare form of *Aspergillus* disease as the epidermis provides a barrier preventing invasion and dissemination [37–40].

Among the most common *Aspergillus* species causing disease, *A. fumigatus* is the most prevalent accounting for up to 90% of IPA. Other common *Aspergillus* species include *A. flavus*, *A. niger*, *A. terreus*, and *A. nidulans* [41].

Aspergilli produce allergens and are therefore often implicated in asthma and allergic bronchopulmonary aspergillosis (ABPA). Increased serum levels of IgE and IgG are characteristic of ABPA. Increased levels of IgG are associated with chronic aspergillosis whereas IgE indicates allergic aspergillosis. Diseases emanating from invasive aspergillosis are much less frequent [42]. In asthma and ABPA patients, allergic reactions mediated by *A. fumigatus* occur when the fungal allergens f2, f3, and f6 react with IgE. The molecular structure of the allergens also influences the way the immune system responds in allergic patients [43].

Detection of *Aspergillus* encounters several limitations, e.g., hyphae in bronchoalveolar lavage (BAL) have shown limited credibility, unless accompanied by other tests as analysis of biopsy specimens and evident necrosis in the affected tissues [44]. Also, overlapping of symptoms of chronic and allergic aspergillosis can render accurate diagnosis difficult, thus underlining the importance of more precise diagnostic methods [32, 45, 46].

The introduction of immunodetection technologies brought new insights on more accurate and sensitive diagnostics of *Aspergillus* infections. Using hybridoma technology, Thornton and colleagues were able to generate an *Aspergillus*-specific mAb (JF5) for the development of an immune-chromatographic lateral-flow device (LFD) for diagnosis of IPA [10]. This technique allows the detection of *Aspergillus* during active growth since the mAb JF5 binds to an extracellular antigen secreted exclusively during growth [10, 47]. Compared to other more complex and time-consuming laboratory-based technologies such as PCR or plate-based enzyme immunoassays (EIAs), LFD is a cheap and unsophisticated test that can be performed *in situ*. Although it has a time advantage, results should be accurately confirmed by other more sophisticated methods [48]. However, LFD has shown higher sensitivity and specificity levels compared to commercially available tests such as Platelia GM and Fungitell (1→3)-β-D-glucan [49].

A low molecular weight antigen, 18 kDa protein, is a major antigen possible to detect in the urine of patients with IPA. Since urine specimens have the advantage of being noninvasive, Dufresne and colleagues proceeded to test the possibility of using lateral-flow technology by using urine specimens to detect *Aspergillus* galactomannans [50].

Antibodies against *Aspergillus* can be formed in healthy people, but aspergillosis can develop in immunocompromised patients with underlying disease [32, 51]. Levels of *Aspergillus*-specific antibodies may vary in patients with high risk of developing aspergillosis compared to healthy individuals [52]. The enzyme-linked immunosorbent assay (ELISA) is a specific-antibody method where results can be analyzed regarding a raised level above a cut-off of antibody levels compared to healthy individuals [53–55]. One of the limitations is the fact that by using healthy individuals to establish cut-offs for the determination of *Aspergillus*-specific antibody levels might be unappropriated to diagnose patients with high-risk of developing aspergillosis [42]. ELISA is a technique that allows the detection of several single types of antibodies such as IgG, IgM, IgA, and IgE. Patient sera antibodies bind to specific antigens which are then detected by anti-human antibodies. The resulting reaction produces a color-change that can be quantified through a spectrophotometer. Improvements on this technique over the years resulted in fully automated equipment which has the advantage in reducing diagnostics costs and time as two hours total [42, 56, 57]. An ELISA was developed specifically to detect *A. fumigatus* [58]. No major cross-reactivities were found; however, only 53% of patients were Afmp1p antigen positive because of other *Aspergillus*-infections rather than *A. fumigatus* or simply due to the low sensitivity of the method [58, 59]. A well-characterized marker for invasive aspergillosis is the cell wall component galactomannan. *Aspergillus* galactomannan has been included in commercially available diagnostic tests with more relevance for the Bio-Rad Platelia *Aspergillus* GM-EIA [60, 61].

Antibody detection in invasive aspergillosis disease might be an issue due to the immunosuppression setting that results in low or absent antibody response [42]. As an alternative, since *Aspergillus* is angiotropic and angioinvasive, invasive infections can be tracked through detection of circulating antigens in any body fluids, including the bloodstream [48]. Initially, techniques for detection of circulating antigens were based on rabbit or goat antisera to react against antigens present on the surface of spores and hyphae circulating in the body fluids [1]. However, the results are not always successful since the polyclonal antibodies (pAbs) have poor specificity, leading to false-positives. Cross-reactivity observed in the antisera implies difficulties in differentiating the correct fungal species [3–6]. Although improved methods followed to avoid cross-reactivities, still no greater success was achieved until Richardson and colleagues introduced the concept of fungal antigens as potential markers for infection [8]. The introduction of hybridoma technology to produce monoclonal antibodies (mAbs) allowed a more specific and targeted diagnostics by use of antibodies. Hybridomas are hybrid cell lines, result of the fusion of an antibody-producing B cell with a myeloma cell (B cancer cell) for cell immortalization. By this way, hybrid cells retain the characteristics

of the B cell and the constant growth features of the myeloma. The resulting antibodies are characterized by having single specificity, binding to the same epitope, and therefore, called mAbs [7]. By producing murine mAbs, it was possible to differentiate the fungi up to the genus and species level [10, 11, 62, 63]. However, hybridoma technology also has some limitations since many mAbs recognize carbohydrate residues which are found to be conserved among several fungal species. Such limitations imply that many hybridoma cell lines must be screened for the specificity of single epitopes. Recombinant DNA or phage display technologies could overcome this issue [64]. For example, antisera against *Aspergillus* species cross-reacts within the same genera and also with other species such as *Penicillium* [3, 5, 65]. Unspecificity on *Aspergillus*-related infections detection makes it more difficult to diagnose [66–68]. However, success in detecting IPA has been shown by the use of antisera against somatic antigens and recombinant cell wall galactomannan from *A. fumigatus* [58, 59, 69].

Overall, studies recommend the use of both galactomannan and 1,3-β-D-glucan detection assays in parallel with analysis of clinical and microbiological data to avoid misinterpretations [70, 71].

1.3 *Cryptococcus* spp.

Cryptococcal meningitis disease caused by the encapsulated yeast *Cryptococcus* is responsible for approximately 625,000 deaths per year [72]. *Cryptococcus neoformans* and *Cryptococcus gattii* are among the most prevalent *Cryptococcus* pathogen species [73].

This life-threatening disease affects both immunocompetent and immunosuppressed patients, especially patients with T-cell deficiency, such as the human immunodeficiency virus (HIV) [74–76]. The infection occurs through inhalation and further colonization of the respiratory system. Then, it can disseminate to different parts of the body, particularly to the brain [77, 78].

Traditional methods used for diagnosis of Cryptococcosis include direct examination with India ink staining and antigen detection, which is based on mAbs for the capsule [73]. *Cryptococcus* capsule acts as a barrier, showing antiphagocytic properties, and the capsule components can also modulate the immune response, such as inhibition of proinflammatory cytokines production [79, 80]. The capsule is mainly composed of two polysaccharides, glucuronoxylomannan (GXM) and galactoxylomannan (GalXM). A minor part of the capsule is composed by mannoproteins (MP), corresponding to less than 1 % of the total mass [81, 82]. The basic structure of GXM is a tri-mannose repeat with a glucuronic acid residue. GXM varies in *Cryptococcus* strains from the levels of xylose addition and O-acetylation of mannose [83]. These variations lead to different antibody reactivity, in which can be differentiated into five serotypes (A, B, C, D, and A/D) [82]. Therefore, GXM is considered as the major epitope for antibodies production. In fact, there are several reports referring to GXM for production of

monoclonal antibodies [84–90]. Several kits, such as latex agglutination (LA), enzyme immunoassay (EIA) and Lateral Flow (LF) are commercially available. In these kits, antibodies against GXM are used for diagnosis of *Cryptococcus* infections [91].

In brief, *Cryptococcus* infections particularly affect immunosuppressed patients with T-cell deficiency. GXM has particularly been shown to be an indicator of *Cryptococcus* virulence, and therefore, it has also been successfully used as an epitope for immunodetection.

1.4 Zygomycetes

Zygomycetes are a group of fungi that comprise the orders of Entomopthorales and the Mucorales, known as being associated with human pathogens. Zygomycetes affect predominantly those immunocompromised patients who are diabetic, steroid-treated patients, trauma and burns and the haematopoietic stem cell transplant (HSCT) recipients [48, 92]. This class of fungal pathogens is associated with high mortality rates of up to 80 %, and current diagnostic techniques are not accurate or efficient enough to prevent such outputs [93, 94]. The most common human infections and lately considered as emergent human fungal pathogens are the genera *Rhizopus* (*R. arrhizus*), *Mucor* (*M. circinelloides*), *Rhizomucor* (*R. pusillus*), *Cunninghamella* (*C. bertholletiae*), and *Absidia* (*A. corymbifera*), all belonging to the Mucorales order [93–96]. Zygomycosis is mainly characterized by rhinocerebral, pulmonary and disseminated disease. Clinical features include facial swelling with ocular involvement and progression to the brain, often complicated by pulmonary or disseminated infection [48, 96].

Infection by Zygomycetes occurs mainly through inhalation of airborne spores but also, percutaneous inoculation or ingestion constitute other ways of infection [96, 97].

Diagnostics of zygomycosis can be confused with those of mould infections such as invasive aspergillosis, as both share similarities on CT scans of HSCT patients [96, 98]. Other diagnostic techniques are currently used such as cultures although they may indicate negative results. Identification at the species or genera level is performed by cultures and comparison with documented and described morphological features. Zygomycosis, when possible, can be confirmed by tissue biopsy. One of the most distinctive features of these infections is the invasion of blood vessels by the non-septated hyphal structures, resulting in hemorrhage and tissue necrosis. Many times, these pathogens disseminate from the original site of infection into other distal organs [94, 96]. However and often, the diagnosis of such infections is only made at the autopsy state which is already too late [96, 99].

Immunodetection of *Mucor mucedo* was described by Schimek and colleagues where 4-Dihydromethyltrisporate dehydrogenase (TDH), an enzyme of the trisporic acid synthesis pathway in Zygomycetes, is able to convert a mating type sex pheromone into

methyltrisporate [100, 101]. Expression of TDH, which encodes *TSP1* gene can be analyzed by RT-qPCR. Antibodies developed against *Mucor mucedo* TDH and immunodetection of TDH have shown promising results in a rabbit model [101].

1.5 *Fusarium* spp.

Fusarium comprises a genus of fungi that includes around 100 species from which only around 12 are considered human pathogens. *Fusarium* species represent an emergent fungal threat for immunocompromised patients, although it can also occur in immunocompetent individuals [102–106]. *F. solani* is one of the most prevalent species among the genus *Fusarium*, which can be accounted for up to 50 % of human infections [48]. Other *Fusarium* species capable of infecting humans are *F. anthophilum*, *F. oxysporum*, *F. verticillioides*, and *F. proliferatum* [107, 108]. Fusariosis can occur as superficial infections, keratitis and other eye infections, deep localized and disseminated infections [104]. The main infection route of *Fusarium* spp. is the airways and less frequent through the skin and mucosal membranes [104]. Morphological detection is insufficient to differentiate *Fusarium* species and the rDNA internal transcribed spacer (ITS) region, which is generally used to identify fungal species, is also not enough to distinguish many *Fusarium* species [72]. Current diagnostic tools include culture and biopsy of local tissue [104] and more recently multilocus sequence typing [72, 105, 109, 110].

Currently, there are only a few mAbs developed against *Fusarium*. Jensen and colleagues developed the *Fusarium*-specific mAb IgM, which recognizes 51 and 63 kDa antigens [111]. Thornton and colleagues used *F. solani* strains to develop a genus-specific mAb (ED7) that binds to a 120 kDa antigen, localized to the spores and hyphal cell walls of *Fusarium* [48].

In the next sections, materials and methods, we describe in detail a methodology currently used in our laboratory for immunodetection of *Candida* spp. This methodology comprises the production of antibodies in an *in vivo* mouse model, identification of antigens and purification techniques. The same methodology could be applied for detection of other human fungal pathogens, but details are not described here.

2 Materials

2.1 Media

1. YPD medium: 25 g/l Bacto™ peptone, 12.5 g/l Bacto™ yeast extract, 2 % glucose. Dissolve the powdered contents in 800 ml deionized water and fill up to exactly 1000 ml once all contents are dissolved. Autoclave or filter sterile the YPD medium immediately.
2. Dulbecco's Modified Eagle Medium (DMEM) (D5671; Sigma-Aldrich).

3. HAT medium: 400 ml DMEM medium, 50 ml fetal bovine serum—ultra low IgG (16250-086; Life Technologies), 25 ml BM Condimed H1 Hybridoma Cloning Supplement (11088947001; Roche), 5 ml Penicillin-Streptomycin (P4333; Sigma-Aldrich), 5 ml GlutaMAX (35050-038, Carlsbad), 5 ml Sodium-pyruvate (S8636; Sigma-Aldrich), and 10 ml HAT supplement (21060-017; Gibco).

2.2 Buffers

1. PBS buffer for *in vivo* use (Phosphate Buffered Saline) (P5368; Sigma-Aldrich).
2. Binding buffer for antibody purification: 20 mM sodium biphosphate.
3. Elution buffer: 0.1 M glycine–HCl, pH 2.7.
4. Lysis buffer: Tris–HCl, pH 8.0, 150 mM Sodium chloride NaCl, 0.1% Nonidet P-40, Protease inhibitor cocktail—EDTA-free (04693132001; Roche).
5. 2× Lämmli buffer for immunoprecipitation: 125 mM Tris–HCl, pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate, 0.002% bromophenol blue, 10% 2-mercaptoethanol. Dissolve all components in distilled water and add the 2-mercaptoethanol immediately prior to usage.
6. Cross-linking buffer for immunoprecipitation: 0.2 M triethanolamine set to pH 8.2 with HCl.
7. Cross-linking buffer for immunoprecipitation with dimethyl pimelimidate dihydrochloride (DMP): 25 mM DMP in cross-linking buffer (0.2 M triethanolamine, pH 8.2 with HCl).
8. Blocking buffer for cross-linking: 0.1 M ethanolamine pH 8.2, adjusted with HCl.
9. Running buffer for SDS-PAGE: 25 mM Tris, 192 mM glycine and 0.1% sodium dodecylsulfate.

2.3 Consumables

1. Injection needles: 27G×½″ for subcutaneous injections, 29G×½″ for intravenous injections, 23G to puncture the *vena facialis.*
2. Counting chamber.
3. Cell-culture certified consumables: 96-well plates, 12-well plates, 6-well plates, 6 cm dishes and 10 cm dishes.
4. HiTrap Protein G HP column (17-0404-01; GE Heathcare).
5. Luer connector compatible syringe.
6. Dynabeads® (10007D; Thermo Fisher Scientific).

2.4 SDS PAGE Gel Components

1. Loading gel (10%): 4.2 ml H_2O, 2.5 ml of 1.5 M Tris, pH 8.8, 3.3 ml of 30% acrylamide–bis solution, 100 µl of 10% SDS, 10 µl *N,N,N′,N′*-tetramethylethylendiamine (TEMED), and 100 µl of ammonium persulfate (APS).

2. Stacking gel (4.5%): 6 ml H_2O, 2.5 ml of 0.5 M Tris, pH 6.8, 1.5 ml of 30% acrylamide–bis solution, 100 μl of 20% SDS, 10 μl TEMED, and 100 μl of APS.

3 Methods

3.1 Preparation of the Inoculum

1. Inoculate *C. albicans* SC5314 in 5 ml of YPD media in a culture tube and incubate overnight at 30 °C and 220 rpm.
2. Dilute the culture in 50 ml fresh YPD medium in a plastic flask to an OD_{600} of 0.1 and incubate at 30 °C and 220 rpm until the OD_{600} reaches 1.
3. Harvest the cells by centrifugation for 5 min at $1180 \times g$. Wash the cells twice with 40 ml of endotoxin-free PBS and resuspend in 10 ml PBS.
4. Heat-kill the *C. albicans* cells by heating to 70 °C for 5 min.

3.2 Immunization of Mice

1. Settle 4 BALB/C mice of the same sex in a standard cage (*see* **Note 1**).
2. Day 1—Collect pre-immunization sera and then immunize the mice subcutaneous with 5×10^7 heat-killed *C. albicans* cells each (*see* **Note 2**). Resuspend the heat-killed cells in 100 μl PBS and mix with 100 μl of Freund's complete adjuvant (*see* **Note 4**).
3. Day 14—Immunize the mice subcutaneous with 5×10^7 heat-killed *C. albicans* cells each. Resuspend the heat-killed cells in 100 μl PBS and mix with 100 μl of Freund's incomplete adjuvant.
4. Day 24—Draw blood for analysis.
5. Day 35—Immunize the mice subcutaneous with 5×10^7 heat-killed *C. albicans* cells each. Resuspend the heat-killed cells in 100 μl PBS and mix with 100 μl of Freund's incomplete adjuvant.
6. Day 45—Draw blood for analysis.
7. Day 56—Administer the final immunization boost by intravenous injecting 1×10^7 heat-killed *C. albicans* cells into each mouse (*see* **Note 3**). Resuspend the heat-killed cells in 100 μl PBS.
8. Day 66—Draw the final blood sample and isolate the splenocytes (*see* **Note 5**).

3.3 Isolation of Splenocytes and Fusion with Myelomas

The following steps are performed in collaboration with the Monoclonal Antibody Facility (MAF) at the Campus Vienna Biocenter (VBC) in Vienna, Austria. Their methodologies mainly follow previously published protocols for the isolation of splenocytes and the fusion with myelomas [112]. The protocol is here briefly summarized:

1. Disinfect the body of the mouse with 70% EtOH.
2. Carefully remove the spleen and wash it with DMEM.
3. Make sure to remove any tissue adhering to the spleen.
4. Use a sterile pestle to mechanically homogenize the spleen.
5. Pass the suspension through a sterile cell strainer (100 μm).
6. Lyse the erythrocytes using erythrocyte lysis buffer.
7. Mix 1×10^8 splenocytes with 1×10^7 X63-Ag8.653 myeloma cells.
8. Remove the supernatant completely to avoid a dilution of the PEG1500.
9. Slowly add 0.8 ml PEG1500 at 37 °C to the pellet and carefully resuspend the pellet over the course of 1 min.
10. Continue stirring the cells with the pipette for 1 more minute at 37 °C.
11. Dilute the suspension by slowly adding 10 ml DMEM.
    (1 ml/min at first and 2 ml/min later).
12. Add further 12 ml DMEM dropwise at 37 °C.
13. Centrifuge the suspension and remove the supernatant.
14. Gently resuspend the pellet in 50 ml DMEM supplemented with 20% FCS.
15. Distribute the suspension to a 96-well plate, with 200 μl per well.
16. Incubate the plate overnight at 37 °C with 5% CO_2.
17. The following day remove 100 μl supernatant from each well and replace it with HAT medium.
18. Repeat the previous step every 3 days.
19. After 10–14 days, culture supernatants are used or Western blot analysis to confirm the production of mAbs by the hybridoma pools. Positive pools can be frozen or directly used for screening of single mAb producing clones.

3.4 Screening of Hybridomas for Single mAb Producing Clones

1. Thaw frozen stocks and immediately transfer them into 15 ml tubes containing 10 ml of DMEM at room temperature.
2. Centrifuge the sample for 7 min at $300 \times g$ at room temperature.
3. Resuspend the pellet in 2 ml HAT medium at room temperature, transfer the suspension to a 3 cm round dish and incubate the dish at 37 °C with 5% CO_2.
4. Monitor the cell confluency daily using a microscope.
5. Once the cells have reached confluency, carefully resuspend the cells by gentle pipetting and collect the cells in a 15 ml tube (*see* **Note 6**).

6. Count the cells in a counting chamber and place 100, 200 and 600 cells in 25 ml HAT medium, respectively. Distribute the three different cell suspensions to one 96-well plate each. Fill each well with 250 μl of cell suspension.
7. Add HAT medium to the remaining cells to a total volume of 2 ml. Transfer the suspension to a 3 cm dish and incubate at 37 °C with 5% CO_2 until the cells are confluent and can be frozen as a new stock.
8. Control the growth of the cells in the plate daily using a microscope.
9. Remove 80 μl of supernatant on day 7 and replace the volume with fresh HAT medium. Prepare a 1:10 dilution of the supernatant and use it for Western blot analysis with whole-cell extract from *C. albicans*.
10. Select 3–6 clones showing the strongest signal at Western blot analysis for the production of antibodies.

3.5 Upscaling of Culture Volume and Production of Antibodies

1. Resuspend the selected cells by gentle pipetting and transfer them into the wells of a 12-well plate, filled with 0.8 ml of HAT medium each. Incubate the plate a 37 °C with 5% CO_2 and monitor the cell confluency again.
2. Repeat the procedure once the cells have reached 80% confluency and transfer the cells to 6-well plates holding 1 ml of fresh HAT medium in each well.
3. Once the cells have reached sufficient confluency again, repeat the step using a 6 cm dish holding 4 ml of HAT medium and later with a 10 cm dish holding 14 ml of medium (*see* **Notes 7** and **8**).
4. Incubate the 10 cm dishes at 37 °C with 5% CO_2 until the cells have reached 80% confluency.
5. Prepare a series of dilutions ranging from 1:1000 to 1:20000 for each supernatant and use Western blot analysis to determine the antibody amount.
6. Transfer the suspensions to 15 ml tubes and centrifuge them for 7 min at 300 × *g* and 4 °C.
7. Transfer the supernatants to new tubes and sterile-filter them.
8. Resuspend the cells in 1 ml FBS with 10% DMSO at 4 °C and split the volume into 2 cryotubes. Freeze the tubes at −80 °C before moving them to liquid nitrogen for long-term storage.

3.6 Purification of Antibodies Using 1 ml HiTrap Protein G Columns

1. Prepare 5× 1.5 ml collection tubes with 60 μl of 1 M Tris–HCl, pH 9.0 in each tube.
2. Prepare the sample by adding the equal volume of binding buffer (20 mM sodium phosphate) to the previously sterile-filtered cell culture supernatant.

3. Remove and store 50 μl as input sample.
4. Fill a Luer connector compatible syringe with 10 ml binding buffer and connect it to a HiTrap Protein G column without introducing air to the column.
5. Remove the "snap-off" end of the column outlet
6. Wash the column with 10 column volumes (CV) of binding buffer at a flow rate of about 3 ml/min.
7. Fill a Luer connector compatible syringe with the sample and attach it to the column without entering air into the system (*see* **Note 9**).
8. Apply the sample to the column and collect the flow through in a 50 ml falcon tube.
9. Wash the column with 10 CV of binding buffer.
10. Elute the antibody with 5 CV of elution buffer (0.1 M glycine-HCl, pH 2.7) and collect 1 ml aliquots in the previously prepared collection tubes.
11. Store the purified antibody at 4 °C from now on (keep on ice).
12. Wash the column with 10 CV of binding buffer.
13. Determine the antibody concentrations in the elution fractions by measuring the absorbance at 280 nm on a NanoDrop spectrophotometer. An absorbance of 1 at 280 nm corresponds to 0.8 mg/ml.
14. Pool the fraction that contain antibody for the buffer exchange.
15. Exchange the buffer using 3 kDa Amicon Ultra 0.5 ml spin filters. Spin five times for 10 min and fill up to 450 μl PBS each time.
16. Recover the antibody from the spin filters and adjust the concentration to 1 mg/ml. Store the antibody in PBS with 50% glycerol and 0.02% NaN_3 at −20 °C (*see* **Note 10**).
17. Control the antibody purity by running 4 μg on a 12% SDS-PAGE followed by Coomassie staining.
18. Use 3 μl of both the input and the flow through for a dot blot to determine the antibody levels.
19. Wash the protein G column with 5 CV 20% ethanol before storage at 4 °C.

3.7 Optional: Protein G Column Cleaning and Regeneration

Cleaning and regeneration is necessary only if the column is used for a different antibody afterwards. In order to completely avoid the risk of contamination, a new column should be used for each antibody.

1. Clean the column with 10 CV 6M guanidinium-HCl, pH 7.0 following the post-elution washing step (10 CV of binding buffer).

2. Regenerate the column with 10 CV 1 M acetic acid, pH 2.4.
3. Wash the column with 10 CV binding buffer.
4. Store the column at 4 °C.

3.8 Preparation of *C. albicans* Whole Cell Extract (WCE)

1. Grow *C. albicans* overnight in 20 ml YPD medium.
2. Dilute the culture to an OD_{600} of 0.25 in 50 ml YPD (50 ml are sufficient for one IP reaction).
3. Incubate the fresh culture at 30 °C and 120 rpm to an OD_{600} of about 2.0.
4. Harvest the cells by centrifugation at 1500 × *g* and 4 °C for 3 min.
5. Wash the cell pellet in 20 ml of cold sterile H_2O (4 °C) and remove the supernatant.
6. Resuspend the pellet in 0.5 ml lysis buffer with protease inhibitor and transfer the suspension to a screw cap tube.
7. Add an equal amount of glass beads to the screw cap tube.
8. Place the screw cap tubes in a FastPrep and run five times for 30 s at 6 m/s and place the tubes on ice in between each run.
9. Puncture the screw cap tubes with a glowing hot 27G needle close to the bottom, place each tube in a 2 ml Eppendorf tube and spin down for 2 min at 700 × *g* 4 °C (loosen the lid before spin).
10. Transfer supernatant to a 1.5 ml tube, spin down cell debris for 10 min at full speed at 4 °C and transfer the supernatant to a 1.5 ml tube.

3.9 Identification of Antigens Using Immunoprecipitation with Dynabeads

1. Prepare 30 μl Dynabeads® according to the manual. Briefly, resuspend the Dynabeads® in the vial and vortex mix for at least 30 s. Transfer 30 μl of the suspension to a 1.5 ml tube and separate the beads from the solution by placing the tube on a magnet. Remove the supernatant and take the tube off the magnet (*see* **Notes 11–13**).
2. Add 10 μg antibody diluted in 200 μl PBS with 0.05 % Tween 20 to the tube containing the Dynabeads®.
3. Rotate the tube for 10 min at room temperature.
4. Place the tube on a magnet and remove the supernatant.
5. Remove the tube from the magnet and gently resuspend the Dynabeads® in 200 μl PBS with 0.05 % Tween 20 by pipetting.
6. Remove the buffer, add the WCE to the Dynabeads and incubate overnight at 4 °C on a rotary shaker.
7. Centrifuge for 30 s at 1000 × *g* and 4 °C.
8. Remove the supernatant.

9. Wash the beads three times using 700 μl cold lysis buffer without proteinase inhibitor.
10. For non-cross-linked antibodies proceed with the denaturing elution method. Briefly, resuspend the beads in 20 μl 2× Lämmli buffer and place all samples at 70 °C for 10 min. Afterwards, cool the samples on ice, centrifuge for 1 min, and run a SDS-PAGE with silver staining using the whole IP volume.
11. For cross-linked antibodies, add 100 μl of elution buffer and gently resuspend the Dynabeads® by pipetting to avoid the production of foam. Incubate the suspension for 5 min at room temperature with rotation to dissociate the Dynabeads®-Ab-Ag complex. Place the tube on magnet and transfer the supernatant with the eluted protein to a fresh tube. Repeat the elution process once and pool both eluates. Precipitate by adding 30 % TCA and incubation on ice for 10 min. Centrifuge for 10 min at 18,200 × *g* and 4 °C. Wash the protein pellet once with 500 μl cold acetone and air-dry the pellet for 2 min. Resuspend the pellet in 15 μl 2× Lämmli buffer and use the whole IP volume for SDS-PAGE with silver staining. Cross-linking is only necessary if the antigen has the same size as the heavy or the light chain of the mAb (~25 and ~50 kDa, respectively).

3.10 Cross-Linking of Antibody to Dynabeads

1. Add 1 ml of cross-linking buffer to the Protein A/G immobilized antibody and vortex gently to resuspend. If beads are in the lid, always spin down 5 s at 1000 × *g*. Apply to magnet for 30 s to pull beads to the side of the tube and remove supernatant. Repeat this step once.
2. Resuspend in 1 ml cross-linking buffer containing 25 mM dimethyl pimelimidate dihydrochloride (DMP). Mix thoroughly and incubate at room temperature for 45 min with agitation.
3. Apply to magnet for 30 s to pull beads to the side of the tube and remove supernatant.
4. Add 1 ml blocking buffer and vortex gently to resuspend. Apply to magnet for 30 s to pull beads to the side of the tube and remove supernatant.
5. Add 1 ml of blocking buffer and vortex to resuspend. Incubate for 1 h at room temperature with agitation.
6. Apply to magnet for 30 s to pull beads to the side of the tube and remove supernatant. Add 1 ml of PBS, vortex to resuspend, apply to magnet for 30 s to pull beads to the side of the tube and remove supernatant. Repeat wash twice.
7. Add 1 ml elution buffer and vortex gently to resuspend, apply to magnet for 30 s to pull beads to the side of the tube and

remove supernatant. This elutes bound antibody that is not cross-linked with DMP.

8. Wash beads 4 times in 600 μl lysis buffer (without protease inhibitor) prior to incubation with WCE.

4 Notes

1. BALB/C mice are preferable, since splenocytes from C57BL/6 mice show a lower efficacy when it comes to the fusion with myelomas.
2. Sub cutaneous injection: Take the mouse by its neck and restrain the tail as well as one leg with your small finger. This exposes one flank of the mouse (are off the hip with a lot of loose skin) that should be used for the subcutaneous injection. Make sure to penetrate the skin and inject the total volume into the cavity. A G27 × ½″ needle should be used for the injection, thinner needles might cause problems due to the thick fluid. The sides of the mouse should be alternated between the individual immunizations. After the injection there will be a visible bulge under the skin of the mouse. This bulge will diminish as the injected volume is taken up.
3. Intravenous injection: Place the mouse in a suitable restrainer with sufficient ventilation. Use a 29G × ½″ needle for the injection.
4. Freund's adjuvant: For the first immunization Freund's complete adjuvant is used, for the later immunizations Freund's incomplete adjuvant should be used.
5. Blood drawing from mice: Puncture the vena facialis of the mouse and immediately collect the blood drops in a sterile microvette. To isolate the sera centrifuge the tubes for 10 min at 1880 × *g* and room temperature.
6. Cell confluency of hybridoma cells:
   Hybridomas grow as microcolonies and are only loosely attached to the plate. Once the cells have reached confluency, 500 μl of supernatant (includes floating cells) can be used to inoculate a new plate. These cells can then be frozen as a new stock once they have reached confluency as well.
7. Before upscaling the culture volume to 14 ml in a 10 cm dish, 1:100 and 1:1000 dilutions of the supernatants can be screened again for antibodies against *C. albicans* using Western blot analysis.
8. Always heat HAT medium to 37 °C before usage unless otherwise stated.
9. Connect the syringe and the column "drop to drop" to avoid air in the column.

10. Antibody storage:
    Alternatively, to the storage at −20 °C, the antibody can be stored without glycerol in PBS with 0.02 % NaN_3 only at 4 °C.
11. According to the Dynabeads® manual 50 μl of beads should be used, however 30 μl have proven to be sufficient.
12. Make sure that there are no glass beads on the outside of the screw cap tubes or in the sealing of the cap. Beads on the outside of the tube can severely damage the FastPrep machine and beads in the sealing of the cap will cause leakage.
13. Check the breakage of the cells under a microscope. Cells with highly contrasted outlines are not broken. After the FastPrep runs more than 90 % of the cells should be broken.

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Chapter 21

The Molecular Blueprint of a Fungus by Next-Generation Sequencing (NGS)

Christian Grumaz, Philipp Kirstahler, and Kai Sohn

Abstract

Sequencing the whole genome of an organism is invaluable for its comprehensive molecular characterization and has been drastically facilitated by the advent of high-throughput sequencing techniques. Especially in clinical microbiology the impact of sequenced strains increases as resistance and virulence markers can easily be detected. Here, we describe a combined approach for sequencing a fungal genome and transcriptome from initial nucleic acid isolation through the generation of ready-to-load DNA libraries for the Illumina platform and the final step of genome assembly with subsequent gene annotation.

Key words Next-generation sequencing (NGS), Whole-genome shotgun sequencing (WGS), RNA sequencing (RNA-seq), Fungal genome, Assembly, Annotation, Bioinformatics

1 Introduction

The rapid developments of high-throughput sequencing technologies (next-generation sequencing, NGS) reduce costs while increasing capacity. This has opened up completely new dimensions in nucleic acid analytics making it possible to sequence several hundred million fragments simultaneously in contrast to single fragments as in the past. The words once proclaimed by Sydney Brenner in 1980 "*Progress in science depends on new techniques, new discoveries and new ideas, probably in that order*" [1] perfectly reflect the developments enabled by NGS which finally revolutionized countless areas of life sciences research.

1.1 Sequencing Technologies: The Next Generation

These days, the most widely used technology is provided by Illumina and is based on a reversible terminator chemistry which is an advancement of Frederick Sanger's technique from 1970, but in a highly parallelized manner [2, 3]. In principle, a DNA polymerase incorporates fluorescently labeled deoxyribonucleotide triphosphates (dNTPs), for each base a specific fluorophore, into the DNA template strand in consecutive cycles. The number of

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cycles determines read lengths of the sequenced fragments. When incorporated during each cycle, the nucleotides are identified by their specific fluorophore excitation for millions of fragments simultaneously. Currently, this sequencing-by-synthesis (SBS) chemistry delivers the highest accuracy, the highest yield of error-free reads and the highest percentage of high-quality reads above a phred score of Q30 [2, 4, 5]. The technology is available in different configurations ranging from bench top sequencers (MiSeq) with relatively low throughput or a medium sized sequencer (NextSeq) up to an ultra-high-throughput series (HiSeq). Table 1 shows a summary including the major technical specifications—read lengths and throughput in amount of reads and gigabases (Gb). The method of choice is dependent on experimental demands. By way of example, for conventional RNA-seq approaches for differential gene expression profiling one might sequence around 30 mio. 50 bp long reads in single-read mode on a HiSeq for the human transcriptome. For *de novo* sequencing of a fungal genome one might prefer longer reads of up to 150–300 bp in paired-end mode on a HiSeq or MiSeq dependent on genome size and required sequence coverage.

In addition, there are also alternative techniques available which can be applied for specific questions but which are based on completely different sequencing principles with respective advantages and limitations. The Ion Torrent PGM from Life Technologies by way of example measures the release of hydrogen ions during DNA replication in a semiconductor chip while the single molecule sequencer from the 3rd generation of sequencing technologies, PacBio RSII from Pacific Biosciences, detects phospholinked hexaphospate nucleotides in real-time during incorporation with an immobilized DNA polymerase in so called zero-mode waveguides (ZMWs) [6]. A second third-generation technology is provided by Oxford Nanopore Technologies which is not based on SBS chemistry like the other techniques mentioned above, but deduces sequence composition out of the base-specific magnitude of a current measured across nanopores [7].

1.2 De Novo Genome Assembly and Gene Annotation via NGS

There are many different protocols of generating libraries suitable for assembling a genome *de novo*, but the first step is always similar: genomic DNA (gDNA) has to be sheared randomly in multiple copies. This procedure generates shorter fragments now able to be sequenced following the ligation of technology-specific adapters required for the sequencing reaction. However, the crucial point of random shearing is that these fragments share overlapping sequences just enabling one to put the short sequences piece by piece together into so called contigs (short for *contiguous*). Summarized, this is the principle of whole-genome shotgun (WGS) sequencing, the golden standard for *de novo* genome assemblies [8–10]. Depending on the sequencing platform, an adapted library

Table 1
Specification summary of selected sequencing technologies according to manufacturer's homepages in July 2015

NGS platform	Technology	Read length (bases)	Sequences per run[a]	Capacity	Comments
"2nd generation"					
Illumina MiSeq	Irreversible dye terminator	2 × 300	~25 mio.	~15 Gb/run	Benchtop system, for targeted and small genome sequencing
Illumina NextSeq 500	~	2 × 150	~400 mio.	~120 Gb/run	Larger benchtop system, for middle-scaled genomics
Illumina HiSeq 2500—high output mode	~	2 × 150	~4000 mio.	~1000 Gb/run	High throughput system but also flexible with an internal rapid mode, for large-scale genomics or for targeted and small genome sequencing
Illumina HiSeq 2500—rapid output mode	~	2 × 250	~600 mio.	~300 Gb/run	
Illumina HiSeq 4000	~	2 × 150	~5000 mio.	~1,500 Gb/run	Maximum throughput system with lowest costs, for production-scale genomics
Life Technologies Ion Torrent PGM	Ion semiconductor sequencing	Up to 400	~5,5 mio.	~2 Gb/run	Benchtop system, for targeted and small genome sequencing
"3rd generation"					
PacBio RS II	Phospholinked fluorescent nucleotides	Ø > 4000	Ø 60,000	Ø 1.6 Gb/run	High performance system with ultra long reads, for *de novo* sequencing
Oxford Nanopore Series	Ion conductor nanopore sequencing	tba	tba	tba	tba

[a]Quality passed single-reads

preparation specific to the requirements of the technology is necessary including an optimal fragment size distribution according to the maximum read length specifications, for example. Generally, the longer the sequenced reads are the better is the corresponding assembly. However, a high sequence quality and an adequate coverage of sequenced bases also play crucial roles.

This workflow for the *de novo* sequencing of fungal genomes is optimized for Illumina's technology as it shows most accurate sequence quality with highest possible sequence depth at reasonably low costs. Read lengths of 250 bp from HiSeq rapid output mode or 300 bp provided by MiSeq might be sufficient for an assembly. Beyond that, a special feature of this sequencing technology is the possibility to sequence fragments from both sides by the so called paired-end mode (e.g., 2 × 250 bp), revealing two important benefits: (1) shorter "difficult-to-sequence" regions, e.g., with high GC contents which are located in the middle of a paired-end sequenced fragment of up to 1–2 kb can be handled and still be assembled to one fragment, and (2) longer "difficult-to-assemble" regions, e.g., with repetitive sequence content which per se are almost impossible to assemble properly might be managed by the so-called mate pair library protocol with insert sizes up to roughly 10 kb.

However, the optimal *de novo* assembly just starts with the sample preparation followed by the sequencing reaction considering the already mentioned factors (library protocol, sequence quality and coverage) and can only be accomplished with adequate bioinformatics tools and workflows. Generally, a sequence quality check and correction are needed before starting the assembly usually based on de Bruin graphs for short reads [11]. Briefly, the reads are split into k-mers and from these k-mers all possible (k-1)-mers are generated representing the nodes in a de Brujin graph. Directed edges are then added between the nodes if they cover one of the k-mers. For example, from the 4-mer AGCC the two 3-mers AGC and GCC are defined while from GCCT the two 3-mers GCC and CCT are present. Thus, our graph will contain three nodes (AGC, GCC and CCT) and two directed edges (AGCC and GCCT). To determine the assembled sequence a path has to be found which passes each edge exactly once, the so called Euler Path. This path would proceed from AGC to GCC to CCT resulting in the sequence AGCCT. The contigs generated this way or even scaffolds, if mate pair information was used additionally, represent the draft genome. This draft can then further be finished either by targeted Sanger sequencing and chromosomal walking methods or by long read NGS technologies like PacBio RSII to achieve chromosomal resolution.

Once having the genome assembled, the genomic content can now be characterized and annotated. Usually, an in silico gene prediction searching for longest possible open reading frames (ORFs)

coding for proteins is performed through sequence homology searches in collections of protein databases. However, one of the major challenges here is the recognition of splice sites which might vary in sequence and in frequency per ORF from species to species. In this context, recent studies of fungal transcriptomes revealed that utilization of additional RNA-seq data drastically improves the predictions of up to 45 % corrected ORFs which were based exclusively on in silico methods [12, 13]. Beside the main advantage of the exact identification of splice sites through intron-spanning reads, also untranslated regions and noncoding genes can be determined. Thus, following a combined approach of *de novo* genome sequencing and RNA-seq is a highly sophisticated way to reveal the molecular blueprint of a fungus. A summary of the workflow described in this chapter is illustrated in Fig. 1.

2 Materials

2.1 Wet Lab Materials

2.1.1 Extraction of Genomic DNA and RNA

1. Tabletop centrifuge (Heraeus Biofuge pico).
2. Tabletop vortexer (Neolab).
3. Retsch mill with a 10 ml Teflon grinding jar and a tungsten carbide ball with 7 mm diameter (Retsch MM200).
4. Magnetic stand 2 ml reaction tubes (Life Technologies).
5. AllPrep DNA/RNA Mini Kit (Qiagen).
6. Agencourt RNAClean XP Beads (Beckman Coulter).
7. TURBO DNase (Thermo Fisher).
8. 2-mercaptoethanol (Carl Roth).
9. 70 % EtOH in RNase-free water.
10. Liquid nitrogen.
11. 50 ml tubes (Greiner).
12. Block heater (Neolab).
13. Molecular biology grade water (5 Prime).

2.1.2 Quantification and Quality Check of Nucleic Acids

1. Qubit (Life Technologies) (*see* **Note 1**).
2. Qubit dsDNA HS Assay Kit (Life Technologies).
3. Qubit assay tubes (Life Technologies).
4. Ethidium bromide (10 mg/ml) (Carl Roth).
5. Agarose (Biozym).
6. Tris-acetate-EDTA buffer, TAE: 40 mM Tris, 20 mM acetic acid, 1 mM EDTA.
7. 0.8 % agarose gel: Prepare a 0.8 % agarose solution in TAE by boiling. Cool down to 50–60 °C and add 2.5–5 μl ethidium bromide. Mix gel until it is still warm and pour it onto the gel casting device.

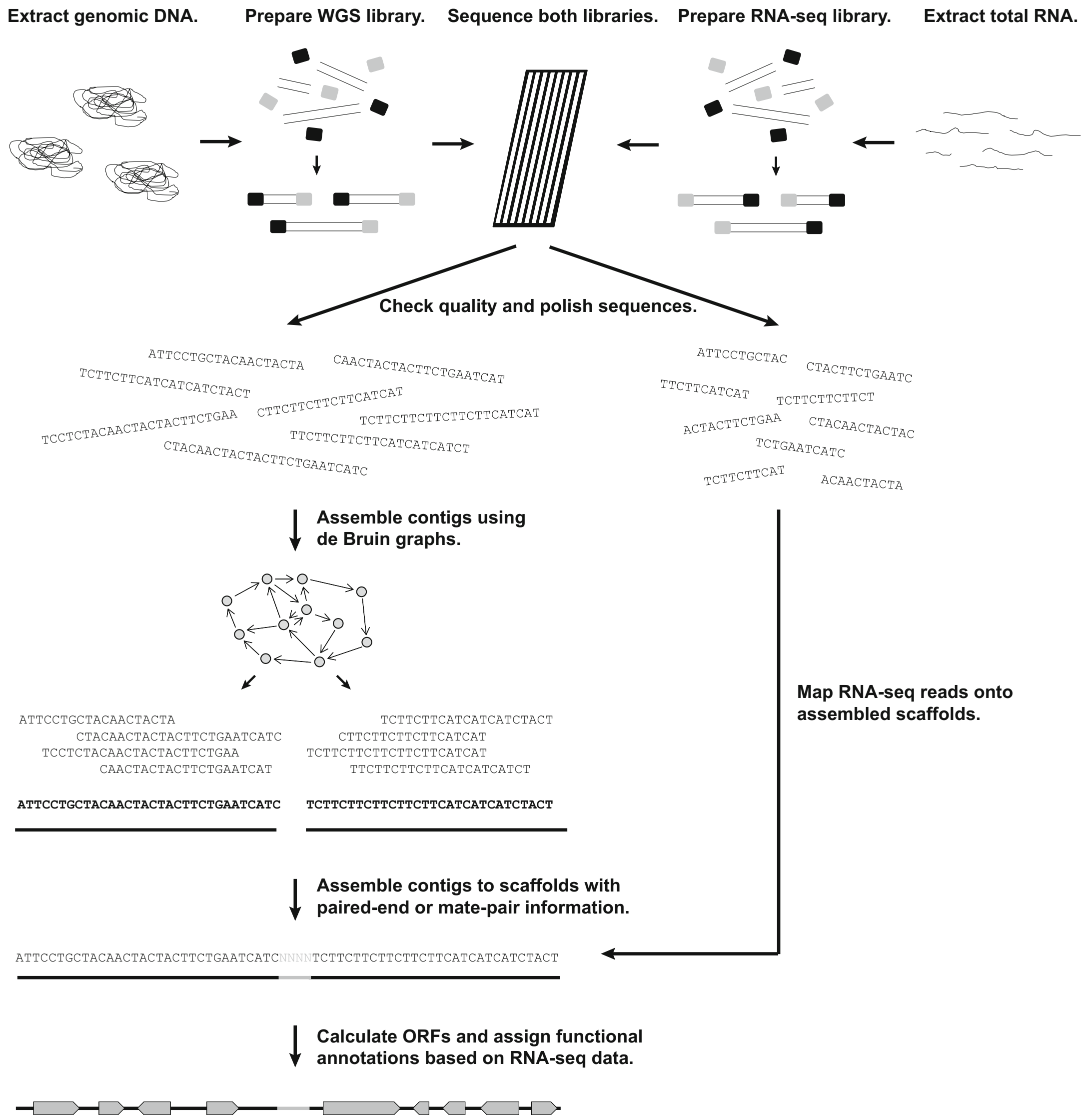

Fig. 1 Workflow of WGS *de novo* genome and transcriptome sequencing via NGS. WGS whole-genome shotgun, NGS next-generation sequencing, ORF open reading frames

8. Gel loading dye and DNA ladder (NEB).
9. Gel electrophoresis and documentation system with power supply (Peqlab) (*see* **Note 2**).
10. 2100 Bioanalyzer (Agilent Technologies) (*see* **Note 3**).
11. Agilent RNA 6000 Nano Kit (Agilent Technologies).
12. Agilent High Sensitivity DNA Kit (Agilent Technologies).

2.1.3 Library Preparation for NGS

1. Tabletop centrifuge (Heraeus Biofuge pico).
2. Nextera DNA Sample Preparation Kit (Illumina) (*see* **Note 4**).
3. Nextera DNA Sample Preparation Index Kit (Illumina).

4. DNA Clean & Concentrator-5 (Zymo Research) (*see* **Note 5**).
5. TruSeq Stranded mRNA Sample Preparation Kit (Illumina) and SuperScript II (Thermo Fisher) (*see* **Note 6**).
6. Agencourt AMPure XP Beads (Beckman Coulter).
7. 80% always freshly prepared EtOH (Carl Roth).
8. Magnetic stand 2 ml reaction tubes (Life Technologies) (*see* **Note 5**).
9. Magnetic stand for 96-well plates (Thermo Fisher).
10. PCR strips or tubes with at least 0.2 ml (Biozym).
11. 96-well plates (Eppendorf, twin-tec, semi-skirted).
12. Microseal "B" adhesive seals (Bio-Rad).
13. Thermocycler (Bio-Rad).
14. DNA low-bind reaction tubes (Eppendorf).

2.2 Bioinformatics Analysis Software

Install bioinformatics tools according to the instructions of the developers.

1. Minimal system requirements.
   (a) Linux Machine.
   (b) At least 250 GB free space.
   (c) 16 GB RAM.
   (d) 2-Core 2 GHz system (additional cores will speed up the computation significantly).
2. Fastqc (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) [14].
3. bbmaps (http://sourceforge.net/projects/bbmap/).
4. Abyss (http://www.bcgsc.ca/platform/bioinfo/software/abyss/) [15].
5. HISAT (https://ccb.jhu.edu/software/hisat/manual.shtml) [16].
6. BRAKER1 (http://bioinf.uni-greifswald.de/augustus/downloads/).
7. GeneMark-ET (http://exon.gatech.edu/genemark/) [17].
8. Augustus (http://bioinf.uni-greifswald.de/augustus/downloads/) [18].
9. NCBI BLAST+ (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastDocs&DOC_TYPE=Download) [19].
10. InterProScan (http://www.ebi.ac.uk/interpro/interproscan.html) [20].
11. ANNIE (http://genomeannotation.github.io/annie/).
12. GAG (http://genomeannotation.github.io/GAG/).

3 Methods

3.1 Simultaneous DNA and RNA Isolation

3.1.1 Extraction of Nucleic Acids

1. Pellet fungal cells or spores for 3 min at 3000 × *g* at RT in a 2 ml reaction tube.
2. Resuspend pellet in 300 μl PBS.
3. Transfer resuspended cells drop by drop in a 50 ml tube filled with liquid nitrogen to freeze.
4. Decant liquid nitrogen without losing the frozen beads.
5. Transfer frozen beads to the precooled (liquid nitrogen) Teflon grinding jar loaded with a corresponding tungsten carbide ball of the Retsch mill (*see* **Note 7**).
6. Applying a shaking frequency of 30 per second for 2 min to disintegrate the fungal cells.
7. Transfer frozen powder to 350 μl RLT Plus buffer supplemented with 3.5 μl 2-mercaptoethanol and vigorously vortex for 1 min.
8. Then proceed according to the manufacturer's instruction protocol of the AllPrep DNA/RNA Mini Kit.
9. Elute DNA in 50 μl EB buffer into a 1.5 ml reaction tube and store at −20 °C up to 12 months.
10. Elute RNA in 50 μl RNase-free water into a 1.5 ml reaction tube and directly proceed with additional DNase treatment.

3.1.2 Additional DNase Treatment of RNA

1. Add 5.6 μl 10X TURBO DNase buffer and 1 μl TURBO DNase.
2. Incubate at 37 °C for 30 min.
3. To purify DNA-free RNA add 90 μl RNAClean XP beads previously brought to RT.
4. Mix the solution by gently pipetting up and down ten times.
5. Incubate for 5 min at RT.
6. Place the 1.5 ml reaction tube on a magnetic stand for 2 min or until the supernatant has cleared.
7. Carefully remove and discard the supernatant without disturbing the beads.
8. Wash the beads on the stand with freshly prepared 70% EtOH without resuspending them by 30 s of incubating.
9. Carefully remove and discard the supernatant without disturbing the beads.
10. Repeat **steps 8** and **9**.
11. Allow beads to air-dry for at least 5 min on the stand.
12. Take the 1.5 ml reaction tube off the stand and resuspend beads in 33 μl RNase-free water by gently pipetting up and down ten times.

13. Incubate for 2 min at RT.
14. Place the 1.5 ml reaction tube on the magnetic stand for 2 min or until the supernatant has cleared.
15. Carefully transfer 30 µl of the supernatant into fresh 1.5 ml reaction tube.
16. RNA can be stored at −70 °C up to 12 months.

3.1.3 DNA Quantification and Quality Check

1. Measure dsDNA concentration using the Qubit dsDNA HS Assay Kit according to the instruction protocol of the manufacturer (*see* **Notes 1** and **8**).
2. Check DNA for integrity on a 0.8 % agarose gel: Mix 100 ng of DNA with an appropriate DNA loading dye and load solution onto the gel. In parallel load a DNA ladder. Run the gel at 120 V for 1 h (*see* **Note 2** and **9**) (*see* Fig. 2a).
3. Store DNA at −20 °C.

3.1.4 RNA Quantification and Quality Check

1. Measure RNA concentration using a UV spectroscopic photometer. For later calculation of input material for library preparation, use this quantification measurement.

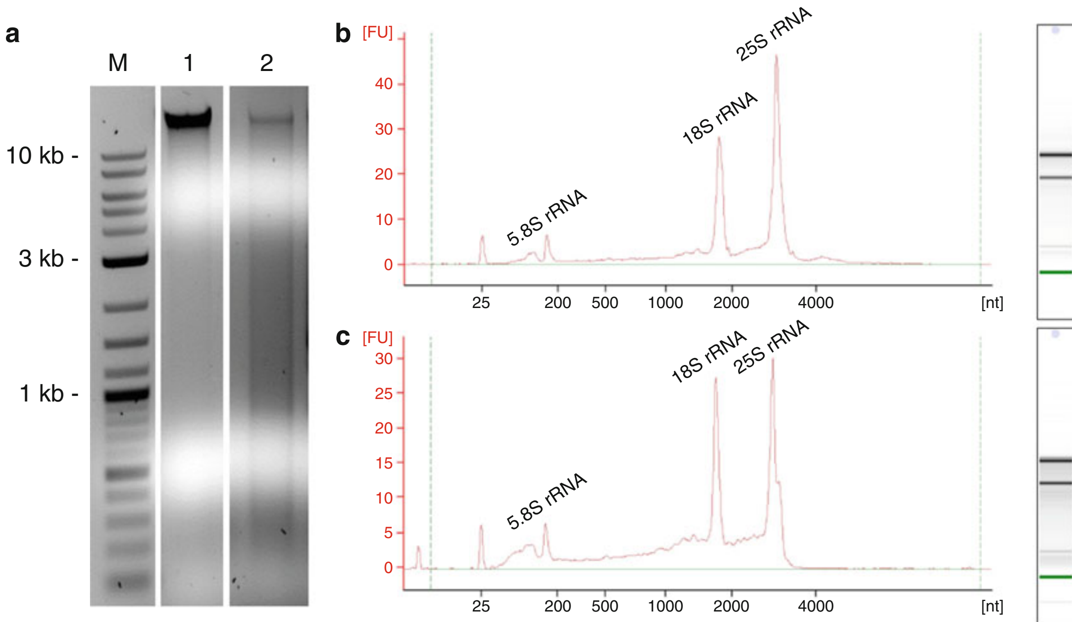

Fig. 2 Quality control of genomic DNA on an agarose gel (**a**) and total RNA on the Agilent Bioanalyzer (**b**, **c**). M = marker, 2-log-ladder; 1 = high quality DNA with nearly no degradation visible; 2 = medium quality DNA with degradation products. A gel extraction of the high molecular weight band in sample 2 would be an option to prevent further DNA isolation (*see* **Note 9**) (**a**). Two examples of total RNA are presented in electropherograms and gel-like images while the upper sample represents high-quality RNA with a RIN of 9 (**b**) and the lower sample already shows slight degradation indicated by the weaker 25S rRNA signal and an overall higher background resulting in a lower RIN of 7

2. Check RNA integrity with the RNA 6000 Nano Kit on the Agilent Bioanalyzer according to the instruction protocol of the manufacturer (*see* **Note 10**) (*see* Fig. 2b, c).
3. Store at −70 °C.

3.2 Whole-Genome Shotgun (WGS) Library Preparation

More information and some practical notes about the sample preparation steps can be viewed in the instructor's manual describing the standard procedures. Basically following those instructions, we still propose some minor modifications facilitating the original protocol. For all the incubation and amplification steps in the thermocycler use a heated lid at 100 °C and insert the tubes/strips after reaching initial temperature. Make sure that all frozen solutions are thoroughly mixed up after thawing and centrifuged briefly before using them. If solutions have to be mixed by pipetting, make sure to gently pipette up and down ten times.

3.2.1 Tagmentation of Genomic DNA

1. Thaw TD (tagment DNA buffer), TDE1 (tagment DNA enzyme) and genomic DNA on ice.
2. Dilute DNA to a concentration of 2.5 ng/μl and add 20 μl of it into a PCR tube.
3. Add 25 μl TD buffer and 5 μl TDE1.
4. Mix the solution by pipetting.
5. Incubate the PCR tube for 5 min at 55 °C, then hold at 10 °C.

3.2.2 Cleanup of Tagmented DNA

1. Thaw RSB (resuspension) buffer on RT.
2. Add 180 μl Zymo DNA Binding Buffer into a fresh 1.5 ml reaction tube.
3. Add 50 μl of tagmented sample and mix the solution by pipetting.
4. Transfer 230 μl of sample mixture on a fresh Zymo-Spin column in a collection tube.
5. Centrifuge for 30 s at 10,000 × *g* at RT and discard the flow-through.
6. Add 200 μl Zymo DNA Wash Buffer on the column (make sure EtOH was added prior to use as indicated on the label).
7. Centrifuge for 30 s at 10,000 × *g* at RT and discard the flow-through.
8. Repeat **steps 6** and 7 once.
9. Centrifuge for 1 min at 10,000 × *g* at RT to make sure no residual wash buffer is present.
10. Transfer the column to fresh 1.5 ml reaction tube.
11. Add 25 μl RSB buffer to the column matrix and incubate for 2 min.
12. Centrifuge for 30 s at 10,000 × *g* at RT (*see* **Note 11**) (*see* Fig. 3a, c).

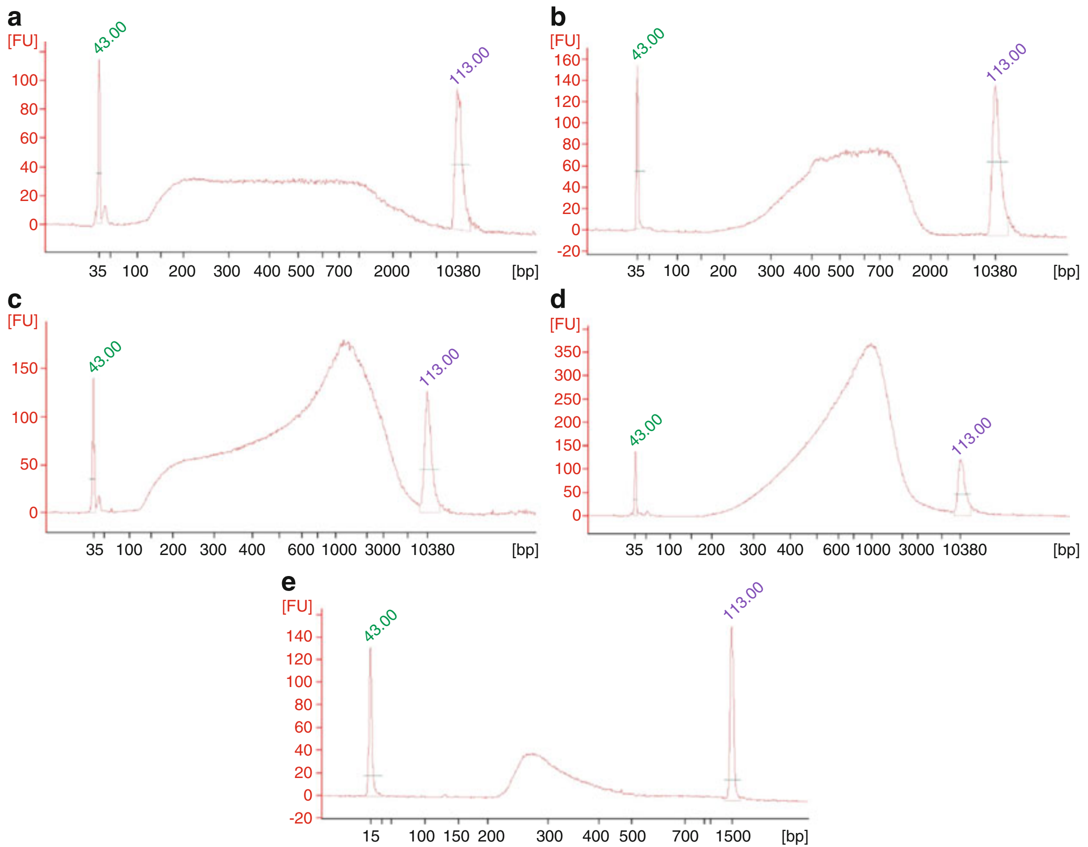

Fig. 3 Generation of successful WGS libraries after tagmentation (**a**, **c**) and after PCR (**b**, **d**) and of one exemplary RNA-seq library (**e**). Nextera libraries are quite variable in size distribution depending on the genomic template: bacterial genome of 5 MB (**a**, **b**) and a fungal genome of 20 MB (**c**, **d**) (*see* **Note 12**). In contrast, RNA-seq libraries should always result in a quite similar quality ranging from 250 to 600 bp (**e**)

3.2.3 PCR Amplification

1. Thaw NPM (Nextera PCR master mix), PPC (PCR primer cocktail) and the index primers (N7xx and N5xx) at RT.
2. Add index primers N7xx and N5xx each 5 μl into fresh PCR tube (*see* **Note 12**).
3. Add 15 μl NPM and 5 μl PPC to the primers.
4. Transfer 20 μl of purified tagmented DNA to the PCR mix.
5. Mix solution by pipetting.
6. Place reaction tube in a thermocycler and subject to PCR with the following conditions: 72 °C for 3 min, 98 °C for 30 s, 5 cycles each at 98 °C for 10 s, 63 °C for 30 s, and 72 °C for 3 min; hold at 10 °C.

3.2.4 PCR Cleanup

1. Bring AMPure XP beads to RT (prepare an aliquot with 30 μl/sample) and vortex them properly.

2. Add 30 μl AMPure XP beads into fresh 1.5 ml reaction tube.
3. Transfer 50 μl of the PCR product to the beads.
4. Mix the solution by pipetting and incubate for 5 min at RT.
5. Place the 1.5 ml reaction tube on a magnetic stand for 2 min or until the supernatant has cleared.
6. Carefully remove and discard the supernatant without disturbing the beads.
7. Wash the beads on the stand with freshly prepared 80% EtOH without resuspending them by 30 s of incubating.
8. Carefully remove and discard the supernatant without disturbing the beads.
9. Repeat **steps 8** and **9**.
10. Allow beads to air-dry for at least 5 min on the stand.
11. Take the 1.5 ml reaction tube off the stand and resuspend beads in 33 μl RSB buffer by pipetting.
12. Incubate for 2 min at RT.
13. Place the 1.5 ml reaction tube on the magnetic stand for 2 min or until the supernatant has cleared.
14. Carefully transfer 30 μl of the supernatant into fresh 1.5 ml DNA low-bind reaction tube.
15. The ready-to-load WGS library can be stored at −20 °C up to several months.

3.2.5 Quality Analysis and Quantification of the WGS Library

1. Dilute your ready-to-load WGS library 1:5.
2. Check DNA library size distribution with the High Sensitivity DNA Kit on the Agilent Bioanalyzer according to the instruction protocol of the manufacturer (*see* **Notes 3** and **13**) (*see* Fig. 3b, d).
3. Measure DNA library concentration with the Qubit dsDNA HS Assay Kit according to the instruction protocol of the manufacturer.

3.3 RNA-Seq Library Preparation

For RNA sample preparation with the TruSeq Stranded mRNA Kit, manufacturer's instructions are very easy to follow and elaborately guide you through the enrichment and fragmentation of mRNA, the first strand and second strand synthesis, the adenylation of 3′-ends, the adapter ligation and a final PCR amplification step by step.

Here again, we basically follow those instructions, but with some minor modifications facilitating the original protocol. Although input recommendation for total RNA ranges from 0.1 to 4 μg, rather use lower amounts between 100 and 500 ng and only 11–13 PCR cycles to avoid over-amplification in final PCR. Also, during sample preparation rather use the outer wells of the

96-well plate to have visual control while pipetting. For all the incubation and amplification steps in the thermocycler use a heated lid at 100 °C and insert the plate after reaching initial temperature. Make sure that all frozen solutions are thoroughly mixed up after thawing and centrifuged briefly before using them and that AMPure XP beads are brought to room temperature (RT) before use. If solutions have to be mixed by pipetting, make sure to gently pipette up and down 10 times. To properly seal the plate always use the Microseal "B" adhesive seal.

3.3.1 Enrichment and Fragmentation of mRNA

1. Bring RPB (RNA purification beads), BBB (bead binding buffer), ELB (elution buffer), and BWB (bead washing buffer) to RT.
2. Dilute 100–500 ng total RNA in a final volume of 50 μl nuclease-free water in a 96-well plate.
3. Add 50 μl RPB and mix by pipetting.
4. Seal the plate and incubate for 5 min at 65 °C in a thermocycler.
5. Remove the plate and place on ice for 1 min.
6. Place the plate on the bench, remove the seal and incubate for 5 min at RT.
7. Place the plate on the magnetic stand (for 96-well plates) for 5 min.
8. Discard the supernatant.
9. Remove the plate and wash the beads with 200 μl BWB by pipetting until beads are thoroughly dispersed.
10. Place the plate on the magnetic stand for 5 min.
11. Discard the supernatant.
12. Remove the plate and elute the mRNA with 50 μl ELB by pipetting until beads are thoroughly dispersed.
13. Seal the plate and incubate for 2 min at 80 °C in a thermocycler.
14. Remove the plate and place on ice for 1 min.
15. Place the plate on the bench and add 50 μl BBB after removing the seal.
16. Mix solution by pipetting and incubate for 5 min at RT.
17. Repeat **steps 7–11**.
18. Remove the plate and elute mRNA with 19.5 μl FPF.
19. Mix by pipetting until beads are thoroughly dispersed and seal the plate.
20. Incubate for 8 min at 94 °C, then hold at 7 °C in a thermocycler.
21. Briefly centrifuge the plate and immediately proceed with first strand synthesis.

3.3.2 First Strand cDNA Synthesis

1. Thaw FSA (first strand synthesis act D mix) and add 50 µl SuperScript II before first use (FSA+).
2. Place the plate on the magnetic stand for 5 min.
3. Transfer 17 µl into a fresh well of the plate and add 8 µl FSA+.
4. Mix by pipetting and seal the plate.
5. Incubate for first strand cDNA synthesis with the following conditions: 25 °C for 10 min, 42 °C for 15 min, 70 °C for 15 min, hold at 7 °C.
6. Take the plate and immediately proceed with second strand synthesis.

3.3.3 Second Strand Synthesis

1. Thaw SMM (second strand marking master mix) and RSB (resuspension buffer).
2. Bring AMPure XP beads to RT (prepare an aliquot with 90 µl/sample) and vortex them properly.
3. Remove seal, add 5 µl RSB and 20 µl SMM.
4. Mix by pipetting and seal the plate.
5. Incubate for 60 min at 16 °C in a thermocycler.
6. Remove seal and add 90 µl AMPure XP beads.
7. Mix by pipetting and incubate for 5 min at RT.
8. Place the plate on a magnetic stand for 2 min or until the supernatant has cleared.
9. Carefully remove and discard the supernatant without disturbing the beads.
10. Wash the beads on the stand with freshly prepared 80% EtOH without resuspending them by 30 s of incubating.
11. Carefully remove and discard the supernatant without disturbing the beads.
12. Repeat **steps 8** and **9**.
13. Allow beads to air-dry for at least 5 min on the stand.
14. Remove the plate and resuspend beads in 20 µl RSB buffer by pipetting.
15. Incubate for 2 min at RT.
16. Place the plate on the magnetic stand for 2 min or until the supernatant has cleared.
17. Carefully transfer 17.5 µl of the supernatant into fresh well of the plate.
18. Proceed with 3′-end adenylation or store the plate at −20 °C up to 7 days.

3.3.4 Adenylation of 3′-Ends

1. Thaw ATL (A-tailing mix).
2. Add 12.5 µl ATL and mix by pipetting.

3. Seal the plate and incubate reaction for 30 min at 37 °C immediately followed by a second incubation step for 5 min at 70 °C.
4. Take the plate, remove the seal and immediately proceed with adapter ligation.

3.3.5 Adapter Ligation

1. Thaw desired RNA adapter indices (AR001-AR027), STL (stop ligation buffer) and RSB.
2. Immediately before use, remove LIG (ligase) from storage.
3. Bring AMPure XP beads to RT (prepare an aliquot with 92 μl/sample) and vortex them properly.
4. Add 2.5 μl RSB, 2.5 μl AR0xx, and 2.5 μl LIG to the reaction in that order.
5. Mix reaction by pipetting and seal the plate.
6. Incubate for 10 min at 30 °C.
7. Take the plate to your bench and remove the seal.
8. Add 5 μl STL and mix by pipetting.
9. For an effective purification of adapter-ligated fragments and removal of residual adapters two sequential cleanups with AMPure XP beads are necessary. Thus please exactly follow the instructions in Subheading 3.3.3 according to **steps 6–17** with the following modifications:
   (a) Cleanup: … use 42 μl AMPure XP beads
   … elute in 53 μl RSB and then transfer 50 μl into the second cleanup.
   (b) Cleanup: … use 50 μl AMPure XP beads
   … elute in 23 μl RSB and the transfer 20 μl into a fresh well of the plate.
10. Either proceed with PCR amplification or store the plate at –20 °C up to 7 days.

3.3.6 PCR Amplification

1. Thaw PMM (PCR master mix), PPC (PCR Primer cocktail), and RSB.
2. Bring AMPure XP beads to RT (prepare an aliquot with 50 μl/sample) and vortex them properly.
3. Add 5 μl PPC and 25 μl PMM to the purified adapter-ligated fragments to reach a final volume of 50 μl.
4. Mix by pipetting and seal the plate.
5. Place the plate in a thermocycler and subject to PCR with the following conditions: 98 °C for 30 s, 11–13 cycles each at 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s; 72 °C for 5 min, hold at 7 °C.
6. Take the plate to your bench and remove the seal.

7. Exactly follow the instructions in Subheading 3.3.3 according to **steps 6–17** with the following modifications: … use 50 μl AMPure XP beads

   … elute in 33 μl RSB and then transfer 30 μl into a into fresh 1.5 ml DNA low-bind reaction tube.
8. The ready-to-load RNA-Seq library can be stored at −20 °C up to several months.

3.3.7 Quality Analysis and Quantification of the RNA-Seq Library

1. Quality analysis and quantification of the RNA-seq library is identical to the WGS library (compare Subheading 3.2.5. and *see* Fig. 3e).

3.4 Sequencing

1. Libraries might be sequenced by a commercial provider or using proprietary sequencing facilities.
2. For the WGS libraries, perform a paired-end Illumina run with at least 2×250 bp on HiSeq in rapid output mode or with 2×300 bp on a MiSeq depending on the options of your provider.
3. You should get at least a 100-fold coverage of sequence reads based on the expected genome size of the respective fungus, e.g., having a genome size of 20 MB you should get at least 2 Gb of high quality data with a phred score > Q30.
4. For the RNA-Seq libraries, perform a single-read Illumina run with at least 20 million reads and a minimum read length of 50 bp.
5. Take the raw *.fastq-files and proceed with the bioinformatics part.

3.5 Bioinformatic Analysis

Install all programs according to developer instructions and add them to your path if necessary. You need to configure Abyss with a higher maximum k-mer size using ./configure --enable-maxk=256 instead of ./configure, because the default value is 65 bp (*see* **Note 14**).

3.5.1 Check Sequencing Run Performance

1. Open terminal and move to folder containing your fastq.gz files:

   ```
   cd<path_to_fast.gz_files>
   ```

   You should now be in a folder containing the fastq.gz files from the DNA and RNA sequencing run.
2. Run commands:

   ```
   fastqc -o fastqc_out --noextract -t 1<file-Name_dna_read_1>.fastq.gz
   fastqc -o fastqc_out --noextract -t 1<file-Name_dna_read_2>.fastq.gz
   fastqc -o fastqc_out --noextract -t 1<file-Name_rna>.fastq.gz
   ```
3. Open the .html files in the fatqc_out folder.

4. Inspect quality of the sequencing run (*see* **Note 15**).

3.5.2 Polishing Reads

1. Trim reads from adapter sequences and low quality called bases below a phred score of Q10 with bbduk (*see* **Note 16**). The adapter file should contain the adapter sequences used in your library preparation kit. For Nextera preparation, trim these two sequences:

   CTGTCTCTTATACACATCTCCGAGCCCACGAGAC (remove from R1)

   CTGTCTCTTATACACATCTGACGCTGCCGACGA (remove from R2)

   Run commands to trim DNA:

```
bbduk.sh -Xmx1g in1=<fileName_dna_read_1>.fastq.gz in2=<fileName_dna_read_2>.fastq.gz out1=<fileName_dna_read_1>_AQ10.fastq.gz out2=<fileName_dna_read_2>_AQ10.fastq.gz minlen=50 qtrim=rl trimq=10 ktrim=r k=25 mink=11 ref=<path_to_adapter_file>/adapter.fasta hdist=1
```

   Run commands to trim DNA:

```
bbduk.sh -Xmx1g in=<fileName_RNA>.fastq.gz out=<fileName_RNA>_AQ10.fastq.gz minlen=50 qtrim=rl trimq=10 ktrim=r k=25 mink=11 ref=<path_to_adapter_file>/adapter.fasta hdist=1
```

2. Filter reads for contamination from Illumina sequencing control. Download the *Enterobacteria phage phiX174 sensu lato* reference genome in fasta-format from NCBI. Remove phiX contamination or other known contamination from the reads.

   Run commands to clean DNA:

```
bbduk.sh -Xmx1g in1=<fileName_dna_read_1>_AQ10.fastq.gz in2=<fileName_dna_read_2>_AQ10.fastq.gz out1=<fileName_dna_read_1>_clean.fastq.gz out2=<fileName_dna_read_2>_clean.fastq.gz ref=<path_to_phix_file>/phix.fasta k=31 hdist=1
```

   Run commands to clean RNA:

```
bbduk.sh -Xmx1g in=<fileName_rna>_AQ10.fastq.gz out=<fileName_rna>_clean.fastq.gz ref=<path_to_phix_file>/phix.fasta k=31 hdist=1
```

3. Check if the trimming and filtering was successful with following fastqc commands:

```
fastqc -o fastqc_out_clean --noextract -t 1<fileName_dna_read_1>_clean.fastq.gz
```

```
fastqc -o fastqc_out_clean --noextract -t 1<fileName_dna_read_2>_clean.fastq.gz
fastqc -o fastqc_out_clean --noextract -t 1<fileName_rna>_clean.fastq.gz
```

3.5.3 Assembly with Abyss

1. Assemble the DNA reads using the Abyss assembler. The optimal k-mer length for assembly can vary strongly; however, it should be between half to two-third of the read length (*see* **Note 17**). Run commands:

```
mkdir assembly_abyss
cd assembly_abyss
export k
for k in {125..165}; do
   mkdir k$k
   abyss-pe -C k$k name=<organismName>
   in='<../fileName_dna_read_1>_clean.fastq.gz
   ../<fileName_dna_read_2>_clean.fastq.gz
done
abyss-fac k*/<organismName>-contigs.fa
cd ..
```

2. *A*byss-fac will create a file containing the assemble stats for each k-mer. Choose the best assembly based on the given values: a large N50, a low number of contigs and an adequate number of assembled bases around the predicted genome length.
3. Copy the <organismName>-contigs.fa to the folder containing your cleaned reads and rename it to <*organismName*>.fasta.

3.5.4 Genome Annotation

1. Generate index from the assembled genome for the HISAT mapper with following commands:

```
mkdir index_hisat
hisat-build <organismName>.fasta hisat_index/<organismName>
```

2. Map RNA-seq reads onto assembled genome:

```
hisat -x index_hisat/<organismName>-U<fileName_rna>_clean.fastq.gz | samtools view -bS -><organismName>.bam
```

3. Sort bam file so we can use it as input for BRAKER1:

```
samtools sort<organismName>.bam<organismName>_sorted
```

4. Run BRAKER1 on the assembled genome and the RNA-seq reads using the following command:

```
braker.pl --fungus --species=<organismName>-genome=<organismName>.fasta --bam=<organismName>_sorted.bam -workingdir=<organismName>_braker
```

5. Extract protein sequences from the BRAKER1 output:

```
<path_to_augustus>/scripts/getAnnoFasta.pl
../braker/<organismName>/augustus.gff
mv /braker/<organismName>/augustus.aa <organ-
ismName>.faa
```

6. Parse output file from BRAKER1 to gff format.

```
perl <path_to_augustus>/scripts/gtf2gff.
pl < ../braker/<organismName>/augustus.gff
--gff3 --out=<organismName>.gff
```

7. Run InterProScan to gather information about our predicted genes with following command:

```
interproscan.sh -i organismName.faa --goterms
-pathways -b<organismName>
```

8. Blast against SwissProt to retrieve gene names from verified genes which share a high similarity with our predicted genes (*see* **Note 18**):

```
blastp -db<path_to_swissProt_db>/uniprot_sprot.
fasta -query organismName.faa -out organismName.
blast -evalue 1e-06 -outfmt 6
```

9. Run ANNIE to generate the input for the next step to get a fused file from blast and InterProScan output compatible with subsequent converter GAG by using following command:

```
python3<path_to_annie>/annie.py -b<organ-
ismName>.blast -ipr<organismName>.tsv
-g<organismName>.gff -db<path_to_swis-
sProt_db>/uniprot_sprot.fasta -o<organis-
mName>.annie
```

10. GAG merges the prepared ANNIE file with the gff file from BRAKER1 to create a tbl file format usable as input for tbl2asn to create a submission for GenBank and two fasta files containing the assembled genome and the protein sequences (*see* **Note 19**).

```
python2<path_to_gag>/gag.py --fasta<organ-
ismName>.fasta --gff<organismName>.gff
--anno<organismName>.annie --out final_anno-
tation --fix_start_stop
```

4 Notes

1. Alternatively, measuring DNA with UV spectroscopic photometers like a NanoDrop is also possible. However, an exact concentration of double-stranded DNA (dsDNA) given by the Qubit dsDNA HS Assay Kit is necessary to prepare an optimal library with Nextera method [21].

2. Alternatively, the application of a Fragment Analyzer (Advanced Analytical Technologies) to check the integrity of genomic DNA with the Genomic DNA Analysis Kit is more accurate than is the analysis using conventional agarose gel electrophoresis.
3. Alternatively, DNA library or RNA integrity measurement on a Fragment Analyzer (Advanced Analytical Technologies) can also be performed using the NGS Fragment or RNA Analysis Kit, respectively.
4. There are far more protocols to prepare DNA libraries for WGS sequencing using Illumina, but we chose the Nextera method because of its simplicity mainly reflected in the tagmentation step where fragmentation and adapter ligation take place in one reaction directly followed by final PCR. Nearly each other protocol requires a separate fragmentation step either by sonification (Covaris) or by a biochemical reaction with a fragmentase (NEB) followed by end repair plus optionally A tailing enabling the ligation of adapters. Independent of how many steps a library preparation would take, a PCR-free protocol should be applied for genomes with high GC contents of above 65% (TruSeq DNA PCR-Free, Illumina). Alternatively, for assembling genomes to scaffold or even to chromosome level with Illumina short reads, a more comprehensive protocol for mate pairs should be considered to possibly overcome repetitive regions of up to 10 kb or even a long read technology like PacBio or Oxford Nanopore could be considered.
5. For large-scale genome projects, there is a high throughput option for preparing Nextera libraries in 96-well plates. Use ZR-96 DNA Clean & Concentrator-5 Kit, 96-well PCR plates, a magnetic stand for 96-well plates and Microseal "B" adhesive seals.
6. For the preparation of libraries out of RNA one can also use alternative sample preparation protocols, just make sure they enrich for polyA$^+$ RNA and that they contain strand-specificity.
7. Particular care is necessary to avoid any contamination with RNAses. Therefore, any parts of the Teflon grinding jar and ball should be pretreated with 0.5% sodium hypochlorite for 10 min. Afterwards, wash with 70% EtOH and blow dry using nitrogen.
8. If concentration is too high, make sure to dilute the DNA to be in the range for the Qubit dsDNA HS Assay Kit between 10 pg/μl and 100 ng/μl.
9. High quality of genomic DNA for *de novo* genome sequencing is an indispensable prerequisite. Thus, if it is not possible to isolate DNA without already degraded fragments, you can extract the high molecular weight band from an agarose gel if still present, e.g., with the MinElute Gel Extraction Kit

(Qiagen). Just make sure to load enough DNA onto the gel as loss of DNA might be up to 50%.

10. Make sure to use RNA with an RNA integrity number (RIN) > 7. Integrity below this value indicates already degraded molecules. Using such a template might already decrease quality of RNA-seq results.
11. Here, you can check the tagmentation step on a Fragment Analyzer (Advanced Analytical Technologies) or Bioanalyzer (Agilent) with the corresponding high sensitivity DNA kits (compare Fig. 3a, c).
12. Make sure you do not cross-contaminate index primers if preparing more than one sample simultaneously, e.g., in 96-well plates, as this step is crucial for the purity of a sample. It is also recommended to exchange index primer caps with new ones (included in the kit) after using them once. Make your notes immediately when preparing the samples which barcode combination is assigned to which sample.
13. If fragment size distribution of the ready-to-load library is either too short or too long with an average size significantly below 500 bp or significantly above 2000 bp, respectively, you might adapt initial input amount of DNA slightly: for too short libraries use 70 ng or even more and for too long libraries use 30 ng or even less (compare Fig. 3b, d). Depending on the genome and the quantification method used, this optimization helps in getting optimal results from the sequencing run.

Sometimes co-isolated contaminants might disturb tagmentation reaction. To possibly prevent such disturbance, do an additional purification of DNA preferably with a bead-based method (e.g., AMPure XP Beads, Beckman Coulter).

14. Many tools are multi-threaded, look for --threads/--cpus/-p parameter in the respective manual.
15. Significant deviation of “per sequence GC content” from the normal distribution, especially additional peaks or bumps can be a hint for contaminations. Further, be aware that if many bases are below a phred score of Q30 there is a higher chance of errors in the assembly. At the fastqc website you can also find examples for good and bad sequencing runs.
16. Alternatively, you can use also other trimming tools like cutadept [22], trimmomatic (http://www.usadellab.org/cms/index.php), or fqtrim (https://ccb.jhu.edu/software/fqtrim/index.shtml).
17. Most assembler for Illumina reads uses the de Bruijn graph method like Abyss, Velvet, SPAdes, and SOAPdenovo [15, 23–25]. In our workflow, we use the Abyss assembler optimal for Illumina paired-end reads for 2 × 150–2 × 250 bp. However,

there are also other assemblers using different algorithms like Celera [9, 10, 26] which can be utilized for long read technologies like PacBio. Those long reads are optimal for scaffolding or gap closing of for example already assembled Illumina contigs or can also be used exclusively if sequence coverage is high enough (>50- to 100-fold) [27].

18. To search exclusively for fungal proteins or domains you can also use a custom database containing only fungal sequences, e.g., created from the RefSeq database.
19. GAG ignores lines in the gff file which does not contain an ID tag. The –fix_start_stop parameter fixes this by adding the start and stop codons again. Introns are ignored by GAG even with ID tag.

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Chapter 22

Microarray Technologies in Fungal Diagnostics

Steffen Rupp

Abstract

Microarray technologies have been a major research tool in the last decades. In addition they have been introduced into several fields of diagnostics including diagnostics of infectious diseases. Microarrays are highly parallelized assay systems that initially were developed for multiparametric nucleic acid detection. From there on they rapidly developed towards a tool for the detection of all kind of biological compounds (DNA, RNA, proteins, cells, nucleic acids, carbohydrates, etc.) or their modifications (methylation, phosphorylation, etc.). The combination of closed-tube systems and lab on chip devices with microarrays further enabled a higher automation degree with a reduced contamination risk. Microarray-based diagnostic applications currently complement and may in the future replace classical methods in clinical microbiology like blood cultures, resistance determination, microscopic and metabolic analyses as well as biochemical or immunohistochemical assays. In addition, novel diagnostic markers appear, like noncoding RNAs and miRNAs providing additional room for novel nucleic acid based biomarkers. Here I focus an microarray technologies in diagnostics and as research tools, based on nucleic acid-based arrays.

Key words DNA microarray, SNP analyses, Molecular diagnostics

1 Introduction

Microarrays are defined as a set of ordered probes on a solid support. Such arrayed probes exist since the mid to late seventies when Gergen et al. [1] created the first documented ordered arrays of *E. coli* containing individual plasmids, the precursor of ordered cDNA libraries contained in *E. coli* in the 1980s. Identification of the DNAs of interest at that time was done using radioactively labeled nucleic acids, which hybridized to the clone of interest. The development of fluorescence-based detection methods, the progress in DNA synthesis and the availability of sequenced genomes enabled the development of a novel genome wide technology based on microarrays. In 1995, Schena and colleagues published a revolutionizing method for transcriptional analysis using multiple cDNAs spotted in high density onto glass

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slides [2]. This pioneering work may be considered as the birth of modern microarray technologies. It enabled differential expression measurements using simultaneous, two-color fluorescence hybridization as well as species identification or genotyping. This development cannot be separated from the genome-wide sequencing programs which started in the late 80ties and resulted in the publication of the first genomes in the mid-nineties, with the genome of *S. cerevisiae* being the first eukaryotic genome published [3]. This led to the decoding of the human genome only 5 years later [4, 5]. The knowledge of the genomes made it possible to represent each predicted gene or transcriptional unit of a genome as a probe on a microarray. Prior to microarray technology, genome wide transcriptional profiling was not possible. Consequently, the early publications focused on the generation and analysis of genome wide transcriptional profiling in *S. cerevisiae* [6, 7]. Array technologies initially developed for multiparametric nucleic acid detection from there on rapidly developed towards a tool for the detection of all kind of biological compounds (DNA, RNA, proteins, cells, nucleic acids, carbohydrates, etc.) or their modifications (methylation, phosphorylation, etc.) within the last 20 years. The basic principle as indicated above is simple. An array consists of a defined number of ordered probes immobilized on a solid support targeting molecules like for example mRNA, genomic DNA, proteins, or lectins. The biological interaction of these molecules now can be monitored by detecting their interaction with the respective, generally labeled ligands at the defined spot on the array, like the interaction of complementary bases of nucleic acids, antibody–antigen interactions or the interaction of carbohydrates with lectins for example. In addition to these two dimensional or planar arrays suspension bead arrays have been developed which are based for example on the immobilization of the probes on microscopic polystyrene beads as the solid support and flow cytometry for bead and target detection [8]. By that they follow a concept significantly different from planar arrays. Here reactions take place in solution and not on a planar surface; however, the multiplexing capacity is limited. Multiplexing here is accomplished for example by using different microsphere sets based on a mix of two colors. This enables the generation of a clearly distinguishable set of about 100 different microbeads, which can be analyzed individually after coupling to the respective probe. Originally established for detection of antibodies and antigens [9], nowadays several FDA-cleared nucleic acid based assays for pathogen detection exist. I provide some protocols on fabrication and application of microarrays, focusing on infection diagnostics and SNP detection using planar oligonucleotide arrays.

1.1 DNA Microarrays: Development, Production, and Application

1.1.1 Microarray Development

Microarrays enable the parallel analysis of several tens of thousands of analytes including nucleic acids, proteins and carbohydrates among others in contrast to most classical biological assays. Already the application possibilities of nucleic acid based arrays range from a focused set of several genes for diagnostics for example to detect pathogens or SNPs (single nucleotide polymorphisms) for determining resistance phenotypes, up to the transcriptomic profiling or resequencing of entire genomes [10–14]. To set up a microarray oligonucleotides complementary to the respective target molecule like mRNA, miRNA, or genomic DNA are immobilized or synthesized directly on solid supports like glass, silicon, nylon, or other polymers in an ordered manner. These oligonucleotides may correspond to several defined diagnostic targets, e.g., for pathogen identification or to all open reading frames of an organism to allow transcriptional profiling. They might even contain overlapping fragments of an entire genome, the so-called tiling arrays for monitoring transcriptional activities over entire chromosomes [15, 16] or detecting interaction of transcription factors with DNA (Chromatin IP, ChIP-on-chip analyses [17, 18]). In order to detect the hybridization event, the most commonly used method is to label the RNA using nucleic acid polymerizing enzymes and fluorescently labeled nucleotides. Hybridization to the microarray then leads to a specific molecular interaction at the location where the complementary strand is immobilized and thereby fluorescent labeling of the respective spot. Read out using a fluorescence scanner with a photomultiplier tube or imaging with a CCD camera will give the information if the target molecule is present in the analyzed sample or not in a semiquantitative manner [14] (*see* also Fig. 1). Next to the

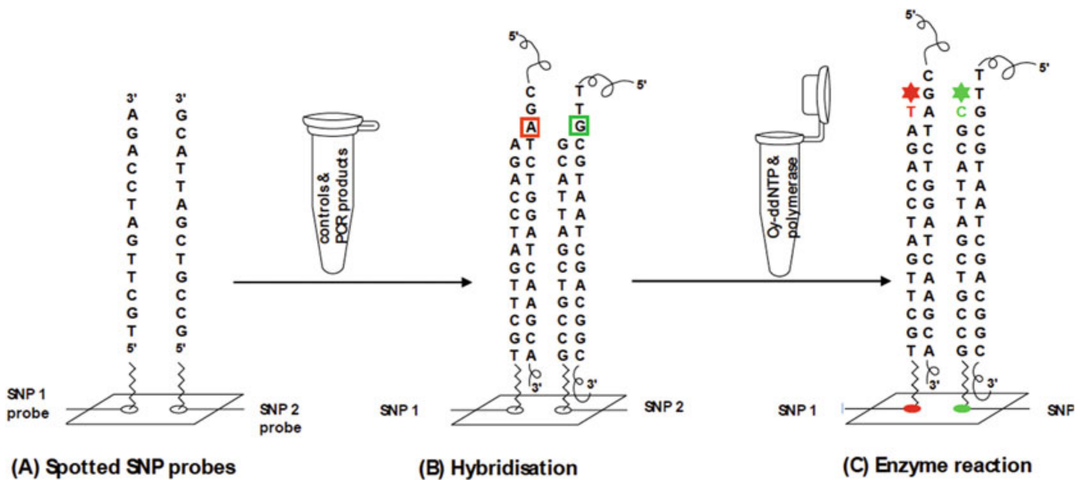

Fig. 1 Illustration of APEX-reaction scheme shown for two SNP positions. (**a**) Two probes (SNP probe 1 and 2) are spotted on glass-slides. (**b**) Hybridization of labeling-control-templates and DNA sample (e.g., PCR products). (**c**) Enzymatic incorporation of one corresponding ddNTP using thermosequenase. The SNP nucleotides are marked with *green* and *red asterisk*, respectively

direct labeling, also indirect labeling techniques have been used. For example biotin-labeled nucleotides are incorporated during PCR und afterwards detected using streptavidin or antibody-conjugates. Another example is the colorimetric Silverquant®-technology used by Eppendorf for their DualChip®-microarrays. Biotin-labeled nucleotides are used in the PCR-reaction. Gold-coupled antibodies against biotin are added after hybridization and the colorimetric reaction is started by adding silver nitrate and a reducing agent. This leads to silver precipitation at the gold particles. Furthermore, streptavidin–horseradish peroxidase conjugate for colorimetric detection [19] has been used (for review *see* [20]).

In the last century the number of sequenced organisms was rather small, including 38 bacteria, one fungus (*S. cerevisiae*), two invertebrates (*Caenorhabditis elegans* and *Drosophila melanogaster*), and one plant (*Arabidopsis thaliana*), all with relatively small and simple genomes. About 10 years later the catalog of sequenced organisms literally exploded giving ample opportunities to set up highly specific microarrays. The NCBI update from October 2013 reported that the number in public archives accounted for 24,788 prokaryotic registered genome projects representing 4528 different species; 14,311 of them have assembled genomes either complete (2670) or draft (11,641), and the remainder either do not have submitted sequence data yet or have only raw sequence reads uploaded to Sequence Reads Archive [21]. The major reason for this expansion in genome sequencing is the development of next-generation sequencing technologies (NGS) at the beginning of the twenty-first century [22] which is described in detail in another chapter of this book. This huge amount of genome sequences now enables completely new approaches to medical research and diagnostics, including the development of diagnostic microarrays. Other than for proteomics, where proteome wide analyses were in principle possible using high-resolution protein separation and MS-technologies without the knowledge of the genomes, this is indispensable for the setup of DNA-microarrays. Consequently, the knowledge of the genomes of individual organisms set the start for genome-wide analyses based on microarrays. Indeed for *S. cerevisiae* the first genome-wide transcriptional analyses appeared shortly after the publication of its genome sequence [6, 23, 24]. Therefore, *S. cerevisiae* as one of the major model organisms was central in developing both the technology and biochemistry, as well as the bioinformatic methods required for transcriptional profiling. Recognizing the wealth of data generated from genome-wide analyses, including thousands of transcription profiles from almost all sequenced species, methods to analyze the data are a prerequisite for making meaningful use of these data-sets. Therefore, microarray technologies introduced a wealth of mathematic and statistical analyses into biology. This trend is further boosted by the generation of even larger amounts of data using

NGS-technologies. Several books on Microarray technology and data analysis have been written which introduce perfectly into these topics [12, 25].

1.1.2 DNA-Microarray Production

Although there are many different ways to produce microarrays, three mayor ways to manufacture DNA microarrays have been dominating the field: light directed synthesis (incl. photolithography), piezoelectric ink jet printing, and robot spotting [12, 26, 27]. The two methods used initially were the robot-based spotting of premade oligonucleotides or PCR-products onto a solid support using split- or solid pins. Using split pins, DNA is fed in by capillary forces and deposited at defined locations on the microarray using robot technology, enabling the spotting of around 100 slides in a row with a few 10 thousands of spots per slide. This is sufficient for a genome wide analysis of most microbial genomes [2, 6]. This method was developed and used directly by many research labs and later was provided by companies offering the respective services. Due to improvements in oligonucleotide synthesis and the concomitant dropping in costs, the next generations of these arrays generally used oligonucleotides as probes. This also resulted in a higher accuracy in detecting the individual gene or transcriptional unit using the most discriminative sequence selected by bioinformatics [28, 29]. At the same time as the first printed arrays appeared, arrays based on light directed synthesis using photolithographic masks were presented by Affymetrix [30, 31]. The photolithographic masks have to be generated for each nucleotide in an oligomer (four times) and are the cost determining factor for this type of array. Probes in general are 20–25 nt in length, and 22–40 probes per gene are synthesized. Nimblegen introduced micro-mirror devices for this type of photolithographic technology, directing the light for cleaving protective groups directly on the desired spot on the array. This rendered the need for photolithographic masks dispensable and thereby the technology much cheaper. Shortly thereafter, the piezoelectric ink jet printing technology was used for either directly spotting DNA molecules or synthesizing them step by step on a solid support using standard phosphoramidite chemistry [26]. This technology was commercialized by Rosetta Inpharmatics and licensed to Agilent which is currently running this technology (Agilent sure print technology, https://www.chem.agilent.com/Library/technicaloverviews/Public/5988-8171en.pdf).

In addition to arrays where the target molecule directly interacts with the probe on the surface of the array, methods have been developed where the interaction of target and probe takes place in solution. Only later, after a completed in solution reaction it is transferred to a solid support, where it addresses a defined spot via a defined bar-code on the probe, the so called ZIP code. These ZIP codes can be covalently linked to probes addressing targets like RNA, DNA or even peptides/epitopes or chemical molecules.

This method enables setting up an universal array platform using uniform sets of defined ZIP-code oligonucleotides onto which any type of probe can be applied to [29, 32].

Not only natural DNA but also modified nucleic acids have been used as probes or capture molecules on DNA-microarray systems. The main reasons for these developments were (1) to reduce cross-reactivity and (2) to increase interaction with the targets in the samples applied. Nonnatural variants that have been used as capture molecules include L-DNA [33] and PNA-variants [34]. L-DNA probes have the great advantage that they interact with the same kinetics with its antiparallel L-DNA strand to an L-DNA duplex. Thus all the knowledge gained with D-DNA-microarrays can be used for the L-DNA array. The L-DNA probes for this purpose are fused to a D-DNA oligomer complementary to the respective target. The L-DNA part of the oligomer (fused to the D-DNA) addresses an immobilized complementary L-DNA oligonucleotide on the microarray surface, as described for the ZIP-code arrays mentioned above. This allows a literally background-free hybridization on the L-DNA arrays, since D-DNA and L-DNA do not hybridize at all [33]. These so-called ZIP-code arrays have been used for example for transcriptional profiling, using natural D-DNA oligomers complementary to the target DNA/RNA which is fused to an L-DNA. Unfortunately, the cost for synthesizing L-DNA oligomers is rather high, hampering further development. In addition to L-DNA arrays synthetic nucleic acid analogs based on a pseudo-peptide backbone, PNAs, instead of a phospho-diester backbone have been developed. They show excellent sequence specific recognition properties and are less susceptible to changes in ionic strength. Since PNAs are not charged, repulsion between the negatively charged backbone present in DNA-duplexes does not occur, resulting in higher affinities and therefore also higher melting curves of PNA/DNA or PNA/RNA duplexes if compared to their natural counterparts [34–36].

In addition to planar array systems on solid support, bead based systems involving synthesizing DNA on small polystyrene beads have been developed, like random bead arrays or suspension arrays. If beads are marked for example with different amounts of fluorescent dye they are individually identifiable. After coupling for example oligonucleotides to such beads, a specific binding event can be detected using fiber optics [37] or FACS as in the Luminex xMAP technology.

Since the beginning of this century, DNA microarrays have been used widely as a research tool and were developed as diagnostic tools. This is reflected in the huge amount of literature that has been published in this field. End of August 2015 more than 2200 reviews and more than 40,000 original research publications are listed in PubMed with the search strings “microarray” and “gene expression analysis” (“Microarray” by itself gives almost 70,000 hits)

that deal with topics like, cancer research [38–42], molecular karyotyping [43], chromosomal microarray analysis [11] phylogenetics/microbiome [44], or gene regulation via ChIP-Chip [45, 46] just to name a few applications. DNA microarrays have been used to study heart diseases [47], aspects in dermatology [48] or mental disorders like autism [49] and many other topics. Looking at "microarray" and "diagnostic" as search terms more than 20,000 hits are given in PubMed, indicating that a significant part of microarray development is devoted to setting up diagnostic systems mostly for medical diagnostics.

1.1.3 Fungal Diagnostics

Fungal pathogenesis has been thoroughly investigated using microarrays. DNA microarrays for the major fungal pathogens have been designed and produced shortly after the technology was established. A comprehensive review on the early developments in array development to unravel fungal pathogenicity can be found at [50]. Some of the major findings, facilitated by DNA-microarrays were the identification of the mechanism of mating in *C. albicans*, transcriptional regulatory networks governing morphogenesis, regulation of cell wall biogenesis, the concept of rewiring transcriptional circuits between organisms, unraveling resistance mechanisms, drugs discovery and target identification, and host–pathogen interaction just to name a few [51–66]. These studies revealed not only mechanistic insight into fungal pathogenesis but also novel biomarkers for diagnostics like markers related to drug resistance for example.

The first generation of diagnostic arrays developed for fungal diagnostic focus on species detection via conserved regions of the genome. They are based in general on either the 28S fungal rRNA gene or the intergenic transcribed spacer (ITS). Focusing on one target allows using broad range PCRs amplifying all potential pathogens with one or just a few primer pairs. This strongly reduces the complexity of the PCR and the complications associated with it. The key for such a broad range PCR is to target the right gene or genomic region. On one hand this gene should be conserved enough to cover all microorganisms targeted with one amplification reaction. On the other hand it should be polymorphic enough to provide sufficient discriminatory power for the individual species. The rRNA genes and the ITS region are currently the most appropriate genes for this purpose. In addition enough sequence information from many isolates of one species is available to ensure specificity of the selected probes.

One of the earliest diagnostic arrays focusing solely on pathogenic fungi was designed to detect the 12 most common pathogenic *Candida* and *Aspergillus* species [67]. Oligonucleotide probes were designed based on sequence variations of the internal transcribed spacer (ITS) regions using a universal PCR amplifying the fungal ITS1 and ITS2 target regions. The array was validated by

using 21 clinical isolates as blinded samples. Several other arrays based on ITS or ribosomal genes have been published since then [68–71].

Arrays focusing on resistance of fungi to antimycotica are sparse. One reason for this is that resistance in fungi is either an intrinsic property of the species or gained by accumulating individual SNPs. For example it is known that *C. krusei* is generally resistant to Fluconazole, which is also true in a similar way for *C. glabrata* [72], whereas resistance in generally susceptible strains, like *C. albicans* occurs via gain of function mutations often by a combination of several individual point mutations during long term treatment of patients which are more difficult to characterize [73, 74]. Therefore only few attempts to develop diagnostic arrays for detecting resistance mechanisms by analyzing the respective SNPs in the genomes of *C. albicans* and *C. glabrata*, based on the known gain of function mutations have been undertaken [75].

Several diagnostic assays to detect fungal pathogen have been cleared by the FDA. For example a suspension array based diagnostic test detecting the five most relevant Candida spp. (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, and *C. krusei*) uses supramagnetic nanoparticles coated with defined Candida capture molecules. The sensitivity of this assay has been given with 3–11 CFUs per ml, depending on the species. This technology enables the identification of Candida within patient blood samples without the need for purification or extraction of target molecules and takes about 3–5 h [76]. The FDA and based its approval on a clinical study of 1500 patients, in which this test correctly categorized nearly 100 % of the negative specimens as negative for the presence of yeast [77]. In a separate clinical study of 300 blood samples with specific concentrations of yeast, T2Candida correctly identified the organism in 84–96 % of the positive specimens. The system represents a fully automated assay, requiring only the blood sample (several ml) to be plugged into the respective cartridge. T2Candida is manufactured by T2 Biosystems, Inc. in Lexington, Mass., USA.

These examples already show that fungal diagnostics using microarrays has been demonstrated successfully, including the first commercial applications.

1.1.4 Methods for SNP Analysis

Several SNPs in *C. albicans* and *C. glabrata* are known to be linked to resistance to azoles. In order to detect these SNPs the APEX (Arrayed primer extension) method is a suitable method for detection. It allows a combination of many resistance associated SNP in addition to fungal species identification on a single diagnostic device for monitoring of acquired and intrinsic resistance. The APEX reaction, initially called minisequencing, was first described by Syvänen et al. [78]. It consists of an annealing reaction between probes spotted on a solid support and the targets, which were chosen for analysis. The basic principle makes use of

differently labeled ddNTPs which are incorporated by DNA polymerases at the following 3′ nucleotide after correct hybridization of probes and target ssDNA. The use of ddNTPs prevents a further elongation, resulting in only one fluorescently labeled nucleotide to be integrated (Fig. 1). Using four differently labeled dyes, each nucleotide in a sequence can be identified clearly. Depending on the Array scanner four different dyes can be used. However, this requires careful examination of laser excitation wavelength and excitation and emission properties of the dyes selected in order to avoid cross talk of the dyes. The sequenced position can be analyzed using fluorescence scanners in a similar way as for other microarray applications. The APEX-reaction is illustrated in Fig. 1. To control hybridization and enzymatic incorporation, labeling-controls are spotted and their corresponding synthetic templates are added to the sample to be analyzed prior to hybridizations. Both the hybridization reaction and the enzymatic reaction can be performed in one step.

2 Materials and Equipment

The methods described here have been developed mainly within the PhD-theses of Stefan Hartmann, Michaela Mai, and Sonja Weishaupt [75, 79, 80].

2.1 Equipment

1. Microarray spotter, e.g., Microgrid II (Biorobotics), equipped with SMP3 splitpins (Array It) or MicroSpot 2500 Splitpins (Fig. 3) (*see* **Note 1**).
2. Microarray scanner, e.g., Genepix 4300A (Molecular Devices).
3. UV-stratalinker 1800; Stratagene.
4. Cabinet dryer; Memmert GmbH.
5. Heat-controlled water bath (Memmert, GER).
6. Reaction chambers (GeneFrames, ABGene) for hybridization and on chip reactions.
7. Thermomixer (Eppendorf).
8. Megafuge 1.0, Heraeus.

3 Materials

1. Coated microscopic slides (Epoxy-, amino-, aldehyde-coated slides).
2. Spotting buffer according to the slide manufacturer (see below).
3. Modified oligonucleotides (see below, e.g., probes require a 5′ amino-C6 modification for covalent immobilization on epoxy slides).

4. Blocking solution according to the slide manufacturer (see below).
5. 0.1 % (v/v) Triton X-100.
6. 1 mM HCl.
7. 100 mM KCl.
8. ddH_2O.
9. 0.2 % SDS.
10. Aminosilane prehybridization buffer (3× SSC, 0.1 % SDS, and 10 mg/ml BSA).
11. Aldehyde blocking solution (1.0 g $NaBH_4$, 300 ml PBS, 100 ml ethanol; freshly prepared 30 min ahead of use).
12. Primer for ERG11 amplification in 5′-phosphorylated form or without modification:
    ERG11-(rv) 5′-ACACTGAATCGAAAGAAAGTTGCCG-3′.
    ERG11-(fw) 5′-AATAGACAAAGAAAGGGAATTCAAT CG-3′.
13. Qiagen Phusion polymerase.
14. dNTPs, PCR grade, 10 mM.
15. $MgCl_2$, 50 mM.
16. λ exonuclease.
17. DNAse I.
18. 10× annealing buffer (1 mM EDTA (pH 8.0), 2 M NaCl, 1 % (v/v) Triton X-100).
19. Thermosequenase (GE Healthcare).
20. 1 mM DTT.
21. 0.1 mM cy3-ddCTP (PerkinElmer).
22. 0.1 mM cy5-ddUTP (PerkinElmer).
23. 0.1 mM fluorescein-ddGTP (PerkinElmer).
24. Wash buffer I: 2× SSC, 0.2 % (w/v) SDS.
25. Wash buffer II: 2× SSC.
26. Wash buffer III: 0.2× SSC.

4 Methods

4.1 Design of Oligonucleotides for SNP Detection

As a prerequisite the association of the SNP with a resistance phenotype has to be known. We could rely on an array of SNPs determined within the EURESFUN FP6 project (e.g., [74]) and from literature. For probe design, sequence alignments have to be performed for each gene separately using a standard DNA-software tool, like ClustalW/Clustal X for example. It is advisable to collect all available sequence information (e.g., in NCBI) for the gene to be analyzed and align it in order to identify variabilities which

Fig. 2 SNP-flanking region of DNA double strand. SNP position marked in *red*. Sense probe is highlighted in *blue*, antisense probe in *orange*

could be detrimental to probe design. For each SNP two independent oligomers, a sense and an antisense probe can be designed as shown in Fig. 2 below.

The probe sequence for sense probe(s) marked in blue and for antisense probe (as) marked in orange frame the SNP flanking region (Fig. 2). Hence, two completely independent probes with different sequences and thermodynamic parameters may be developed, acting as internal controls (*see* **Note 2**).

For a set of *ERG11* and *TAC1* probes [74] designed this resulted in oligonucleotide length between 19 and 30 nucleotides. All developed probes require a 5′ amino-C6 modification for covalent immobilization on epoxy slides (or other modifications for other slides, see below) to facilitate hybridization and on chip enzymatic reactions. As control oligomers, which are helpful in validating the array-system and for quality control, the corresponding synthetic templates of the probes may be designed as reverse complementary oligonucleotides with an overhang of three nucleotides on their 5′-end. Several software tools exist to check the quality of designed probes in silico as mentioned above. Furthermore, all probes should be analyzed for potential cross talk with fungal or human DNA, for example using blast analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Positively evaluated probes are immobilized on the respective modified glass slide as described below in order to create the array.

4.2 Spotting of Oligonucleotides on Microarrays

Spotting should be performed at RT and with a relative humidity (rh) of 50–60 % to achieve spot diameters between 100 and 120 μm and a spot to spot distance of 320 μm (between spot centers) using SMP3 splitpins. The oligos in general are provided in 384-well plates (preferably in a cooled cabined within the spotter), from which they are transferred onto the slides by the robot (*see* also **Note 1**) It is highly advisable to test the combination of spotting buffer and slides used with fluorescently labeled oligonucleotides to ensure optimal quality of spotting. Figure 3 shows two different outcomes of amounts and size of the immobilized oligonucleotides resulting from differences in the spotting buffer only.

4.2.1 General Procedure for Spotting

1. Select slides, e.g., epoxy-coated slides (Schott Nexterion) (*see* **Note 3** and Fig. 4).
2. For epoxy-coated slides use amino-modified oligonucleotides (5′ amino-C6 oligomers provided in 384-well plates).

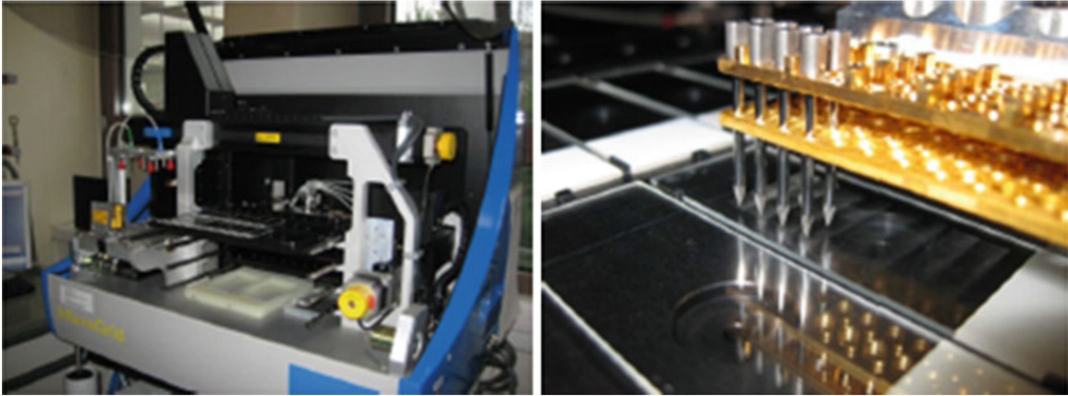

Fig. 3 BioRobotics MicroGrid II Spottingroboter (*left*); Pintool using 8 SMP3 splitpins in parallel during the spotting process on epoxysilane-coated slides

Fig. 4 Evaluation of spotting buffer. Two distinct spotting buffers were tested in spotting of fluorescently labeled amino-modified oligonucleotides on epoxy-coated slides. Slides were treated after spotting as described in Subheading 5.3. and scanned immediately afterwards

3. Select spotting buffer as indicated above. For epoxy coated slides from Schott Nexterion use the provided buffer as indicated (SpotI/SpotIII (1:3), Schott Nexterion).
4. Generate a spotting plate with a concentration of 50 μM of 5′ amino-C6 oligomers in 384-well plates (e.g., from a stock plate generated in Subheading 2) in the selected spotting buffer.
5. Place all components in your Microarray spotter according to the manufacturer's instructions. Start the spotting process of oligonucleotides onto the slides. Here we use the Microgrid II arrayer with SMP3 splitpins (*see* **Note 4**).

4.3 Post-processing of Microarrays

Subsequently to spotting, probes have to be immobilized by covalently linkages depending on the surface chemistry of the slides. Furthermore, in order to avoid unspecific binding of nucleic acids, the slides have to be pretreated prior to hybridization. For three individual slide types the prehybridization procedure is given below:

4.3.1 Epoxy Coated Slides

1. After printing, slides have to be incubated for 30 min in a humidity chamber (90% rh).

   Then baking at 60 °C, for 30 min is required (cabinet dryer; Memmert GmbH).

2. Prehybridization treatment is performed at RT using three washing steps in washing buffer

   0.1 % (v/v) Triton X-100 for 5 min.
   1 mM HCl for 4 min.
   100 mM KCl for 10 min.

3. Blocking step (1× Nexterion blocking solution, 65 °C, 15 min).
4. Final washing step in ddH_2O for 1 min at RT.
5. After the final washing step slides have to be dried by centrifugation (10,000 × *g*, 3 min) to avoid stains by remaining droplets on the slide.

4.3.2 Aminosilane-Slides

1. Covalent binding of probes is attained by UV-crosslink. Crosslinking is performed at 250 mJ (UV-stratalinker 1800; Stratagene).
2. Prehybridization treatment is performed at RT by rinsing in 0.1 % SDS (20 s, RT), ddH_2O (20 s, RT).
3. Incubation in aminosilane prehybridization buffer (3× SSC, containing 0.1 % SDS and 10 mg/ml BSA) for 45 min at 42 °C.
4. Final washing step in ddH_2O (20 s, RT).
5. Arrays are dried by centrifugation (10,000 × *g*, 5 min).

4.3.3 Aldehyde Slides

1. Slides are incubated in a humidity chamber (90 % rh) at RT for 15 min and incubated at 120 °C for 1 h to complete covalent binding of probes directly after spotting.
2. Slides are rinsed in 0.2 % SDS (4 min, RT) to remove unbound molecules and buffers.
3. Slides are rinsed in ddH_2O (4 min, RT).
4. Blocking/prehybridization is performed for 15 min at RT in aldehyde blocking solution (1.0 g $NaBH_4$, 300 ml PBS, 100 ml ethanol; freshly prepared 30 min ahead of use).
5. Finally, the slides are washed at RT in 0.2 % SDS (4 min) and in ddH_2O for 4 min.
6. Arrays are dried by centrifugation (10,000 × *g*, 5 min).

4.3.4 Storage of Microarrays

We recommend that all arrays are stored at RT in an inert atmosphere (e.g., argon) after post processing until use (maximally 1 year) (*see* **Note 5**).

4.4 Generation of Target DNA for SNP Detection

For hybridizing of the target gene to the array, here *ERG11* as one example, it needs to be amplified from the respective patient sample or colony from blood culture of the *C. albicansf* isolate. Therefore, a PCR protocol needs to be developed, which for a single target might address the respective gene in one PCR or

which requires a multiplex PCR addressing all or as many as possible genes (*see* **Note 6**).

1. For *ERG11* the following primers have been used to amplify the gene from blood culture isolates, resulting in a product of 1700 bb, encompassing all relevant SNPs:

   ERG11-(rv) 5′-ACACTGAATCGAAAGAAAGTTGCCG-3′.
   ERG11-(fw)5′-AATAGACAAAGAAAGGGAATTCAATCG-3′.
   Fw: forward; Rv: reverse.

   Primers are 5′-phosphorylated for the later generation of ssDNA (see below) or not modified (*see* **Note 6**).

2. The following PCR protocol was used for amplification of the *ERG11* gene:
   98 °C for 30 s,
   30 cycles of:

   Denaturation: 98 °C for 10 s,
   Annealing: 53 °C for 30 s,
   Elongation: 72 °C for 30 s,
   Final elongation: 72 °C for 7 min.

3. Components for PCR (50 μl) amplifying *ERG11* are listed below, using Qiagen Phusion polymerase.

Component	Concentration	Volume (μl)	End conc.
Phusion buffer HF5	5×	10	1×
Primer Fw	10 μM	2.5	0.5 μM
Primer Rv	10 μM	2.5	0.5 μM
Genomic DNA	Variable	Variable	1–2 ng/μl
dNTPs	10 mM	1	0.2 mM
$MgCl_2$	50 mM	0.2	0.2 mM
Phusion polymerase	2 U	0.5	0.02 U/μl
ddH_2O		Ad 50 μl	

4. In the case of using the PCR-product directly as dsDNA, the sample was denatured for 2 min at 96 °C prior to hybridization on the arrays (*see* also **Note 6**).

4.5 Preparation of ssDNA

For SNP detection single stranded DNA (ssDNA) has significant advantages with regard to sensitivity and specificity of the results and maximizes the efficiency of hybridization. Therefore generation of ssDNA is able to improve the outcome of the results significantly. To generate ssDNA phosphorylated primers are used which act as a priming site for nucleases. Phosphorylated strands of DNA can be digested enzymatically using λ exonuclease to obtain the respective ssDNA strand. To generate both version of the ssDNA,

each gene has to be amplified in two separate reactions, using either the forward or the reverse primer phosphorylated at its 5′ end together with the appropriate non-phosphorylated primer (as in a regular PCR) generating dsDNA with one 5′-phosphorylated strand, respectively. The protocol for this PCR-reaction is identical to the reaction described above. Also ssDNA may be fragmented to ~100 bp equivalents (*see* **Note 6**).

4.5.1 λ Exonuclease Digestion for ssDNA Preparation

1. The reaction mixture contains in a volume of 40 μl:

   1× λ exonuclease buffer.
   2 U/μl λ exonuclease.
   800 ng of DNA (*see* **Note 7**).
2. Incubation at 37 °C for in 30 min.
3. The enzyme is then heat inactivated at 80 °C for 15 min.
4. Isolation of ssDNA using PCR purification kit (Qiagen) according to the manufacturer.

4.5.2 Fragmentation of ssDNA (See **Note 8**)

1. 800 ng ssDNS.
2. 350 μU Dnase (Fisher Scientific) per 1 ng of DNA (*see* **Note 9**!) in 1× reaction buffer.
3. Add ddH_2O up to 30 μl.
4. Incubate for 10 min at 37 °C.
5. Stop the reaction by adding 3 μl stop-solution for 10 min at 65 °C.

4.6 On-Chip Primer Extension Reaction

The conventional APEX reaction includes two independent reactions, the annealing or probe-target hybridization reaction and the enzyme-enhanced elongation-reaction. Both reactions can be combined also in a single reaction step. The total time for hybridization and enzymatic reaction thereby can be reduced to 30 min. This process requires the appropriated reaction chambers (GeneFrames, ABGene), which can be agitated using suitable shaker equipment with well-defined temperature control (heat-controlled water bath (Memmert, GER) at 50 °C). The following protocol has been established to perform the on chip reaction.

4.6.1 Combined Hybridization/APEX Reaction

1. GeneFrames, ABGene, are added to the microarrays framing the probes on the array. In the generated well the following components were added up to a final volume of 70 μl (*see* **Note 10**):
2. 350–500 ng digested DNA or/and 1 pmol synthetic template.
3. 0.7 μl 10× annealing buffer (1 mM EDTA (pH 8.0), 2 M NaCl, 1% (v/v) Triton X-100).
4. 0.7 μl 10× thermosequenase buffer (GE Healthcare).

Fig. 5 False color overlay of an *ERG11* SNP-analysis of a clinical isolate of *C. albicans* using cy3-ddCTP, cy5-ddUTP, and fluorescein-ddGTP

5. 0.1 M DTT (Promega GmbH).
6. 0.1 mM thermosequenase (GE Healthcare).
7. 0.1 mM cy3-ddCTP (PerkinElmer) (*see* **Note 11**).
8. 0.1 mM cy5-ddUTP (PerkinElmer).
9. 0.1 mM fluorescein-ddGTP (PerkinElmer).
10. Hybridization was performed for 30 min at 50 °C on a Thermomixer (Eppendorf) (*see* **Note 12**).
11. Hybridized slides were washed three times in washing buffers with decreasing salt concentrations at RT for 5 min each
    Wash buffer I: 2× SSC, 0.2% (w/v) SDS.
    Wash buffer II: 2× SSC.
    Wash buffer III: 0.2× SSC.
12. Slides were dried by centrifugation (10,000×*g*, 4 min; Megafuge 1.0, Heraeus) and stored light protected at RT until scanned (*see* **Note 13**).
13. Scanning was performed using Genepix 4300A (Fig. 5)

4.6.2 Dye selection

As described above only three fluorescently labeled nucleotides were used for simultaneous detection of the bases following the 3′-end of the capture probe. This combination worked with our laser-scanner combination with only limited cross talk between the dyes. The phenomenon of cross talk is well known. It is due to the fact that the excitation and emission wavelengths of dyes are not sharp peaks but can be rather broad. For example at an extinction wavelength of 488 nm not only fluorescein but also cy3-dyes will be activated by nearly 18%. Standard filter for fluorescein (band pass: 525/±25 nm) let pass emission-light caused by cy3-dyes of about 18% of total emission at 560 nm resulting in a false positive fluorescein signal at cy3-labeled probes. To control this at least partially we favor the use of only three labeled nucleotides instead of 4, as the problem gets worse, the more dyes are used. Since two probes on the coding and noncoding strand can be designed using only two dyes could potentially suffice to detect all possible SNPs. Depending on the filters and lasers of your scanner, as well as mathematic correction of background signals, some options for four color SNP detection have been published as well, which you

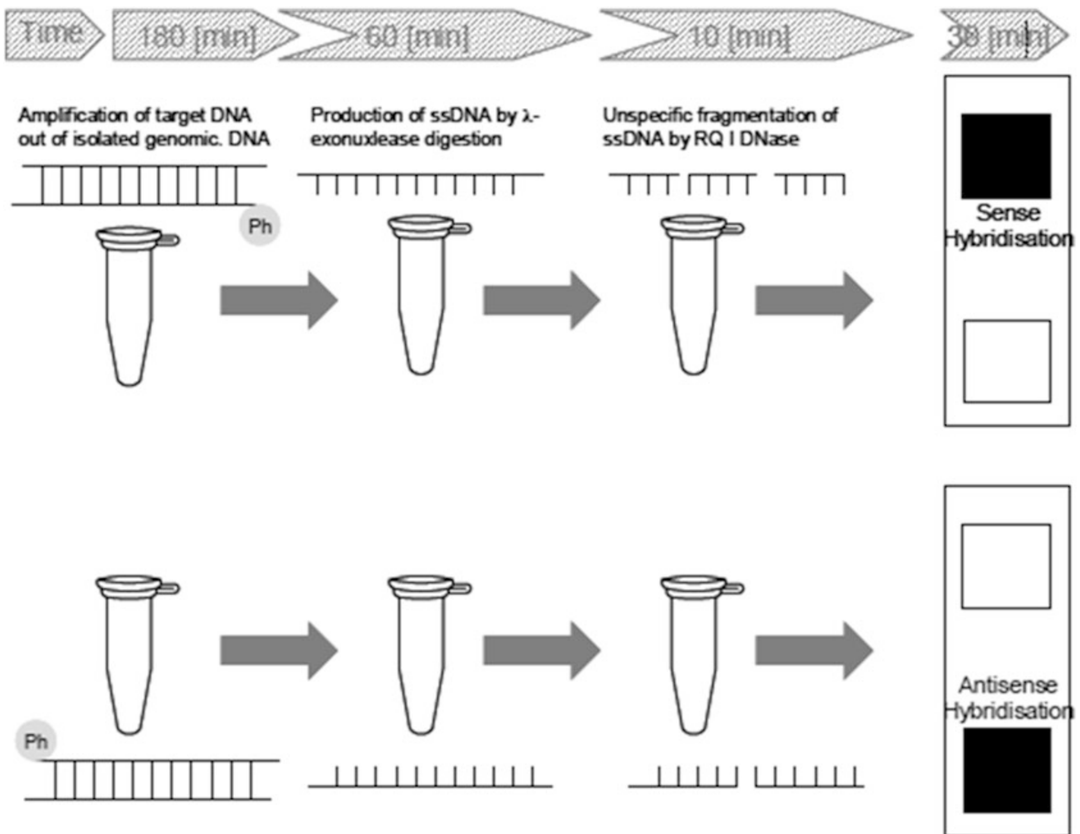

Fig. 6 Illustration of experimental workflow starting with amplification of genomic DNA using phosphorylated primers (P). Second, the phosphorylated strand is digested to obtain sense and antisense DNA of target genes, separately, followed by unspecific fragmentation. Finally, fragmented target DNA is hybridized on the arrays and on chip primer extension reaction is initiated in parallel

should consider if you have the appropriate equipment [81, 82]. In these publications a combination of dyes and the required lasers as given below is described:

Excitation lasers: Blue Argon 488 nm for R110; Green HeNe 543.8 nm for Tamra; Yellow HeNe 594 nm for Texas Red; Red HeNe 632.8 nm for Cy5. The dyes were coupled to the respective ddNTPs and used in the following concentrations: 0.1 μM ddATP-Texas Red, 0.1 μM ddCTP-Tamra, 0.1 μM ddGTP-R110, and 0.2 μM of ddUTP-Cy5 (PerkinElmer Life Sciences) (*see* **Note 11**).

The general experimental workflow for APEX as described above is illustrated in Fig. 6.

5 Notes

1. Homogeneous spots as well as constant and maximal amounts of probes coupled to the surface of the array are required to maximize capacity of hybridized target DNA. These are critical

features for high quality microarray systems. To ensure this quality the microarray production, even for small scale production requires automation. For spotting we use a contact spotter, Microgrid II (Biorobotics), equipped with SMP3 splitpins (Array It) or MicroSpot 2,500 Splitpins (Fig. 3). The pins used are crucial in contact printing, especially with regard to durability and reproducibility of spots over a larger number of slides. It is crucial to exchange the pins from time to time, depending on the type of pin and frequency of usage. Check regularly under the microscope if they are still ok.

2. If several targets are addressed differences in GC-content of the target genes are most likely to be observed. This might imply a larger range of values for length and TM of the individual probes. However, it should be ensured that for all probes that the differences between the TM are within a small range (5–6 °C), e.g., by varying the probe length. To achieve high specificity of probes, melting temperature (TM) and Gibbs free enthalpy for hairpin (ΔGhairpin) and dimer bonding (ΔGDimer) should be calculated for all probes. The melting temperature (TM) should be set to one temperature, depending on the polymerase reaction after hybridization (using Thermosequenase 55 °C has been working for us as TM), values for ΔGhairpin and ΔGDimer were set to 0 kcal/mol. The length of the oligos may vary significantly.
3. Several surfaces for microarray production exist. The most commonly used surfaces are epoxy-coated glass slides or aminosilane- and aldehyde-coated slides (available for example from Schott Nexterion). Depending on the reactive surface, several buffer systems exist, which are adjusted to the surface chemistry. It is advisable to evaluate the spotting-buffer-system in the context of your robotic system, as it is crucial for the reproducible amount of DNA spotted and linked to the array (*see* Fig. 4). Also the modification of the oligonucleotides has to be adjusted accordingly.
4. Optimal spotting parameters for contact spotting on the respective slides should be determined. Optimization includes spotting parameters of the robot (for example dipping depth into the probe solution and touch mode), the humidity (rh) in the spotting chamber (which needs to be constant to receive reproducible amounts) and spot distance, depending on the spot-density required. Therefore, the optimal spotting parameters like soft-touch, soft-touch distance of pins, pin dipping depth, optimal temperature and rh during spotting process need to be evaluated. In order to identify the optimal spotting parameters fluorescently labeled oligonucleotides could be used for establishing the spotting process and as quality controls in the final array.
5. For example in an exsiccator filled with argon. This is especially relevant if spotting controls already containing fluorescent

dyes like Cy3 and Cy5 are used, since the dyes are in general very sensitive to oxygen.

6. Long PCR-products often generate lower signals on microarrays due to more complex hybridization kinetics. Therefore, as an alternative several primer pairs amplifying fragments of 100–300 bp in length can be used especially if only few regions of a gene are relevant for SNP analysis. Another method to improve SNP detection is the generation of single stranded DNA (ssDNA), which has significant advantages with regard to sensitivity and specificity of the results and maximizes the efficiency of hybridization. For this purpose 5′-phosphorylated primer have to be used which enable digestion of one the strands of the PCR product using nucleases.
7. It is advisable to digest each PCR product separately.
8. Since smaller DNA fragments hybridize more efficiently on arrays than larger DNA fragments, ssDNA can further be processed to obtain smaller fragments. For this purpose purified ssDNA is randomly digested with DNAse I (*see* also **Note 6**).
9. The optimal concentration of DNase I has to be identified by analyzing different DNase amounts between 300 and 500 μU DNase per ng DNA. The fragment length after digestion can be determined via capillary gel electrophoresis (Agilent Bioanalyser; Agilent) according to the manufacturer's instruction or by agarose gel-electrophresis. Adding too much DNAse might result in complete loss of the sample, so this is a critical step!
10. Wells generated by gene frames, instead of just adding a cover slip are required to enable a rapid and thorough mixing of the fluid on the probes.
11. Alternatively, depending on the scanning equipment four dyes can be used. In this case replace the given dyes by 0.1 μM ddATP-Texas Red, 0.1 μM ddCTP-Tamra, 0.1 μM ddGTP-R110, and 0.2 μM of ddUTP-Cy5 (PerkinElmer Life Sciences). Exact laser settings are given in the text below.
12. The hybridization and enzymatic incorporation process was controlled by adding 0.5 pmol control-templates (positive and negative APEX-controls) to the mixture.
13. Scanning has to be performed if possible immediately or the latest within 1 day after hybridization to avoid bleaching of the dyes employed.

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Part V

Drug Resistance Testing

Chapter 23

Molecular Detection of Resistance to Echinocandins

Brunella Posteraro*, Antonietta Vella*, Elena De Carolis, and Maurizio Sanguinetti

Abstract

In the last years, life-threatening fungal diseases have increased significantly, due to the rising number of human individuals susceptible to fungal infections, which are in part complicated by the emergence of antifungal drug-resistant pathogens. Among yeasts, *Candida albicans* and *Candida glabrata* are the most common organisms responsible for invasive fungal diseases. The molecular detection of echinocandin resistance in *Candida* species may represent a useful means of monitoring the incidence of clinical isolates with antifungal resistance-associated gene alterations. Here, we describe the current methods that enable researchers and/or clinical microbiologists to accurately detect echinocandin-resistant isolates of *C. albicans* and *C. glabrata*.

Key words Antifungal drug resistance, Fungal pathogens, Molecular analysis, Quantitative real-time RT-PCR, Gene sequencing

1 Introduction

Echinocandins are inhibitors of the 1,3-β-D-glucan synthase, an enzyme coded by the *FKS* gene, that is responsible for the fungal cell-wall β-D-glucan synthesis. Occurrence of clinical isolates exhibiting antifungal drug resistance to these agents is considered as a very rare event in *C. albicans*. By contrast, there is an alarming trend of increased echinocandin resistance in *C. glabrata* that can also regard azole antifungal agents. The mechanisms of echinocandin resistance involve amino acid changes in "hot-spot" regions of the Fks subunits of the 1,3-β-D-glucan synthase. In *C. albicans* mutations occur in two highly conserved "hot-spot" regions of the *FKS1* gene [1], while in *C. glabrata* mutations occur in homologous regions of the *FKS1* and *FKS2* genes [2].

*These two authors have contributed equally for this chapter.

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2 Materials

2.1 Polymerase Chain Reaction (PCR) Reagents

PCR specific primers (Table 1) are usually shipped from the manufacturer as lyophilized powders. Prior to use, centrifuge the primer-containing vials at max speed for 10 min, then suspend the primers in RNase-free water at a 100-μM stock concentration, from which prepare a 10-μM working solution. Store all primer solutions at −20 °C.

2.2 Agarose Gel Electrophoresis Components

(a) Prepare a 1% agarose gel. Pour 20 mL of 50× TAE (Tris-acetate-EDTA; Thermo Fisher Scientific) buffer in a 2-L graduated cylinder, dilute to 1 L with distilled water, and mix. Weight 1 g of UltraPure™ agarose (Life Technologies), transfer to a screw-cap bottle and add 100 mL of 1× TAE buffer. Place the bottle with the cap loose in the microwave oven, and heat the solution until bubbles appear. Remove the flask carefully, and swirl gently to suspend any agarose particles. Reheat the solution until the solution comes to boil, and all agarose particles are dissolved. Mix gently and cool to 50–60 °C (at room temperature for at least 20 min) before pouring the agarose solution into the gel casting tray.

(b) For visualization of DNA in the gel, add to the agarose solution, just prior to pouring it into the tray, a fluorescent dye,

Table 1
Primer sets

Species	Gene region	Primer name	Primer sequence (5′–3′)	PCR product size (bp)
C. albicans	*FKS1* HS1	F2426	CATTGCTGTGGCCACTTTAG	514
		R2919	GATTTCCATTTCCGTGGTAGC	
	FKS1 HS2	F4590	TACTATGGTCATCCAGGTTTCC	384
		R4954	GGTCAAATCAGTGAAAACCG	
C. glabrata	*FKS1* HS1	Cg-fks1HS1f	CCATTGGGTGGTCTGTTCACG	725
		Cg-fks1HS1r	GATTGGGCAAAGAAAGAAATACGAC	
	FKS1 HS2	Cg-fks1HS2f	GGTATTTCAAAGGCTCAAAAGGG	835
		Cg-fks1HS2r	ATGGAGAGAACAGCAGGGCG	
	FKS2 HS1	Cg-fks2HS1f	GTGCTCAACATTTATCTCGTAGG	981
		Cg-fks2HS1r	CAGAATAGTGTGGAGTCAAGACG	
	FKS2 HS2	Cg-fks2HS2f	CGTAGACCGTTTCTTGACTTC	559
		Cg-fks2HS2r	CTTGCCAATGTGCCACTG	

Six primer sets (each composed of forward and reverse sequences) designed for amplifying, respectively, the HS1 and HS2 regions of the *FKS1* gene in *C. albicans*, and the HS1 and HS2 regions of the *FKS1* and *FKS2* genes in *C. glabrata*

such as SYBR® Green nucleic acid gel stain (Invitrogen). Follow the manufacturer's instructions to obtain the appropriate fluorescent dye concentration solution and to manipulate it in safety.

2.3 PCR Product Purification Reagents

Prepare a PE buffer by adding 220 mL of ethanol (96–100%), as indicated on the PE bottle included in the MinElute PCR Purification Kit (Qiagen), to obtain a working solution.

3 Methods

3.1 DNA Extraction

1. Set heating block at 99 °C.
2. With a sterile inoculating loop, put five yeast colonies in 300 μL of ultrapure water, vortex, and centrifuge at max speed for 5 min (*see* **Note 1**).

   Discard supernatant, add to pellet 1 volume of glass beads (0.45–0.55 mm diameter; Sigma-Aldrich) and 2 volumes of ultrapure water (*see* **Note 2**) and vortex the mixture.
3. Shake the sample using a Mini-Beadbeater at max speed for 10 s, wait 10 s and repeat three times.
4. Incubate the sample at 99 °C for 15 min.
5. Centrifuge at max speed for 5 min, transfer the supernatant in a new tube, and store the DNA solution at 4 °C (*see* **Note 3**).

3.2 DNA Measurement

1. Measure the DNA concentration on the basis of the optical density (OD), using a spectrophotometer at 260/280 nm wavelength. Calculate the OD_{260}/OD_{280} ratio to have an indication of nucleic acid purity (pure DNA has an OD_{260}/OD_{280} ratio of ~1.8). Calculate the amount of total DNA using the following formula:

   dsDNA concentration = 50 μg/mL × OD_{260} × dilution factor.

   Example: A sample of dsDNA is diluted 100×. The diluted sample gives a reading of 0.95 on the spectrophotometer at OD_{260}. To determine the concentration of DNA in the original sample, perform the following calculation:

   dsDNA concentration = 50 μg/mL × 0.95 × 100;
   dsDNA concentration = 4.75 mg/mL.
2. Prepare a working solution of DNA to a 2 ng/μL concentration to be used (an aliquot) as a template in PCR reaction, and store the solution at 4 °C (*see* **Note 3**).

3.3 Polymerase Chain Reaction (PCR)

1. The PCR primers, previously described by Slater et al. [3] and by Thompson et al. [4], are listed in Table 1.
2. Program your thermal cycler to perform PCR. Use the thermal profile described in Table 2 (*see* **Note 4**).

Table 2
Thermal profile for PCR amplification

Temperature (°C)	Time	
95	15 min	
95	30 s	
50	30 s	× 35 Cycles
72	40 s	
72	5 min	
4	Hold	

Table 3
Master mix reaction components for PCR amplification

Component	Amount (for one sample) (μL)
Buffer PCR 10×	2.5
$MgCl_2$ 50 mM	0.75
dNTPs 10 mM	0.5
10 μM forward primer	1.25
10 μM reverse primer	1.25
Taq polymerase (5 U/μL)	0.125
Template DNA (2 ng/μL)	5
Nuclease-free water	5

3. Prepare a master mix reaction as described in Table 3 (*see* **Note 5**). If you have more than one sample, prepare a multiple master mix and aliquot 20 μL for each sample.
4. Add DNA template as a second component of the mix. To test for genomic DNA contamination of enzyme/primer mixes, for each set of primers include a negative control without DNA template (*see* **Note 6**).
5. Cap each PCR tube, and gently mix the content. Make sure that all components are at the bottom of the tube; centrifuge briefly if needed.
6. Place reaction tubes in a PCR thermal cycler.

3.4 PCR Products Gel Electrophoresis

1. Check the PCR amplification through a gel electrophoresis. Mix 2.5 μL of the PCR product with 2.5 μL of water, add 1 μL of 6× Mass Ruler DNA Loading dye (Thermo Fisher Scientific), mix by pipetting, and carefully load the sample into a gel well.

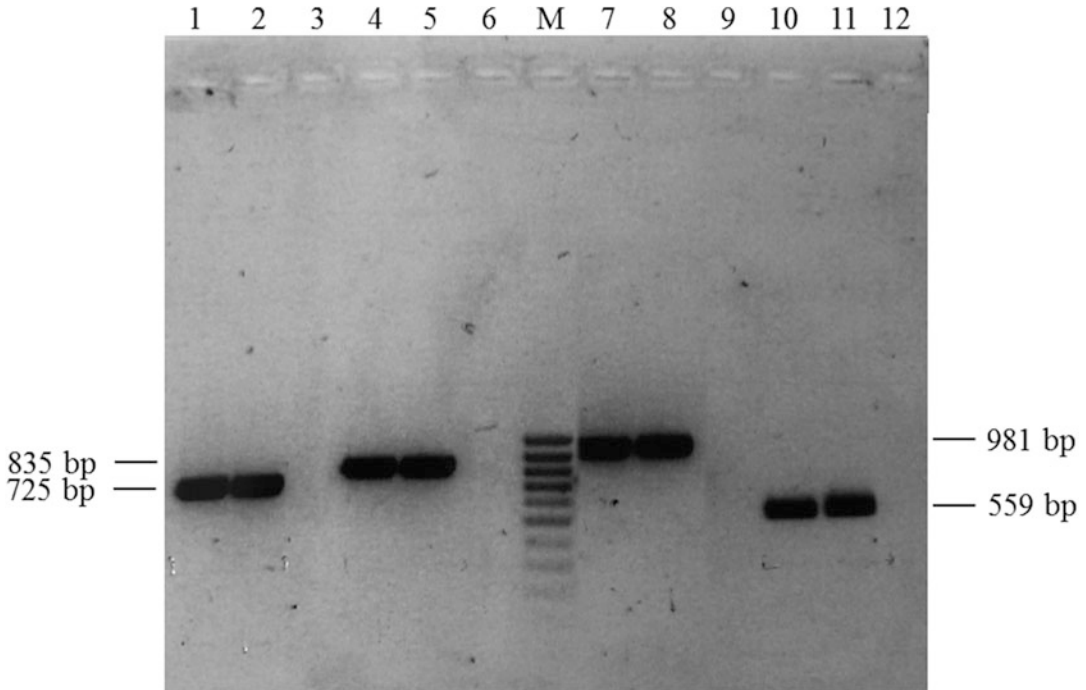

Fig. 1 Agarose gel electrophoresis. PCR products obtained with two *C. glabrata* strains using Cg-fks1 (HS1 and HS2) and Cg-fks2 (HS1 and HS2) primers, as described in Table 1. *Lanes 1* and *2*: Cg-fks1 HS1 products, *lane 3*: negative control, *lanes 4* and *5*: Cg-fks1 HS2 products, *lane 6*: negative control, M: Mass ruler low range, *lanes 7* and *8*: Cg-fks2 HS1 products, *lane 9*: negative control, *lanes 10* and *11*: Cg-fks2 HS2 products, *lane 12*: negative control. The expected size of each PCR product is indicated in Table 1

Finally, load 4 μL of Mass Ruler Low range DNA Ladder (Fermentas, Thermo Fisher Scientific) into a gel control well.

2. Turn on the power supplier and run the gel for 30 min at 80 V.
3. Visualize the gel on the UV light box. It should obtain a picture similar to that shown (Fig. 1).

For more information about the agarose electrophoresis procedure, click on https://www.eeb.ucla.edu/Faculty/Barber/Protocols.htm

3.5 PCR Products Purification

To purify the PCR products obtained as above, use the MinElute PCR Purification Kit (Qiagen) (*see* **Note 7**). Briefly:

1. Add 5 volumes of buffer PBI to 1 volume of the PCR reaction, and mix throughout.
2. Place a MinElute column in a 2-mL collection tube.
3. To bind DNA, apply the sample to the MinElute column, avoiding to touch filter with tip, and centrifuge for 1 min at max speed.

4. Discard flow-through and place the MinElute column back into the same tube.
5. To wash, add 0.75 mL of buffer PE to the MinElute column and centrifuge for 1 min at max speed.
6. Discard flow-through and place the MinElute column back into the same tube.
7. Centrifuge the column for 1 min at max speed to eliminate any ethanol residue.
8. Place the column in a clean 1.5-mL centrifuge tube.
9. To elute DNA, add 10 μL of nuclease-free water to the middle of the column (*see* **Note 8**), wait for 1 min, and then centrifuge for 1 min at max speed.
10. Measure the DNA concentration as indicated in Subheading 3.2, and dilute in nuclease-free water as to obtain 2 ng/μL DNA concentration, which will be used as a template in sequencing reaction.

3.6 Sequencing Reaction and Sequencing Products Purification

1. Program your thermal cycler to perform PCR amplification. Use the thermal profile described in Table 4.
2. To perform the sequencing reaction, use ABI PRISM BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific). Prepare the reaction mixture as described in Table 5 (*see* **Note 9**).

Table 4
Thermal profile for DNA sequencing

Temperature (°C)	Time	
96	1 min	
96	10 s	
50	5 s	× 55 Cycles
60	4 min	
4	Hold	

Table 5
Master mix reaction components for DNA sequencing

Component	Amount (for one sample) (μL)
BigDye® ready reaction pre-mix	2
BigDye® Terminator v1.1 sequencing buffer (5×)	4
10 μM primer forward or reverse	1
Template DNA (2 ng/μL)	5
Nuclease-free water	8

3. For more details about the use of ABI PRISM BigDye Terminator v1.1 Cycle Sequencing Kit and the optimization of thermal cycling conditions, read the BigDye® Terminator v1.1 Cycle Sequencing Kit protocol (Applied Biosystems, Thermo Fisher Scientific).
4. For sequencing reaction purification, use the G50 Dye Terminator Removal Kit (RBC Bioscience) (*see* **Note 7**). Remove the red stopper under the G50 column and centrifuge at 2000 × *g* for 2 min.
5. Transfer the G50 column to a 1.5-mL microcentrifuge tube and carefully load the sample to the middle of the gel bed surface.
6. Centrifuge at 2000 × *g* for 3 min.
7. The purified sample will be recovered at the bottom of collection tube.
8. Load the sample onto the ABI PRISM DNA Analyzer or store it at −20 °C avoiding light.

3.7 ABI PRISM DNA Analyzer Electrophoresis

Details on how to perform sample electrophoresis and data analysis can be found in the ABI PRISM DNA Analyzer instrument manual.

3.8 Hot-Spot Mutation Assessment

In Table 6 are listed the mutations most frequently reported in echinocandin-resistant *Candida* isolates [1, 2]. To assess for the presence of *FKS1* mutation(s) in a *C. albicans* isolate and of *FKS1* and/or *FKS2* mutation(s) in a *C. glabrata* isolate, compare the wild-type gene sequences of the *C. albicans* ATCC MYA-2876 strain (NCBI Reference Sequence: XM_716336.1) or the *C. glabrata* ATCC 90030 strain (NCBI Reference Sequence: HM366440.1, for *FKS1*; GenBank: HM366442.1, for *FKS2*) with those obtained from the test isolates. Select one of charge-free sequence analysis programs available online; for example, the software MEGA version 6 [5] is a nice and simple program to use.

In Fig. 2 is shown an alignment made using the MEGA6 software to detect *C. glabrata FKS2* gene mutations.

4 Notes

1. Handle yeast cultures aseptically, possibly near to a Bunsen flame or under a laminar flow cabinet.
2. For Mini-Beadbeater utilization, use screw-cap vials.
3. If you plan using DNA until 1 week, store DNA at 4 °C, otherwise store at −20 °C.
4. For the annealing temperature (Ta) choose the lowest temperature of melting between the two primers and lower it to 5 °C. For more details on the Ta setting, *see* Rychlik et al. [6].

Table 6
Gene mutations most commonly encountered in echinocandin-resistant isolates of *Candida* species

Species	*FKS1* genotype	Fks1 phenotype	*FKS2* genotype	Fks2 phenotype
C. albicans	T1921C	F641L	–	–
	T1922C	F641S	–	–
	T1933C	S645P	–	–
	C1934A	S645Y	–	–
	C1934T	S645F	–	–
	G1942T	D648Y	–	–
	C1946A	P649H	–	–
	G4082A	R1361H	–	–
C. glabrata	T1874C	F625S	Del1974-1976	F659del
	T1885C	S629P	T1976C	F659S
	A1895G	D632G	T1976G	F659V
	T1896G	D632E	T1987C	S663P
	G1894T	D623Y	C1997G	D666G
			C1998A	D666E
			C1999A	P667T
			G4125A	W1375L

5. As a Taq polymerase, choose an high-fidelity enzyme with low error rate. Prepare the PCR master mix under a laminar flow cabinet; this cabinet should be exclusively dedicated to mix PCR preparation in order to avoid acid nucleic carryover. Use only pipettes and tube racks dedicated.
6. Set PCR conditions on the basis of the kit chosen for use and of the thermal cycler available in the lab; for suggestions about the experimental condition optimization, *see* Kramer et al. [7].
7. For PCR product and sequencing reaction purifications, different homemade protocols have been described that have comparable efficiencies to that of commercial kits, but that are more time consuming.
8. Preheating nuclease-free water at 56 °C can be useful to elute efficiently the DNA from MinElute column filter.
9. For the *Candida* isolate in which an echinocandin resistance mutation has to be detected, plan running two sequencing reactions for each gene under investigation, the one using the primer forward and the other with the primer reverse in order to confirm possible point mutation found.

Fig. 2 Sequence alignments. Alignments obtained with the MEGA6 software for *C. glabrata FKS2* sequences, showing the presence of different point mutations in the genes from echinocandin-resistant strains, compared with the gene from a wild-type strain. In (**a**) is shown nucleotide sequences and in (**b**) the relative amino acid sequences

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Chapter 24

Molecular Detection of Resistance to Azole Components

Brunella Posteraro*, Antonietta Vella*, Elena De Carolis, and Maurizio Sanguinetti

Abstract

Fungal infections have increased significantly in the last few years, and their outcomes are in part complicated by the emergence of antifungal drug-resistant pathogens. Together with *Candida* species, the mould *Aspergillus fumigatus* is one of the most prevalent organisms to cause invasive fungal disease. The molecular detection of (tri)azole resistance in both *Candida* and *Aspergillus* species may represent a useful means of monitoring the incidence of clinical isolates with antifungal resistance-associated gene alterations. Here, we describe molecular methods that have been developed to allow for accurate detection of azole-resistant isolates among *C. glabrata* and *A. fumigatus* fungal species.

Key words Antifungal drug resistance, Fungal pathogens, Molecular analysis, Quantitative real-time RT-PCR, Gene sequencing

1 Introduction

Azoles (fluconazole, FCZ; itraconazole; ITZ, voriconazole; VRC; posaconazole, PSC) have as their target the ergosterol, the principal sterol of the fungal cell membrane. Specifically, azoles bind the 14-alpha lanosterol demethylase, an enzyme of the ergosterol biosynthesis pathway that is codified by the *ERG11* gene in yeasts and by the *cyp51* gene in moulds. Unfortunately, decreased susceptibility to FCZ is very common in the yeast *Candida glabrata*, whereas fatal infections due to (tri)azole-resistant isolates of the mould *Aspergillus fumigatus* are increasingly reported. Although azole resistance in *C. glabrata* can also result from the appearance of point mutations in, or from the overexpression of, the *ERG11* target gene, it is known to be almost exclusively mediated by enhanced efflux of azole drugs. This is, in turn, due to the overexpression of ATP-binding cassette (ABC) transporter genes, such as *CgCDR1*,

*These two authors have contributed equally for this chapter.

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CgCDR2, and *CgSNQ2* [1, 2]. Conversely, azole resistance in *A. fumigatus* is known to be mainly associated with the presence of point mutations in the *cyp51A* gene [3].

2 Materials

2.1 YEAST: Microorganism Growth Medium

1. Suspend 65 g of yeast-extract peptone dextrose (YEPD) broth medium (Sigma-Aldrich) in 1 L of distilled water.
2. Heat to boiling while stirring to dissolve all the medium powder completely.
3. Autoclave for 15 min at 121 °C. Cool down at room temperature and store at 4 °C.

2.2 YEAST: RNA Extraction

1. Use only RNase-free materials, aerosol-resistant pipette tips and RNase-free water or UltraPure DEPC-treated water. Wear latex gloves while handling reagents and RNA to prevent RNase contamination from the skin surface (*see* **Note 1**).
2. RLT and RPE buffers are supplied in the RNeasy Mini kit (Qiagen). RPE buffer: add 4 volumes of ethanol (96–100%) as indicated on the RPE bottle to obtain a working solution. RLT buffer: add 10 μL of β-mercaptoethanol (β-ME) per 1 mL of RLT buffer. Work in a fume hood and wear appropriate protective clothing. The RLT buffer containing β-ME can be stored at room temperature for up 1 month.
3. Prepare acid-washed glass beads (0.45–0.55 mm diameter; Sigma-Aldrich) by soaking in concentrated nitric acid for 1 h in a fume hood, followed by extensive washing with deionized water and by drying in a baking oven.
4. For Mini-Beadbeater utilization (*see* Chapter 23), use screw-cap microvials.

2.3 YEAST: Quantitative Real-Time RT-PCR (qRT-PCR)

1. Primers and probes are shipped from the manufacturer as lyophilized powders. Prior to use, centrifuge at max speed for 10 min the primer-containing vials, and suspend the contents in RNase-free water at a 100-μM stock concentration, from which prepare a 20-μM working solution. Store all primer solutions at −20 °C.
2. For fluorescent probes, use the buffer equipped from the manufacturer and follow the recommended procedure. Generally, a qRT-PCR kit contains all the components ready to use; thus, follow carefully the manufacturer's instructions for use and storage.

2.4 MOULD: DNA Extraction

1. AP3/E and AW buffers are supplied in the DNeasy Plant Mini Kit (Qiagen). AP3/E buffer: add 60 mL of ethanol (96–100%) as indicated on the AP3/E bottle to obtain a working solution.

AW buffer: add 40 mL of ethanol (96–100%) as indicated on the AW bottle to obtain a working solution.

2. For Mini-Beadbeater utilization, use screw-cap microvials.
3. Preheat a water bath or heating block to 65 °C.
4. Ensure to have ice.

2.5 DNase I treatment

RiboPureTM-Yeast Kit (Ambion).

2.6 qRT-PCR

EXPRESS One-Step SuperScript® qRT-PCR kit (Invitrogen).

2.7 Fungal DNA Extraction

DNeasy Plant Mini Kit (Qiagen).

2.8 Agarose Gel Electrophoresis Components

See description provided in the Chapter 23.

3 Methods

3.1 YEAST: Microorganism Growth Culture

1. With a sterile inoculating loop, put a single yeast colony in 5 mL of YEPD broth (*see* **Notes 2** and **3**) in a 50-mL conical tube. Mix and incubate under constant agitation (240 rpm) at 30 °C overnight.
2. Prepare a sub-inoculum by adding 1 mL of overnight broth culture to 8 mL of fresh broth medium in a 50-mL conical tube. Mix and incubate under constant agitation (240 rpm) at 30 °C until to reach the yeast mid-exponential growth phase.
3. After two hours, transfer 800 μL of broth culture in a micro-cuvette and measure the optical density (OD) using a spectrophotometer (*see* **Note 4**) at 600 nm wavelength. If OD value is near to 0.6 continue as described below (*see* Subheading 3.2); otherwise, if OD is lower than 0.6, incubate again and make the OD measurement every 30 min.

3.2 YEAST: RNA Extraction and Quantitation

1. For RNA extraction use the RNeasy Mini kit.
2. Collect the cells in a 15-mL centrifuge tube and harvest them by centrifuging at 1000 × *g* for 5 min at 4 °C.
3. Discard supernatant and suspend the pellet in 600 μL of RLT buffer, vortex and add the sample to the acid-washed glass beads (*see* Subheading 2.2, **item 3**).
4. Vortex and agitate the sample using a Mini-Beadbeater at max speed for 20 s and place sample on ice for 1 min. Repeat this step 4 times.
5. Wait for the beads to deposit at the bottom, and transfer the lysate to a RNase-free microcentrifuge tube; then centrifuge

for 2 min at full speed, and transfer the supernatant to a new RNase-free microcentrifuge tube. From this step, use only RNase-free-material.

6. Add 1 volume of 70% ethanol to the lysate and mix well pipetting.
7. Transfer the sample to an RNeasy spin column placed in a 2-mL collection tube. Centrifuge for 15 s at ≥8000 × *g*. Discard the flow-through.
8. Add 700 μL of RW1 buffer to the RNeasy spin column. Centrifuge for 15 s at ≥8000 × *g*. Discard the flow-through.
9. Add 500 μL of RPE buffer to the RNeasy spin column. Centrifuge for 15 s at ≥8000 × *g*. Discard the flow-through.
10. Add 500 μL of RPE buffer to the RNeasy spin column. Centrifuge for 2 min at ≥8000 × *g*. Discard the flow-through.
11. Place the RNeasy spin column in a new collection tube and centrifuge at full speed for 1 min.
12. Place the RNeasy spin column in a microcentrifuge tube, add 50 μL of RNase-free water and centrifuge for 1 min at ≥8000 × *g*. Repeat this step using the same tube.
13. Further experimental details are available into the manufacturer's protocol "Purification of total RNA from yeast."
14. For DNase I treatment, use RiboPureTM-Yeast Kit and proceed as follows.
15. Prepare the DNase digestion reaction as indicated in Table 1. Mix by pipetting and incubate at 37 °C for 30 min.
16. Mix DNase inactivation reagent by vortexing and add 0.1 volume to sample. Mix by vortexing and incubate at room temperature for 5 min.
17. Centrifuge at max speed for 2–3 min and transfer the supernatant in a new tube.
18. Store RNA sample at −20 °C for up to 1–2 months or at −80 °C for longer than 2 months.
19. For RNA quantity and quality assessment, measure RNA concentration by spectrophotometer at 260/280 nm wavelength.

Table 1
Components for DNase digestion reaction

Component	Amount (for one sample) (μL)
RNA sample (from Subheading 3.2, **step 12**)	100
10× DNase 1 buffer	10
DNase I (8 U)	4

20. Calculate the amount of total RNA using the following formula:

Total RNA (μg) = OD_{260} × [40 μg/(1 OD_{260} × 1 mL)] × dilution factor × total sample volume (mL)

Example: Total RNA was eluted in 100 μL. A 25-μL aliquot of the eluate was diluted to 300 μL in 10 mM Tris–HCl, pH 7.5. An OD_{260} of 0.25 was obtained. The amount of RNA in the sample is:

Total RNA (μg) = 0.25 × [40 μg/(1 OD_{260} × 1 mL)] × 12 × 0.1 = 12 μg

21. Calculate the ratio OD_{260}/OD_{280} value; a value in the range of 1.8–2.1 is usually considered an acceptable indicator of good RNA quality [4].
22. To check for integrity, RNA sample can be analyzed by formaldehyde agarose gel electrophoresis according to Aranda et al. [5] or by using an automated gel electrophoresis system.
23. For the next step, dilute RNA sample as to obtain a 4 ng/μL solution, and store until use as indicated (*see* Subheading 3.2, **item 18**). For example, if the initial RNA concentration is 50 ng/μL, add 8 μL in 100 μL of RNase-free water.

3.3 YEAST: Quantitative Real-Time RT-PCR (qRT-PCR)

1. For qRT-PCR, use EXPRESS One-Step SuperScript® qRT-PCR kit. The PCR primers and probes, previously described by Sanguinetti et al. [6] and by Li et al. [7], are listed in Table 2.
2. Program the PCR real-time instrument to perform cDNA synthesis and subsequently PCR amplification (*see* **Note 5**). The cycling program is described in Table 3.
3. First of all, the real-time PCR efficiencies must be evaluated. For each gene to be analyzed, perform the real-time RT-PCR reaction with a serial dilution of standard RNA template. Prepare a dilution series (e.g., 50, 5, 0.5, and 0.05 ng) of template to construct a standard curve.
4. Set up reactions on ice using the components described in Table 4. A standard 20-μL reaction size is made; always prepare a master mix of common components for multiple reactions (*see* **Note 6**). Add RNA template as a second component of the mix.
5. Prepare no-RT and no-template controls reactions; the first is useful to test for genomic DNA contamination of the RNA sample, so do not add the EXPRESS SuperScript® Mix; the latter is useful to test for genomic DNA contamination of the enzyme/primer mixes, so do not add template RNA.
6. Cap or seal each PCR tube/plate, and gently mix. Make sure that all components are at the bottom of the tube/plate; centrifuge briefly if needed.

Table 2
Primers and probes for qRT-PCR

Gene (GenBank accession no.)	Primer or probe	Sequence[a]	Reference
CgCDR1 (AF109723)	CDR1a	TAGCACATCAACTACACGAACGT	[6]
	CDR1b	AGAGTGAACATTAAGGATGCCATG	[6]
	CDR1pr	6FAM-TGCTGCTGCTTCTGCCACCTGGTT-TAMRA	[6]
CgCDR2 (AF251023)	CDR2a	GTGCTTTATGAAGGCTACCAGATT	[6]
	CDR2b	TCTTAGGACAGAAGTAACCCATCT	[6]
	CDR2pr	6FAM-TACCTTTGCGTGCTGGGCGTCACC-TAMRA	[6]
CgSNQ2 (AF251022)	SNQ2a	ACCATGTGTTCTGAATCAATCAA	[6]
	SNQ2b	TCGACATCATTACAATACCAGAAA	[6]
	SNQ2pr	6FAM-AACTAATCGCCGCAGGTTGTGACA-TAMRA	[6]
CgERG11 (L70389)	ERGa	ATTGGTGTCTTGATGGGTGGTC	[6]
	ERGb	TCTTCTTGGACATCTGGTCTTTCA	[6]
	ERGpr	6FAM-ACTTCCGCTGCTACCTCCGCTTGG-TAMRA	[6]
CgACT1 (CAGL0K12694g)	ACT1fw	TTGGACTCTGGTGACGGTGTTA	[7]
	ACT1rv	AAAATAGCGTGTGGCAAAGAGAA	[7]
	ACT1pr	6FAM-CCACGTTGTTCCAATTTACGCCGG-TAMRA	[7]

[a] *6FAM* 6-carboxyfluorescein, *TAMRA* 6-carboxy-*N,N,N′,N′*tetramethylrhodamine

Table 3
Thermal profile for qRT-PCR

Temperature (°C)	Time	
50	15 min	cDNA synthesis
95	2 min	
95	15 s	× 40 Cycles
60	1 min	

Table 4
Master mix reaction components for qRT-PCR

Component	Amount (μL)
EXPRESS SuperScript® qPCR SuperMix Universal	10
EXPRESS SuperScript® Mix for One-Step qPCR	2
20 μM forward primer	0.9
20 μM reverse primer	0.9
20 μM fluorescent probe	0.5
Water RNase-free	5
RNA template	5

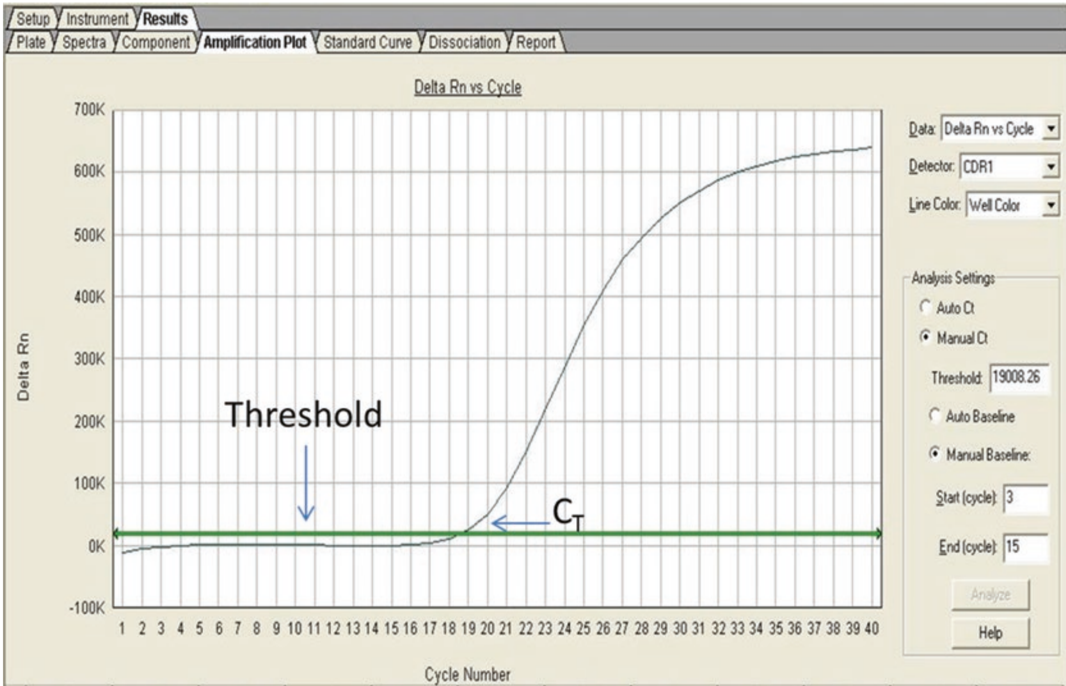

Fig. 1 Detection of the real-time product by qRT-PCR

7. Place reactions in a real-time PCR instrument.
8. When the real-time reactions are finished, for each gene set an arbitrary baseline, so that it is at 10 standard deviations above the mean emission-baseline calculated from the cycle 1–15 [8]. Generally, the instrument software allows for automatic baseline setting (Fig. 1). The point at which the amplification curve crosses the threshold is defined as the C_T, and this value is predictive of the quantity of target input.
9. Plot the C_T value versus the RNA concentration input to calculate the slope (Fig. 2). Generally, the software calculates the slope value automatically. The slope of a standard curve provides an indication of the efficiency of a real-time PCR run. A slope of −3.322 means that the PCR has an efficiency of 1, with the amount of PCR product that doubles at each cycle.
10. Calculate the PCR efficiency according to the following equation: $E = 10^{(-1/\text{slope})}$

    To determine the relative quantification of a target gene in comparison with a reference gene, use the method described by Pfaffl [9], and calculate the relative expression ratio using the following equation:

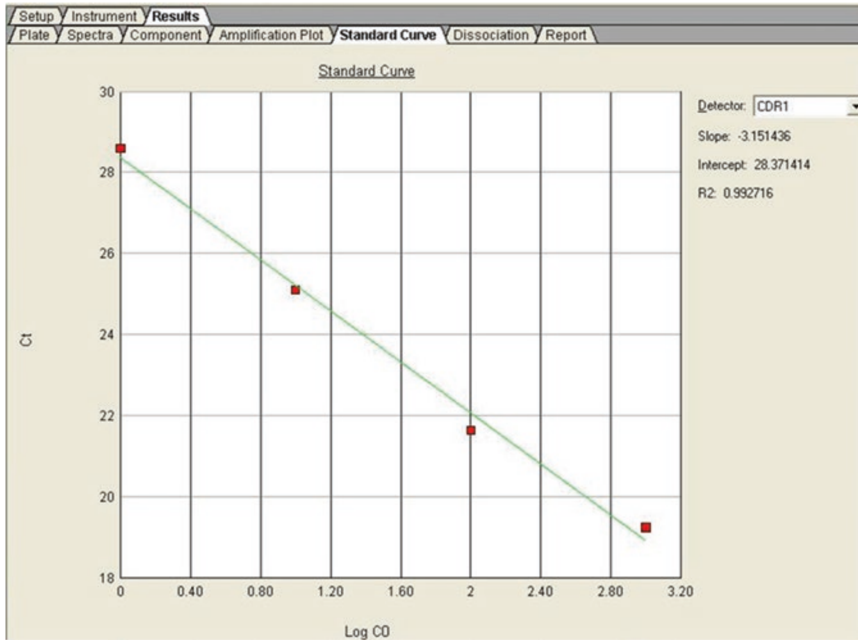

Fig. 2 RNA sample concentration. Indicated as log CO plotted versus C_T. All points represent the mean of triplicate PCR amplifications. Slope and other parameters are shown automatically

Table 5
Efficiencies of qRT-PCRs for the expression of azole resistance-associated *C. glabrata* genes

Gene	Slope	–1/Slope	Efficiency
CDR1	–3.15	0.32	2.08
CDR2	–3.61	0.28	1.91
SNQ2	–3.28	0.30	2.00
ERG11	–3.37	0.30	2.00
ACT1	–3.41	0.29	1.95

$$\text{ratio} = \frac{\left(E_{\text{target}}\right)^{C_T \text{ target(control sample)}}}{\left(E_{\text{ref}}\right)^{C_T \text{ ref(control sample)}}}$$

11. A twofold increase in the level of expression of the target gene is considered significant.

Example:
Sample: A FCZ-resistant clinical *C. glabrata* isolate.
Control: The FCZ-susceptible *C. glabrata* DSY564 [10].

Table 5 shows the efficiency values for qRT-PCR reactions performed to assess the expression of azole

Table 6
Relative expression of *C. glabrata* target genes in comparison with the *ACT1* reference gene

Gene	Efficiency	Control		Sample		ΔC_T ($C_{T\text{control}} - C_{T\text{ sample}}$)	$E^{\Delta C_T}$	Ratio
		Mean C_T[a]	% CV[b]	Mean C_T	% CV			
CDR1	2.08	24.40	2.00	19.93	0.60	4.47	26.41	25.39
CDR2	1.89	19.34	0.50	18.46	1.21	0.88	1.75	1.68
SNQ2	2.02	21.79	0.19	22.02	0.18	−0.23	0.85	0.81
ERG11	1.98	23.28	0.28	22.89	0.57	0.39	1.30	1.25
ACT1	1.96	22.59	0.38	22.53	0.14	0.06	1.04	

[a]Mean C_T value was calculated from the values obtained for three replicated reactions with the same RNA sample
[b]*CV* coefficient of variance

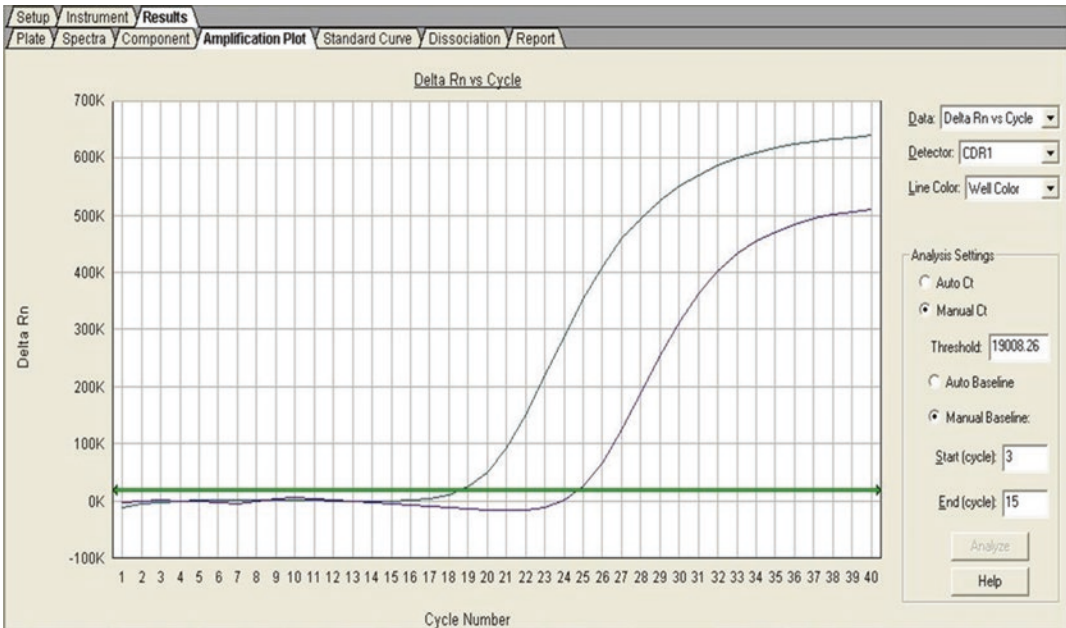

Fig. 3 Amplification plots. The plots show increases in the fluorescence level that appear sooner in the sample (C_T 19) than in the control (C_T 25)

resistance-associated *C. glabrata CDR1*, *CDR2*, *SNQ2*, *ERG11*, and *ACT1* genes, whereas Table 6 shows the relative expression of these target genes in comparison with the *ACT1* reference gene. On the basis of the expression ratio values obtained with the sample (FCZ-resistant clinical *C. glabrata* isolate) RNA, it argues that only the *CDR1* gene is overexpressed. In addition, the fluorescence curves obtained for the sample and control RNAs with respect to the *CDR1* target gene are shown (Fig. 3).

3.4 MOULD: DNA Extraction

1. For fungal DNA extraction, use the DNeasy Plant Mini Kit. The following protocol is essentially that described in the kit, unless some slight modifications.
2. Add 400 μL of AP1 buffer, 4 μL of RNase A and 1 volume of glass beads (0.45–0.55 mm diameter) in a microvial.
3. Add ≤100 mg of mycelium and disrupt with a tip (*see* **Note 7**). Vortex and incubate for 10 min at 65 °C.
4. Add 130 μL of AP2 buffer and incubate for 5 min on ice.
5. Vortex and agitate the sample using a mini-Beatbeater at max speed for 10 s, incubate for 2 min at 65 °C and afterwards for 2 min on ice. Repeat for two times.
6. Centrifuge the lysate for 5 min at 16,000 × *g*.
7. Pipet the supernatant into a QIAshredder Mini spin column (pink column) in a 2-mL collection tube. Centrifuge for 2 min at 16,000 × *g*.
8. Transfer the flow-through fraction into a new tube, without disturbing the pellet. Add 1.5 volumes of AP3/E buffer, and mix by pipetting.
9. Transfer 650 μL of the mixture into a DNeasy Mini Spin column (white column) in a 2-mL collection tube. Centrifuge for 1 min at 10,000 × *g*. Discard flow-through. Repeat this step with the remaining sample.
10. Place the spin column into a new 2-mL collection tube. Add 500 μL of AW buffer and centrifuge for 1 min at 10,000 × *g*. Discard flow-through.
11. Add another 500 μL of AW buffer and centrifuge for 2 min at 16,000 × *g*. Discard flow-through.
12. Transfer the spin column to a new 1.5-mL microcentrifuge tube, and add 140 μL of DNase-free water (*see* **Note 8**). Incubate for 5 min at room temperature. Centrifuge for 1 min at 10,000 × *g*. Store DNA at 4 °C.
13. Further details are available in the DNeasy Plant Mini Kit protocol.

3.5 MOULD: DNA Measurement

See description provided in the Chapter 22.

3.6 MOULD: Polymerase Chain Reaction (PCR) and Sequencing Reaction

1. Primers for PCR amplification of the *cyp51A* gene and primers for sequencing of PCR products [11] are listed in Table 7.
2. Instructions about the primers and PCR setting, the gel electrophoresis of PCR products, the PCR products purification, the sequencing reaction and purification, the electrophoresis on the ABI PRISM DNA Analyzer can be found in the Chapter 22.

Table 7
Primers for the *cyp51A* gene PCR amplification and sequencing

Primer	Sequence (5′-3′)	Use
0F	TCATATGTTGCTCAGCGG	PCR
4R	CCTATTCCGATCACACCAAA	
1R	CATTGAGCAAGATTGCCG	Sequencing
2F	CGGCAATCTTGCTCAATG	
2R	GGTGAATCGCGCAGATAGT	
3F	ACTATCTGCGCGATTCACC	
3R	GTCAAGATCCTTGTACTGGAGC	
4F	CTCCAGTACAAGGATCTTGAC	

Table 8
***Cyp51A* gene mutations most commonly associated with patterns of azole resistance or reduced susceptibility in *A. fumigatus* isolates**

Mutation	Azole			Reference
	ITZ	PSC	VRZ	
G54	–[a]	–	+[b]	[12, 13]
M220	–	+/–[c]	+/–	[14]
G464S	–	–	–	[15]
G138C	–	–	–	[16]
TR34/L98H	–	–	–	[17]
TR46/Y121F/T289A			–	[18–20]

[a]Isolates harboring this mutation are azole-resistant
[b]Isolates harboring this mutation are azole-susceptible
[c]Isolates harboring this mutation are low-level susceptible to azoles

3.7 MOULD: Hot Spot Mutation Assessment

To assess for the presence of mutations in the *cyp51A* gene, the wild-type gene sequences from *A. fumigatus* reference strain (GenBank: AF338659.1) are compared with those obtained from the clinical *A. fumigatus* isolate under study. For details about the sequence analysis, *see* Chapter 22. Table 8 shows the most common mutations associated with azole susceptibility patterns in *A. fumigatus* isolates [12–20].

4 Notes

1. Prior to proceeding with RNA extraction, use RNaseZap® RNase Decontamination Solution.
   (Thermo Fisher Scientific) in order to decontaminate accurately the surface of laminar flow cabinet, the centrifuge and the pipettes from RNase.
2. Before use, keep the growth medium at room temperature.
3. To prepare fungal cultures, work aseptically near to a Bunsen flame or under a laminar flow cabinet.
4. For the spectrophotometer use, follow carefully the instructions reported in the instrument manual.
5. Prepare the qRT-PCR master mix under a laminar flow cabinet dedicated only for mix PCR preparation, in order to avoid any acid nucleic contamination. Use only pipettes and rack tube dedicated.
6. Each RNA sample must be tested in triplicate.
7. Work with fungal cultures under a laminar flow cabinet.
8. Preheating nuclease-free water at 56 °C is useful to elute DNA from DNeasy Mini Spin column filter efficiently.

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Part VI

Novel Experimental Approaches

Chapter 25

Immune Cell-Supplemented Human Skin Model for Studying Fungal Infections

Andreas Kühbacher, Kai Sohn, Anke Burger-Kentischer, and Steffen Rupp

Abstract

Human skin is a niche for various fungal species which either colonize the surface of this tissue as commensals or, primarily under conditions of immunosuppression, invade the skin and cause infection. Here we present a method for generation of a human *in vitro* skin model supplemented with immune cells of choice. This model represents a complex yet amenable tool to study molecular mechanisms of host–fungi interactions at human skin.

Key words 3D skin model, Immune reaction to fungi, Host–fungi interaction, Infection model

1 Introduction

Interaction of fungi with human epithelial surfaces like the skin or mucosa can be of commensal or pathogenic nature. In either case, molecular interactions between the two organisms are complex and involve not only the epithelium itself but also various types of immune cells in a tissue specific manner. These include neutrophils, dendritic cells and different types of T cells [1]. Therefore such mechanisms can only be studied with complex host models. Several mouse models of fungal infection exist [2, 3]; however, drawbacks of such models are substantial differences to the human host [4, 5] as well as ethical considerations. Different epithelial *in vitro* models like recombinant human epithelia (RHE) or basic 3D tissue models have been used in the past for infection studies [6–8]. Yet these models do usually not comprise epithelium, a subepithelial layer and components of the immune system at the same time. More complex models containing keratinocytes, fibroblasts, and specific types of immune cells exist as well, which are however not constructed in a way that allows for easy separation of individual cell types for downstream analysis [9, 10]. We therefore

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present here an *in vitro* 3D skin model that can be supplemented with different types of immune cells and is constructed in a modular way. This infection model is amenable to various types of subsequent analysis methods including histological staining and next-generation sequencing. It further allows for analyzing individual cell types separately as they are arranged in layers that are separable after the experiment. The immune cell supplemented human skin model presented here combines the advantages of cultured cells like relative ease of handling, robustness, and human genetic background with some of the assets of whole animal models like three-dimensional tissue architecture and a microenvironment comprising multiple cell types including defined components of the immune system.

We describe a protocol using immortalized cells for 3D skin reconstruction and supplementation with T cells. The use of immortalized cells highly improves reproducibility of the tissue architecture and facilitates increased throughput as cell availability is no limitation. In addition, since they are derived from primary cells by well-defined genetic manipulation the general architecture of the 3D model is phenotypically indistinguishable from 3D models generated from primary cells from donors. However, in particular the immune cells may be replaced by primary cells isolated from human blood or buffy coat if required, due to the lack of appropriate immortalized cells. The protocol is divided in three parts: First, a basic skin model consisting of fibroblasts embedded in a collagen matrix and keratinocytes growing on top is generated. After proliferation and differentiation of the epidermal keratinocytes, the tissue model can be supplemented with immune cells. In this example we use Jurkat T cells that are embedded in a lower concentration collagen gel underneath the skin model. Embedding of immune cells prevents these cells from getting diluted in the surrounding medium. Finally the immune cell supplemented model can be infected with pathogens, like *Candida albicans* (Fig. 1). Analysis of the infection process can be performed for each cell type individually,

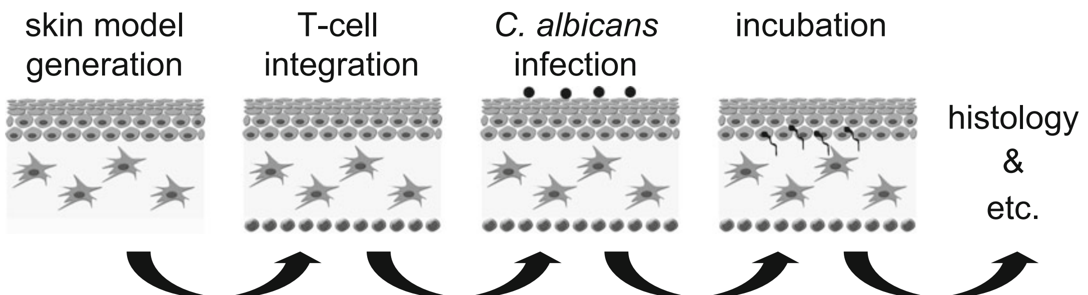

Fig. 1 Scheme of skin model construction and infection. Scheme depicting the three steps of the protocol presented here: construction of a basic skin model with dermal fibroblasts and keratinocytes, integration of immune cells below the tissue, and infection with *C. albicans*

by separating the individual layers of the model skin. This enables the identification of the individual contribution of each cell type to the response against invading pathogens.

2 Materials

The protocol presented here is optimized for immortalized keratinocytes (Ker-CT) and fibroblasts (S1F). Integration of immune cells is shown using the example of the Jurkat T cell line (*see* **Note 1**). Other cell lines as well as primary cells may be used; however, adaptations may be necessary especially for other types of immune cells.

2.1 Cells and Fungi

1. Immortalized human fibroblasts S1F [11].
2. Immortalized human keratinocytes Ker-CT (American Type Culture Collection, ATCC) [12].
3. Jurkat human T cells (American Type Culture Collection, ATCC).
4. *Candida albicans* strain SC5314.

2.2 Media, Cell Culture Components, and Buffers

1. Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} supplemented with 1 Mm EDTA.
2. DMEMcomplete: DMEM, 10% heat-inactivated FCS, 2 mM L-glutamine.
3. Keratinocyte growth medium 2 (KGM2, Promocell): Keratinocyte basal medium (KBM2), all supplements provided with the supplement pack: bovine pituitary extract (BPE) 0.004 ml/ml, epidermal growth factor (hEGF) 0.125 ng/ml, hydrocortisone 0.33 μg/ml, insulin 5 μg/ml, epinephrine 0.39 μg/ml, transferrin 10 μg/ml, $CaCl_2$ 0.06 mM.
4. KGMairlift medium: like KGM2 but without BPE and hEGF and additional 1.88 mM $CaCl_2$.
5. RPMIcomplete: RPMI1640, 10% heat-inactivated FCS, 2 mM L-glutamine.
6. Trypsin–EDTA solution.
7. Accutase solution.
8. Gel neutralization solution (GNS) 250 ml: 232.5 ml 2× concentrated DMEM, 7.5 ml heat-inactivated FCS, 7.5 ml 3 M HEPES, 1.25 ml chondroitin-4-sulfate 5 mg/ml stock solution, 1.25 ml chondroitin-6-sulfat 5 mg/ml stock solution. Either the individual components or the final GNS should be sterile-filtered.
9. Fibronectin solution 50 μg/ml in H_2O.
10. Yeast extract broth (YPD) supplemented with 2% glucose.

2.3 Consumables

1. Serological plastic pipettes.
2. T-75 cell culture flasks.
3. 24-well cell culture plate.
4. 12-well cell culture plate.
5. 6-well cell culture plates.
6. Cell culture inserts with 0.47 cm^2 surface and 8 μm pore size (ThermoFisher).
7. Type I collagen solution 6 mg/ml either commercial or extracted from rat tails.

3 Methods

The basic human skin model is built up from S1F immortalized dermal fibroblasts and Ker-CT keratinocytes. All cells and tissue models are cultured in a 37 °C incubator with a humidified 5% CO_2-containing atmosphere. Cells need to be handled under sterile conditions in a laminar flow hood and all media, solutions, and consumables must be sterile. Fibroblasts are cultured in DMEM supplemented with 10% FCS and keratinocytes are cultured in KGM with all supplements shipped with the medium. Cells should be subcultured twice a week at 70–80% confluency. For subculturing, fibroblasts are detached with trypsin while keratinocytes are detached with accutase (*see* **Note 2**) Jurkat T cells are kept in RPMI1640 supplemented with 10% FCS and subcultured twice a week. All numbers in this protocol are calculated for 6 tissue models. Three of these models are supplemented with Jurkat T cells and three models serve as an immune cell free control.

3.1 Construction of the Basic Skin Model

1. Invert the tube with collagen (*see* **Note 3**) from the fridge several times and if necessary spin down at 200 × *g* at 4 °C for 10 min to remove bubbles.
2. Transfer 6 cell culture inserts to a 24-well plate.
3. Prepare a 15 ml plastic tube with 3 ml collagen and keep on ice (*see* **Notes 4** and **5**).
4. Detach fibroblasts from T-75 flask. To do so, wash cells once with 5 ml Mg^{2+}—and Ca^{2+} free PBS, 1 mM EDTA and detach for 3 min with 1 ml trypsin at 37 °C/5% CO_2.
5. Resuspend cells in DMEMcomplete in a total volume of 10 ml.
6. Count cells and prepare a 15 ml plastic tube with 9×10^5 cells (*see* **Note 5**).
7. Spin down cells at 200 × *g* for 5 min.

8. Aspirate the medium and resuspend the cell pellet in 1.5 ml ice-cold GNS without making bubbles.
9. Transfer the cells/GNS suspension in the tube with collage on ice using a 1000 μl pipette tip or a 5 ml plastic pipette if larger volumes are prepared, and mix by pipetting up and down carefully and stirring with the pipette. Avoid making bubbles. Cell suspension and collagen are mixed at a ratio of 1:2 to get a final cell suspension of 2×10^5 cells per ml.
10. Transfer 500 μl of the cell-containing collagen gel in each insert using a 1000 μl pipette (*see* **Note 6**).
11. Transfer the inserts for 30 min to a 37 °C/5 % CO_2 incubator to allow the collagen to polymerize.
12. If the collagen is polymerized add 2 ml DMEM complete to each well for submerse culture (*see* **Note 7**).
13. Transfer the plate back to the incubator for 24 h.
14. Aspirate medium from the insert and the outer well without injuring the collagen gel and transfer the inserts to a 12-well tissue culture plate.
15. Add 25 μl of a 50 μg/ml fibronectin solution on top of each collagen gel and transfer the plate back to the incubator at 37 °C/5 % CO_2 and let there for 30 min.
16. Meanwhile Detach keratinocytes from 2T-75 flasks of 70–80 % cell confluence. To do so, wash cells once with 5 ml Mg^{2+}—and Ca^{2+} free PBS, 1 mM EDTA and detach for 10–15 min with 1 ml accutase solution at 37 °C/5 % CO_2.
17. Resuspend cells in a total volume of 10 ml KGM2 medium.
18. Count cells and prepare a 15 ml plastic tube 3.3×10^6 cells.
19. Spin down cells at $200 \times g$ for 5 min.
20. Aspirate the medium and resuspend the cell pellet in 660 μl KGM2 medium to a final concentration of 5×10^6 cells/ml. Add 100 μl cell suspension on top of each collagen gel in the insert.
21. Transfer the plate with the tissue culture inserts to the incubator at 37 °C/5 % CO_2 and let there for at least 2 h to let the keratinocytes attach to the collagen matrix.
22. Add 4.5 ml KGM2 (*see* **Notes 7** and **8**) medium for submerse culture and keep for 3 days in the incubator at 37 °C/5 % CO_2.
23. Aspirate the medium and transfer each insert to one well of a 6-well plate (*see* **Note 9**).
24. Carefully detach the gel from the border of the insert using a 200 μl pipette tip (*see* **Note 10**).
25. Add KGMairlift (*see* **Note 11**) medium to the outer well but keep the inside of the insert dry. The meniscus of medium

should be as high as the gel, so that nutrient supply is optimal but the surface of the model stays dry (*see* **Note 12**).

26. A few hours after airlift make sure that the surface of the models is still dry and remove excess of medium if necessary.
27. Replace airlift medium every 2–3 days. Adjust the volume to the shrinking tissue models (*see* **Note 13**) so that the meniscus is constantly at the same level as the tissue surface which must stay dry. The tissues are cultured at the air–liquid interface for 10 days.

3.2 Integration of Immune Cells

1. Invert the tube with collagen from the fridge several times and if necessary spin down at 200 × *g* at 4 °C for 10 min to remove bubbles.
2. Transfer six new cell culture inserts to a new 6-well plate (*see* **Note 14**).
3. Prepare two times a 2 ml plastic tube with 300 μl collagen and keep on ice.
4. Count Jurkat T cells and transfer 4.5×10^6 cells to a 15 ml plastic tube (*see* **Note 15**).
5. Spin down cells for 5 min at 200 × *g*.
6. Take the inserts with the skin models out of the incubator and aspirate the medium from the outer well.
7. Take the skin models out of the inserts with tweezers and place them besides the insert in the outer well of the 6-well plate.
8. Wash Jurkat cells once with 5 ml KGMairlift medium (*see* **Note 16**) and resuspend the cell pellet in 600 μl cold GNS.
9. Transfer the cell suspension to the prepared collagen gel and mix well without making bubbles. 600 μl GNS without cells in collagen are mixed with the other aliquot of collagen and serve as control for 3 of the tissue models (*see* **Note 17**). The final GNS–collagen ratio should be 2:1 with a final cell concentration of 5×10^6 cells/ml.
10. Transfer 200 μl of the Jurkat cell–collagen mix or the cell free mix to each insert and transfer to the incubator at 37 °C/5 % CO_2 for 2–3 min. As soon as the collagen polymerizes take the plate out of the incubator and place the skin models on top of the fresh collagen gel (*see* **Note 18**).
11. Transfer the plate back to the incubator at 37 °C/5 % CO_2 and let polymerization go on for another 30 min.
12. Add KGMairlift medium in the outer well and keep for 24 h at 37 °C/5 % CO_2. Normally 2–2.25 ml medium is required per well. The meniscus of the medium should be at the same level as the tissue surface which needs to stay dry.

3.3 Infection of Skin Models

1. The evening prior to infection prepare a *Candida albicans* overnight culture (*see* **Note 19**) by inoculating 15 ml YPD supplemented with glucose in a 50 ml falcon tube with *C. albicans* strain SC5314 from a frozen stock (*see* **Note 20**).
2. Make a small whole in the lid with a hot syringe needle to allow for air exchange.
3. Incubate in a shaker in tilted position at 30 °C and 160 rpm overnight.
4. 3–5 h before infection replace the medium in the outer wells of the tissue cultures by fresh KGMairlift medium.
5. Directly before infection dilute the *C. albicans* overnight culture 1:4 in fresh medium and measure the OD_{600} (*see* **Note 21**).
6. Calculate the fungal cell number based on the assumption that an OD_{600} of 1 equals 3×10^7 cells/ml (*see* **Note 22**).
7. Spin down 500 μl culture for 3 min at $2500 \times g$ and wash once in 500 μl PBS (*see* **Note 23**).
8. Prepare a fungal cell suspension of 20,000 cells/ml sterile PBS.
9. Add 5 μl (100 fungal cells) (*see* **Note 24**) of the fungal suspension on top of each tissue model or add PBS on top of uninfected control tissues. Be careful to place the drop on the center of the model in order to avoid leaking.
10. Transfer the plate back to the incubator at 37 °C/5 % CO_2 and let the infection go on for 2 days or the desired time period (*see* **Note 24**).
11. For histological analysis, infected or non-infected tissue models (Fig. 2) should be formalin-fixed and paraffin-embedded according to standard procedures. Alternatively, keratinocytes, fibroblasts, and immune cells may be separated with tweezers for molecular or biochemical analysis of individual cell types (*see* **Note 25**).

4 Notes

1. Primary cells can be used for generation of skin models; however, there may be significant variation between donors. Moreover cell numbers obtained from one donor may be limited. Therefore this protocol has been optimized for immortalized cells. In this way it can be guaranteed the best that others are able to reproduce construction of the skin model. The protocol can however easily be adapted to primary cells.
2. Accutase should be removed by centrifugation after cell detachment since it strongly decreases cell attachment even when diluted in fresh KGM2.

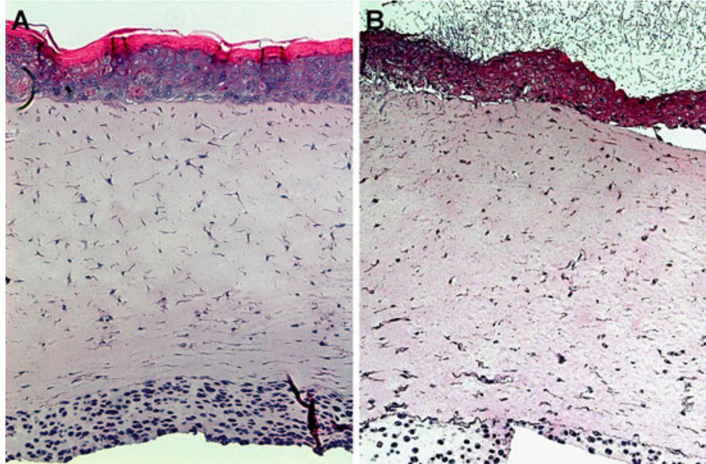

Fig. 2 Non-infected and infected human skin model supplemented with Jurkat T cells. Hematoxylin/Eosin stained skin model sections either non-infected (**a**) or infected for 2 days with 100 colony forming units *C. albicans* (**b**) are shown

3. Commercial collagen may be used or collagen may be extracted from rat tail tendons. To do so rat tail tendons are disinfected for 30 min in 70 % ethanol. After removing the skin and removing 2 to 3 cm of the tail at each end cut the tail in three pieces and isolate the tendons using a scalpel. Weight the tendons, disinfect them with 70 % ethanol, and wash several times with PBS until they are clean. Then transfer 1 g of tendons to 10 ml 0.1 % acetic acid in water and incubate for at least 1 week at 4 °C and 500 rpm on a magnetic stirrer. For purification of the collagen centrifuge at 4 °C first at $3000 \times g$ for 15 min, then transfer the supernatant to new tubes and centrifuge again at $17{,}000 \times g$ for 1 h. The collagen can be aliquoted and stored at −20 °C. Purity may be determined by SDS-PAGE.
4. Since pipetting collagen is very unprecise it is recommended to compare the volume in the 15 ml tube with the required volume of water in an equal tube in order to make sure that the right volume has been pipetted.
5. Only 0.33 ml collagen and 1×10^5 fibroblasts are required per insert. Because of the high viscosity of collagen solution, there is however a high pipetting loss. Therefore always prepare cell suspensions in collagen in excess (at least 15 %).
6. At room temperature collagen quickly polymerizes. Therefore it is important to handle it on ice. Air bubbles should be avoided in the collagen solution as they cannot be removed easily from the viscous material.
7. During submerse culture it is important that the insert is fully immersed in medium.

8. After the keratinocytes are added the whole model is grown in keratinocyte growth medium since it is compatible with both, fibroblasts and keratinocytes. DMEM supplemented with FCS would be optimal for fibroblasts; however, it cannot be used for keratinocytes.
9. During the airlift phase the tissue models get access to medium only from below. The medium level cannot be higher than the tissue model. Therefore the inserts need to be moved to a 6-well plate with a larger radius in order to be able to provide enough volume for the medium.
10. The tissue models substantially decrease in volume during airlift growth. If the collagen matrix is still attached to the inner wall of the insert, shrinkage would not be homogeneous and the model would be asymmetric. To avoid this, the collagen gel is detached from the insert wall by holding the insert with tweezers and carefully pulling away the gel from the wall all around with a 200 μl pipette tip. Like this symmetric and flat tissue models are obtained that develop a small well in the center which is ideal later on for treatment of the skin model surface or for infection.
11. In order to switch keratinocyte growth from proliferation to differentiation and to obtain a stratified and cornified epithelium, keratinocyte medium needs to be supplemented with additional calcium and it needs to be devoid of BPE and hEGF.
12. During airlift growth the tissue models access medium only from underneath. It can happen that an air bubble is trapped directly below the insert. Such bubbles need to be avoided and the plate should be checked carefully before transferring it to the incubator.
13. If the tissue models don't shrink during airlift growth make sure that there is no bubble below the insert bottom preventing access to the medium.
14. When the immune cell-containing collagen is transferred to the old wet insert, it runs through the pored before polymerizing. Therefore we recommend using a fresh and dry 6-well plate and new cell culture inserts at this step.
15. The number of a certain type of immune cells added to the tissue model may be varied. The concentration of the respective cell type in blood may be a first approximation.
16. Before integration of the immune cells into the tissue model they are grown in a different medium that for example often contains FCS which is not compatible with keratinocytes or may alter the tissue in any way. We therefore recommend washing immune cells once with KGMairlift before using them for tissue models.

17. For comparisons of tissue models with and without a certain type of immune cells we advise to use tissue models transferred on top of empty collagen gel as a control in order to exclude the possibility that the observed effect is not solely caused by the presence of the additional collagen in the immune cell compartment.
18. In order to achieve ideal attachment of the dermal layer of the skin model to the new collagen gel containing the immune cells, the skin model should be transferred to the immune cell compartment before it is completely polymerized. It should however also not be transferred too early when the collagen of the immune cell gel is still liquid. In this case the skin model would sink. One can transfer the rest of the cell/collagen mix in the tube also to the incubator and use it as indicator for polymerization.
19. *C. albicans* is grown over night. We use this stationary phase culture for infection. Alternatively the culture can be diluted in the morning and let grown for another few hours in order to obtain a culture in exponential growth phase for infection.
20. Here we show the protocol for skin model infection with *C. albicans* strain SC5314. The tissue models may however be as well infected with other *C. albicans* strains of other relevant fungal species. Infection dose and time should be optimized in this case.
21. The OD_{600} of an overnight grown *C. albicans* culture is higher than 1. In order to be able to measure the optical density in a linear range it should be diluted in YPD to an OD_{600} of below 0.9. We use then this diluted aliquot directly for the experiment.
22. The correlation of OD_{600} value and density of viable fungal cells should be determined for the individual species with the spectroscope used. This can be done with a standard curve correlating OD_{600} with colony forming units.
23. Before using the fungal cells for infection experiments they should be washed with PBS in order to remove residual YPD which may influence the host tissue by itself.
24. For *C. albicans* we infect skin models with 100 colony forming units per tissue culture insert. At this infection dose one should infect for 24 h or longer in order to observe invasion. Alternatively the infection dose may be increased and the infection time decreased.
25. For separation of the individual layers of the skin models we advise shorter infection times as the epithelium may not be easily pulled off the dermal layer if it is already too much destroyed. In our experience the individual layers can be well separated up to 24 h post infection with *C. albicans*. Proper separation should be confirmed by microscopy of tissue sections of the dermal layer.

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