AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology

MINIREVIEW

Molecular Diagnostic Testing for *Aspergillus*

Margaret V. Powers-Fletcher,[a,b] Kimberly E. Hanson[a,b,c]

Department of Pathology, Clinical Microbiology Division, University of Utah, Salt Lake City, Utah, USA[a]; Institute for Clinical and Experimental Pathology, ARUP Laboratories, Salt Lake City, Utah, USA[b]; Department of Medicine, Infectious Diseases Division, University of Utah, Salt Lake City, Utah, USA[c]

The direct detection of *Aspergillus* nucleic acid in clinical specimens has the potential to improve the diagnosis of aspergillosis by offering more rapid and sensitive identification of invasive infections than is possible with traditional techniques, such as culture or histopathology. Molecular tests for *Aspergillus* have been limited historically by lack of standardization and variable sensitivities and specificities. Recent efforts have been directed at addressing these limitations and optimizing assay performance using a variety of specimen types. This review provides a summary of standardization efforts and outlines the complexities of molecular testing for *Aspergillus* in clinical mycology.

Aspergillosis, defined as an infection caused by organisms included in the genus *Aspergillus*, constitutes a spectrum of diseases that range from allergic reactions to disseminated disease in immunocompromised hosts. *Aspergillus fumigatus* is the predominant organism causing invasive aspergillosis (IA), but other species have become increasingly recognized as important opportunistic pathogens (1).

Despite recent advances in antifungal therapy, IA continues to cause significant morbidity and mortality, in part due to difficulties and delays in making a microbiologic diagnosis (2). Diagnosis has relied historically on the isolation of *Aspergillus* in culture combined with compatible histopathologic or radiographic features of disease. Fungal culture, however, is relatively slow and insensitive, while histopathology and radiographic imaging are not organism specific. The use of fungal cell wall biomarkers, such as 1,3-β-D-glucan or *Aspergillus* galactomannan antigen, has improved early diagnosis of IA, but these methods also have significant limitations, including poor sensitivity in certain patient groups (3) and issues with nonspecificity (4). There has been significant recent interest in the use of molecular diagnostics to aid in the rapid and accurate diagnosis of aspergillosis. Additionally, the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) composite definitions for invasive fungal infections are being updated (5). The question of including molecular detection techniques for definitions of IA will be revisited. Although EORTC/MSG definitions were designed primarily for clinical and epidemiologic research involving hematology/oncology patients (6), the inclusion of *Aspergillus* nucleic acid amplification testing (NAAT) in the IA definitions may foster increased use of these tests for clinical care in the near future. This review highlights some of the complexities associated with DNA sequence-based identification strategies and summarizes current approaches for the direct detection of *Aspergillus* nucleic acids in clinical specimens.

ASPERGILLUS TAXONOMY AND MOLECULAR SPECIES RECOGNITION

The genus *Aspergillus* is subdivided into eight subgenera, with each subgenus subdivided into sections that include many related species (7). The section *Fumigati*, for example, includes more than 30 species, with at least 10 isolated from clinical specimens (8). *A. fumigatus* is the most important pathogen within the *Fumigati* section. However, phenotypic identification of a cultured isolate to the species level can be problematic because of the overlapping morphological features of these organisms. As a result, it has been proposed that closely related species within the medically important sections *Fumigati*, *Flavi*, *Nidulantes*, *Usti*, and *Terrei* be reported by the clinical laboratory as a "species complex" (8). This approach confers the added benefit of minimizing taxonomic confusion and potentially decreases the likelihood that rarer pathogenic species will be overlooked or dismissed as insignificant.

Confident identification of a cultured *Aspergillus* isolate to the species level requires molecular interrogation. Identifying isolates to the species level is useful for epidemiologic studies and may be clinically relevant, since newly described species within the *Fumigati* complex (*A. lentulus*) and the *Usti* complex (*A. calidoustus*) reportedly have elevated MICs to several antifungal drugs, including azoles (9, 10). At the molecular genetics level, the nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS) region, which spans ITS-1, 5.8S, and ITS-2, was selected as the official DNA barcode for fungal identification because of the completeness of available sequence databases and the fact that universal primers have been designed to target this region (11). ITS sequences, however, may lack sufficient variation for resolution of some *Aspergillus* species, and a secondary barcode or identification marker is usually needed to identify an isolate to the species level (12). On the basis of these observations, the International Society for Human and Animal Mycology-sponsored *Aspergillus* Working Group has recommended use of the ITS region for identification of cultured isolates to the species complex level and a protein-coding locus, such as for β-tubulin (*BenA*) or calmodulin (*CaM*), for the identification of individual species within the complex.

Accepted manuscript posted online 3 August 2016

Citation Powers-Fletcher MV, Hanson KE. 2016. Molecular diagnostic testing for *Aspergillus*. J Clin Microbiol 54:2655–2660. doi:10.1128/JCM.00818-16.

Editor: C. S. Kraft, Emory University

Address correspondence to Kimberly E. Hanson, kim.hanson@hsc.utah.edu.

MOLECULAR METHODS FOR DIRECT DETECTION OF *ASPERGILLUS* IN CLINICAL SPECIMENS

A variety of research-use-only and/or CE-marked commercial kits for the direct detection of *Aspergillus* nucleic acid have been described. Performance of these assays varies on the basis of specimen type and patient population and the matter in which infection status was determined in the study (13–23). So far, no molecular assays for *Aspergillus* have been approved by the U.S. Food and Drug Administration (FDA) for use in the diagnosis of IA. Molecular testing in the United States has, therefore, relied on laboratory-developed tests (LDTs), which also vary widely in terms of test formats and performance. Recent work has focused on identifying the critical components of assay design with the aim of improving standardization of LDTs across assays and clinical laboratories.

DNA versus RNA amplification platforms. NAAT has the potential to be highly sensitive and specific for the detection of *Aspergillus* DNA or RNA. Amplification of DNA by PCR has been the most widely applied NAAT method, in large part because this platform is routinely used for bacterial and viral organisms. Nested PCR protocols have been developed in an attempt to optimize sensitivity (24), but caution is required with these formats because minor contaminants from the first round of PCR can be amplified and lead to false-positive results.

Multiple different DNA amplicon detection methods have been described and include fluorescently labeled probes (25, 26), electrospray ionization mass spectrometry (ESI-MS) (25, 26), and enzyme linked immunosorbent assays (ELISAs) (27, 28). Additionally, real-time PCR can be used to quantify fungal burden (29), while multiplexed arrays allow for the differentiation of larger numbers of pathogenic species (30).

Isothermal amplification techniques, such as nucleic acid sequence-based amplification (NASBA), have also been used to detect *Aspergillus* nucleic acids (25, 31). NASBA offers several potential advantages over PCR, including more efficient amplification, the potential to assess cell viability, and lack of concern over contamination with fungal DNA (31, 32). Additionally, the use of RNA templates may increase sensitivity by capitalizing on the fact that highly expressed genes produce thousands of transcripts within a cell.

In situ hybridization (ISH) probes targeting fungal rDNA have been used for the detection of *Aspergillus* in fresh and formalin-fixed paraffin-embedded (FFPE) tissues without the requirement for nucleic acid extraction or amplification. ISH enables direct visualization of organisms with the use of labeled probes that bind to complementary fungal sequences. Genus-specific probes are potentially useful for differentiating *Aspergillus* spp. from other fungal pathogens that produce septate branching hyphae *in vivo*. However, ISH sensitivity may be limited compared to that of traditional histopathology stains (33), and results have sometimes been difficult to interpret due to the autofluorescence of hyphae or high background produced by necrotic tissue (34).

Gene targets. The rDNA gene cluster is the most common target of assays designed for direct detection in clinical specimens (35) due to the presence of highly conserved (18S and 28S) and variable (ITS and D1/D2) regions. In addition to robust rDNA sequence databases and the availability of universal primers, an additional advantage of using the rDNA genes as a diagnostic target is their copy numbers. Experiments evaluating different strains of *A. fumigatus* observed 38 to 91 copies of the 18S rDNA subunit per genome (36), which may increase the sensitivity of detection. A potential limitation of rDNA targets, however, is the close phylogenetic relationship between *Aspergillus*, *Penicillium*, and *Paecilomyces* spp. (37). Depending on the primer and probe sequences, cross-reactivity among these species can be predicted and is an important consideration when testing respiratory specimens.

In general, genus-level assay designs often target 18S, while *A. fumigatus*-specific assays target ITS-1, mitochondrial DNA, alkaline protease, or *Aspergillus* collagen-like (*acl*) genes. A gene encoding a hemolysin that is overexpressed *in vivo* during infection (*aspHS*) may also have promise as an alternative target, with improved specificity for active infection due to its increased detection in germinated conidia compared to that in nongerminated conidia (38).

Nucleic acid extraction method. The quality and quantity of nucleic acid that are available for amplification greatly influence the performance of molecular detection assays. Significant differences in extraction efficiency have been observed across specimen types. For example, extracting high-quality nucleic acid from FFPE can be difficult due to the DNA degradation that occurs after formalin fixation (39). Using contrived whole-blood samples seeded with *Aspergillus* conidia, the European *Aspergillus* PCR Initiative (EAPCRI) showed that extraction-related factors, including specimen volume, use of mechanical disruption (bead beating) of fungal cell walls, lysis of white cells, and elution volumes, had statistically significant impacts on assay sensitivity (40). On the basis of these observations, the EAPCRI proposed a standardized fungal DNA extraction protocol for use with *Aspergillus* PCR whole-blood assays (41). A follow-up study was conducted to evaluate the impact of combining the EAPCRI recommendations with four different automated, commercially available nucleic acid extraction platforms (42). PCR efficiency, procedure duration, ease of use, and cost were all considered in the selection of a preferred system. Whole-blood specimens with known quantities of *Aspergillus* conidia were analyzed, and all four platforms extracted as little as 10 CFU/ml. However, instruments varied in their ability to reproduce extraction efficiency, contamination rates, and ease of use. The optimal automated extraction platform will likely vary somewhat by the needs and workflow of individual laboratories and require local verification.

An important limitation of these studies is the use of conidia as the reference material for assay comparisons. Intact conidia are not thought to circulate in the blood of infected patients or to be the predominant form of the organism present in tissue-invasive disease. From a practical perspective, however, this has been the only way to standardize fungal inoculum and simultaneously challenge the diagnostic method with the various complexities presented by the fungal cell wall.

Specimen type. Blood and respiratory and tissue specimens have been used predominantly for direct detection of *Aspergillus*. Testing fractions of blood is particularly desirable because these specimens can be obtained noninvasively. In a meta-analysis of 16 studies that combined the results of more than 10,000 serum, plasma, or whole-blood samples from 1,618 at-risk patients, the overall sensitivity of a single positive *Aspergillus* PCR was 88%, with a specificity of 75% for proven or probable IA (43). Requiring two sequential positive PCRs increased the specificity from 75% to 87% without impacting the overall clinical sensitivity.

Which blood fraction is optimal for testing has not been set-

tled. Few studies have directly compared test performance using whole blood, sera, and plasma collected simultaneously. Furthermore, heterogeneities in assay design, patient population, and the criteria used to define a positive test have impeded direct comparisons across studies. While recognizing these limitations, there are several general observations to note. First, experiments using *Aspergillus* conidia spiked in whole-blood and plasma samples have shown identical limits of detection (10 CFU/ml and 100 fg DNA) in both matrixes (44). When specimens were obtained from allogeneic hematopoietic stem cell transplant (HSCT) recipients with histologically proven IA, however, *Aspergillus* DNA was detected more often in whole blood than in plasma (44). Circulating *Aspergillus* nucleic acid was also detected earlier in the course of disease using whole blood than when using sera (44). These studies suggest that testing whole blood may be more sensitive than testing either sera or plasma.

A meta-analysis of whole-blood and serum studies also found that PCR assays of whole blood yield a higher sensitivity (86% versus 76%, respectively) and lower specificity (73% versus 85%, respectively) than those of sera, but this difference did not reach statistical significance (35). EAPCRI-compliant studies (i.e., no more than one deviation from the EAPCRI criteria) showed a statistically significantly higher PCR specificity (98% versus 85%) with an additional nonsignificant increase in sensitivity (67% versus 61%) relative to studies that did not follow EAPCRI guidance. When at least two positive PCRs were used to define a positive result, PCR specificity was increased (95% versus 73%), whereas sensitivity decreased (85% versus 64%).

Most recently, a study of hematology patients with probable IA compared all three specimen types (whole blood, plasma, and serum) using EAPCRI-recommended protocols (45). PCR performed with plasma resulted in the highest sensitivity (91%), followed by PCR from serum and whole blood (sensitivities of 80% and 55%, respectively). These observations stand in contrast to those of previous reports, possibly because of differences in processing (i.e., plasma was removed from the whole-blood pellet) and prolonged specimen storage methods for the whole-blood specimens used in the study. Specificity was actually significantly higher using whole-blood pellets (96%) than when using either serum (69%) or plasma (53%). When at least two sequential specimens were required to define a positive result, the specificity of plasma increased to 92%, with a decline in sensitivity to 82%. The authors suggested that testing multiple specimen types can optimize test characteristics, but this may not be practical in a routine clinical setting.

In sum, EAPCRI protocols have been shown to enhance *Aspergillus* PCR test characteristics. Requiring at least two positive PCR results to define a positive blood test improves assay specificity, potentially at the expense of sensitivity. Lastly, testing serum or plasma appears to result in acceptable sensitivity and specificity. These specimen types are easier to process than whole blood, which requires preanalytical cell lysis steps, and using serum confers the added advantage of allowing biomarker testing of the same sample.

Respiratory specimens are the next most commonly studied specimen type. A meta-analysis of 15 different studies of bronchoalveolar lavage (BAL) specimens reported a pooled sensitivity and specificity of 79% and 94%, respectively, for proven/probable IA (46). An important limitation of testing respiratory specimens is the inability to differentiate airway colonization from invasive disease on the basis of nucleic acid detection alone (47). High fungal burdens determined by quantitative PCR (qPCR) may be more suggestive of IA, but there remains significant overlap in the fungal loads measured in cases of airway colonization versus those of invasive pulmonary disease (48). Additional confounders affecting accurate fungal quantitation include differences in assay design, the inherent variability of BAL fluid sampling, and variable rDNA target gene copy numbers.

In an attempt to address qPCR assay variability, the EAPCRI collaborated with the *Aspergillus* Technology Consortium (AsTeC) and Invasive Aspergillosis Animal Models (IAAM) group to create an *A. fumigatus* DNA calibrator (49). Twelve clinical and research laboratories in the United States and Europe independently determined the concentration of DNA in the calibrator by performing replicate testing of a dilution series. Results were then combined to assign a value to the undiluted stock material that was derived from conidia. Similar to the international quantitation standards for HIV, hepatitis C virus, Epstein-Barr virus, and cytomegalovirus, the use of an *A. fumigatus*-specific calibrator may help harmonize assessments of fungal load across assays and clinical laboratories.

Molecular testing for pathogenic fungi has also been applied to biopsy material consisting of fresh or FFPE tissues. Panfungal approaches, rather than pathogen-specific assays, are used more commonly in this context to broaden the diagnostic yield. These LDTs result in sensitivities that have ranged from 86% to 94% in culture-proven cases and 64% to 89% in histopathology-confirmed cases (34, 50–52). *Aspergillus*-specific PCRs have also been applied to tissue specimens. In one study that included 59 fresh tissue samples from 49 at-risk patients, a genus-level nested PCR had 89% sensitivity with 100% specificity for proven/probable disease (53). Tissue-based diagnostic approaches are limited by the need for invasive procedures, however, which may not be possible in critically ill patients.

SURVEILLANCE STRATEGIES

Molecular testing for IA has been deployed as either a standalone test for at-risk patients with compatible signs/symptoms or a part of screening algorithms. Several studies involving hematology/HSCT patients evaluated serial surveillance with blood collected at weekly intervals during defined periods of risk. The first report was from a randomized study conducted in 409 HSCT recipients that compared an *Aspergillus* PCR-based preemptive treatment approach to empirical antifungal therapy triggered by febrile neutropenia (54). Twice-weekly PCR surveillance was continued until day 30 posttransplant, and then once-weekly testing continued until day 90 posttransplant. A single positive PCR result was required to initiate preemptive treatment. Patients in both groups received antifungal therapy after 120 h of febrile neutropenia that did not respond to broad-spectrum antibacterial therapy, regardless of PCR results, or if pulmonary infiltrates were detected. An improved 30-day survival rate was observed in the group that received preemptive treatment compared to that for those treated on the basis of symptoms alone (mortality rate, 1.5% versus 6.3%, respectively; $P = 0.015$).

Combining nucleic acid and antigen detection is an alternative strategy. In one study, combination monitoring led to earlier diagnosis and a lower incidence of IA in high-risk hematology patients receiving fluconazole prophylaxis than did galactomannan surveillance alone (55). Another open-label study of 240 adult

hematologic malignancy patients and HSCT recipients randomized subjects to receive preemptive therapy based on *Aspergillus* PCR plus galactomannan testing or empirical antifungal therapy that was driven by clinical symptoms, culture, and/or histology (56). Overall, the preemptive group had less antifungal drug exposure than did the empirical therapy group (difference, 17%; $P = 0.002$), with no significant differences observed in the incidence of proven IA, all-cause mortality, or *Aspergillus*-attributable mortality between groups.

Altogether, these studies support the concept that preemptive therapy based on molecular diagnostic testing is safe and effective. The combination of *Aspergillus* PCR and galactomannan testing may be better than the use of either test alone. The cost-effectiveness of preemptive strategies compared to that of universal antifungal prophylaxis in high-risk patients, however, requires additional study.

MOLECULAR METHODS FOR DETECTION OF ANTIFUNGAL DRUG RESISTANCE

Over the last decade, an increasing number of *A. fumigatus* isolates recovered from both the environment and clinical specimens have been found to display decreased susceptibility to azole antifungal drugs. Available evidence suggests that resistance may be emerging as a result of the widespread usage of these compounds in agriculture and clinics. The predominant resistance mechanism involves mutations in the *cyp51A* gene, which encodes a protein targeted by azole antifungal drugs, and a variety of these mutations have been shown to confer azole resistance (57). Molecular resistance testing has been applied to cultured isolates as a part of epidemiologic drug resistance surveillance studies (58) and to clinical isolates as a supplement to phenotypic susceptibility testing (59). Multiplex real-time PCR has also been used to detect azole-resistant strains directly in BAL fluid from patients at risk for IA (17). With serum samples spiked with *A. fumigatus* DNA, this multiplex assay was shown to achieve a limit of detection of 75, 100, 500, and 75 genomes per sample for the L98H, T289A, TR34, and Y121F mutations, respectively. When applied to clinical specimens, however, no resistance mutations were detected, likely due to a lack of resistant strains in the studied patient population (15). Central registries of treatment and outcome data for patients with molecularly and phenotypically defined azole-resistant *Aspergillus* infection are needed to determine the potential utility of molecular resistance testing for use in clinical care.

ASSAY QUALITY CONTROL

Fungi are ubiquitous in the environment; therefore, there is significant risk for fungal DNA contamination resulting in a false-positive reaction. Contamination of clinical specimens, collection devices (60), and reagents (61) should be closely monitored by the laboratory and considered by the clinician when interpreting results. Conversely, the potential for false-negative results is also a concern. Inhibitory substances in complex sample matrixes, such as in FFPE tissues, can interfere with the assay chemistry and result in a failure to amplify the target sequence (39). The inclusion of negative, positive, and internal controls is essential for *Aspergillus* assay quality control programs (40).

SUMMARY AND CONCLUSIONS

Molecular diagnostics for *Aspergillus* have significant potential to improve clinical outcomes by promoting a more rapid and accurate diagnosis of invasive disease than is possible with classical methods. Molecular assays for *Aspergillus* are highly complex, and nuances in assay design, as well as in result interpretation, significantly impact test performance. In addition to the complexity inherent in these tests, a significant barrier to their use has been the lack of commercially available standardized assays. Recent works by the EAPCRI, AsTeC, and IAAM have advanced the *Aspergillus* LDT field, but an FDA-approved assay will likely be required to bring *Aspergillus* molecular testing to mainstream clinical care in the United States. In the meantime, the importance of nucleic acid extraction procedures and quality control programs should be emphasized for laboratories that elect to develop their own tests.

REFERENCES

1. **Torres HA, Rivero GA, Lewis RE, Hachem R, Raad II, Kontoyiannis DP.** 2003. Aspergillosis caused by nonfumigatus *Aspergillus* species: risk factors and *in vitro* susceptibility compared with *Aspergillus fumigatus*. Diagn Microbiol Infect Dis **46:**25–28. http://dx.doi.org/10.1016/S0732-8893(03)00013-0.
2. **Denning DW.** 1996. Therapeutic outcome in invasive aspergillosis. Clin Infect Dis **23:**608–615. http://dx.doi.org/10.1093/clinids/23.3.608.
3. **Pfeiffer CD, Fine JP, Safdar N.** 2006. Diagnosis of invasive aspergillosis using a galactomannan assay: a meta-analysis. Clin Infect Dis **42:**1417–1427. http://dx.doi.org/10.1086/503427.
4. **Sulahian A, Porcher R, Bergeron A, Touratier S, Raffoux E, Menotti J, Derouin F, Ribaud P.** 2014. Use and limits of (1-3)-beta-d-glucan assay (Fungitell), compared to galactomannan determination (Platelia *Aspergillus*), for diagnosis of invasive aspergillosis. J Clin Microbiol **52:**2328–2333. http://dx.doi.org/10.1128/JCM.03567-13.
5. **De Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, Pappas PG, Maertens J, Lortholary O, Kauffman CA, Denning DW, Patterson TF, Maschmeyer G, Bille J, Dismukes WE, Herbrecht R, Hope WW, Kibbler CC, Kullberg BJ, Marr KA, Munoz P, Odds FC, Perfect JR, Restrepo A, Ruhnke M, Segal BH, Sobel JD, Sorrell TC, Viscoli C, Wingard JR, Zaoutis T, Bennett JE.** 2008. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin Infect Dis **46:**1813–1821. http://dx.doi.org/10.1086/588660.
6. **Ascioglu S, Rex JH, de Pauw B, Bennett JE, Bille J, Crokaert F, Denning DW, Donnelly JP, Edwards JE, Erjavec Z, Fiere D, Lortholary O, Maertens J, Meis JF, Patterson TF, Ritter J, Selleslag D, Shah PM, Stevens DA, Walsh TJ.** 2002. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. Clin Infect Dis **34:**7–14. http://dx.doi.org/10.1086/323335.
7. **Klich MA.** 2006. Identification of clinically relevant aspergilli. Med Mycol **44:**127–131. http://dx.doi.org/10.1080/13693780600796546.
8. **Balajee SA, Houbraken J, Verweij PE, Hong SB, Yaghuchi T, Varga J, Samson RA.** 2007. *Aspergillus* species identification in the clinical setting. Stud Mycol **59:**39–46. http://dx.doi.org/10.3114/sim.2007.59.05.
9. **Balajee SA, Gribskov JL, Hanley E, Nickle D, Marr KA.** 2005. *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. Eukaryot Cell **4:**625–632. http://dx.doi.org/10.1128/EC.4.3.625-632.2005.
10. **Varga J, Houbraken J, Van Der Lee HA, Verweij PE, Samson RA.** 2008. *Aspergillus calidoustus* sp. nov., causative agent of human infections previously assigned to *Aspergillus ustus*. Eukaryot Cell **7:**630–638. http://dx.doi.org/10.1128/EC.00425-07.
11. **Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W.** 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. Proc Natl Acad Sci U S A **109:**6241–6246. http://dx.doi.org/10.1073/pnas.1117018109.
12. **Samson RA, Visagie CM, Houbraken J, Hong SB, Hubka V, Klaassen CH, Perrone G, Seifert KA, Susca A, Tanney JB, Varga J, Kocsube S, Szigeti G, Yaguchi T, Frisvad JC.** 2014. Phylogeny, identification and nomenclature of the genus *Aspergillus*. Stud Mycol **78:**141–173. http://dx.doi.org/10.1016/j.simyco.2014.07.004.
13. **White PL, Hibbitts SJ, Perry MD, Green J, Stirling E, Woodford L,**

McNay G, Stevenson R, Barnes RA. 2014. Evaluation of a commercially developed semiautomated PCR-surface-enhanced Raman scattering assay for diagnosis of invasive fungal disease. J Clin Microbiol **52:**3536–3543. http://dx.doi.org/10.1128/JCM.01135-14.

14. **White PL, Perry MD, Moody A, Follett SA, Morgan G, Barnes RA.** 2011. Evaluation of analytical and preliminary clinical performance of *Myconostica* MycAssay *Aspergillus* when testing serum specimens for diagnosis of invasive aspergillosis. J Clin Microbiol **49:**2169–2174. http://dx.doi.org/10.1128/JCM.00101-11.
15. **White PL, Posso RB, Barnes RA.** 2015. Analytical and clinical evaluation of the PathoNostics AsperGenius assay for detection of invasive aspergillosis and resistance to azole antifungal drugs during testing of serum samples. J Clin Microbiol **53:**2115–2121. http://dx.doi.org/10.1128/JCM.00667-15.
16. **Torelli R, Sanguinetti M, Moody A, Pagano L, Caira M, De Carolis E, Fuso L, De Pascale G, Bello G, Antonelli M, Fadda G, Posteraro B.** 2011. Diagnosis of invasive aspergillosis by a commercial real-time PCR assay for *Aspergillus* DNA in bronchoalveolar lavage fluid samples from high-risk patients compared to a galactomannan enzyme immunoassay. J Clin Microbiol **49:**4273–4278. http://dx.doi.org/10.1128/JCM.05026-11.
17. **Chong GL, van de Sande WW, Dingemans GJ, Gaajetaan GR, Vonk AG, Hayette MP, van Tegelen DW, Simons GF, Rijnders BJ.** 2015. Validation of a new *Aspergillus* real-time PCR assay for direct detection of *Aspergillus* and azole resistance of *Aspergillus fumigatus* on bronchoalveolar lavage fluid. J Clin Microbiol **53:**868–874. http://dx.doi.org/10.1128/JCM.03216-14.
18. **Huttner A, Emonet S, Harbarth S, Renzi G, Kaiser L, Schrenzel J.** 2014. Polymerase-chain reaction/electrospray ionization-mass spectrometry for the detection of bacteria and fungi in bronchoalveolar lavage fluids: a prospective observational study. Clin Microbiol Infect **20:**O1059–O1066. http://dx.doi.org/10.1111/1469-0691.12749.
19. **Orsi CF, Bettua C, Pini P, Venturelli C, La Regina A, Morace G, Luppi M, Forghieri F, Bigliardi S, Luppi F, Codeluppi M, Girardis M, Blasi E.** 2015. Detection of *Pneumocystis jirovecii* and *Aspergillus* spp. DNA in bronchoalveolar lavage fluids by commercial real-time PCR assays: comparison with conventional diagnostic tests. New Microbiol **38:**75–84.
20. **Pini P, Bettua C, Orsi CF, Venturelli C, Faglioni L, Forghieri F, Bigliardi S, Luppi F, Girardis M, Blasi E.** 2015. Clinical performance of a commercial real-time PCR assay for *Aspergillus* DNA detection in serum samples from high-risk patients: comparison with a galactomannan enzyme immunoassay. Eur J Clin Microbiol Infect Dis **34:**131–136. http://dx.doi.org/10.1007/s10096-014-2211-y.
21. **Aslan M, Oz Y, Aksit F, Akay OM.** 2015. Potential of polymerase chain reaction and galactomannan for the diagnosis of invasive aspergillosis in patients with febrile neutropenia. Mycoses **58:**343–349. http://dx.doi.org/10.1111/myc.12322.
22. **Oz Y, Aslan M, Aksit F, Metintas S, Gunduz E.** 2016. The effect of clinical characteristics on the performance of galactomannan and PCR for the diagnosis of invasive aspergillosis in febrile neutropenic patients. Mycoses **59:**86–92. http://dx.doi.org/10.1111/myc.12438.
23. **Danylo A, Courtemanche C, Pelletier R, Boudreault AA.** 2014. Performance of MycAssay *Aspergillus* DNA real-time PCR assay compared with the galactomannan detection assay for the diagnosis of invasive aspergillosis from serum samples. Med Mycol **52:**577–583. http://dx.doi.org/10.1093/mmy/myu025.
24. **Yamakami Y, Hashimoto A, Tokimatsu I, Nasu M.** 1996. PCR detection of DNA specific for *Aspergillus* species in serum of patients with invasive aspergillosis. J Clin Microbiol **34:**2464–2468.
25. **Zhao Y, Park S, Kreiswirth BN, Ginocchio CC, Veyret R, Laayoun A, Troesch A, Perlin DS.** 2009. Rapid real-time nucleic acid sequence-based amplification-molecular beacon platform to detect fungal and bacterial bloodstream infections. J Clin Microbiol **47:**2067–2078. http://dx.doi.org/10.1128/JCM.02230-08.
26. **Alonso M, Escribano P, Guinea J, Recio S, Simon A, Pelaez T, Bouza E, Garcia de Viedma D.** 2012. Rapid detection and identification of *Aspergillus* from lower respiratory tract specimens by use of a combined probe-high-resolution melting analysis. J Clin Microbiol **50:**3238–3243. http://dx.doi.org/10.1128/JCM.00176-12.
27. **Jones ME, Fox AJ, Barnes AJ, Oppenheim BA, Balagopal P, Morgenstern GR, Scarffe JH.** 1998. PCR-ELISA for the early diagnosis of invasive pulmonary *Aspergillus* infection in neutropenic patients. J Clin Pathol **51:**652–656. http://dx.doi.org/10.1136/jcp.51.9.652.
28. **Florent M, Katsahian S, Vekhoff A, Levy V, Rio B, Marie JP, Bouvet A, Cornet M.** 2006. Prospective evaluation of a polymerase chain reaction-ELISA targeted to *Aspergillus fumigatus* and *Aspergillus flavus* for the early diagnosis of invasive aspergillosis in patients with hematological malignancies. J Infect Dis **193:**741–747. http://dx.doi.org/10.1086/500466.
29. **Khot PD, Fredricks DN.** 2009. PCR-based diagnosis of human fungal infections. Expert Rev Anti Infect Ther **7:**1201–1221. http://dx.doi.org/10.1586/eri.09.104.
30. **Buelow DR, Gu Z, Walsh TJ, Hayden RT.** 2012. Evaluation of multiplexed PCR and liquid-phase array for identification of respiratory fungal pathogens. Med Mycol **50:**775–780. http://dx.doi.org/10.3109/13693786.2012.666681.
31. **Loeffler J, Hebart H, Cox P, Flues N, Schumacher U, Einsele H.** 2001. Nucleic acid sequence-based amplification of *Aspergillus* RNA in blood samples. J Clin Microbiol **39:**1626–1629. http://dx.doi.org/10.1128/JCM.39.4.1626-1629.2001.
32. **Compton J.** 1991. Nucleic acid sequence-based amplification. Nature **350:**91–92. http://dx.doi.org/10.1038/350091a0.
33. **Hayden RT, Isotalo PA, Parrett T, Wolk DM, Qian X, Roberts GD, Lloyd RV.** 2003. *In situ* hybridization for the differentiation of *Aspergillus*, *Fusarium*, and *Pseudallescheria* species in tissue section. Diagn Mol Pathol **12:**21–26. http://dx.doi.org/10.1097/00019606-200303000-00003.
34. **Rickerts V, Khot PD, Myerson D, Ko DL, Lambrecht E, Fredricks DN.** 2011. Comparison of quantitative real-time PCR with sequencing and ribosomal RNA-FISH for the identification of fungi in formalin fixed, paraffin-embedded tissue specimens. BMC Infect Dis **11:**202. http://dx.doi.org/10.1186/1471-2334-11-202.
35. **Arvanitis M, Ziakas PD, Zacharioudakis IM, Zervou FN, Caliendo AM, Mylonakis E.** 2014. PCR in diagnosis of invasive aspergillosis: a meta-analysis of diagnostic performance. J Clin Microbiol **52:**3731–3742. http://dx.doi.org/10.1128/JCM.01365-14.
36. **Herrera ML, Vallor AC, Gelfond JA, Patterson TF, Wickes BL.** 2009. Strain-dependent variation in 18S ribosomal DNA copy numbers in *Aspergillus fumigatus*. J Clin Microbiol **47:**1325–1332. http://dx.doi.org/10.1128/JCM.02073-08.
37. **Hope WW, Walsh TJ, Denning DW.** 2005. Laboratory diagnosis of invasive aspergillosis. Lancet Infect Dis **5:**609–622. http://dx.doi.org/10.1016/S1473-3099(05)70238-3.
38. **Abad-Diaz-De-Cerio A, Fernandez-Molina JV, Ramirez-Garcia A, Sendino J, Hernando FL, Peman J, Garaizar J, Rementeria A.** 2013. The *aspHS* gene as a new target for detecting *Aspergillus fumigatus* during infections by quantitative real-time PCR. Med Mycol **51:**545–554. http://dx.doi.org/10.3109/13693786.2012.756989.
39. **Babouee Flury B, Weisser M, Prince SS, Bubendorf L, Battegay M, Frei R, Goldenberger D.** 2014. Performances of two different panfungal PCRs to detect mould DNA in formalin-fixed paraffin-embedded tissue: what are the limiting factors? BMC Infect Dis **14:**692. http://dx.doi.org/10.1186/s12879-014-0692-z.
40. **White PL, Bretagne S, Klingspor L, Melchers WJ, McCulloch E, Schulz B, Finnstrom N, Mengoli C, Barnes RA, Donnelly JP, Loeffler J.** 2010. *Aspergillus* PCR: one step closer to standardization. J Clin Microbiol **48:**1231–1240. http://dx.doi.org/10.1128/JCM.01767-09.
41. **White PL, Perry MD, Loeffler J, Melchers W, Klingspor L, Bretagne S, McCulloch E, Cuenca-Estrella M, Finnstrom N, Donnelly JP, Barnes RA.** 2010. Critical stages of extracting DNA from *Aspergillus fumigatus* in whole-blood specimens. J Clin Microbiol **48:**3753–3755. http://dx.doi.org/10.1128/JCM.01466-10.
42. **Perry MD, White PL, Barnes RA.** 2014. Comparison of four automated nucleic acid extraction platforms for the recovery of DNA from *Aspergillus fumigatus*. J Med Microbiol **63:**1160–1166. http://dx.doi.org/10.1099/jmm.0.076315-0.
43. **Mengoli C, Cruciani M, Barnes RA, Loeffler J, Donnelly JP.** 2009. Use of PCR for diagnosis of invasive aspergillosis: systematic review and meta-analysis. Lancet Infect Dis **9:**89–96. http://dx.doi.org/10.1016/S1473-3099(09)70019-2.
44. **Loeffler J, Hebart H, Brauchle U, Schumacher U, Einsele H.** 2000. Comparison between plasma and whole blood specimens for detection of *Aspergillus* DNA by PCR. J Clin Microbiol **38:**3830–3833.
45. **Springer J, White PL, Hamilton S, Michel D, Barnes RA, Einsele H, Loffler J.** 2016. Comparison of performance characteristics of *Aspergillus* PCR in testing a range of blood-based samples in accordance with international methodological recommendations. J Clin Microbiol **54:**705–711. http://dx.doi.org/10.1128/JCM.02814-15.
46. **Tuon FF.** 2007. A systematic literature review on the diagnosis of invasive

aspergillosis using polymerase chain reaction (PCR) from bronchoalveolar lavage clinical samples. Rev Iberoam Micol **24:**89–94. http://dx.doi.org/10.1016/S1130-1406(07)70020-7.

47. **Hayette MP, Vaira D, Susin F, Boland P, Christiaens G, Melin P, De Mol P.** 2001. Detection of *Aspergillus* species DNA by PCR in bronchoalveolar lavage fluid. J Clin Microbiol **39:**2338–2340. http://dx.doi.org/10.1128/JCM.39.6.2338-2340.2001.
48. **Luong ML, Clancy CJ, Vadnerkar A, Kwak EJ, Silveira FP, Wissel MC, Grantham KJ, Shields RK, Crespo M, Pilewski J, Toyoda Y, Kleiboeker SB, Pakstis D, Reddy SK, Walsh TJ, Nguyen MH.** 2011. Comparison of an *Aspergillus* real-time polymerase chain reaction assay with galactomannan testing of bronchoalvelolar lavage fluid for the diagnosis of invasive pulmonary aspergillosis in lung transplant recipients. Clin Infect Dis **52:**1218–1226. http://dx.doi.org/10.1093/cid/cir185.
49. **Lyon GM, Abdul-Ali D, Loeffler J, White PL, Wickes B, Herrera ML, Alexander BD, Baden LR, Clancy C, Denning D, Nguyen MH, Sugrue M, Wheat LJ, Wingard JR, Donnelly JP, Barnes R, Patterson TF, Caliendo AM.** 2013. Development and evaluation of a calibrator material for nucleic acid-based assays for diagnosing aspergillosis. J Clin Microbiol **51:**2403–2405. http://dx.doi.org/10.1128/JCM.00744-13.
50. **Muñoz-Cadavid C, Rudd S, Zaki SR, Patel M, Moser SA, Brandt ME, Gomez BL.** 2010. Improving molecular detection of fungal DNA in formalin-fixed paraffin-embedded tissues: comparison of five tissue DNA extraction methods using panfungal PCR. J Clin Microbiol **48:**2147–2153. http://dx.doi.org/10.1128/JCM.00459-10.
51. **Lau A, Chen S, Sorrell T, Carter D, Malik R, Martin P, Halliday C.** 2007. Development and clinical application of a panfungal PCR assay to detect and identify fungal DNA in tissue specimens. J Clin Microbiol **45:**380–385. http://dx.doi.org/10.1128/JCM.01862-06.
52. **Buitrago MJ, Aguado JM, Ballen A, Bernal-Martinez L, Prieto M, Garcia-Reyne A, Garcia-Rodriguez J, Rodriguez-Tudela JL, Cuenca-Estrella M.** 2013. Efficacy of DNA amplification in tissue biopsy samples to improve the detection of invasive fungal disease. Clin Microbiol Infect **19:**E271–E277. http://dx.doi.org/10.1111/1469-0691.12110.
53. **Reinwald M, Spiess B, Heinz WJ, Heussel CP, Bertz H, Cornely OA, Hahn J, Lehrnbecher T, Kiehl M, Laws HJ, Wolf HH, Schwerdtfeger R, Schultheis B, Burchardt A, Klein M, Durken M, Claus B, Schlegel F, Hummel M, Hofmann WK, Buchheidt D.** 2013. *Aspergillus* PCR-based investigation of fresh tissue and effusion samples in patients with suspected invasive aspergillosis enhances diagnostic capabilities. J Clin Microbiol **51:**4178–4185. http://dx.doi.org/10.1128/JCM.02387-13.
54. **Hebart H, Klingspor L, Klingebiel T, Loeffler J, Tollemar J, Ljungman P, Wandt H, Schaefer-Eckart K, Dornbusch HJ, Meisner C, Engel C, Stenger N, Mayer T, Ringden O, Einsele H.** 2009. A prospective randomized controlled trial comparing PCR-based and empirical treatment with liposomal amphotericin B in patients after allo-SCT. Bone Marrow Transplant **43:**553–561. http://dx.doi.org/10.1038/bmt.2008.355.
55. **Aguado JM, Vazquez L, Fernandez-Ruiz M, Villaescusa T, Ruiz-Camps I, Barba P, Silva JT, Batlle M, Solano C, Gallardo D, Heras I, Polo M, Varela R, Vallejo C, Olave T, Lopez-Jimenez J, Rovira M, Parody R, Cuenca-Estrella M.** 2015. Serum galactomannan versus a combination of galactomannan and polymerase chain reaction-based *Aspergillus* DNA detection for early therapy of invasive aspergillosis in high-risk hematological patients: a randomized controlled trial. Clin Infect Dis **60:**405–414. http://dx.doi.org/10.1093/cid/ciu833.
56. **Morrissey CO, Chen SC, Sorrell TC, Milliken S, Bardy PG, Bradstock KF, Szer J, Halliday CL, Gilroy NM, Moore J, Schwarer AP, Guy S, Bajel A, Tramontana AR, Spelman T, Slavin MA, Australasian Leukaemia Lymphoma Group Australia and New Zealand Mycology Interest Group.** 2013. Galactomannan and PCR versus culture and histology for directing use of antifungal treatment for invasive aspergillosis in high-risk haematology patients: a randomised controlled trial. Lancet Infect Dis **13:**519–528. http://dx.doi.org/10.1016/S1473-3099(13)70076-8.
57. **Bader O, Weig M, Reichard U, Lugert R, Kuhns M, Christner M, Held J, Peter S, Schumacher U, Buchheidt D, Tintelnot K, Gross U, Myko-LabNet-D Partners.** 2013. *cyp51A*-Based mechanisms of *Aspergillus fumigatus* azole drug resistance present in clinical samples from Germany. Antimicrob Agents Chemother **57:**3513–3517. http://dx.doi.org/10.1128/AAC.00167-13.
58. **van der Linden JW, Arendrup MC, Warris A, Lagrou K, Pelloux H, Hauser PM, Chryssanthou E, Mellado E, Kidd SE, Tortorano AM, Dannaoui E, Gaustad P, Baddley JW, Uekotter A, Lass-Florl C, Klimko N, Moore CB, Denning DW, Pasqualotto AC, Kibbler C, Arikan-Akdagli S, Andes D, Meletiadis J, Naumiuk L, Nucci M, Melchers WJ, Verweij PE.** 2015. Prospective multicenter international surveillance of azole resistance in *Aspergillus fumigatus*. Emerg Infect Dis **21:**1041–1044. http://dx.doi.org/10.3201/eid2106.140717.
59. **Garcia-Effron G, Dilger A, Alcazar-Fuoli L, Park S, Mellado E, Perlin DS.** 2008. Rapid detection of triazole antifungal resistance in *Aspergillus fumigatus*. J Clin Microbiol **46:**1200–1206. http://dx.doi.org/10.1128/JCM.02330-07.
60. **Harrison E, Stalhberger T, Whelan R, Sugrue M, Wingard JR, Alexander BD, Follett SA, Bowyer P, Denning DW.** 2010. *Aspergillus* DNA contamination in blood collection tubes. Diagn Microbiol Infect Dis **67:**392–394. http://dx.doi.org/10.1016/j.diagmicrobio.2010.02.028.
61. **Loeffler J, Hebart H, Bialek R, Hagmeyer L, Schmidt D, Serey FP, Hartmann M, Eucker J, Einsele H.** 1999. Contaminations occurring in fungal PCR assays. J Clin Microbiol **37:**1200–1202.

Margaret V. Powers-Fletcher, Ph.D., is a Medical and Public Health Laboratory Microbiology Fellow at the University of Utah/ARUP Laboratories. She attended graduate school at the University of Cincinnati, where she earned her Ph.D. after studying the pathogenesis of the opportunistic fungal pathogen *Aspergillus fumigatus*. During her fellowship, she expanded her research interests in the field of clinical mycology to include the improvement of diagnostics for invasive fungal infections and the appropriate interpretation of antifungal susceptibility testing. She has coauthored seven manuscripts and one book chapter related to clinical mycology and has received awards from the American Heart Association and the Medical Mycological Society of the Americas for her work related to the pathogenesis of *A. fumigatus*.

Kimberly E. Hanson, M.D., M.H.S., is Head of the Mycology Section within ARUP Laboratories and Director of the Transplant Infectious Diseases and Immunocompromised Host Service at the University of Utah. She attended Northwestern University Medical School and completed an internal medicine residency at Dartmouth-Hitchcock Medical Center. Dr. Hanson went to Duke University for adult infectious diseases and medical microbiology fellowships, followed by a master's degree program in Health Sciences Research, after which she served as the Associate Director of Molecular Microbiology at Duke University before being recruited to the University of Utah. Currently, Dr. Hanson is an Associate Professor in the Departments of Medicine and Pathology. Her research is focused on the diagnosis and management of opportunistic infections affecting transplant recipients. Dr. Hanson has published more than 50 manuscripts, review articles, and book chapters. She is actively involved in laboratory administration, patient care, and medical education.