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(New) Methods for Detection of *Aspergillus fumigatus* Resistance in Clinical Samples

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Abstract

Purpose of Review—The incidence of invasive aspergillosis has increased substantially over the past few decades, accompanied by a change in susceptibility patterns of *Aspergillus fumigatus* with increasing resistance observed against triazole antifungals, including voriconazole and isavuconazole, the most commonly used antifungal agents for the disease. Culture-based methods for determining triazole resistance are still the gold standard but are time consuming and lack sensitivity. We sought to provide an update on non-culture-based methods for detecting resistance patterns to *Aspergillus*.

Recent Findings—New molecular-based approaches for detecting triazole resistance to *Aspergillus*, real-time polymerase chain reaction (PCR) to detect mutations to the *Cyp51A* protein, have been developed which are able to detect most triazole-resistant *A. fumigatus* strains in patients with invasive aspergillosis.

Summary—Over the last few years, a number of non-culture-based methods for molecular detection of *Aspergillus* triazole resistance have been developed that may overcome some of the limitations of culture. These molecular methods are therefore of high epidemiological and clinical relevance, mainly in immunocompromised patients with hematological malignancies, where culture has particularly limited sensitivity. These assays are now able to detect most triazole-resistant *Aspergillus fumigatus* strains. Given that resistance rates vary, clinical utility for these assays still depends on regional resistance patterns.

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Conflict of Interest Dieter Buchheidt reports being a consultant to Basilea, Gilead Sciences, and Merck Sharp & Dohme/Merck; receiving research grants from Gilead Sciences and Pfizer; serving on the speakers' bureau of Astellas, Basilea, Gilead Sciences, Merck Sharp & Dohme/Merck, Pfizer, and TEVA; and receiving travel grants from Astellas, Gilead Sciences, Merck Sharp & Dohme/Merck, and Pfizer. Martin Hoenigl reports untied research funding from Gilead. Jeffrey Jenks and Birgit Spiess declare no conflict of interest relevant to this manuscript.

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Keywords

Invasive aspergillosis; Triazoles; Cyp51A gene; Voriconazole; Isavuconazole

Introduction

Infections caused by *Aspergillus fumigatus* (*A. fumigatus*) affect patients with immune impairment, including those with hematologic malignancies and those with pre-existing lung conditions or liver cirrhosis, and are associated with devastating mortality rates [1••, 2••, 3]. Early and reliable diagnosis of invasive aspergillosis (IA) and subsequent rapid initiation of appropriate antifungal therapy has been shown to improve survival significantly [4, 5•]. However, IA progresses rapidly and is difficult to diagnose at early stages [6, 7•], and once diagnosed, emergence of triazole resistance complicates selection of appropriate antifungal treatment [8, 9, 10••].

Optimally, diagnostic tests for IA would therefore detect not only the presence or evidence of invasive growth of *Aspergillus* spp. but also triazole resistance. While most cases of IA are now diagnosed by fungal biomarkers, including galactomannan and 1,3- β -D-glucan [4, 11–13], these fail to provide information on antifungal susceptibility of the causative pathogen. Culture-based approaches have the potential to both detect the causative fungal species and detect resistance. However, culture-based approaches are limited by low sensitivities—in particular during early phases of infection—and long turnaround time [11]. Importantly, triazole resistance in *A. fumigatus* is caused by point mutations in certain genes, such as the *cyp51A* gene, which encodes the triazole target enzyme required for the biosynthesis of ergosterol [14]. These point mutations may be detected via molecular methods, overcoming the limitations of low sensitivity and long turnaround time that affect culture-based methods and—potentially—improving clinical management and survival in patients infected with triazole resistant *A. fumigatus*. Several polymerase chain reaction (PCR) assays have been developed that can detect triazole resistance mechanisms [15–20], and two are commercially available, AsperGenius® (PathoNostics, Maastricht, the Netherlands) and MycoGenie® (Adamtech, Pessac, France). In this review, we will discuss established and new methods for detection of *A. fumigatus* resistance in clinical samples.

Culture-Based Methods for Detection of Resistance in *Aspergillus fumigatus*

Susceptibility patterns of *A. fumigatus* to the most commonly used antifungal agents have changed over recent years, some of which may also be attributed to increased environmental use of azole fungicides [21] given their relatively low cost and effectiveness against a broad range of fungi [22]. For instance, in the European Union, half of all cereals and grapevine are treated with fungicides, including triazoles [23], and it is estimated that if azoles were no longer used in Europe, a significant fall in wheat production would result with an economic loss in the billions of Euros [22]. Thus, in vitro susceptibility testing for initial antifungal selection and monitoring while on antifungal therapy has become increasingly important.

In addition, antifungal susceptibility testing is important in monitoring background resistance and when comparing the in vitro activity of new antifungal agents to existing agents. Increasing resistance of *A. fumigatus* against triazoles is being documented, including resistance to isavuconazole, the newest triazole which was recently approved for first line treatment of IA [24]. Isavuconazole resistance has been emerging also among non-*A. fumigatus* species [25] and even among wild-type isolates [26],

Collaboration between investigators and the Clinical and Laboratory Standards Institute (CLSI) Subcommittee on Antifungal Susceptibility Testing has generated consensus documents detailing standardized methods for broth- and agar-based antifungal susceptibility testing [27]. A number of culture-based diagnostic tests exist to detect antifungal resistance, and we describe the most commonly used methods here.

Broth and Agar Dilution Methods

Dilution methods using broth and agar are considered the reference standard methods for determining the minimum inhibitory concentration (MIC) of antibiotic and antifungal agents. During antifungal testing with broth microdilution, varying concentrations of antifungal agents are placed in microdilution plate wells filled with broth culture media containing serial dilutions of antifungal agents. Following incubation, the plates are removed and examined for fungal growth, indicated by the presence of cloudy broth. The MIC to the antifungal agent is defined as the lowest concentration, in milligrams per liter, of the agent that inhibits growth of the fungus. In agar dilution, varying concentrations of antifungal agents are combined with melted agar to produce plates with varying serial dilutions of the antifungal agents being tested. *A. fumigatus* is added to spots on the plate, and following incubation, the plates are examined to determine if growth has occurred at the inoculated spots. The lowest concentration of antifungals that prevent *A. fumigatus* growth is considered to be the MIC of the antifungal agent. Although these methods are labor-intensive and expensive, they are the most frequently used method to determine the efficacy of new antibiotic or antifungal agents.

Etest® Method

Etest® (Biomérieux, Marcy-l'Étoile, France) is a commercially available, pre-formed test that consists of a predefined gradient of antibiotic concentrations on a plastic strip. After placement of the test strip and incubation of *A. fumigatus* on an agar plate for 24 to 48 h, an ellipse of growth inhibition occurs that can be used to determine the minimum inhibitory concentration (MIC) of the antifungal agents being tested. This method can be used to determine in vitro activity of a variety of antifungal agents including amphotericin B, flucytosine, caspofungin, and a number of triazoles including voriconazole and posaconazole. While not approved for use by the U.S. Food and Drug Administration (FDA) in the detection of antifungal resistance by filamentous fungi, including *A. fumigatus*, it is still a commonly used test and results have been shown to be in general agreement with the broth dilution reference method for determining susceptibility to amphotericin B [28, 29], itraconazole [29], posaconazole [28, 30, 31], isavuconazole [32, 33], and voriconazole [34], although correlation between broth dilution and caspofungin has proven to not be as robust [35].

Sensititre® Method

Sensititre® (TREK Diagnostic Systems, West Sussex, UK) is a commercially available colorimetric microdilution test that is used for susceptibility testing of yeast and fungi including *Candida* species, *Cryptococcus* species, and *Aspergillus* species. Similar to the broth microdilution method, individual plates are dosed with varying concentrations of antifungal agents as well as a colorimetric indicator. Unlike the broth dilution method, growth or lack of fungal growth is determined by color change rather than presence of turbidity in the plates. Following incubation for 24 h, MIC results are read at the first well that demonstrates a color change of red (indicating growth), purple (inhibition of growth), and blue (no growth). Thus, the lowest MIC is interpreted as the plate with the lowest concentration of antifungal agent that still retains a blue color. This method can be used to determine antifungal activity of amphotericin B, flucytosine, a number of echinocandin agents, and a number of triazoles including voriconazole and posaconazole. Compared to the Etest® method and broth dilution, the Sensititre® method has similar efficacy in determining susceptibility to amphotericin B and itraconazole against *Aspergillus* spp. in one study [29] but was inferior to the Etest® in another [36, 37].

MALDI-TOF MS Method

Newer approaches for identifying pathogens and performing antimicrobial and antifungal testing, such as matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), are being explored. By analyzing the composite correlation index (CCI), or similarities between spectra generated by microorganisms treated with different concentrations of drug, the minimal profile change concentration (MPCC) can be determined [38], which is the lowest drug concentration where the spectra are more similar to the maximum drug concentration than the null drug concentration. The MPCC has been shown to correlate well with the MIC determined by broth dilution in drug susceptibility testing of *Candida albicans* [39]. MALDI-TOF MS has shown good agreement with the CLSI reference method in the detection of resistance patterns to caspofungin with a number of *Candida* and *Aspergillus* spp. including *A. fumigatus* [40]. In a proof-of-concept study, MALDI-TOF MS was shown to accurately detect strains of *A. fumigatus* with reduced voriconazole susceptibility, although this method did not prove to be more rapid or simpler compared to testing with PCR and the Sensititre® microdilution panel [41]. Still, this method needs improved standardization, expanded availability of databases, and databases need to be continuously updated to include reference spectra of unusual pathogens [42, 43].

Molecular Approaches for Detection of Resistance of *Aspergillus fumigatus*

Incidence of IA caused by triazole-resistant *A. fumigatus* (ARAF) is increasing, particularly in patients with underlying hematological malignancies. As the diagnosis of IA is rarely based on positive culture in this group of patients, molecular detection of resistance mutations directly from clinical samples is crucial. Furthermore, the high mortality rates associated with ARAF underline the necessity of using non-culture-based assays for the detection of both *Aspergillus* spp. and triazole resistance directly from clinical samples [44–48, 49••].

Real-time PCR testing has been developed to detect triazole resistance to *A. fumigatus*. The Cyp51A protein, a central enzyme with lanosterol-14 α -demethylase activity in the ergosterol biosynthesis pathway, is a target for triazole antifungals with at least 13 mutations at six amino acid positions causing a resistance phenotype to triazoles [20], including itraconazole, posaconazole [50], voriconazole, and isavuconazole. Molecular PCR-based methods for detecting *Cyp51A* alterations directly from clinical samples have to be very sensitive and specific to ensure the amplification of small amounts of *Aspergillus* DNA and to avoid cross-reactivity with human DNA. It is difficult to amplify the whole *Cyp51A* gene from primary clinical samples due to the very small amount of intact fungal molecules in these samples. In this scenario, nested PCR assays using two consecutive PCRs amplifying the same gene region have shown the highest sensitivities. In addition to in-house triazole resistance, such as ARAf PCR assays detecting the frequent mutation combinations TR34/L98H, TR46/Y121F/T289A, and M220 in the *A. fumigatus Cyp51A* gene by subsequent DNA sequence analysis [44, 45], some real-time PCR systems without DNA sequence analysis are commercially available. For the *Cyp51A* amino acid substitutions G54 and M220 and other known and unknown mutations, several in-house PCR assays plus DNA sequencing from clinical *A. fumigatus* isolates have been established [20, 51, 52]. To detect potential mutations in the PCR products analyzed by DNA sequence analysis, the sequence of the products has to be compared to the sequence of the *A. fumigatus Cyp51A* wild-type sequence using the NCBI alignment service Align Sequence Nucleotide Blast (<http://www.ncbi.nlm.nih.gov/>) or e.a. the FunResDB-A [53].

An overview of commercially available DNA sequence analysis independent real-time PCR systems for detection of *Cyp51A* mutations was published by Rath and Steinmann in 2018 [54••]. The diagnostic performance for the detection of *Aspergillus* DNA of five commercial tests was described, including the MycAssay *Aspergillus*@ system, Microgen Bioproducts Ltd. (Camberley, UK), the AsperGenius@ assay from PathoNostics (Maastricht, the Netherlands), the MycoGenie@ assay from AdamTech (Pessac, France), and the SeptiFast@ system, which is a multiplex-real-time PCR assay for the detection of bacterial and fungal pathogens and last the RenDX Fungiplex@ assay from Renishaw Diagnostics Ltd. (Glasgow, UK).

In addition to the detection of *Aspergillus* DNA, the AsperGenius@ and MycoGenie@ real-time PCR systems are also able to analyze *Cyp51A* mutations. The AsperGenius@ detects the TR34/L98H and Y121F/T289A mutations by melting curve analysis, the MycoGenie@ can identify the TR34/L98H combination. The AsperGenius@ assay was evaluated in bronchoalveolar lavage (BAL) samples with sensitivity and specificity values of 84% and 80%, respectively. Eight samples revealed *Cyp51A* triazole resistance mutations [18, 48]. Schauwvlieghe et al. also analyzed BAL samples from patients with positive galactomannan in BAL and suspected invasive *Aspergillus* infection using the AsperGenius@ system detecting a sensitivity of 79%. Triazole resistance mutations were found in the form of TR34/L98H in eight and T289A/Y121F in three patients [55]. This test was also evaluated in 124 serum samples of 49 hemato-oncological patients (14 proven/probable cases, 33 control patients) and 211 plasma samples from 10 patients with proven or probable IA, two possible cases, and 27 controls. The described sensitivity was 78.6% and the specificity 100% for the first group and 80% and 77.8% for the second group of patients, respectively [56, 57]. The

detection of resistance markers in BAL fluid using the AsperGenius® method was also associated with an increased probability of treatment-failure to triazoles [48]. The AsperGenius® assay had a good diagnostic performance in BAL and serum samples. An advantage of this of the assay is the time saving aspect by detecting triazole resistance mutations without DNA sequence analysis.

The MycoGenie® PCR system is recommended for the testing of biopsy, respiratory, and serum samples. This assay has very high sensitivity by detecting the *28S rRNA* gene of *A. fumigatus* with a LOD of 1 copy/μl and the major triazole resistance mutation combination TR34/L98H with a LOD of 6 copies/μl. The system was evaluated in 2017 by Dannoui et al. in 88 respiratory samples and 69 serum samples of patients with proven or probable IA, revealing a sensitivity of 92.2% for respiratory samples and of 100% for serum samples [58]. A further study was published by Denis et al. comparing the MycoGenie® assay to an *A. fumigatus* Bio-Evolution® system (Bio-Evolution®, Bry-sur-Marne, France) using the *rRNA* gene ITS1 region for *Aspergillus* DNA detection. BAL samples ($n = 73$) of hematological and non-hematological patients were investigated. Patients were classified as proven, probable, and noIA groups for data evaluation. Both assays showed 100% specificity for the detection of *Aspergillus* DNA, and the triazole resistance associated *Cyp51A* alterations TR34/L98H was not detected.

Evaluation of the *A. fumigatus* Bio-Evolution® data revealed a sensitivity of 81% and of the MycoGenie® data of 71%. Apart from promising approaches, the sensitivity and clinical feasibility of the MycoGenie® system have to be further evaluated in different clinical settings.

A further real-time PCR system for the detection of *Aspergillus* DNA and the *Cyp51A* triazole resistance alterations TR34 and TR46 is the Fungiplex *Aspergillus* Azole-R test system (Bruker Daltonik GmbH, Bremen, Germany). The assay is validated for testing of serum, plasma, and BAL fluid samples with different detection limits in different samples. The two *Cyp51A* alterations are detected by using different DNA detection channels of the real-time PCR instruments. In serum, the limit of detection was 50, in plasma 75, and in BAL 25 genome equivalents/500 μl sample for TR34 and in serum 50, in plasma 75, and in BAL 50 *Aspergillus* genome equivalents/500 μl sample for TR46. The assay is not yet validated in a clinical setting.

Triazole resistance in *A. fumigatus* is not only a problem in immunocompromised patients but is also an emerging concern for treating chronically infected and/or colonized patients. A study of Guegan et al. had the aim to evaluate the performance of PCR assays to detect *Aspergillus* fungi together with triazole resistance in sputum samples from cystic fibrosis (CF) patients. The study compared the diagnostic performance of the MycoGenie® assay with the AsperGenius® system and two in-house assays plus DNA sequencing by investigating 119 sputum samples from 87 CF patients. PCR results were also compared to mycological culture. The overall rate of *Aspergillus* detection with the four qPCR assays ranged from 47.9 to 57.1%; the detection rate for positive cultures with *A. fumigatus* was 42/119 (35.3%). Five out of 41 isolated strains were triazole resistant, whereby three revealed *Cyp51A* mutations and only one isolates the TR34/L98H mutation combination.

The authors concluded that “*Cyp51A* targeting was only moderately effective for triazole resistance monitoring” [59].

To perform some comparable analyses between six in-house ARAf PCR assays plus DNA sequence analysis [14, 44, 45] and a commercial realtime PCR assay, Postina et al. investigated in parallel the commercially available AsperGenius® system in detecting the *Cyp51A* alterations TR34/L98H and Y121F/T289A directly from 52 clinical samples (15 biopsies, 22 bronchoalveolar lavage (BAL), 15 cerebrospinal fluid (CSF) samples) and ARAf isolates ($n = 3$) of immunocompromised patients. Both methods were compared concerning amplification and detection of *Aspergillus* DNA and *Cyp51A* alterations. The rate of positive ARAf PCR results plus successful sequencing using the ARAf PCR assays was 61% in biopsies, 29% in CSF, 67% in BAL samples, and 100% in isolates. In comparison, the amount of positive PCRs using the AsperGenius® assays was 47% in biopsies, 42% in CSF, 59% in BAL samples, and 100% in isolates. Altogether, 17 *Cyp51A* alterations were detected using the ARAf PCRs plus subsequent DNA sequencing and therefrom 10 alterations also by the AsperGenius® system. The comparative evaluation of the data revealed that the conventional PCR assays were more sensitive in detecting ARAf in BAL and biopsy samples, whereby differences were not significant. The advantage of the AsperGenius® system was the time saving aspect [14].

Future Perspectives

In the future, knowledge about the epidemiology of *Aspergillus* susceptibility patterns will represent a cornerstone for guiding the appropriate selection of antifungal prophylaxis and treatment. Given that more areas may be burdened with high rates of environmental triazole resistance, triazoles may not be universally recommended as primary antifungal treatment, but instead, treatment choice may depend on local epidemiology of ARAf. While triazole resistance is considered an emerging threat for patients infected by *A. fumigatus* [60] leaving very limited treatment options for those patients, triazole resistance in *Aspergillus terreus* [61] may be even more threatening, because *Aspergillus terreus* is non-susceptible to amphotericin B.

To enable early diagnosis and treatment, more rapid and sensitive methods to diagnose *A. fumigatus* and ARAf need to be developed. Optimally, these methods would be applied as point-of-care tests in blood specimens and detect a broader spectrum of resistance markers including markers for resistance in individuals on prolonged triazole therapy [62].

Conclusion

In summary, non-culture-based molecular detection of *Aspergillus* triazole resistance is of high epidemiological and clinical relevance, mainly in immunocompromised patients with hematological malignancies, where culture has limited sensitivity. Molecular assays are now able to detect most triazole-resistant *A. fumigatus* strains in patients with IA. Given that resistance rates vary, clinical utility for these assays still depends on regional resistance patterns.

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