Development of Novel PCR Assays To Detect Azole Resistance-Mediating Mutations of the Aspergillus fumigatus cyp51A Gene in Primary Clinical Samples from Neutropenic Patients

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The increasing incidence of azole resistance in Aspergillus fumigatus causing invasive aspergillosis (IA) in immunocompromised/hematological patients emphasizes the need to improve the detection of resistance-mediating cyp51A gene mutations from primary clinical samples, particularly as the diagnosis of invasive aspergillosis is rarely based on a positive culture yield in this group of patients. We generated primers from the unique sequence of the Aspergillus fumigatus cyp51A gene to establish PCR assays with consecutive DNA sequence analysis to detect and identify the A. fumigatus cyp51A tandem repeat (TR) mutation in the promoter region and the L98H and M220 alterations directly in clinical samples. After testing of the sensitivity and specificity of the assays using serially diluted A. fumigatus and human DNA, A. fumigatus cyp51A gene fragments of about 150 bp potentially carrying the mutations were amplified directly from primary clinical samples and subsequently DNA sequenced. The determined sensitivities of the PCR assays were 600 fg, 6 pg, and 4 pg of A. fumigatus DNA for the TR, L98H, and M220 mutations, respectively. There was no cross-reactivity with human genomic DNA detectable. Sequencing of the PCR amplicons for A. fumigatus wild-type DNA confirmed the cyp51A wild-type sequence, and PCR products from one azole-resistant A. fumigatus isolate showed the L98H and TR mutations. The second azole-resistant isolate revealed an M220T alteration. We consider our assay to be of high epidemiological and clinical relevance to detect azole resistance and to optimize antifungal therapy in patients with IA.

Invasive aspergillosis (IA) has emerged as a life-threatening infection in immunocompromised patients. An increase in infections due to azole-resistant Aspergillus fumigatus has been observed lately (4, 9, 28), leading to a higher case fatality rate among patients with azole-resistant invasive aspergillosis (27).

The main acquired/observed mechanism conferring azole drug resistance implicates point mutations in the 14α-sterol demethylase gene (cyp51A) and/or increased resistance to azoles (itraconazole, voriconazole, posaconazole) is strictly based on positive cultures from clinical isolates, and the hitherto existing PCR assays to detect both the azole resistance-mediating tandem repeat and the single nucleotide polymorphisms were performed with culture samples and depend on positive culture findings from clinical isolates (7, 8, 14, 17, 26).

Up to now, only one PCR-based assay that detects cyp51A mutations directly in clinical samples (sputum) from patients with pulmonary diseases like allergic bronchopulmonary aspergillosis (ABPA) and chronic pulmonary aspergillosis (CPA) has been described. In this study, samples from a few patients suffering from invasive pulmonary aspergillosis (IPA) were also tested, but these samples did not yield results due to an insufficient amount of sample remaining (6).

From a clinical point of view, at least in patients with hematological malignancies at high risk for IA, evident Aspergillus culture yields are scarce and a culture-based diagnosis of IA is rarely achieved (20, 30). In this context, it may be assumed that azole resistance is underdiagnosed in this group of patients. To overcome culture-based diagnostic limitations, we established PCR assays to detect common azole resistance-mediating mutations of the A. fumigatus cyp51A gene in primary clinical samples from patients with hematological malignancies.

MATERIALS AND METHODS

Strains and growth conditions. An A. fumigatus wild-type strain (DSM 819) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, and the Institute for Medical Microbiology and Hygiene, Mannheim University Hospital, Mannheim, Germany.

One itraconazole-resistant A. fumigatus clinical isolate (strain F14532) and one both itraconazole- and voriconazole-resistant A. fumigatus clinical isolate (strain F16216) were obtained from the Mycology Reference Centre, Manchester Culture Collection, Manchester, United Kingdom. Strain F14532 showed the following MIC values, obtained by the EUCAST reference microdilution method: voriconazole, 1.0 mg/liter; itraconazole, 8.0 mg/liter; posaconazole, 0.5 mg/liter. Strain F16216 had the following EUCAST MIC values: voriconazole, 8.0 mg/liter; itraconazole, 8.0 mg/liter; posaconazole, 2.0 mg/liter.

According to the publication of Verweij et al., we applied the following...
breakpoints for \textit{A. fumigatus} using the EUCAST susceptibility testing methodology; for itraconazole and voriconazole, <2 mg/liter (susceptible), 2-10 mg/liter (intermediate), and >10 mg/liter (resistant); for posaconazole, <0.25 mg/liter, 0.5 mg/liter, and >0.5 mg/liter, respectively (28). Prior to DNA extraction, fungal cultures were grown in BBL malt agar (Becton, Dickinson, \textregistered; components of PCR mixture are on par with those of the L98H PCR mixture; DNA thermal cycler, 2 min initial denaturation at 94°C, 45 cycles of 94°C for 45 s, 52°C for 1 min, and final extension at 72°C for 10 min.

**TABLE 1** Composition of \textit{A. fumigatus} \textit{cyp51A}-specific primer sets and PCR conditions used for amplification of the DNA fragments potentially carrying the L98H, M220, and TR mutations

<table>
<thead>
<tr>
<th>Mutation and primer name</th>
<th>Orientation</th>
<th>Nucleotide sequence (5’-3’)</th>
<th>Fragment length (bp)</th>
<th>Nested Aspergillus PCR result</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L98H mutation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CypA-L98H-S\textsubscript{A}\textsuperscript{a}</td>
<td>Sense</td>
<td>AAAAACCCACAGTCTAACCCTGG</td>
<td>143</td>
<td>+</td>
<td>Total vol, 25 \mu l; 2 \mu l template DNA (approx 100 ng human DNA + unknown amt of \textit{A. fumigatus} DNA), 3 mM MgCl\textsubscript{2}, 0.25 mM each deoxynucleoside triphosphate, 1 U of Taq polymerase (Invitrogen GmbH, Karlsruhe, Germany), 20 pmol of each primer; DNA thermal cycler, 2 min of initial denaturation at 94°C, 40 cycles of 94°C for 45 s, 52°C for 1 min, and final extension at 72°C for 10 min</td>
</tr>
<tr>
<td>CypA-L98H-AS\textsubscript{A,b}</td>
<td>Antisense</td>
<td>GGAATTGGGACAATCATAACAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>M220 mutation</strong></td>
<td></td>
<td></td>
<td></td>
<td>M220 PCR conditions are on par with the L98H conditions</td>
<td></td>
</tr>
<tr>
<td>CypA-M220-S\textsubscript{A}</td>
<td>Sense</td>
<td>GCCAGGAAGTTCGTCCAA</td>
<td>173</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>CypA-M220-AS\textsubscript{A}</td>
<td>Antisense</td>
<td>CTGATTGATGTGCACTGTA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tandem repeat</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Step 1</td>
<td></td>
<td></td>
<td></td>
<td>First step, total vol, 25 \mu l; components of PCR mixture are on par with those of the L98H PCR mixture; DNA thermal cycler, 2 min initial denaturation at 94°C, 23 cycles of 94°C for 45 s, 52°C for 1 min, and final extension at 72°C for 5 min</td>
<td></td>
</tr>
<tr>
<td>CypA-TR-S\textsubscript{1}</td>
<td>Sense</td>
<td>GGAGAAGGGAGAAGGACACTCT</td>
<td>235</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CypA-TR-AS\textsubscript{1}</td>
<td>Antisense</td>
<td>TCACCTACCTACCAATTAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Step 2</td>
<td></td>
<td></td>
<td></td>
<td>Second step, total vol, 25 \mu l; template of 2 \mu l of the first-step PCR mixture; other components are on par with those of the L98H PCR mixture; DNA thermal cycler, 2 min initial denaturation at 94°C, 35 cycles of 94°C for 45 s, 56°C for 1 min, and final extension at 72°C for 5 min</td>
<td></td>
</tr>
<tr>
<td>CypA-TR-S\textsubscript{2}</td>
<td>Sense</td>
<td>AGCACACCCTCACAGTTGCTTA</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CypA-TR-AS\textsubscript{2}</td>
<td>Antisense</td>
<td>TGTATGTTAGTGCTGAACTACCTTT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} S, sense.  
\textsuperscript{b} AS, antisense.
for amplification of a 235-bp DNA fragment. In the second PCR step, a 100-bp DNA fragment is generated using the primer pair CypA-TR-S_A and CypA-TR-AS_A. PCR conditions are also given in Table 1. PCR products were analyzed by ethidium bromide agarose gel analysis.

To exclude cross-reactivity of the designed primer pairs with human genomic DNA, we investigated 100 ng of human DNA as a negative control in the developed PCR assays. An additional negative control for specificity was a sample, adopted in the PCR assays, containing a mixture of 100 ng human genomic DNA and 50 pg of *A. fumigatus* wild-type DNA.

The sensitivities of the different PCR assays were determined using serially diluted *A. fumigatus* wild-type DNA as the template (see Fig. 2A to C)). PCR amplicons were visualized using ethidium bromide agarose gel analysis.

Two azole-resistant *A. fumigatus* strains (strains F16216 and F14532) were obtained from the culture collection at the Mycology Reference Centre, Manchester, United Kingdom. Genomic DNA of both strains was used as a positive control for amplification and detection of the L98H, M220, and TR mutations with our novel developed PCR assays and consecutive DNA sequence analysis.

**Sequence analysis.** The PCR products were used directly for mandatory sequence analysis, without agarose gel extraction, which is time-consuming, usually associated with the loss of a large amount of DNA, and therefore critical when performing PCR directly out of primary clinical samples containing very small amounts of fungal DNA. The PCR products were purified using a MiniElute PCR purification kit (Qiagen, Hilden, Germany), and a minimum of 50 ng DNA was sequenced (Seq-unsere, Vaterstetten, Germany). To detect potential mutations in the PCR products analyzed by DNA sequence analysis, the sequence of the products was compared to the sequence of the *A. fumigatus* cyp51A wild-type sequence using the NCBI alignment service AlignSequenceNucleotideBlast (http://www.ncbi.nlm.nih.gov/).

**Patients.** For the methodological establishment of the three different PCR assays for clinical samples, BAL fluid and tissue samples from 7 immunocompromised neutropenic patients mainly with hematological malignancies were further processed for diagnostic purposes, after the patients gave informed consent. Primary diseases were acute lymphoblastic leukemia (ALL; n = 2), non-Hodgkin's lymphoma (NHL; n = 2), mantle cell lymphoma (n = 1), Waldenstrom's macroglobulinemia (n = 1), and multiple myeloma (n = 1). An additional immunocompromised patient who suffered from chronic obstructive pulmonary disease (COPD; n = 1) was not neutropenic.

**Clinical samples.** For BAL fluid samples, bronchoscopy was performed according to standardized operating procedures as described elsewhere (16), and BAL fluid samples were obtained in a sterile vessel without conservation medium. The mean sample volume was 10 ml. Tissue samples were obtained by needle biopsy (liver, kidney) or surgical procedures (other samples) under sterile conditions.

**RESULTS**

Three distinct PCR assays for the detection of the most common azole resistance-mediating alterations in the *A. fumigatus* cyp51A gene were established.
PCR assays using DNA of clinical samples directly as the template resulted in PCR amplicons. This is exemplarily shown for two patients in Fig. 2A, lanes 8 and 9; B, lanes 11 and 12; and C, lanes 10 and 11.

Fifty picograms of A. fumigatus wild-type DNA and 50 pg of DNA of the two azole-resistant A. fumigatus isolates (strains F16216 and F14532) diluted in 100 ng human genomic DNA resulted in positive signals using the three PCR assays (Fig. 2D). The PCR amplicon of the TR PCR assay for azole-resistant A. fumigatus strain F16216 carried the 34-bp TR in the cyp51A gene promoter region and was therefore 34 bp longer than the wild-type and strain F14532 PCR fragments (Fig. 2D, lane 6).

DNA sequence analysis of the A. fumigatus wild-type PCR amplicons confirmed the wild-type sequence of the cyp51A gene, showing that A. fumigatus cyp51A DNA fragments were amplified by the use of the particular PCR assays.

Azole-resistant A. fumigatus isolate F16216 served as the positive control for the detection of the TR and L98H alterations in the cyp51A gene via PCR and consecutive DNA sequence analysis. The M220 alteration was not found in this sample. In strain F14532, the L98H and TR alterations were not detected, whereas DNA sequence analysis revealed a substitution of base T to C on position 730 of the cyp51A gene, leading to an M220T amino acid alteration. DNA sequence analysis is mandatory to detect the mutations in the amplified cyp51A gene fragments.

Stored samples of 8 hematological patients positive in our nested diagnostic Aspergillus PCR assay (22) were analyzed with the L98H, M220, and TR PCR assays and consecutive DNA sequence analysis to establish and confirm our method for detection of the most common azole resistance-mediating mutations in the A. fumigatus cyp51A gene (Table 2). The samples of patients 1 and 2 shown in Fig. 2A to C did not reveal any mutations after DNA sequence analysis of the PCR amplicons. The samples of patients 3 and 5 showed the L98H alteration, and patient 3 additionally had the TR in the cyp51A promoter region, but in patient 5, the TR alteration was not found in combination with the L98H alteration. Culture results of both patients were negative; neither patient ever received azole therapy or prophylaxis. Patient 5 died; he had been treated with an azole (voriconazole). Patient 3 was treated with an azole (voriconazole) combined with liposomal amphotericin B and survived.

**DISCUSSION**

To overcome culture-based diagnostic limitations, we established PCR assays to detect the most common azole resistance-mediating (key) mutations of the A. fumigatus cyp51A gene in primary clinical samples from patients with hematological malignancies.

PCR assays for the detection of A. fumigatus cyp51A alterations have been described, but the assays investigated fungal DNA only from cultures of clinical isolates (7, 8, 11, 14, 17, 26).

From a clinical point of view, in patients with hematological malignancies at high risk for IA, evident Aspergillus culture yields of blood or BAL, fluid samples are low and a culture-based diagnosis of IA is rarely achieved, because positive culture results from any clinical sample are seldom available, in general, in the early, crucial course of a case of IA. The thereby limited ability of resistance detection led to the development of culture-independent PCR approaches providing a sensitive and rapid methodology of azole resistance identification also in culture-negative clinical samples. Up to now, only one PCR-based assay detecting cyp51A
mutations in clinical samples (sputum) from patients with underlying pulmonary diseases has been described. Only a few patients in the previous study suffered from invasive pulmonary aspergillosis (IPA), but these samples were not tested due to an insufficient amount of sample remaining (6).

Our group has long-standing experience with the development and validation of sensitive and specific PCR–based assays to detect invasive fungal infections in hematological patients, especially infections caused by *Aspergillus* spp. (1–3, 12, 13, 22, 24, 25). We therefore established a molecular, non-culture-based approach to detect the clinically leading azole resistance–mediating key mutations of the *A. fumigatus* cyp51A gene directly from primary clinically relevant samples. In this scenario, BAL fluid and tissue samples are more applicable than blood samples because the fungal load is likely to be higher, particularly in hematological patients undergoing intensive antifungal therapy, so that the ratio of target (fungal) and non-target (human) DNA is more favorable in BAL fluid and tissue samples. The very small amount of included *A. fumigatus* DNA is the limiting factor when investigating primary clinical samples directly. As single PCR assays amplifying short DNA fragments are more sensitive, we established three distinct PCR assays for the detection of cyp51A mutations.

Our nested PCR assay for the detection of the TR alteration in the cyp51A promoter region showed a higher sensitivity than the other two PCR assays. The assays for the detection of the L98H and M220 alterations were established as one-step PCR assays, because nested PCR approaches for detection of these alterations resulted in unspecific PCR amplicons but not in a higher sensitivity. None of the primer combinations showed cross-reactivity with nontarget DNA. In unspecific PCR amplicons but not in a higher sensitivity. None of the primer combinations showed cross-reactivity with nontarget DNA. In this scenario, BAL fluid and tissue samples are more applicable than blood samples because the fungal load is likely to be higher, particularly in hematological patients undergoing intensive antifungal therapy, so that the ratio of target (fungal) and non-target (human) DNA is more favorable in BAL fluid and tissue samples. The very small amount of included *A. fumigatus* DNA is the limiting factor when investigating primary clinical samples directly. As single PCR assays amplifying short DNA fragments are more sensitive, we established three distinct PCR assays for the detection of cyp51A mutations.

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According to Snelders et al. (23), Rodriguez-Tudela et al. (19), van der Linden et al. (27a), Lockhart et al. (15), and Chowdhary et al. (5), azole resistance in *Aspergillus fumigatus* isolates is primarily due to the TR/L98H mutation and would thus be detected by our assay. Howard et al. described more mutation variability in the Manchester, United Kingdom, patient population, so our assay would probably work less well at detecting azole resistance in this group of patients (mostly patients with underlying pulmonary diseases) (10).

Investigating primary clinical samples with our PCR assays, we detected the L98H alteration in patient 5 but not in combination with the TR alteration. The L98H mutation alone does not mediate azole resistance (8). In a second patient suffering from ALL, the L98H mutation was detected in combination with the TR alteration in a brain biopsy specimen. To our knowledge, this is the first patient in Germany showing this molecular finding. The clinical impact of the detected mutation pattern was not assessable, because the patient was treated at the time of brain biopsy with an azole antifungal combined with amphotericin B.

The current study is the first to detect azole resistance directly from primary clinical specimens in a defined group of hematological patients. Both a retrospective epidemiological study and a prospective diagnostic study testing primary clinical samples from hematological patients with a limited or insufficient response to azole treatment or prophylaxis are under way. In addition, we have established novel PCR assays for the detection of other hotspot mutations, e.g., G54.

We consider our rapid molecular assay to be of high epidemiological and clinical relevance to detect azole resistance and to allow significantly faster targeted antifungal therapy in patients with IA. Furthermore, the direct and rapid detection of azole resistance due to cyp51A gene alterations allows the differentiation between the diverse azole resistance mechanisms, as not every case of clinically relevant azole resistance is caused by cyp51A alterations.

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**REFERENCES**


