Azole, polyene and echinocandin MIC distributions for wild-type, TR34/L98H and TR46/Y121F/T289A Aspergillus fumigatus isolates in the Netherlands

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Objectives: To determine the MIC distributions of itraconazole, voriconazole and posaconazole and non-azole drugs for wild-type cyp51A, as well as TR34/L98H and TR46/Y121F/T289A cyp51A mutants of Aspergillus fumigatus.

Methods: We retrieved MIC and cyp51A sequence data for 952 clinical A. fumigatus strains isolated in or referred to our reference laboratory, during the January 2010 to December 2013 period. All MICs were determined using the EUCAST methodology and interpreted using the EUCAST breakpoints.

Results: Three-hundred and sixty-four of the 952 strains (38%) were resistant to azoles. Of these, 225 contained the TR34/L98H mutation, 98 contained the TR46/Y121F/T289A mutation and 39 had no cyp51A mutations. Two isolates harboured other cyp51A mutations, of which one (P216L) has been shown to confer azole resistance. Of the TR34/L98H isolates, 99.6% (224/225) were resistant to itraconazole (MICs ≤ 2 mg/L), 92.4% (208/225) were resistant to voriconazole (MICs ≤ 2 mg/L) and 97.8% (220/225) were resistant to posaconazole (MICs ≤ 0.25 mg/L). All TR 46/Y121F/T289A isolates were resistant to voriconazole (MICs ≥ 16 mg/L), 82.7% (81/98) were resistant to itraconazole with a bimodal MIC distribution and 94.9% (93/98) were resistant to posaconazole. The MICs of amphotericin B, anidulafungin and terbinafine were not affected by the presence of azole-resistance mechanisms.

Conclusions: The TR34/L98H and TR46/Y121F/T289A cyp51A genotypes of A. fumigatus show distinct resistance phenotypes. The mechanisms behind low-level itraconazole resistance in TR46/Y121F/T289A isolates warrant future research. The potential of increased azole dosing for disease caused by low-level resistant strains should be investigated.

Keywords: A. fumigatus, voriconazole, itraconazole, posaconazole, MICs

Introduction

Azole resistance in Aspergillus fumigatus is an emerging health concern in many clinical settings. In the Netherlands, two cyp51A gene mutations are largely responsible for this emerging resistance, and are considered to have arisen throughazole exposure in the environment. These resistance mechanisms consist of combinations of a tandem repeat in the cyp51A promoter and a concomitant mutation in the cyp51A gene itself, i.e. the 34 bp tandem repeat/L98H mutation combination (TR34/L98H) and the 46 bp tandem repeat/Y121F/T289A mutation combination (TR46/Y121F/T289A).

The presence of these mutations and the resulting MICs of the three major azole drugs (itraconazole, voriconazole and posaconazole) have been studied in small case series, often in specific patient populations. To date, the azole MIC distributions for wild-type cyp51A, but particularly TR34/L98H and TR46/Y121F/T289A cyp51A mutants of A. fumigatus, measured by EUCAST methodology, have not been characterized in large strain collections.

We have performed a retrospective analysis of MIC distributions and cyp51A sequence analyses for all A. fumigatus submitted to our national reference laboratory in the 2010–13 period.

Methods

MICs and cyp51A sequence data for A. fumigatus strains isolated from clinical specimens in or referred to our reference laboratory, during the
January 2010 to January 2014 period, were retrieved. Only one isolate per patient per 6 months was included, unless strains exhibited >4-fold differences in the MICs of more than two drugs tested. Identification as *A. fumigatus* was achieved through morphological examination and growth at 48°C and, for azole-resistant isolates, confirmed by the presence and sequence of the cyp51A gene.

All MICs were determined using the EUCAST methodology, i.e. broth microdilution in RPMI1640 medium with 2% glucose and MOPS buffer, with a 1–2.5 × 10⁴ cfu/mL inoculum and a 48 h incubation.² The *A. fumigatus* ATCC 204305 reference strain was used for quality control.

For all strains with azole MICs in the resistant range (i.e. MIC >2 mg/L for itraconazole or voriconazole or MIC >0.25 mg/L for posaconazole), cyp51A gene and promoter region sequence analysis was performed using previously published methods.⁵,⁶ The promoter region and cyp51A gene were amplified separately and sequenced using previously published primer sets, which are specific for *A. fumigatus*.⁷ The obtained cyp51A sequences were compared with the cyp51A sequence available through GenBank (accession number AF338659).⁵–⁷

**Results and discussion**

Nine-hundred and fifty-two *A. fumigatus* strains met our inclusion criteria. The MIC distributions of itraconazole, voriconazole and posaconazole are presented in Figure 1.

Three-hundred and sixty-four strains (38%) were subjected to cyp51A gene mutation analyses because of azole MICs in the resistant range. Of these 364, 225 (62% of all azole-resistant isolates; 24% of the 952 studied isolates) contained the TR₃₄/L₉₈H mutation and 98 (27% of all azole-resistant isolates; 10% of the 952 studied isolates) contained the TR₄₆/Y₁₂₁F/T₂₈₉A mutation. The azole MIC distributions for these strains are given in Figure 2(a) (TR₃₄/L₉₈H) and Figure 2(b) (TR₄₆/Y₁₂₁F/T₂₈₉A).

According to the EUCAST breakpoints, 99.6% (224/225) of TR₃₄/L₉₈H isolates were resistant to itraconazole (MIC >2 mg/L), 92.4% (208/225) were resistant to voriconazole (MIC >2 mg/L) and 97.8% (220/225) were resistant to posaconazole (MIC >0.25 mg/L). For TR₄₆/Y₁₂₁F/T₂₈₉A, all (100%) of the tested isolates were resistant to voriconazole, 82.7% (81/98) were resistant to itraconazole and 94.9% (93/98) were resistant to posaconazole.

The TR₃₄/L₉₈H and TR₄₆/Y₁₂₁F/T₂₈₉A genotypes of *A. fumigatus* show a distinct phenotype. The TR₃₄/L₉₈H mutants show high-level resistance to itraconazole with a lower level of resistance, i.e. MICs just above the breakpoint concentration, to voriconazole and posaconazole. The MIC distributions for TR₃₄/L₉₈H mutants match those reported in small-scale studies of cystic fibrosis patients in Germany and France and a nationwide survey in Germany.²,⁸,⁹

The TR₄₆/Y₁₂₁F/T₂₈₉A mutants show high-level resistance to voriconazole, high-level resistance or, sporadically, low-level resistance to itraconazole and low-level resistance to posaconazole (Figure 2b). These MIC distributions are similar to those

![Figure 1. MIC distributions for 952 clinical *A. fumigatus* isolates. The x-axis shows the MICs and the y-axis shows the percentage of strains in the set with the given MIC.](image)

![Figure 2. MIC distributions according to resistance mechanism. (a) Isolates harbouring the TR₃₄/L₉₈H resistance mechanism (TR₃₄; n = 225). (b) Isolates harbouring the TR₄₆/Y₁₂₁F/T₂₈₉A resistance mechanism (TR₄₆; n = 98). (c) Isolates with an azole-resistance phenotype, but wild-type cyp51A promoter sequences (WT; n = 41). The x-axis shows the MICs and the y-axis shows the percentage of strains in the set with the given MIC.](image)
Recent studies showed a novel mechanism involved in azole resistance, which underlies their azole-resistant phenotype. This is thought to be driven by mutations in the cyp51A gene, but these are probably just the tip of the iceberg. For instance, increased expression of the c1b efflux pump and the cyp51A promoter and F46Y, M172V and E427K mutations in the cyp51A gene; this strain had azole MICs of 16 mg/L (itraconazole), 8 mg/L (voriconazole) and 2 mg/L (posaconazole). The observed increase in MICs has been reported for isogenic isolates recovered over time in azole-treated patients, supporting the development of consecutive resistance mechanisms in A. fumigatus. This study has two important limitations. First, we did not have information on clinical significance or prior azole exposure for the studied isolates; there is likely a referral bias towards azole-resistant isolates or isolates associated with treatment failure. Second, we did not systematically perform molecular identification of isolates identified as A. fumigatus. Although all isolates were able to grow at 48°C, we cannot exclude the presence of closely related sibling species within the A. fumigatus species complex among the azole-susceptible isolates.

In summary, we studied the MIC distributions of azole and non-azole drugs for a large collection of A. fumigatus isolates and were able to further characterize the MIC profiles associated with the TR34/L98H and TR46/Y121F/T289A resistance mechanisms.

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### Transparency declarations

None of the authors has any relation, financial or non-financial, with an entity that holds an interest in the subject matter of this work.

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**Table 1. Values of MIC<sub>50</sub> and MIC<sub>90</sub> (mg/L) of azoles and other antifungals for A. fumigatus**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Itraconazole</th>
<th>Voriconazole</th>
<th>Posaconazole</th>
<th>Amphotericin B</th>
<th>Terbinafine</th>
<th>Anidulafungin</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR34, n=225</td>
<td>&gt;16.0</td>
<td>&gt;16.0</td>
<td>8.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.063</td>
</tr>
<tr>
<td>TR46, n=98</td>
<td>&gt;16.0</td>
<td>&gt;16.0</td>
<td>&gt;16.0</td>
<td>1.0</td>
<td>2.0</td>
<td>0.125</td>
</tr>
<tr>
<td>WT-R, n=41</td>
<td>&gt;16.0</td>
<td>&gt;16.0</td>
<td>4.0</td>
<td>0.5</td>
<td>1.0</td>
<td>0.125</td>
</tr>
<tr>
<td>Azole-S, n=587</td>
<td>0.5</td>
<td>1.0</td>
<td>0.5</td>
<td>0.125</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

TR34, azole-resistant A. fumigatus with a TR<sub>34</sub>/L98H resistance mechanism; TR46, azole-resistant A. fumigatus with a TR<sub>46</sub>/Y121F/T289A resistance mechanism; WT-R, azole-resistant isolates with wild-type cyp51A promoter sequences; Azole-S, A. fumigatus isolates with a wild-type phenotype.
References


