Novel mixed-format real-time PCR assay to detect mutations conferring resistance to triazoles in *Aspergillus fumigatus* and prevalence of multi-triazole resistance among clinical isolates in the Netherlands

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**Objectives:** The aim of this study was: (i) to study the prevalence of triazole-resistant *Aspergillus fumigatus* isolates in the Netherlands; and (ii) to design rapid real-time PCR methods to identify such isolates.

**Methods:** A novel mixed-format real-time PCR assay is described for the detection of mutations leading to triazole resistance in *A. fumigatus*. One set of PCR primers and a probe carrying a single fluorescent label in combination with a double-stranded DNA fluorescent dye allow simultaneous detection of (a) specific mutation(s) as well as of the amplified product that serves as an internal amplification control. The method was applied to a random collection of 209 clinical isolates from throughout the Netherlands and was compared with phenotypic susceptibility testing.

**Results:** A total of four triazole-resistant isolates were identified, resulting in a prevalence of resistant isolates of 2%. All four isolates contained an identical combination of mutations leading to multi-triazole resistance, as reported before by others. Molecular testing results were 100% concordant with phenotypic susceptibility testing.

**Conclusions:** Although in specific patient populations the prevalence of resistance in *A. fumigatus* may be an emerging problem, in the general population it is still relatively low. The novel real-time PCR format allows rapid and reliable identification of such isolates.

**Keywords:** 14α sterol demethylase, susceptibility, L98H, promoter

**Introduction**

Considerable progress has been made in unravelling the molecular mechanisms behind triazole resistance in *Aspergillus fumigatus*. The cyp51A gene product, encoding the 14α sterol demethylase, has been firmly established as the target for triazole-based antifungals.1 Up to the end of 2008, no less than 13 mutations that are causative of a resistance phenotype had been identified at six amino acid positions in this gene.2–7 These positions involve Asn22, Gly54, Leu98, Gly138, Met220 and Gly448. However, mutations at positions Asn22 and Gly448 were reported only after laboratory-induced resistance by random mutagenesis and were not found in clinical isolates. It should be noted that the L98H mutation only leads to triazole resistance in combination with a duplication of a 34 bp DNA fragment in the promoter region of the cyp51A gene.4 These combined changes (L98H+TR) lead to a multi-triazole resistance phenotype. Different mutations may lead to different susceptibility profiles. Therefore, rapid identification of triazole-resistant isolates as well as identification of the underlying mutations are important for optimal patient management and may be valuable in epidemiological surveys. Here, we report the application of a new mixed-format real-time PCR assay for the analysis of mutations at positions Gly54, Leu98, Gly138 and Met220 of the cyp51A gene of *A. fumigatus*. This new assay was used to determine the prevalence of triazole resistance mutations among clinical *A. fumigatus* isolates from the Netherlands.

**Materials and methods**

**Reference samples**

*A. fumigatus* isolates containing a variety of mutations at codons 54 or 220 of the cyp51A gene were provided by Dr E. Mellado (Instituto de...
Detection of the 34 bp duplication in the cyp51A promoter region

Amplifications were performed in a reaction volume of 10 μL using ~1 ng of genomic DNA, 0.5 μM of amplification primers 51APf and 51APR, 0.5 U of FastStart Taq DNA polymerase (Roche Diagnostics), 0.2 mM dNTPs, 1x Resolight dye and 2.5 mM MgCl₂ in 1x reaction buffer. The amplification parameters were denaturation for 10 min at 95°C, followed by 35 cycles of 95°C for 1 s, 60°C for 2 s and 72°C for 5 s. Following amplification, melting curves were recorded by increasing the temperature up to 95°C, with five acquisitions per°C.

MIC determination

MICs of itraconazole (Janssen Research Foundation, Beerse, Belgium), voriconazole (Pfizer Central Research, Sandwich, Kent, UK), posaconazole (Schering-Plough Corp., Kenilworth, NJ, USA) and isavuconazole (Baslea Pharmaceutica International Ltd, Basel, Switzerland) were determined by broth microdilution according to CLSI M38-A2.

Results

Real-time mutation detection

In Figure 1(b and c), typical results are shown for position Leu98 using either a wild-type probe or L98H-specific probe, respectively. In Figure 1(d and e), results are shown using a wild-type probe for Gly54 and Met220, respectively. In both examples, it is clearly visible that different mutations lead to the formation of different melting profiles, allowing the use of a single probe at each position for the discrimination between wild-type products and products containing one of several different mutations. Additional low-intensity peaks are observed in the range of 75–80°C. These peaks strongly correlate to the concentration of the mutation detection probe (Figure 1f) and may be the result of local secondary structures.

Real-time detection of the cyp51A promoter polymorphism

In Figure 1(g), typical amplification curves and melting peaks are shown for the cyp51A promoter region. Presence of the 34 bp duplication in the cyp51A promoter will lead to the generation of a PCR product that is easily distinguished from the wild-type product by its melting peak at an increased temperature (88°C versus 86°C, respectively). Control amplification reactions without template DNA showed no melting peaks (data not shown).

Prevalence of triazole resistance mutations among clinical isolates from the Netherlands

Four out of 209 isolates (1.9%) were resistant to itraconazole (MIC ≥ 16 mg/L) and cross-resistant to voriconazole (MIC ≥ 8 mg/L), posaconazole (two isolates had an MIC of >8 mg/L and two isolates had an MIC of 0.5 mg/L) and isavuconazole (MIC ≥ 4 mg/L). All four isolates contained the L98H+TR mutations. Neither of these two mutations nor a mutation at any of the other interrogated amino acid positions was found in the 205 phenotypically triazole-susceptible isolates.
Figure 1. Principle and examples of the novel mixed-format real-time PCR assay to detect specific point mutations associated with triazole resistance in *A. fumigatus*. (a) Illustration of the principle of the method. Upon hybridization of the probe to the ssDNA target, a dsDNA hybrid molecule is formed. Excitation of the Resolight dye into this dsDNA results in a fluorescence emission that is used to excite the reporter label. This format allows simultaneous detection of the amplified product as well as specific mutations in this target. (b) Detection of a mutation at L98 using a wild-type probe. (c) Specific detection of L98H using an L98H-specific probe. (d) Detection of different mutations at Gly54. (e) Detection of different mutations at Met220. (f) Influence of probe concentration. (g) Detection of the tandem repeat polymorphism in the promoter region (no probe necessary). See text for further details. This figure appears in colour in the online version of *JAC* and in black and white in the printed version of *JAC*. 

Real-time triazole resistance detection in *A. fumigatus*.
Table 1. Overview of primers and probes used in this study

<table>
<thead>
<tr>
<th>CYP51A target</th>
<th>Use</th>
<th>Name</th>
<th>Sequence</th>
<th>Concentration (µM)</th>
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<tr>
<td>Promoter</td>
<td>forward amplification primer</td>
<td>S1APrF</td>
<td>5'-AAATAATCGACGACCACCACCTC-3'</td>
<td>0.5</td>
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<td>Promoter</td>
<td>reverse amplification primer</td>
<td>S1APrR</td>
<td>5'-GGTTGATGCCTGGAACTACACCTT-3'</td>
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<td>Gly54</td>
<td>forward amplification primer</td>
<td>GS4F</td>
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<tr>
<td>Gly54</td>
<td>reverse amplification primer</td>
<td>GS4R</td>
<td>5'-CAAACTTTGAGACTTGCCTTT-3'</td>
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<td>Gly54</td>
<td>wild-type probe</td>
<td>GS4P</td>
<td>5'-LCRed640-ATCAATCCGTAACATGATCCACC-PO4-3'</td>
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</tr>
<tr>
<td>Leu98</td>
<td>forward amplification primer</td>
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<td>L98HPwt</td>
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<tr>
<td>Leu98</td>
<td>mutant probe</td>
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<td>5'-GGCGCTGATTGATGGATGC-3'</td>
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<td>M220R</td>
<td>5'-GTCGCCCGCATGACCAAACAGCACAAGGAT-PO4-3'</td>
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</tr>
<tr>
<td>Met220</td>
<td>wild-type probe</td>
<td>M220P</td>
<td>5'-TGGGGCCCACCAGGTTAGCATAA-PO4-LCRed640-3'</td>
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</tr>
<tr>
<td>Gly138</td>
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<td>G138F</td>
<td>5'-CTATAGTCCATTGACAGCCC-3'</td>
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<tr>
<td>Gly138</td>
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<td>G138R</td>
<td>5'-GCACATAGACCTTACGCC-3'</td>
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</tr>
<tr>
<td>Gly138</td>
<td>wild-type probe</td>
<td>G138P</td>
<td>5'-LCRed640-TACTGTGATCTCCAGTCTGCGT-PO4-3'</td>
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Discussion

We report the use of a novel mixed-format real-time mutation detection format to identify point mutations known to be involved in triazole resistance in A. fumigatus. Identification of resistance mutations in the cyp51A gene was typically performed using DNA sequence analysis. Since these assay formats require multiple post-PCR steps (involving massive amounts of PCR products), they are also more prone to contamination and, thus, require rigorous protocols and dedicated pre- and post-PCR facilities. Any additional post-PCR step also increases the workload, and is associated with higher personnel costs and increased turnaround time. The switch to closed-tube real-time PCR formats may overcome most of these disadvantages.

Unlike most other real-time PCR formats, the new assay format has a built-in internal amplification control. Theoretically, with certain real-time formats, the presence of polymorphisms underneath a specific probe could lead to an uninformative signal (e.g. using TaqMan/hydrolysis probes or Molecular Beacons). Such a result could also be the consequence of probe failure or a lack of amplification (e.g. due to nucleotide polymorphisms in the primer region or by the presence of inhibitors of the PCR process). Without additional tests, it is impossible to distinguish between these options. In the mixed-format assay described here, the additional amplicon-specific melting peak (at ~85°C) confirms successful amplification in a probe-independent manner and, thus, serves as a convenient internal amplification control.

In this study, 4 out of 209 clinical A. fumigatus isolates were found that contained the L98H+TR mutations. Phenotypic resistance testing led to the identification of the same four isolates as being resistant to multiple triazoles. Thus, the results of the new real-time PCR assay format were 100% concordant with phenotypic resistance determinations. Whereas other mutations leading to triazole resistance may be an emerging problem during triazole therapy, none of these mutations was found in this collection from the Netherlands. Our novel approach can easily be expanded to include additional mutations leading to triazole resistance in A. fumigatus. It is now believed that isolates containing the L98H+TR mutations are acquired from the environment. Consequently, our results provide an estimate of the a priori risk of acquiring a multi-triazole-resistant isolate in the Netherlands of <2%.

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Transparency declarations

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References


