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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

Salud Carlos III, Majadahonda, Madrid, Spain). Isolates containing the L98H+TR mutations as well as phenotypically triazole-susceptible control isolates were from the clinical isolate collection at the Canisius Wilhelmina Hospital. Af293/CBS101355 was used as a reference strain in all experiments.

Clinical isolates

The *A. fumigatus* isolates were collected during a 6 month period in 2005 from non-selected patient populations in 14 major hospitals throughout the Netherlands. Initially, 242 isolates were collected, but 27 isolates proved to be non-*A. fumigatus*. If more than one isolate was provided per patient, only the first isolate was used for analysis. In total, 209 isolates were included in this study. Most isolates were from respiratory specimens, and a few were from cerebral and sinus aspirates. Identification was done by macroscopic and microscopic growth characteristics, and growth at 48°C.

Principle of the mixed-format real-time PCR assay

The principle of the new assay format is illustrated in Figure 1. Hybridization of the single-stranded DNA (ssDNA) detection probe to the ssDNA target leads to a partially double-stranded DNA (dsDNA) hybrid molecule. Upon embedding in the dsDNA molecule, the fluorescent Resolight dye is excited by the blue light (~480 nm) and emits a bright green light (~530 nm). The energy from the emitted green light (~530 nm) is transferred, by a process known as fluorescence resonance energy transfer (FRET), to a fluorescent reporter label that emits a red light (~640 nm) (Figure 1a). When the temperature is increased, the partially dsDNA hybrid molecule is denatured, leading to a loss of signal at 640 nm, allowing the generation of so-called 'melting peaks'. The probes are designed so that a perfect hybrid will yield a melting peak at ~65°C. Any mismatch to the probe will become apparent by a melting peak at a lower temperature. Amplification reactions are performed in an asymmetrical fashion. This generates a mixture of dsDNA and ssDNA target molecules. The dsDNA targets are detected through the presence of the Resolight dye that is also detected at 640 nm. Their presence is verified by the formation of a melting peak at ~85°C (temperature may vary for amplicons of different sizes and/or different GC content). The extent of asymmetry during PCR directly influences the ratio between the ssDNA and dsDNA targets. Optimal conditions involve primer ratios of 1:1.5 to 1:5. If the amount of asymmetry is too high, poor amplification is achieved (leading to low overall signals); if the amount of asymmetry is too low, the amount of ssDNA targets is too low (leading to low probe-specific signals).

Real-time mutation detection

DNA was isolated from sporulating cultures grown for 48 h on Sabouraud glucose agar using established procedures. Amplifications were performed on a LightCycler 480 (Roche Diagnostics, Almere, The Netherlands) in a final volume of 10 µL containing ~1 ng of fungal DNA, amplification primers (Eurogentec, Seraing, Belgium) and mutation detection probe (TIB Molbiol, Berlin, Germany) (primer and probe concentrations according to Table 1), 1 U of FastStart *Taq* DNA polymerase (Roche Diagnostics), 0.2 mM dNTPs and 1× Resolight dye (Roche Diagnostics) in 1× reaction buffer containing 2 mM MgCl$_2$. The cycling parameters were as follows; after an initial denaturation step for 10 min at 95°C, 45 amplification cycles of 2 s of denaturation at 95°C, 5 s of annealing at 60°C and 20 s of extension at 72°C were applied. The melting protocol consisted of denaturation at 95°C for 2 s and 45°C for 30 s, after which the temperature was increased to 95°C, with 10 acquisitions per °C under continuous monitoring of the fluorescence at 640 nm.

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 51APrF and 51APrR, 0.5 U of FastStart *Taq* DNA polymerase (Roche Diagnostics), 0.2 mM dNTPs, 1× Resolight dye and 2.5 mM MgCl$_2$ in 1× 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 (Basilea Pharmaceutica International Ltd, Basel, Switzerland) were determined by broth microdilution according to CLSI M38-A2.[8]

Results
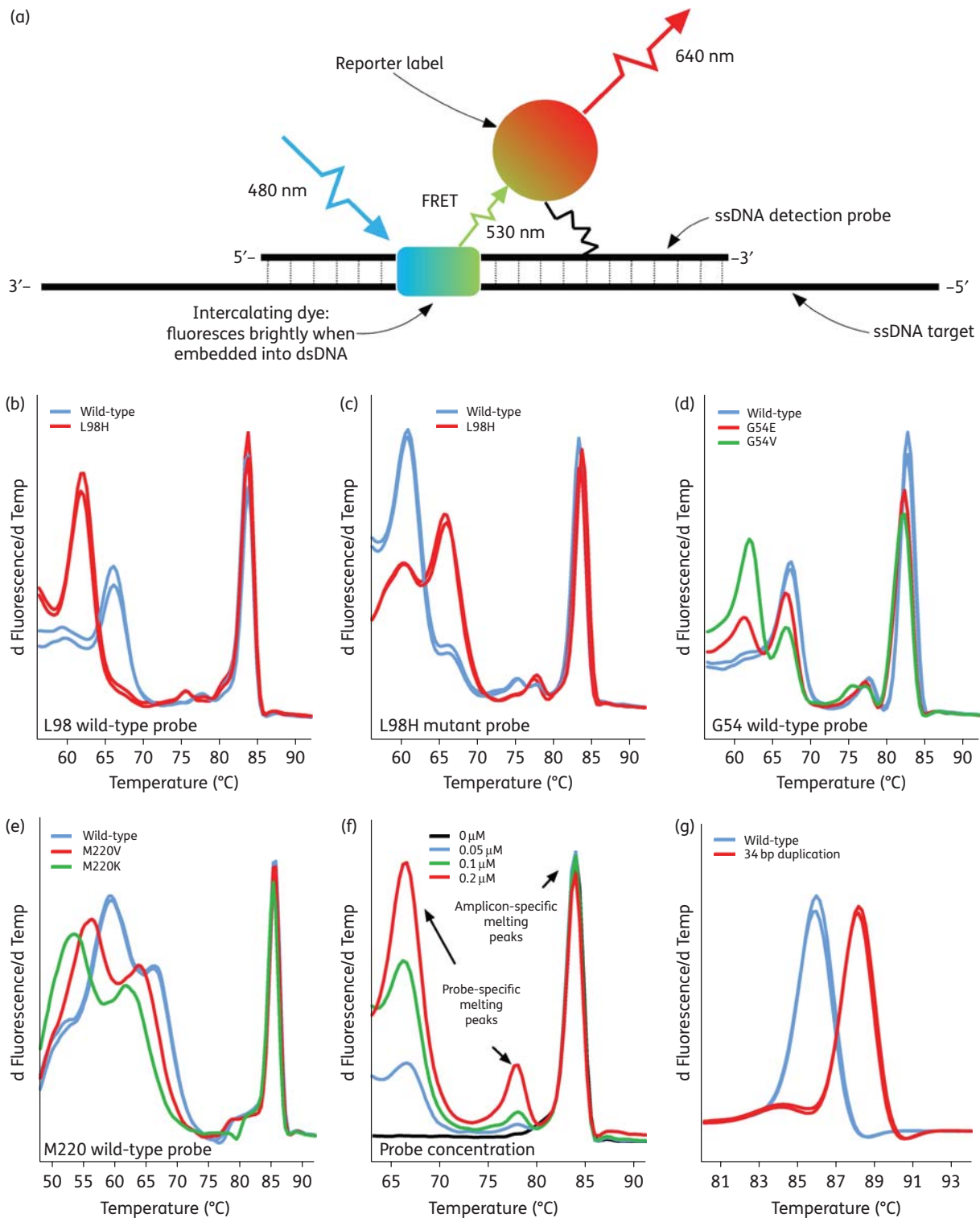
Real-time mutation detection

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*.

Table 1. Overview of primers and probes used in this study

CYP51A target	Use	Name	Sequence	Concentration (μM)
Promoter	forward amplification primer	51APrF	5′-AATAATCGCAGCACCACTCC-3′	0.5
Promoter	reverse amplification primer	51APrR	5′-TGGTATGCTGGAACTACACCTT-3′	0.5
Gly54	forward amplification primer	G54F	5′-AATGGTCTTTCATTGGGTCC-3′	0.3
Gly54	reverse amplification primer	G54R	5′-ACAATCTTGAGACTTGCCTT-3′	0.2
Gly54	wild-type probe	G54P	5′-LCRed640-ATCAATCCCGTAACTGATGGTACTACC-PO$_4$-3′	0.2
Leu98	forward amplification primer	L98HF	5′-CAGTATGGCGATATCTTCACTTT-3′	0.1
Leu98	reverse amplification primer	L98HR	5′-ACTATAGACCTCTTCCGCA-3′	0.5
Leu98	wild-type probe	L98HPwt	5′-LCRed640-TTCTCAACGGCAAGCTCAAGGAT-PO$_4$-3′	0.2
Leu98	mutant probe	L98HPmut	5′-LCRed640-TTCTCAACGGCAAGCACAAGGAT-PO$_4$-3′	0.2
Met220	forward amplification primer	M220F	5′-CAAGGGCTTTACTCCCATCA-3′	0.5
Met220	reverse amplification primer	M220R	5′-GGCGCTGATTGATGATGTC-3′	0.2
Met220	wild-type probe	M220P	5′-TGGGGCCCACGGTAGCATAAA-LCRed640-3′	0.2
Gly138	forward amplification primer	G138F	5′-CTATAGTCCATTGACGACCC-3′	0.2
Gly138	reverse amplification primer	G138R	5′-GCACATGAGACTCTAACGC-3′	0.5
Gly138	wild-type probe	G138P	5′-LCRed640-TACTGCTTGACTCAGTCTGCGT-PO$_4$-3′	0.2

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.[2,4,9] 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 under-neath a specific probe could lead to an unintended loss of 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 resist-ance 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 phenoty-pic resistance determinations. Whereas other mutations leading to triazole resistance may be an emerging problem during triazole therapy by induction,[10] 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 contain-ing the L98H+TR mutations are acquired from the environment.[11]

Consequently, our results provide an estimate of the *a priori* risk of acquiring a multi-triazole-resistant isolate in the Netherlands of <2%.

Transparency declarations

J. F. M. has undertaken consultancy for and received travel grants and research grants from Merck and Co. and its subsidiary Merck, Sharpe & Dohme, Basilea Pharmaceutica, Zeneus Pharmaceutica, Gilead, Pfizer and Schering-Plough. C. H. W. K. has received a research grant from Pfizer. Other authors: none to declare.

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