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Use of a High-Resolution Melt Assay To Characterize Codon 54 of the *cyp51A* Gene of *Aspergillus fumigatus* on a Rotor-Gene 6000 Instrument▽

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A high-resolution melt (HRM) assay using a Rotor-Gene 6000 instrument was developed to characterize the codon for glycine 54 in the *cyp51A* genes from 13 reference isolates and 12 clinical isolates of *Aspergillus fumigatus*. Mutations in this codon confer reduced susceptibility to itraconazole and posaconazole. The assay is simple to perform, and a result of "wild type" or "mutant" is available after approximately 1 h following DNA extraction using commercially available reagents and conventional primers.

Aspergillus fumigatus infections cause high rates of mortality and morbidity in immunocompromised hosts (15, 16). Strains are intrinsically resistant to fluconazole, but itraconazole, voriconazole, posaconazole, and ravuconazole are typically active (10, 18, 23, 26). Recent reports suggest that resistance rates are rising (13, 26, 29). Since azoles can be both fungicidal and fungistatic, infected patients treated with azole drugs may develop resistant strains (2, 5, 6, 7, 10, 12). Resistant strains, possibly environmental strains exposed to agricultural azole compounds, have infected patients who have not had previous exposure to azole drugs (28, 29, 33). Azole drugs block the biosynthesis of ergosterol, a bulk sterol component of the cell walls of fungi. Although not found in human cells, it is nearly identical to cholesterol. Triazole drugs bind to the active site of the fungal cytochrome P450 enzyme 14-alpha lanosterol demethylase, which catalyzes the demethylation of ergosterol precursors (1, 19) and is encoded by the *cyp51A* and *cyp51B* genes in *A. fumigatus* (1, 21). Amino acid substitutions encoded at several codon sites in the *cyp51A* gene, including glycine 54, methionine 220, glycine 138, and leucine 98, have been recently shown to result in reduced susceptibility to itraconazole and posaconazole or azole cross-resistance (1, 2, 7, 10, 12, 20, 22, 23, 24, 34). Molecular assays have been used to rapidly identify mutations in these codons from clinical isolates and/or specimens using probes or sequencing (1, 10, 32). We have developed a novel high-resolution melt (HRM) assay to characterize the codon for glycine 54 in the *cyp51A* gene for isolates of *A. fumigatus*. Mutations in this codon confer reduced susceptibility to itraconazole and posaconazole.

HRM is a recent enhancement of traditional melting analysis of a PCR product. Using specialized instrumentation, highly controlled temperature transitions, and data acquisition during the melt phase allows the detection of single nucleotide polymorphisms with the use of low-cost fluorescent intercalating dyes (4, 8). Reagent costs are similar to those of traditional PCR. HRM assays have been recently used for microbiological applications using various platforms (3, 8, 9, 11, 14, 17, 25, 27, 31). We evaluated the Rotor-Gene 6000 instrument, which can perform both real-time PCR and HRM, to characterize the codon for glycine 54 in the *cyp51A* gene of *A. fumigatus* isolates. Pyrosequencing (Qiagen, Hilden, Germany) was used for confirmation.

Thirteen well-characterized clinical and laboratory mutant strains of *A. fumigatus* and chromosomal DNA of strain R7-1 were used to develop our assay. These strains represented 7 different genotypes, including those corresponding to itraconazole-susceptible wild-type Gly54 (encoded by GGG) and itraconazole-resistant mutant alleles of the *cyp51A* gene, including Glu54 (encoded by GAG and GAA), Lys54 (AAG), Arg54 (AGG), Val54 (GTG), and Trp54 (TGG) (1). Twelve clinical isolates were tested as unknowns. A 10-μl loopful from 48-h growth on potato dextrose agar was suspended in 500 μl lysis buffer (1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA) containing approximately 100 mg of 710- to 1,180-μm glass beads (Sigma, Deissenhogen, Germany). After vortexing for 15 min and heating for 10 min at 100°C, 200 μl was extracted on a MagNA Pure LC system using the MagNA Pure total nucleic acid kit (Roche Diagnostics, Mannheim, Germany). PCR primers were designed to amplify a 107-bp fragment of the *cyp51A* gene (GenBank accession no. AF338659) covering codon 54. Amplicon sequences representing the different genotypes were analyzed by the Poland melting software program to predict melting behavior (http://www.biophys.uni-duesseldorf.de/local/POLAND//poland.html) (30). The reverse primer was biotinylated for pyrosequencing. SensiMix HRM master mix (Quantace, London, England) containing EvaGreen fluorescent intercalating dye was used according to the manufacturer's instructions. The forward primer (5′ GAACCGAACAGAAC CGCCAAT 3′) and reverse primer (5′ biotin CCTTTTCTCT

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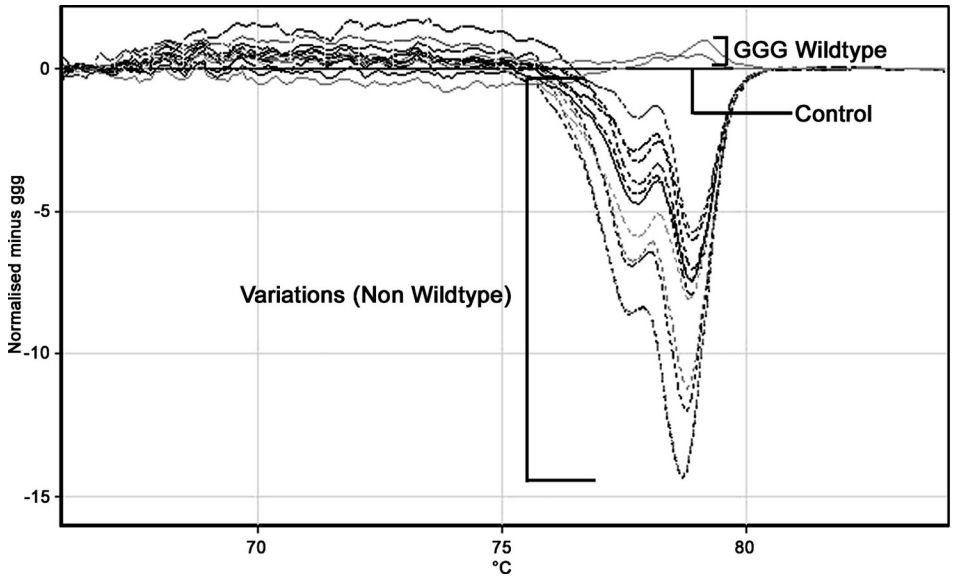

FIG. 1. HRM difference plot comparing the Gly54 wild-type GGG reference control strain to all other strains. The control is displayed as a straight line. Strains are identified as having wild-type GGG or as variations (mutants) based on melt differences as determined by the software. Two samples were correctly identified as wild-type GGG. Eleven mutant strains, including those with Glu54 (GAG and GAA), Lys54 (AAG), Arg54 (AGG), Val54 (GTG), and Trp54 (TGG), were correctly identified as variations.

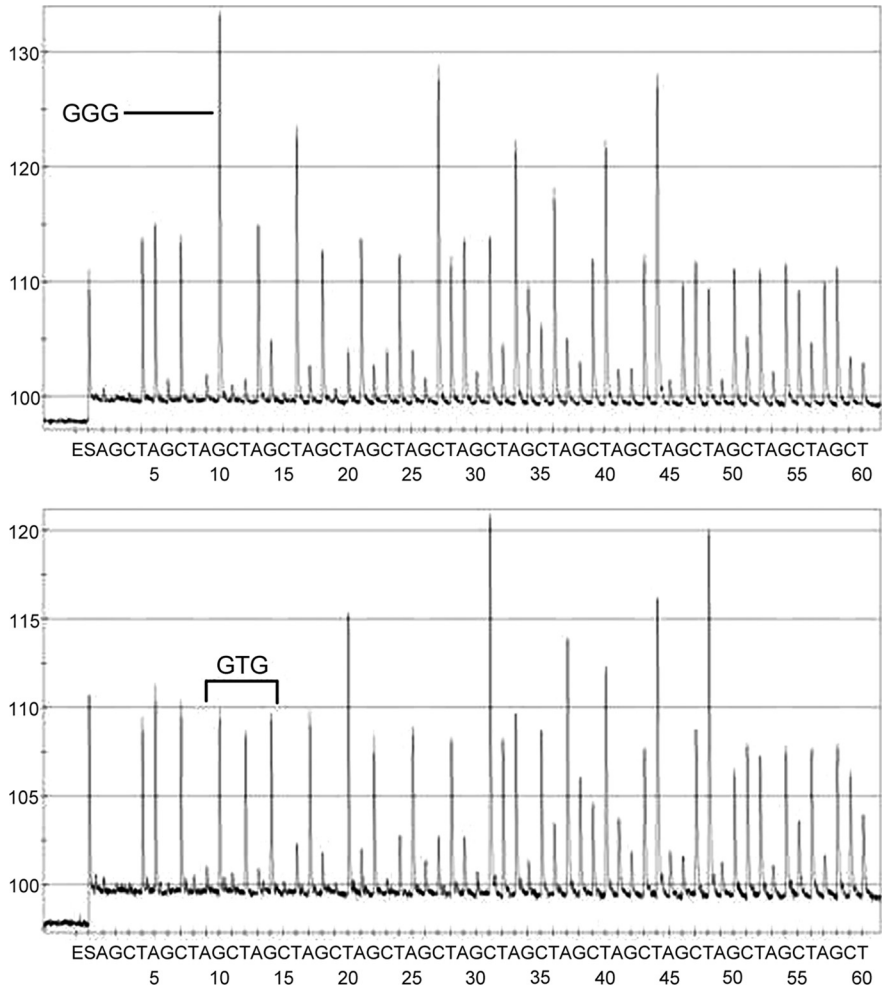

FIG. 2. Representative pyrosequencing pyrograms of a wild-type isolate with GGG and a GTG mutant isolate. HRM-amplified products were directly tested with pyrosequencing using a previously described sequencing primer (32).

TABLE 1. Comparison of results of HRM assay with pyrosequencing and itraconazole susceptibilities of reference *A. fumigatus* strains[a]

Strain	54th codon of *cyp51A*	Amino acid	HRM genotype	HRM confidence (%)	Pyrosequence	Itraconazole[c] MIC (μg/ml)
H11-20[b]	GGG	Gly	GGG	100	GGG	0.5
Af41	GGG	Gly	GGG	98.27	GGG	0.25
RIT12	GAG	Glu	Variation	51.58	GAG	>16
Af72	GAG	Glu	Variation	69.66	GAG	>16
RIT18	GAA	Glu	Variation	24.08	GAA	>16
RIT32	GAA	Glu	Variation	19.82	GAA	>16
RIT15	AAG	Lys	Variation	8.24	AAG	>16
RIT38	AAG	Lys	Variation	8.11	AAG	>16
RIT41	AGG	Arg	Variation	53.22	AGG	>16
RIT51	AGG	Arg	Variation	50.59	AGG	>16
SO/3827A	GTG	Val	Variation	65.0	GTG	>16
SO/3829	GTG	Val	Variation	75.98	GTG	>16
R7-1	TGG	Trp	Variation	41.11	TGG	>16

[a] Characterized strains including itraconazole MIC data were obtained from Balashov et al. (1).
[b] Used as GGG (wild-type) control for HRM assay. The HRM data of all other strains were compared to those of this wild-type control for identification as GGG (wild type) at ≥90% confidence or as a variation (mutant) if <90% confidence.
[c] Posaconazole MICs were not determined. Itraconazole and posaconazole are structurally similar. Resistance to itraconazaole is usually associated with a reduction in posaconazole susceptibility (13, 26, 34).

GCACGCAAAGAAGA 3′) were used at a concentration of 300 nM in a volume of 25 μl with 1× Sensimix HRM, 1× EvaGreen dye, and 2 μl of DNA target (approximately 10 to 100 ng). Instrument parameters were as follows: 95°C for 10 min, 35 cycles (95°C 10 s, 60°C 20 s), acquiring on the green channel, HRM (65 to 85°C), rising by 0.1° each step, 2-s hold at each increment, and hold at 40°C for 1 min. The template was normalized so that samples would have similar cycle thresholds. The PCR was optimized so that samples had similar amplification efficiencies (4). Our method was not able to discriminate all 7 genotypes. However, an isolate identification as non-wild type irrespective of specific genotype is enough to suspect reduced susceptibility to itraconazole and posaconazole. A control of wild-type GGG was selected, and the HRM melts of all other samples were compared to this control using a difference plot (Fig. 1) generated by the instrument software. A confidence threshold of 90% was selected for software analysis. A score of 90% or above identified the sample as genotype GGG, while a score below 90% identified the sample as a variation (mutant). The amplified products were directly tested with pyrosequencing. Fifteen microliters of PCR amplicon was used with a previously described sequencing primer (5′ TCT GGGTAGTACCATCAGT 3′) (31). Ten base pairs were analyzed using pyrosequencing software. Pyrograms (Fig. 2) were verified by manual reads.

Using one wild-type reference strain, H11-20, with GGG, as the control, 12 reference strains were correctly identified as having wild-type GGG (≥90% confidence) or a *cyp51a* mutant (variation) at a ≤90% confidence level. All were confirmed by pyrosequencing (Table 1). Twelve clinical isolates were tested as unknowns. Controls were reference strain H11-20 (wild-type GGG) and mutant (variation) strains RIT12 (GAG) and SO/3829 (GTG). The GTG control was chosen specifically because it had the melt pattern closest to the wild-type pattern during the assay optimization. All 12 clinical isolates were identified as having wild-type GGG, with a confidence range of 93.51% to 99.43%, confirmed by pyrosequencing. The two mutant controls were identified as variations at 43.09% and 82.32% confidence ranges, respectively.

Genetic mutations in codon 54 of the *cyp51A* gene of *A. fumigatus* can be detected using our novel HRM assay. Although we were unable to specifically genotype the 6 mutant alleles, the characterization of an isolate as mutant is sufficient to suspect reduced susceptibility to itraconazole and posaconazole. This assay could be used to screen for wild-type genotypes of codon 54 of the *cyp51A* gene, and variations could be pyrosequenced directly to identify specific mutant *cyp51A* codon sequences if necessary.

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We have no conflicts of interest to disclose.

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