Rapid, High-Throughput, Multiplex, Real-Time PCR for Identification of Mutations in the cyp51A Gene of Aspergillus fumigatus That Confer Resistance to Itraconazole

Sergey V. Balashov, Rebecca Gardiner, Steven Park, and David S. Perlin

Aspergillus fumigatus is an important cause of life-threatening invasive fungal disease in patients with compromised immune systems. Resistance to itraconazole in A. fumigatus is closely linked to amino acid substitutions in Cyp51A that replace Gly54. In an effort to develop a new class of molecular diagnostic assay that can rapidly assess drug resistance, a multiplexed assay was established. This assay uses molecular beacons corresponding to the wild-type cyp51A gene and seven mutant alleles encoding either Arg54, Lys54, Val54, Trp54, or G161A. Molecular beacon structure design and real-time PCR conditions were optimized to increase the assay specificity. The multiplex assay was applied to the analysis of chromosomal DNA samples from a collection of 48 A. fumigatus clinical and laboratory-derived isolates, most with reduced susceptibility to itraconazole. The cyp51A allelic identities for codon 54 were established for all of the strains tested, and mutations altering Gly54 in 23 strains were revealed. These mutations included G54W (n = 1), G54E (n = 12), G54K (n = 3), G54R (n = 3), and G54V (n = 4). Molecular beacon assay results were confirmed by DNA sequencing. Multiplex real-time PCR with molecular beacons is a powerful technique for allele differentiation and analysis of resistance mutations that is dynamic and suitable for rapid high-throughput assessment of drug resistance.

Aspergillus fumigatus is a common cause of invasive mold infections in humans resulting from an alteration of immune status due to AIDS, cancer, or solid organ or bone marrow transplantation. High rates of morbidity and mortality for patients with invasive aspergillosis, despite conventional antifungal therapy, are reported (14). Itraconazole, a triazole antifungal drug approved in 1992, has been widely used for treatment and prophylaxis of fungal infections (6, 41). Although newer triazole drugs, such as voriconazole, ravuconazole, and posaconazole, have recently become available (9, 36), Aspergillus infections are still commonly treated with itraconazole (8).

Triazole drugs are fungistatic, which can result in the development of secondary resistance in infecting strains. Itraconazole resistance in clinical isolates as well as in laboratory mutant strains has been reported (3, 5, 18, 24, 32). Triazole drugs bind to the active site of the fungal cytochrome P450 14α-sterol demethylase, which catalyzes the 14α demethylation of ergosterol precursors (22) and which is encoded by cyp51A in Aspergillus (29). Several amino acid substitutions in Cyp51A resulting in itraconazole resistance in A. fumigatus have been described (7, 26, 33). Yet, unlike the multiplicity of triazole resistance mutations found in Candida spp. (19, 48), itraconazole resistance mutations in the A. fumigatus cyp51A gene are tightly linked to amino acid substitutions at residue 54, corresponding to glycine (7, 26, 33). A. fumigatus strains with G54R, G54V, G54W, and G54E substitutions were detected in clinical isolates as well as in spontaneous and UV-induced itraconazole-resistant laboratory mutants (7, 26, 30, 33). The G54K amino acid change conferred cross-resistance to both itraconazole and posaconazole (26). The replacement of the wild-type chromosomal cyp51A allele by mutant allele bearing the G54K nucleotide change in codon 54 led to the acquisition of resistance to itraconazole de novo (7).

Early diagnosis of invasive aspergillosis by conventional procedures utilizing blood or bronchial fluid specimens is difficult. The use of cell wall components such as galactomannan (GM) as an indicator of disease is a major advancement (10, 23, 27), but it has some deficiencies as well (27, 31, 47).

Early detection of fungi in blood or bronchial alveolar lavage fluid, with a rapid assessment of drug susceptibility, could improve the survival of patients with invasive disease by accelerating the initiation of appropriate antifungal treatment while the fungal loads are still low. Genomic differences among fungi offer an alternative to culturing for detection and identification, and nucleic acid-based amplification assays for the detection of fungal nucleic acids may be the optimal diagnostic approach because they are more rapid and sensitive than current culture-based and biochemical methods (21, 34).

We have exploited the power of molecular beacon technology to develop a new multiplex real-time PCR assay suitable for rapid detection of itraconazole resistance mutations in codon 54 of A. fumigatus cyp51A. Molecular beacons are small, self-reporting, single-stranded nucleic acid hairpin probes that brightly fluoresce when bound to their targets (44). They are particularly well suited for allele discrimination (1, 43) and multiplex applications (37, 45). The molecular beacon-based
assay developed in this study provides the basis for rapid identification of drug resistance in *A. fumigatus* associated with Cyp51A alterations, which can serve as a platform for a more comprehensive rapid diagnostic tool to more routinely assess invasive aspergillosis.

### MATERIALS AND METHODS

#### Strains, culture conditions, and susceptibility testing

Itraconazole-resistant RIT strains were obtained by mutagenesis of parental wild-type H11-20 strain 20 (33). Clinical isolates of *A. fumigatus* were obtained from D. Denning (Manchester University, Manchester, United Kingdom) (7). All the strains were grown and maintained in yeast-extract-peptone-dextrose or Sabouraud dextrose agar medium as previously described (33). Itraconazole (Janssen Pharmaceutica, Titusville, N.J.) was dissolved in dimethylformamide (Sigma-Aldrich Corp., St. Louis, Mo.) prior to addition to culture media. MICs of itraconazole were determined according to the NCCLS M38-P microdilution methodology, as previously described (33, 34).

#### Molecular beacon and primer design

**The sequence of cyp51A gene**

*A. fumigatus* chromosomal DNA was extracted from cells grown for 24 h in Sabouraud dextrose agar medium, as previously described (33). Chromosomal DNA of strain R7-1 was provided by P. Mann (26). Amplification of a 500-bp fragment of cyp51A was performed on an iCycler thermal cycler (Bio-Rad Laboratories), 0.5 mM dideoxynucleotides, and 5 pmol of molecular beacons. The test reaction mixtures were 1 cycle of 3 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C; and 1 cycle of 3 min at 72°C. PCR products were purified with the Montage PCR purification kit (Millipore). PCR products for sequencing were obtained and purified with the CEQ dye terminator cycle sequencing-Quick Start kit (Beckman Coulter, Inc., Fullerton, Calif.) according to the manufacturer's recommendations on an iCycler thermal cycler. AE210F or AE709R primers, 2.5 U of Taq DNA polymerase (Bio-Rad Laboratories), 0.5 mM dideoxynucleoside triphosphates, 50 mM KCl, 4 mM MgCl2, 20 mM Tris-HCl (pH 8.4), and about 100 ng of *A. fumigatus* chromosomal DNA. The cycling conditions were 1 cycle of 3 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C; and 1 cycle of 3 min at 72°C. PCR products were purified with the Montage PCR purification kit (Millipore). PCR products for sequencing were obtained and purified with the CEQ dye terminator cycle sequencing-Quick Start kit (Beckman Coulter, Inc., Fullerton, Calif.) according to the manufacturer's recommendations on an iCycler thermal cycler. AE210F or AE709R primers were used for the sequencing reaction. The cycling conditions for sequencing PCR were 1 cycle of 3 min at 95°C and 30 cycles of 30 s at 96°C, 20 s at 50°C, and 1 min at 72°C. All DNA sequencing was performed on CEQ 8000 genetic analysis system (Beckman Coulter). CEQ 8000 genetic analysis system software (Beckman Coulter) was used for hardware control as well as for analysis of postrun sequencing results.

**Real-time PCR.** Real-time PCR experiments were performed on a Stratagene Mx4000 multiplex qualitative PCR system with the “quantitative PCR (multiple standards)” setting. Reagents from the Brilliant QPCR core reagent kit were used for all reactions. Each 50-μl PCR mixture contained 1× Stratagene Core PCR buffer, 20 pmol of molecular beacons, 25 pmol of each of the cys51AS and cys51AA primers (Table 1), 2.5 U of Stratagene SureStart Taq DNA polymerase, 0.4 mM dideoxynucleoside triphosphates, and 3 or 5 mM MgCl2. In multiplex PCR

### TABLE 1. DNA oligonucleotides used in work

<table>
<thead>
<tr>
<th>Primer or molecular beacon</th>
<th>Sequence*</th>
<th>5′ end modification</th>
<th>3′ end modification</th>
<th>Purpose</th>
</tr>
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<tbody>
<tr>
<td>AE210F</td>
<td>GTCTCTCATTCGTCTGTCTCT</td>
<td>None</td>
<td>None</td>
<td>Sequencing PCR primer</td>
</tr>
<tr>
<td>AF709R</td>
<td>CGGTGAATAAACTCCTGCC</td>
<td>None</td>
<td>None</td>
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<td>None</td>
<td>Real-time PCR primer</td>
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<tr>
<td>CYP51A4</td>
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<td>None</td>
<td>None</td>
<td>Real-time PCR primer</td>
</tr>
<tr>
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<td>CCGGATCATCAGTACAGGATGCCATCCATGCCG</td>
<td>FAM</td>
<td>Dabcyl</td>
<td>AAG allele probe</td>
</tr>
<tr>
<td>GGG-FAM</td>
<td>CCGGATCATCAGTACAGGATGCCATCCATGCCG</td>
<td>FAM</td>
<td>Dabcyl</td>
<td>Wild-type allele probe</td>
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<tr>
<td>GAA-Hex</td>
<td>CCGGATCATCAGTACAGGATGCCATCCATGCCG</td>
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<td>Dabcyl</td>
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<td>AAG-Hex</td>
<td>CCGGATCATCAGTACAGGATGCCATCCATGCCG</td>
<td>HEX</td>
<td>Dabcyl</td>
<td>AAG allele probe</td>
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<tr>
<td>GAG-Hex</td>
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<td>GAG allele probe</td>
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<td>Dabcyl</td>
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<td>Dabcyl</td>
<td>TGG allele probe</td>
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<td>CGG-Hex</td>
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<td>Dabcyl</td>
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<td>ROX</td>
<td>Dabcyl</td>
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<td>None</td>
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<td>AAAGGATCATACCAGTACCTGATGAAA</td>
<td>None</td>
<td>None</td>
<td>GTG allele target</td>
</tr>
</tbody>
</table>

*Probe domains of molecular beacons and target domains of allele targets are underlined. Sequences for molecular beacon probe domains corresponding to codon 54 in *A. fumigatus* gene cyp51A and complement codons in allele targets are in boldface.

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experiments 20 pmol of each molecular beacon (Table 1) was added to the reaction mixture. Amplification of a 70-bp fragment from *A. fumigatus* chromosomal DNA utilized 100 ng of DNA, while use of the 500-bp PCR fragment as a template required 10 pg of DNA (about 300 nmol) per reaction. PCRs were performed with the following parameters: 1 cycle of 10 min at 95°C and 45 cycles of 30 s at 95°C, 30 s at 61°C, and 30 s at 72°C. Annealing temperatures of 55 and 57°C were used when PCR experiments were performed in multiplex format. The fluorescence was measured three times during the annealing step.

**Table 2.** *T<sub>m</sub>*<sup>a</sup> for CYP51A molecular beacons

<table>
<thead>
<tr>
<th>Beacon</th>
<th>3 mM for target</th>
<th>5 mM for target</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GGG-T GAA-T AAG-T GAG-T AGG-T GTG-T TGG-T CGG-T</td>
<td>GGG-T GAA-T AAG-T GAG-T AGG-T GTG-T TGG-T CGG-T</td>
</tr>
<tr>
<td>AAG-FAM</td>
<td>48.7 44.7 62.2 52.7 53.2 48.2 50.7 49.7 49.7 45.2 63.7</td>
<td>54.2 54.7 54.2 52.2 52.2 52.2</td>
</tr>
<tr>
<td>GGG-FAM</td>
<td>65.2 57.2 57.2 57.2 57.2 57.2 57.2 57.2 57.2 57.2</td>
<td></td>
</tr>
<tr>
<td>GAA-HEX</td>
<td>45.7 61.2 41.7 50.7 35.2 45.2 41.7 36.2 46.7 63.7</td>
<td>47.7 52.7 41.2 46.7 42.2</td>
</tr>
<tr>
<td>AAG-HEX</td>
<td>46.2 39.7 61.2 52.2 51.7 49.7 48.2 47.2 48.7 44.7</td>
<td>63.2 53.2 53.2 51.2 50.7 49.7</td>
</tr>
<tr>
<td>GAG-HEX</td>
<td>55.7 55.7 63.2 50.2 57.7 50.7 50.7 57.2 57.2 64.2</td>
<td></td>
</tr>
<tr>
<td>AGG-HEX</td>
<td>54.7 42.2 55.2 49.7 61.7 49.7 56.2 58.2 56.7 44.7</td>
<td>57.2 51.7 63.7 52.2 58.2 59.7</td>
</tr>
<tr>
<td>GTG-HEX</td>
<td>54.7 47.7 48.7 54.7 49.7 63.7 51.7 49.7 56.2 48.7</td>
<td>51.2 57.2 50.7 65.2 53.7 52.2</td>
</tr>
<tr>
<td>TGG-HEX</td>
<td>56.2 47.2 53.7 52.7 58.7 53.7 63.7 60.2 57.7 48.2</td>
<td>54.7 53.2 59.7 55.7 65.2 61.7</td>
</tr>
<tr>
<td>CGG-HEX</td>
<td>54.7 40.7 47.7 48.2 54.2 50.2 54.7 65.2 55.2 42.7</td>
<td>49.2 50.2 55.2 51.7 57.2 66.2</td>
</tr>
<tr>
<td>GAG-RX</td>
<td>52.7 39.2 49.7 46.2 56.2 48.2 62.7 38.2 54.2 39.7</td>
<td>51.2 49.2 57.7 50.2 63.7 59.7</td>
</tr>
<tr>
<td>GGG-RX</td>
<td>52.7 40.7 47.7 46.2 53.7 49.7 53.2 64.2 54.2 42.2</td>
<td>48.7 49.7 54.7 51.2 55.2 65.7</td>
</tr>
<tr>
<td>GAA-Q670</td>
<td>42.2 58.2 42.7 48.7 39.7 42.7 39.2 41.2 46.7 61.7</td>
<td>42.7 51.2 40.2 48.2 44.7 43.2</td>
</tr>
<tr>
<td>GTG-Q670</td>
<td>44.7 46.2 47.7 55.2 42.7 61.2 49.7 48.2 49.7 50.2</td>
<td>49.2 64.2 51.7 50.2</td>
</tr>
</tbody>
</table>

*<sup>a</sup>*<sup>T</sup>*<sub>m</sub> of complement molecular beacon-target hybrids are in boldface.

The sequence of the probe domain was identical to the sequence of *cyp51A* starting from nucleotide position 150 and ending at position 170. Codon GGG, corresponding to wild-type Gly54, occupied nucleotide positions 11 to 13 of the beacon probe domain. The beacon was tested with oligonucleotides representing the wild-type allele and seven known *cyp51A* itraconazole resistance alleles (Table 1). The molecular beacon showed appropriate thermal behavior and hybridization with DNA targets. The efficiency of molecular beacon annealing to different targets varied depending on target nucleotide content and divalent cation concentration, with experimentally derived *T<sub>m</sub>* values for each of the eight beacon-target hybrids shown in Table 2. The stability of intermolecular hybrids of GGG-FAM molecular beacons and artificial oligonucleotide targets decreased in the order of GGG-T > GTG-T > AAG-T > GAG-T > TGG-T > CGG-T > AAG-T > GAA-T. As expected, hybrids with double mismatches possessed the lowest stability. Among noncomplement targets, the GGG-FAM beacon formed the most stable single-mismatched hybrid with GTG-T. The temperature interval between *T<sub>m</sub>* of the most stable mismatched beacon-target hybrid and *T<sub>m</sub>* of the complement beacon-target hybrid represented the condition allowing specific allele-discriminative binding of the molecular beacon or window of discrimination. As shown in Table 2, the window of discrimination for GGG-FAM molecular beacon was within the temperature ranges of 61.7 to 65.2°C under conditions of 3 mM Mg<sup>2+</sup> and 63.7 to 67.2°C at 5 mM Mg<sup>2+</sup>.

**RESULTS**

Itraconazole resistance has been tightly linked to *cyp51A* mutations in the codon for Gly54, resulting in five different amino substitutions (G54K, G54V, G54R, G54E, and G54W) (7, 26, 33), and such linkage provides an ideal opportunity to develop a nucleic acid-based diagnostic for *A. fumigatus* that can rapidly assess the drug resistance status of an infecting organism. Since multiple mutations at codon 54 confer resistance, including single and multiple nucleotide changes, it was important that the nucleic acid-based diagnostic assay possess intrinsic allele specificity and the feasibility of simultaneously detecting a range of mutations in multiplex format. Molecular beacon technology was chosen because it is a superior platform relative to other self-reporting probes for allele discrimination and multiplex assay development (44, 45).

**Design and validation of molecular beacon to wild-type Gly54.** A molecular beacon complementary to the *cyp51A* wild-type allele was synthesized with a 21-nucleotide probe target sequence (hairpin loop) domain and a 6-nucleotide stem domain with the 5' end of the beacon labeled with FAM as the fluorophore and the 3' end modified with a dabcyl quencher. The sequence of the probe domain was identical to the sequence of *cyp51A* starting from nucleotide position 150 and ending at position 170. Codon GGG, corresponding to wild-type Gly54, occupied nucleotide positions 11 to 13 of the beacon probe domain. The beacon was tested with oligonucleotides representing the wild-type allele and seven known *cyp51A* itraconazole resistance alleles (Table 1). The molecular beacon showed appropriate thermal behavior and hybridization with DNA targets. The efficiency of molecular beacon annealing to different targets varied depending on target nucleotide content and divalent cation concentration, with experimentally derived *T*<sub>m</sub> values for each of the eight beacon-target hybrids shown in Table 2. The stability of intermolecular hybrids of GGG-FAM molecular beacons and artificial oligonucleotide targets decreased in the order of GGG-T > GTG-T > AAG-T > GAG-T > TGG-T > CGG-T > AAG-T > GAA-T. As expected, hybrids with double mismatches possessed the lowest stability. Among noncomplement targets, the GGG-FAM beacon formed the most stable single-mismatched hybrid with GTG-T. The temperature interval between *T*<sub>m</sub> of the most stable mismatched beacon-target hybrid and *T*<sub>m</sub> of the complement beacon-target hybrid represented the condition allowing specific allele-discriminative binding of the molecular beacon or window of discrimination. As shown in Table 2, the window of discrimination for GGG-FAM molecular beacon was within the temperature ranges of 61.7 to 65.2°C under conditions of 3 mM Mg<sup>2+</sup> and 63.7 to 67.2°C at 5 mM Mg<sup>2+</sup>.

**Real-time PCR evaluation of wild-type molecular beacon.** DNA templates for real-time PCR evaluation of molecular beacons were obtained by amplification of 0.5-kb PCR fragments of *cyp51A* from chromosomal DNA of *A. fumigatus* strains H11-20 (wild-type GGG allele), RIT12 (GAG), RIT15 (AAG), RIT18 (GAA), RIT51 (AGG), R7-1 (TGG), and Br181 (GTG) (Table 3). The wild-type GGG-FAM molecular beacon was tested in real-time PCR experiments against the above *cyp51A* alleles. At an annealing temperature of 61°C and 3 mM Mg<sup>2+</sup>, the GGG-FAM molecular beacon formed a stable hybrid with the wild-type DNA target, which was detected by observing a high level of fluorescence that increased during the amplification process (Fig. 1A). These experimental con-
Design and evaluation of molecular beacons to Gly54 mutant sequences. After the suitability of a wild-type GGG-FAM molecular beacon for target discrimination was confirmed in real-time PCR experiments, seven molecular beacons targeted to all known Gly54 codon mutations were synthesized. The mutant beacons comprised single- or double-nucleotide substitutions identical to that found in the Gly54 GGG codon of cyp51A of itraconazole-resistant A. fumigatus strains. The cyp51A alleles considered in beacon design were the GAG (7, 26, 33), GAA (33), AAG (7, 26, 33), AGG (7, 26, 33), TGG (26), GTG (7), and CGG (P. Mann, personal communication) alleles. The compositions of all mutant beacons were identical to that of the GGG-FAM beacon except for allele-specific nucleotide substitutions in the probe sequence (Table 1). All seven beacons were labeled with HEX at the 5' end. The temperature profiles for eight DNA targets were determined, and $T_m$ values are listed in Table 2. Most molecular beacons recognizing mutant sequences had thermal profiles different from the GGG-FAM beacon profile. In general, they formed less-stable intramolecular hybrids with mismatched DNA targets and possessed lower $T_m$s. For beacons GAA-HEX, AAG-HEX, and AGG-HEX, temperatures for duplex formation were so low that the corresponding window of discrimination did not overlap with that of the wild-type probe GGG-FAM, which would pose a problem for multiplex applications. However, an overall increase in probe-target hybrid stability and a concomitant increase in $T_m$ values were achieved by increasing the concentration of Mg$^{2+}$ from 3 to 5 mM in the reaction mixture. Under this condition, all molecular beacons (mutant and wild type) could be run in a real-time PCR experiment at the same annealing temperature of 61°C and showed excellent discrimination against both wild-type and mutant alleles (Fig. 1). Non-specific hybridization was not observed for molecular beacons recognizing mutant sequences under these conditions.

### Multiplex real-time PCR detection of Gly54 mutations

Once target-specific hybridization with complement DNA target was validated for each beacon, we investigated the possibility of combining them in a multiplex real-time PCR format. A single-reaction real-time PCR assay with eight different molecular beacons (one wild type beacon labeled with FAM and seven mutant beacons labeled with HEX) and a 0.5-kb template corresponding to each cyp51A allele was performed. The reaction conditions included a 5 mM Mg$^{2+}$ concentration and an annealing temperature of 55°C. In spite of such a low temperature at the annealing step, only specific hybridization was observed for molecular beacons in multiplex real-time PCRs. HEX fluorescence (mutant beacons) was observed only when one or more of the mutant templates were present, while the FAM signal from the GGG-FAM molecular beacon was detected only when the wild-type allele was present. The layout of the single-tube multiplex PCR format is shown in Fig. 2A. In this format, the fluorescence signal indicated the presence of either wild-type or mutant sequences corresponding to itraconazole susceptibility or resistance, respectively. The advantage of this format is that, in a single assay, mutant (resistant) and wild-type (susceptible) strains can be easily distinguished. However, this format cannot be used to identify specific mutations of Gly54 because each mutant beacon was labeled with the same fluorophore.

To expand the applicability of the multiplex assay to distin-
FIG. 1. $cyp51A$ allele discrimination by molecular beacons. Real-time PCR was used to evaluate the specificity of eight different molecular beacons designed to distinguish the wild-type allele and seven mutant alleles. Each panel shows an individual molecular beacon and its relevant Gly54 allele recognition sequence. Real-time PCRs were performed with separate 500-bp templates corresponding to the GGG (●), GAA (■), AAG (□), GAG (○), AGG (■), GTG (●), and TGG (▲) $cyp51A$ alleles and a blank (no DNA; ○) with the indicated specific molecular beacons. Each panel shows a composite representation of eight separate template reactions with the same molecular beacon.
guish separate alleles, the molecular beacons were labeled with different fluorophores: FAM, HEX, ROX, or the CY5 analog Q670 (Table 1). This configuration required two PCRs to assess all seven mutations, since only four colors could be distinguished in a single reaction. The first PCR mixture contained molecular beacons with high $T_m$s against complement DNA targets: GGG-FAM, GAG-HEX, GTG-Q670, and CGG-ROX. The second PCR mixture contained molecular beacons with somewhat lower $T_m$s: AAG-FAM, AGG-HEX, GAA-Q670, and TGG-ROX. The system specificity was further optimized by adjusting the Mg$^{2+}$ concentration up to 3 mM for the first reaction and to 5 mM for the second reaction. A uniform annealing temperature of 57°C was used for both reactions. The layout of this double-tube multiplex PCR format is shown in Fig. 2B. The assay system was tested by adding individual 0.5-kb cyp51A templates to each of two reaction mixtures. Under these conditions, specific hybridization with complement cyp51A alleles was observed for all molecular beacons except the GGG-FAM wild-type beacon, which exhibited some level of nonspecific hybridization with the GTG allele. To avoid any possible false-positive results coming from nonspecific hybridization of the wild-type GGG-FAM beacon, the threshold fluorescence level was adjusted to values close to those obtained for complement beacon-target pairs. This multiplex format allowed all seven specific cyp51A alleles bearing mutations in the Gly54 codon, along with the wild-type allele, to be distinguished in a real-time assay.

Application of an allele-specific panel. The multiplex real-time PCR assay was applied to the analysis of a collection of 48 A. fumigatus isolates comprising itraconazole-susceptible ($n=1$; parental strain) and -resistant ($n=33$) laboratory strains obtained during previous in vitro studies (26, 33) and itraconazole-susceptible ($n=4$) and -resistant ($n=11$) clinical isolates (Table 3). Chromosomal DNA from each strain was isolated and used as a template in both single-tube and double-tube formats. The assay in the single-tube format revealed only the presence of wild-type or mutant cyp51A alleles, while the system in the double-tube format specified the particular Gly54 mutations. Out of 48 total strains tested, 23 isolates bearing mutant cyp51A alleles with GAG, GAA, AAG, AGG, and GTG at codon 54 were found. All 23 strains with G54 mutations exhibited elevated levels of itraconazole resistance. Of the 14 clinical isolates, 7 were found to have resistance mutations (Table 3). In all cases but one, the presence of a resistant allele detected by the assay correlated with drug susceptibility testing. The single negative result came from the sample of RIT19 DNA, where a secondary mutation in codon 55 of cyp51A was revealed. For this reason, none of the eight molecular beacons (mutant and wild type) could hybridize specifically with the RIT19 DNA. All strains were subjected to DNA sequencing to validate the real-time determination.

DISCUSSION

The multiplex PCR assay for itraconazole resistance presented in this study provides a foundation for rapid analysis of target site drug resistance mutations that has the potential to extend molecular diagnostics beyond pathogen identification to include simultaneous evaluation of drug susceptibility. This approach represents a natural progression from earlier studies utilizing individual LightCycler probes for rapid identification of triazole resistance mutations in Aspergillus and Candida spp. (15). Microbial drug resistance remains a complicating factor for treatment of fungal infections. While fluconazole resistance in yeasts has remained largely constant (39, 42), resistance to newer triazole drugs (38) and the echinocandins (4) poses new challenges, since in some cases novel molecular mechanisms contribute to resistance (12). Mutations in cyp51A conferring resistance to itraconazole, especially at the Gly54 codon, are well established (7, 26, 33) and represent suitable targets for molecular analysis that have been exploited in this study. The relevance of cyp51A mutations for clinically observed itraconazole resistance needs more thorough investigation, but it is significant, as demonstrated by the fact that 7 of 14 clinical isolates with reduced itraconazole susceptibility were found to contain Gly54 mutations (Table 3). The strong linkage between target site mutations and phenotypic in vitro resistance provided the primary rationale for focusing on these mutations as a surrogate marker for triazole resistance. In fact, of the 48 examined laboratory and clinical isolates for which itraconazole MICs were elevated, 23 were found to contain mutations in codon 54 of the cyp51A gene (Table 3).

The development of fast, accurate, and sensitive diagnostic assays for the identification of invasive aspergillusosis remains an important goal to overcome current deficiencies associated with standard microbiological identification. Rapid clinical diagnosis and aggressive preemptive therapy can limit morbidity and morality associated with invasive fungal disease. Yet, most clinical laboratories still rely on culture-based technology with phenotypic end points that can take several days for positive identification and even longer to determine drug susceptibility. In addition to causing time delays, these techniques often lack adequate sensitivity and specificity, and organisms such as Aspergillus are difficult to culture from blood. This often means that empirical therapy must begin in the absence of positive
pathogen identification. To circumvent this problem, non-culture-based techniques are emerging rapidly and include tests such as the enzyme-linked immunosorbent assay for the GM antigen and (1→3)-β-D-glucan (BDG) (13), as well as real-time PCR-based assays for Aspergillus-specific DNA (17). Nucleic acid-based diagnostics provide rapid and sensitive results, reducing the time needed for diagnostic work-up to a few hours, and rapid pathogen identification can be achieved with high fidelity from both culture and primary specimens (17, 37). Real-time PCR assays have been reported to have a sensitivity higher than those of the GM and BDG tests (11), although such a determination is controversial since conclusive studies are lacking.

PCR-based amplification of highly conserved rRNA genes and intergenic sequences is the most reliable approach for identification of fungi (20). The fidelity of these assays has improved markedly with the emergence of real-time self-reporting nucleic acid probes, which can be used to detect one to five organisms per milliliter of blood (16), and include LightCycler (16), TaqMan (2), and molecular beacon (35, 37) probes. Real-time PCR with high-fidelity self-reporting probes enables both PCR amplification and detection to be performed in a sealed tube, which reduces the possibility of contamination and allows product formation to be continuously monitored and validated. The fidelity of authentic target recognition by self-reporting real-time probes is critical to a clinical microbiology laboratory because PCR amplification has the potential to amplify small amounts of target DNA from contaminating organisms and even human DNA. Furthermore, real-time probes are quantitative and have a large dynamic range, exceeding 1 million times that of the starting target.

Molecular beacons were selected in this study because they can accurately distinguish allelic differences in a DNA sequence by detecting single nucleotide differences (28). This property is derived from inherent energetic properties of the hairpin structure that make mismatched probe-target hybrids less thermodynamically stable than hybrids between corresponding linear probes, resulting in a wider temperature range for discrimination between perfect matches and single-nucleotide changes (1). Molecular beacon technology has been successfully applied to mutational analysis of bacteria and viruses for single-nucleotide polymorphism genotyping, allele differentiation, qualitative microorganism identification, and quantitative gene expression and viral-load assays (25, 35, 37, 40, 46). The versatility of such a high-fidelity probe system for nucleic acid-based detection now extends beyond simple pathogen detection and includes a rapid assessment of drug resistance where specific changes in the DNA are linked to resistance.

The ultimate goal of actively discriminating between cyp51A alleles differing in a single nucleotide was achieved by optimizing molecular beacon design and real-time PCR conditions. Since mutations conferring resistance to itraconazole in A. fumigatus are clustered at a single locus corresponding to codon 54 of the cyp51A gene, the analysis was restricted by surrounding this locus. The probe domains of synthesized molecular beacons reproduced sequences of eight known cyp51A alleles (the wild-type allele and seven mutant alleles) starting from nucleotide position 150 and ending at 170. The probe length of 21 nucleotides and a stem sequence of 6 nucleotides provided sufficient specificity for detection of point mutations.

Application of molecular beacons in individual PCRs showed specificity suitable for allele differentiation (Fig. 1); only a minor nonspecific hybridization was noted for the GGG-FAM wild-type beacon with respect to the GTG allele. Importantly, no nonspecific interactions were observed for the beacons recognizing mutant sequences. Such high discriminating power is especially important for prospective application of the assay for analysis of total-DNA samples from potentially mixed A. fumigatus populations where a small amount of mutant cyp51A DNA could be accurately detected in a background of wild-type DNA.

A single multiplex reaction assay that combines numerous probes and that is capable of identifying multiple pathogens is an efficient and cost-effective approach for a clinical microbiology laboratory. Such assays require that probes representing different targets be reliably resolved in the same reaction vessel. Multiplexing in its simplest format required a single tube and included all eight (wild-type plus seven mutant) molecular beacons. The wild-type beacon was labeled with FAM, while all mutant beacons were labeled with HEX. The assay was robust and detected either the wild-type sequence or a mutant sequence. Combining all eight allele-specific molecular beacons in a single multiplex PCR assay made possible the differentiation of DNA from wild-type and itraconazole-resistant strains with mutations in cyp51A. Although the assay was allele specific, it was designed to give a wild-type (FAM fluorescence) or mutant (HEX fluorescence) output, and it could not be used to distinguish which mutant allele was present in the template. This assay format is powerful because many probes can be included in a single reaction with a common set of PCR primers, and it accurately determines resistance if a mutant beacon signal is detected. For clinical laboratories, such an output may be sufficient.

The multiplex format utilizing individually labeled molecular beacons made possible genotyping of specific cyp51A itraconazole resistance mutations. The Max4000 real-time system capabilities were restricted to analysis of no more than four fluorophores per tube, so the assay of eight beacons labeled by FAM, HEX, ROX, and Q670 was split between two PCR tubes. In both multiplex formats, the PCR amplification of individual wild-type or mutant DNA target produced distinct and robust fluorescence signals. No false-negative or false-positive results due to hybridization or PCR failure and no nonspecific hybridization were ever observed. The only case in which no fluorescence signal was obtained was for a DNA sample of the RT19 strain, which had two missense mutations G161A and T163A in codons 54 and 55 of cyp51A gene. This result provided evidence of specificity not only with respect to known mutations at codon 54 but also to other potential mutations at the cyp51A locus covered by molecular beacons.

It is important to recognize that, although the multiplex assay developed in this work focused on resistance-associated mutations in codon 54 of cyp51A, in fact, the platform is dynamic and can be expanded to include mutations, once identified, at other loci. These include the recently described Met220 mutations described by Mellado and colleagues (30). In principle, a broader platform that includes a comprehensive inventory of target site mutations, along with a quantitative
assessment of drug efflux transporter gene expression levels, could then accurately identify most, if not all, resistant strains in a rapid assay. Thus, the overall robustness and ultimate clinical value of the assay will depend on the characterization of resistance alleles and the prevalence of certain mutations or mutation “hot spots.”

In conclusion, we have developed a multiplex molecular beacon real-time PCR assay that permits simple, rapid, and reliable diagnosis of the itraconazole resistance mutations in the *A. fumigatus* *cyp51A* gene. Thus, the notion of identifying an invasive pathogen such as *Aspergillus* from a primary specimen and simultaneously assessing its drug susceptibility status is entirely feasible and has important implications for therapeutic management of patients.

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