We employed an endpoint genotyping method to update the prevalence rate of positivity for the TR34/L98H mutation (a 34-bp tandem repeat mutation in the promoter region of the cyp51A gene in combination with a substitution at codon L98) and the TR46/Y121F/T289A mutation (a 46-bp tandem repeat mutation in the promoter region of the cyp51A gene in combination with substitutions at codons Y121 and T289) among clinical *Aspergillus fumigatus* isolates obtained from different regions of Iran over a recent 5-year period (2010 to 2014). The antifungal activities of itraconazole, voriconazole, and posaconazole against 172 clinical *A. fumigatus* isolates were investigated using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) broth microdilution method. For the isolates with an azole resistance phenotype, the cyp51A gene and its promoter were amplified and sequenced. In addition, using a LightCycler 480 real-time PCR system, a novel endpoint genotyping analysis method targeting single-nucleotide polymorphisms was evaluated to detect the L98H and Y121F mutations in the cyp51A gene of all isolates. Of the 172 *A. fumigatus* isolates tested, the MIC values of itraconazole (≥16 mg/liter) and voriconazole (≥4 mg/liter) were high for 6 (3.5%). Quantitative analysis of single-nucleotide polymorphisms showed the TR34/L98H mutation in the cyp51A genes of six isolates. No isolates harboring the TR46/Y121F/T289A mutation were detected. DNA sequencing of the cyp51A gene confirmed the results of the novel endpoint genotyping method. By microsatellite typing, all of the azole-resistant isolates had genotypes different from those previously recovered from Iran and from the Dutch TR34/L98H controls. In conclusion, there was not a significant increase in the prevalence of azole-resistant *A. fumigatus* isolates harboring the TR34/L98H resistance mechanism among isolates recovered over a recent 5-year period (2010 to 2014) in Iran. A quantitative assay detecting a single-nucleotide polymorphism in the cyp51A gene of *A. fumigatus* is a reliable tool for the rapid screening and monitoring of TR34/L98H- and TR46/Y121F/T289A-positive isolates and can easily be incorporated into clinical mycology algorithms.
TABLE 1 Distribution of azole-resistant and azole-susceptible (wild-type) *Aspergillus fumigatus* isolates examined in this study according to year of isolation

<table>
<thead>
<tr>
<th>Year of isolation</th>
<th>No. of isolates with each phenotype and resistance mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>2010</td>
<td>24</td>
</tr>
<tr>
<td>2011</td>
<td>35</td>
</tr>
<tr>
<td>2012</td>
<td>37</td>
</tr>
<tr>
<td>2013</td>
<td>38</td>
</tr>
<tr>
<td>2014</td>
<td>32</td>
</tr>
<tr>
<td>Total</td>
<td>166</td>
</tr>
</tbody>
</table>

In the current study, we therefore evaluated the prevalence of TR34/L98H- and TR46/Y121F/T289A-positive isolates among clinical *Aspergillus fumigatus* isolates obtained from patients with *Aspergillus* diseases in Iran over a recent 5-year period (2010 to 2014), using PCR sequencing and the novel endpoint genotyping assay targeting SNPs in the cyp51A gene of *A. fumigatus*.

**MATERIALS AND METHODS**

**Fungal isolates.** One hundred seventy-two clinical *A. fumigatus* isolates obtained from 142 patients with *Aspergillus* diseases were investigated. These patients included 88 patients with chronic pulmonary aspergillosis (CPA; 61.97%), 23 patients with allergic bronchopulmonary aspergillosis (ABPA; 16.19%), 20 patients with aspergilloma (14.08%), and 11 patients with invasive pulmonary aspergillosis (7.75%). Patient-related data were collected in accordance with the applicable rules concerning the review of research ethics committees at the Tehran University of Medical Sciences, and informed consent was obtained from all patients. The isolates were stored in 10% glycerol broth at −80°C at the Tehran University Mycology Reference Centre in Iran (Tables 1 and 2).

The isolates were submitted to various fungus culture collections across Iran over the last 5 years (2010 to 2014) for species identification and antifungal susceptibility testing and were then submitted to the Mycology Reference Centre at the School of Hygiene & Institute of Public Health Research, Tehran University of Medical Sciences, Tehran, Iran. The isolates were subcultured on Sabouraud dextrose agar (SDA) supplemented with 0.02% chloramphenicol for 5 days at 35 to 37°C. The isolates were originally identified by experienced technicians on the basis of macroscopic colony morphology, the microscopic morphology of the conidia and conidium-forming structures, and the ability to grow at 48°C, and their identities were further confirmed by sequence-based analysis of parts of the β-tubulin and calmodulin genes, as described previously (21, 22).

**DNA extraction.** DNA was isolated as described previously (26); in brief, the isolates were cultured on Sabouraud dextrose agar slants. Conidia were harvested and added to 200 μl of breaking buffer (100 mM NaCl, 10 mM Tris-HCL, pH 8, 2% Triton X-100, 1% sodium dodecyl sulfate, 1 mM EDTA, pH 8) with −0.1-g glass beads (diameters, 0.4 to 0.6 mm). After shaking by vortexing, the conidia were incubated at 70°C for 30 min while they were shaken. Then, 200 μl of phenol-chloroform-isoamyl alcohol (25:24:1) saturated with pH 8.0 aqueous buffer was added, and the samples were incubated for 5 min while they were shaken. After centrifugation for 5 min, the upper phase containing the DNA was transferred to a new tube. One microliter of DNA was used per PCR mixture.

**Strain identification and cyp51A sequence analysis.** All isolates were identified using sequence-based analysis of the calmodulin and β-tubulin genes, as described previously (21, 22). The sequence of the promoter region and the full coding sequence of the cyp51A gene were determined by amplification and subsequent sequencing as described

**TABLE 2 Underlying disease and in vitro susceptibilities of six clinical *Aspergillus fumigatus* isolates that grew on the 4-well plates**

<table>
<thead>
<tr>
<th>Azole-resistant <em>Aspergillus fumigatus</em> isolate</th>
<th>Underlying disease</th>
<th>MIC (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-IR-AF 1002</td>
<td>CPA</td>
<td>0.5 Amphotericin B</td>
</tr>
<tr>
<td>T-IR-AF 1088</td>
<td>CPA</td>
<td>0.5 Amphotericin B</td>
</tr>
<tr>
<td>T-IR-AF 1143</td>
<td>CPA</td>
<td>0.5 Amphotericin B</td>
</tr>
<tr>
<td>T-IR-AF 1416</td>
<td>CPA</td>
<td>0.5 Amphotericin B</td>
</tr>
<tr>
<td>T-IR-AF 1499</td>
<td>ABPA</td>
<td>0.5 Amphotericin B</td>
</tr>
<tr>
<td>T-IR-AF 1521</td>
<td>CPA</td>
<td>0.5 Amphotericin B</td>
</tr>
</tbody>
</table>

a All isolates were positive for the 34-bp tandem repeat in the promoter region of the cyp51A gene and the L98H amino acid substitution (nucleotides are numbered from the translation start codon ATG of cyp51A) in the cyp51A gene, and all patients had previously been exposed to azoles.
b CPA, chronic pulmonary aspergillosis; ABPA, allergic bronchopulmonary aspergillosis.
The following dyes were used as 5'-quenchers: 6-carboxyfluorescein (6FAM), LightCycler Red 610 dye (LC610), cyanine 3 (Cy3), and 3'-quencher Blackberry quencher (BBQ).

Antifungal resistance phenotypic analysis. Table 2 shows the underlying disease of the patients and the in vitro susceptibilities of six clinical A. fumigatus isolates that grew on the 4-well plates. All six isolates showed a multiresistant phenotype, and the MICs of itraconazole (≥16 mg/liter) and voriconazole (≥2 mg/liter) for these isolates were high. Five of these isolates were recovered from patients with chronic pulmonary aspergillosis (CPA), and one was from a patient with allergic bronchopulmonary aspergillosis (ABPA).

Resistance mechanism. As shown in Fig. 1, quantitative analysis of the single-nucleotide polymorphisms showed the presence of the TR34/L98H mutation in the cyp51A gene of the 6 out of the 172 A. fumigatus isolates for which the MICs of itraconazole and voriconazole were elevated. However, no A. fumigatus isolates harboring the TR46/Y121F/T289A mutation were detected. Sequence analysis of the cyp51A gene confirmed the presence of the TR34/L98H mutation in those 6 isolates, yet no other polymorphisms were identified in any of the 172 isolates tested.

Genotypic analysis. Microsatellite typing of six STR loci showed identical patterns for two out of the six azole-resistant isolates, proving that the two isolates had a similar phylogenetic origin and, possibly, the same origin. Of note, the two patients from whom these isolates were recovered lived in the same geographical area. Comparison of genetic relatedness by the generation of dendrograms of the STR profiles showed that the 6 Iranian clinical isolates clustered apart from the 20 Dutch TR34/L98H control isolates and those previously isolated in Iran between 2003 and 2009 (5).

RESULTS

Prevalence of azole-resistant A. fumigatus isolates in Iran from 2010 to 2014. The global distribution of azole-resistant and azole-susceptible (wild-type) A. fumigatus isolates examined in this study is shown in Table 1 according to the year of isolation. All isolates were identified to be A. fumigatus by sequence analysis of the ITS1, ITS2, and ß-tubulin genes. Of the 172 A. fumigatus isolates, 6 isolates (recovered from separate patients) grew on the wells containing itraconazole and voriconazole, indicating a prevalence of 3.5%.

TABLE 3 Sequences of primers and probes used for detection of L98H and Y121F mutations in cyp51A gene of Aspergillus fumigatus

<table>
<thead>
<tr>
<th>Assay</th>
<th>Primer or probe</th>
<th>Sequence (5’–3’)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyp51A amplification</td>
<td>Forward primer</td>
<td>GCCGGTTCAAGGGAACGAG</td>
</tr>
<tr>
<td>cyp51A amplification</td>
<td>Reverse primer</td>
<td>CTTGATGAACTTTTTCTGCTCCATCAG</td>
</tr>
<tr>
<td>cyp51A L98 detection</td>
<td>L98 probe</td>
<td>6FAM-AACGGCAAG+C+T+CAAGGATGTC-BBQ</td>
</tr>
<tr>
<td>cyp51A L98H detection</td>
<td>L98H probe</td>
<td>CY5-CAACCGCGAAG+C+T+CAAGGATGTC-CA-BBQ</td>
</tr>
<tr>
<td>cyp51A Y121 detection</td>
<td>Y121 probe</td>
<td>LC610-TTGGGACAATC+A+T+ACACCGATCGG-BBQ</td>
</tr>
<tr>
<td>cyp51A Y121F detection</td>
<td>Y121F probe</td>
<td>6HEX-TTGGGACAATC+A+T+CACACCGATCGG-BBQ</td>
</tr>
</tbody>
</table>

†The following dyes were used as 5’-fluorophores: 6-carboxyfluorescein (6FAM), LightCycler Red 610 dye (LC610), cyanine 3 (Cy3), and 3’-quencher Blackberry quencher (BBQ).
The acquisition of azole resistance in *A. fumigatus* is an emerging public health problem which definitely needs continued surveillance at the national and international levels (9). The main molecular mechanism of azole resistance in *A. fumigatus* is explained by several mutations in the *cyp51A* gene (38). Two common genetic variants associated with resistance to azoles are the TR34/L98H mutation and the TR46/Y121F/T289A mutation (1, 2). Both mutations are predominantly found in the environment, show a strong tendency to migrate, and have now been reported in many countries from several continents (3, 23, 40–42). In addition, the clinical implications of infection due to *A. fumigatus* isolates harboring these mutations are significant, as they cause both diagnostic challenges and azole treatment failure (8, 43).

Since effective monitoring of azole resistance requires effective detection methods, rapid diagnostic tools are warranted to obtain a better understanding of the scale of this emerging problem and to detect the emergence of new resistance mechanisms early (7, 8). In the current study, we employed for the first time a rapid and simple one-step endpoint genotyping quantitative PCR assay (11–20) to detect the L98H and Y121F mutations in TR 34/L98H- and TR46/Y121F/T289A-positive azole-resistant *A. fumigatus* isolates. Interestingly, all of the *A. fumigatus* isolates in which the L98H mutation was confirmed by PCR sequencing of the *cyp51A* gene were correctly found to be mutated from the endpoint fluorescence plots (Fig. 1). The quantitative SNP assay used in the current study is based on the competition between probes detecting the wild type and the mutants (11, 16–18, 44). Endpoint measurements of the fluorescent signal for the mutant probe versus that for the wild-type probe were used for target detection and SNP discrimination (16–20). Importantly, this is a rapid method that is technically simple to perform and can easily be employed in clinical diagnostic laboratories.

Of note, molecular techniques are a promising tool to rapidly provide information about the azole resistance genotype of *A. fumigatus* isolates. Mellado et al. used PCR amplification of the *cyp51A* region followed by DNA sequencing (45); PCR assays were performed using primers generated from the unique sequence of the *A. fumigatus cyp51A* gene, and the *A. fumigatus cyp51A* gene was further evaluated by consecutive DNA sequence analysis to detect and identify point or tandem repeat mutations (45). Using real-time quantitative PCR, Klaassen et al. applied a mixed-format assay and analyzed the melting curves obtained with specific probe primers to detect clinically related mutations at positions Gly54, Leu98, Gly138, and Met220 of the *cyp51A* gene of *A. fumigatus* (38). The L98H and TR34 mutations have also been detected using specific PCR assays targeting each mutation (46), a nested PCR assay followed by DNA sequencing (47), and a PCR-restriction fragment length polymorphism (RFLP) assay (39). In addition, PCR-based assays were also tested to detect *cyp51A* gene mutations directly in clinical samples (48, 49). Moreover, two commercial multiplex real-time PCR assays which are able to differentiate susceptible from resistant *A. fumigatus* strains and identify various mutations of the *cyp51A* gene directly in serum and bronchoalveolar lavage fluid samples were recently introduced (50, 51).

In conclusion, our data show that there was not a significant increase in the prevalence of azole-resistant *A. fumigatus* isolates harboring the TR34/L98H resistance mechanism over a recent 5-year period in Iran. The quantitative assay detecting a single-nucleotide polymorphism in the *cyp51A* gene of *A. fumigatus* is a powerful surveillance method with high epidemiological and clin-

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**FIG 1** Endpoint fluorescence plot of single-nucleotide variance for detection of the L98H mutation in clinical *Aspergillus fumigatus* isolates using a quantitative PCR assay. Relative L98 (6-carboxyfluorescein [6FAM]) and L98H (cyanine 5 [Cy5]) fluorescence levels are plotted on the y and x axes, respectively. Blue diamonds, control isolates; purple circles, clinical *A. fumigatus* isolates without a mutation in the *cyp51A* gene at L98; black circles, clinical *A. fumigatus* isolates harboring the L98H substitution in the *cyp51A* gene; X, nuclease-free water, which was used as a negative control.
cial relevance to determine whether *A. fumigatus* isolates have acquired the TR_{G} /L98H and or TR_{E} /Y121F/T289A mutations and can easily be incorporated into clinical mycology algorithms.

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