High prevalence of clinical and environmental triazole-resistant *Aspergillus fumigatus* in Iran: is it a challenging issue?

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Triazole antifungal agents are the mainstay of aspergillosis treatment. As highlighted in numerous studies, the global increase in the prevalence of triazole resistance could hamper the management of aspergillosis. In the present three-year study, 513 samples (213 clinical and 300 environmental samples) from 10 provinces of Iran were processed and screened in terms of azole resistance (4 and 1 mg l\(^{-1}\) of itraconazole and voriconazole, respectively), using selective plates. Overall, 150 *A. fumigatus* isolates (71 clinical and 79 environmental isolates) were detected. The isolates were confirmed by partial sequencing of the \(\beta\)-tubulin gene. Afterwards, *in vitro* antifungal susceptibility tests against triazole agents were performed, based on the Clinical and Laboratory Standards Institute (CLSI) M38-A2 document. The CYP51A gene was sequenced in order to detect mutations. The MIC of itraconazole against 10 (6.6 %) strains, including clinical (\(n=3, 4.2\ %\)) and environmental (\(n=7, 8.8\ %\)) strains, was higher than the breakpoint and epidemiological cut-off value. Based on the findings, the prevalence of azole-resistant *A. fumigatus* in Iran has increased remarkably from 3.3 % to 6.6 % in comparison with earlier epidemiological research. Among resistant isolates, TR\(_{34}/L98H\) mutations in the CYP51A gene were the most prevalent (\(n=8, 80\ %\)), whereas other point mutations (F46Y, G54W, Y121F, G138C, M172V, F219C, M220I, D255E, T289F, G432C and G448S mutations) were not detected. Although the number of patients affected by azole-resistant *A. fumigatus* isolates was limited, strict supervision of clinical azole-resistant *A. fumigatus* isolates and persistent environmental screening of azole resistance are vital to the development of approaches for the management of azole resistance in human pathogenic fungi.

**INTRODUCTION**

Recently, emergence of triazole-resistant *Aspergillus fumigatus* infections has become a major medical concern due to the long-term use ofazole antifungals. Frequent exposure of *A. fumigatus* to fungicides can lead to the development of
azole-resistant species in the environment over time (Verweij et al., 2009b). The first case of azole-resistant A. fumigatus was reported in the UK (Denning et al., 1997). The frequency of azole resistance has increased over the past decade, resulting in the emergence of triazole-resistant A. fumigates infections in European countries (e.g. Austria, Belgium, Denmark, France, Germany, the Netherlands, Norway, Spain and Turkey), the USA, South America and Asian countries (e.g. China, India, Iran, Japan and Kuwait), with an incidence rate of 3.3–38% (Badali et al., 2013; Bader et al., 2013; Bueid et al., 2010; Burgel et al., 2012; Chowdhary et al., 2011; Chryssanthou, 1997; Howard et al., 2009; Lockhart et al., 2011; Mortensen et al., 2010; Pham et al., 2014; Seyedmousavi et al., 2013; Snelders et al., 2008; van der Linden et al., 2011; van Ingen et al., 2014). According to a recent international surveillance study on the clinical isolates of azole-resistant A. fumigatus, an incidence rate of 3.2% was reported, which introduced a novel phase in the management of invasive aspergillosis (van der Linden et al., 2015). In addition, according to a nationwide surveillance study, prevalence of azole resistance was estimated at 5.5% in patients with aspergillosis in Belgium (Vermeulen et al., 2015). In a previous study in the Netherlands, prevalence of azole resistance ranged between 5% and 10% in different hospitals (Lestrade et al., 2016). Additionally, in Denmark, 4.5% of A. fumigatus isolates from patients showed triazole resistance, while in France, resistant isolates were detected in 8% of patients with cystic fibrosis. Additionally, in a worldwide survey of medical centers, a resistance rate of 5.8% was reported, with most resistant isolates reported from China (Bowyer & Denning, 2014).

**Fig. 1.** Distribution of A. fumigatus isolates in Iran. A total of 513 clinical and environmental isolates were obtained from 10 provinces. Clinical and environmental azole-resistant A. fumigatus strains harbouring TR34/L98H mutations are marked with red and green circles, respectively.
One of the major mechanisms involved in the azole resistance of *A. fumigatus* is the modification of sterol 14α-demethylation, which is a key enzyme in the ergosterol biosynthetic pathway, encoded by CYP51A and CYP51B genes (Chen et al., 2005; Gulshan & Moye-Rowley, 2007). In fact, mutations at codons TR34/C241L, G54, M220, G138C and Y121F/T289A have been identified in azole-resistant *A. fumigatus* strains (Chen et al., 2005; Gulshan & Moye-Rowley, 2007). Over 90% of azole-resistant *A. fumigatus* isolates harbour a leucine-to-histidine substitution at codon 98, along with a 34 bp tandem repeat in the CYP51A promoter region. This issue, which is associated with high rates of treatment failure, has become a major medical concern (Howard et al., 2009; Snelders et al., 2009; van der Linden et al., 2011; Verweij et al., 2007). However, a novel CYP51A-promoter duplication mutation, known as TR46/Y121F/T289A, has recently been introduced, which is considered responsible for the elevated MIC of voriconazole (Chowdhary et al., 2013; van der Linden et al., 2013). Considering the rapid expansion of triazole resistance in various regions and the undesirable outcomes for patient management, we aimed to study the epidemiology of triazole-resistant *A. fumigatus* and CYP51A mutations in viable clinical and environmental isolates in Iran during 2013–2015.

**METHODS**

**Fungal strains.** In the present study, 213 clinical specimens were obtained from the lower respiratory tract (n=144; 67.6%), sinus (n=24; 11.2%), cerumen (n=24; 11.2%), nails (n=12; 5.6%) and biopsy samples (n=9; 4.2%) via routine diagnostic procedures at hospital laboratories in 10 provinces of Iran. In addition, 300 environmental samples from the soil of gardens surrounding the hospitals (n=190; 63.3%) and indoor air of hospital wards (n=110; 36.6%) were collected and examined in terms of the growth of triazole-resistant *A. fumigatus* isolates.

Cultures were prepared on a Sabouraud dextrose agar plate (SDA; Difco), supplemented with 4 and 1 mg l⁻¹ of itraconazole and voriconazole, respectively, at 45 °C for 72 h in the dark. All colonies growing on the plates, mimicking *A. fumigatus* complexes, were sub-cultured. Strain identities were reconfirmed by DNA sequencing of the partial β-tubulin gene using TUB2a (5'-TGACCCACAGAGTT-3') and TUB2b (5'-GTTGTGGGAATCCACTC-3') as previously described (Badali et al., 2013; Khodavaisy et al., 2016).

**In vitro antifungal susceptibility tests.** The minimum effective concentration (MEC) of caspofungin (Merck Sharp & Dohme) and MICs of itraconazole (Janssen Research Foundation), voriconazole (Pfizer Central Research), posaconazole (Schering-Plough) and amphotericin B (Bristol-Myers Squibb) were determined according to the Clinical and Laboratory Standards Institute document M38-A2 (CLSI, 2008). Antifungal agents including amphotericin B, itraconazole, voriconazole, posaconazole and caspofungin were dispensed into the microdilution trays at final concentrations of 0.016–16 mg l⁻¹. Inoculum suspensions were prepared on potato dextrose agar (MERCK, Germany) for 2–3 days by slightly scraping the surface of mature colonies with a sterile cotton swab, soaked in sterile saline solution containing Tween 40 (0.05%). The supernatants were adjusted spectrophotometrically to an OD range of 0.09–0.13 (0.5×10⁶ to 3.1×10⁸ cf.u. ml⁻¹) at a wavelength of 530 nm, as determined by the quantitative colony count for specifying the viable cf.u. per milliliter.

Conidial suspensions, which mostly consisted of conidia, were diluted 1:50 in RPMI 1640 medium (GIBCO, UK). The microdilution plates

### Table 1. In vitro antifungal susceptibility of 150 clinical and environmental *A. fumigatus* strains to five antifungal drugs

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>MIC range</th>
<th>MIC</th>
<th>G</th>
<th>M ( \text{GM} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMB</td>
<td>0.063–16</td>
<td>1.0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ITC</td>
<td>0.063–2</td>
<td>0.25</td>
<td>1</td>
<td>0.125</td>
</tr>
<tr>
<td>VRC</td>
<td>0.031–4</td>
<td>1</td>
<td>1</td>
<td>0.16</td>
</tr>
<tr>
<td>POS</td>
<td>0.008–2</td>
<td>1</td>
<td>1</td>
<td>0.0125</td>
</tr>
<tr>
<td>CAS</td>
<td>0.008–5</td>
<td>1</td>
<td>1</td>
<td>0.0125</td>
</tr>
</tbody>
</table>

**GM, geometric mean MIC.** *AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; CAS, caspofungin.*
Table 2. Characteristics of clinical and environmental isolates with decreased drug susceptibility and mutations

<table>
<thead>
<tr>
<th>Number</th>
<th>Isolate name</th>
<th>Source of isolate</th>
<th>City</th>
<th>MIC range (mg l⁻¹)</th>
<th>CYP51A substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ITC VRC POS CAS AMB TR34/L98H</td>
<td>Clinical (bronchoalveolar lavage) Tehran</td>
<td>&gt;16</td>
<td>4 4 0.25 1</td>
<td>- - - - - - -</td>
</tr>
<tr>
<td>2</td>
<td>IFRC 442</td>
<td>Environmental (hospital soil) Ahvaz</td>
<td>&gt;16</td>
<td>0.5 0.25 0.125 0.5</td>
<td>+ - - - - - -</td>
</tr>
<tr>
<td>3</td>
<td>IFRC 443</td>
<td>Environmental (hospital soil) Ahvaz</td>
<td>&gt;16</td>
<td>0.25 0.016 0.25 1</td>
<td>+ - - - - - -</td>
</tr>
<tr>
<td>4</td>
<td>IFRC 444</td>
<td>Clinical (bronchoalveolar lavage) Tehran</td>
<td>&gt;16</td>
<td>4 4 0.25 1</td>
<td>- - - - - - -</td>
</tr>
<tr>
<td>5</td>
<td>IFRC 450</td>
<td>Clinical (bronchoalveolar lavage) Tehran</td>
<td>&gt;16</td>
<td>4 4 0.25 1</td>
<td>- - - - - - -</td>
</tr>
<tr>
<td>6</td>
<td>IFRC 451</td>
<td>Clinical (bronchoalveolar lavage) Tehran</td>
<td>&gt;16</td>
<td>4 4 0.25 1</td>
<td>- - - - - - -</td>
</tr>
<tr>
<td>7</td>
<td>IFRC 452</td>
<td>Clinical (bronchoalveolar lavage) Tehran</td>
<td>&gt;16</td>
<td>4 4 0.25 1</td>
<td>- - - - - - -</td>
</tr>
<tr>
<td>8</td>
<td>IFRC 453</td>
<td>Clinical (bronchoalveolar lavage) Tehran</td>
<td>&gt;16</td>
<td>4 4 0.25 1</td>
<td>- - - - - - -</td>
</tr>
<tr>
<td>9</td>
<td>IFRC 454</td>
<td>Clinical (bronchoalveolar lavage) Tehran</td>
<td>&gt;16</td>
<td>4 4 0.25 1</td>
<td>- - - - - - -</td>
</tr>
<tr>
<td>10</td>
<td>IFRC 455</td>
<td>Clinical (bronchoalveolar lavage) Tehran</td>
<td>&gt;16</td>
<td>4 4 0.25 1</td>
<td>- - - - - - -</td>
</tr>
</tbody>
</table>

IFRC, Invasive Fungi Research Center, Sari, Iran.*AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; CAS, caspofungin.

DNA analysis of CYP51A gene. Conventional PCR assay was carried out to determine the presence of the TR4/L98H mutation in the CYP51A gene of triazole-resistant *A. fumigatus* isolates (MIC > 2 mg l⁻¹) in a total volume of 25 µl, containing 12.5 µl Taq 2× Master Mix Red [0.1 M Tris/HCl, pH 8.5, (NH₄)₂SO₄, 4 mM MgCl₂, 0.2% Tween 20, 0.4 mM deoxynucleotides, 0.2 units µl⁻¹ Taq DNA Polymerase (Ampliqon), inert red dye and stabilizer], 10 pmol AFCYPFF (5′-AATATCGCAGGCACGACCTTC-3′) and AFCYPPR (5′-TGGTATGC TGGAACTACACCTT-3′) primers, 2 µl template DNA and 8.5 µl distilled water.

PCR amplification started with an initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 94°C (60 s), 60°C (30 s) and 72°C (60 s) and a final 10 min extension at 72°C. PCR products were run on 2% agarose gel (Ahmad et al., 2014). Azole-resistant *A. fumigatus* isolates, containing TR4 in the CYP51A promoter region, were expected to yield a 139 bp amplicon, whereas the WT sequence (no tandem repeat) was speculated to yield a 105 bp amplicon (Ahmad et al., 2014).

For isolates with reduced susceptibility to itraconazole and voriconazole (MIC > 2 mg l⁻¹), according to CLSI breakpoints (Verweij et al., 2009a), the whole CYP51A gene was amplified, using three pairs of primers (Chen et al., 2005). The primers used were as follows: CYP1-L (5′-CACCCCTCCCTGTTGTCTCCT-3′), CYP1-R (5′-AGCCCTGAAAGTT-CGGTGAA-3′), CYP2-L (5′-CATGTCGCCACCTTATGGAAGG-3′), CYP2-R (5′-CTTGGCGGTAGTATAGTGTA-3′), CYP3-L (5′-TCCGGCTCCTAGCCTAAG-3′) and CYP3-R (5′-CCTTGGTAAGTTCC TCGATGGT-3′) (Chen et al., 2005). Each fragment was sequenced from both ends. PCR conditions were similar to the conditions of tandem repeat amplification in the CYP51A promoter region. To detect possible mutations in the PCR products, which were assessed via DNA sequence analysis, the sequence of the products was compared with WT *A. fumigatus* CYP51A sequence, using MEGA software version 5 (Tamura et al., 2011).

**Mutation and microsatellite genotypic analysis.** All resistant *A. fumigatus* strains were subjected to mixed-format real-time PCR assay for the detection of mutations responsible for triazole resistance (Khodavaisy et al., 2016). Genotyping of azole-resistant *A. fumigatus* and WT isolates was performed with a panel of nine short tandem repeats (Chowdhary et al., 2012). For phylogenetic analysis, clinical (n=3) and environmental (n=4) *A. fumigatus* isolates (WT) were used. Also, the resistant strains with or without mutations at TR4/L98H were both clinical (n=2) and environmental (n=6) (Fig. 2).

**Statistical analysis.** MIC values were calculated for clinical and environmental samples and the strains were compared. For statistical
Table 1 summarizes the results of in vitro antifungal susceptibility tests, i.e. MIC range, geometric mean MIC, MIC<sub>50</sub> and MIC<sub>90</sub>. Basically, the geometric mean MIC of posaconazole for all clinical and environmental strains was low (0.049 and 0.087 mg l<sup>-1</sup>, respectively); a similar finding was reported for the MIC of caspofungin (0.062 and 0.063 mg l<sup>-1</sup>, respectively). Remarkably, for clinical and environmental strains, the highest MICs in an increasing order were reported in voriconazole (0.085 and 0.241 mg l<sup>-1</sup>, respectively), itraconazole (0.520 and 0.442 mg l<sup>-1</sup>, respectively) and amphotericin B (0.567 and 0.707 mg l<sup>-1</sup>, respectively). Although clinical <i>A. fumigatus</i> isolates were more susceptible to azoles, compared with the environmental isolates, no statistically significant difference was detected (<i>P</i> > 0.05). On the other hand, a significant difference was observed between the strains (<i>P</i> < 0.05). According to in vitro antifungal susceptibility tests, MIC values above the clinical cut-off points against 10 (6.6%) strains, both clinical (4.2%) and environmental (7.6%) strains, were reported in at least one of the antifungal agents (Table 2). Based on the findings, only five (3.3%) strains were cross-resistant to itraconazole, voriconazole and posaconazole. The high MIC distributions of itraconazole and voriconazole were shifted approximately more than two log dilution steps apart.

**DNA analysis of CYP51A gene**

PCR amplification of the TR<sub>34</sub>/L98H mutation in the CYP51A gene of resistant isolates (MIC > 2 mg l<sup>-1</sup> for itraconazole and voriconazole) yielded an amplicon of 139 bp (8/10; 80%), while susceptible isolates (MIC < 2 mg l<sup>-1</sup>) yielded an amplicon of 105 bp (142/150). For confirmation and observation of mutations in the entire CYP51A gene, DNA sequencing was performed for resistant isolates (MIC > 2 mg l<sup>-1</sup>). It was confirmed that all resistant isolates contained a CYP51A mutation. The only mutation found in eight (5.3%) isolates was the TR<sub>34</sub>/L98H (leucine-to-serine) mutation.
histidine substitution). In contrast, the TR<sub>46</sub>/Y121F/T289A mutation was not detected in the CYP51A promoter region (Table 2). Microsatellite analysis of all Iranian itraconazole-resistant <i>A. fumigatus</i> isolates revealed a single TR<sub>34</sub>/L98H genotype. However, resistant isolates were genetically different from their azole-susceptible counterparts (Fig. 2).

**DISCUSSION**

According to the European Centre for Disease Control (ECDC), the overall burden of aspergillosis is estimated at 2.4 million cases each year in European countries. Azoles, specifically voriconazole and itraconazole, are the recommended treatment options for such infections (Kleinkauf, 2013). In recent years, clinical and environmental isolates of triazole-resistant <i>A. fumigatus</i> have been reported in European countries including the Netherlands (38%), the UK (20%), Italy (13%), Turkey (10.2%), France (8%), Denmark (4.5%), Spain (4.2%) and Germany (3.2%). Similarly, the high prevalence of triazole-resistant <i>A. fumigatus</i> has been revealed in other countries such as Australia (1%), China (5.8%), India (1.9%), Iran (3.4%), Taiwan (7.9%), Tanzania (13.8%) and the USA (<1%) (Chowdhary et al., 2014). According to previous research in Iran, the prevalence of clinical and environmental azole-resistant <i>A. fumigatus</i> isolates has been estimated at 3.2% and 3.3%, respectively (Badali et al., 2013; Seyedmousavi et al., 2013). In contrast with the present findings, in previous research, with the increased rate ofazole resistance (4.2% and 7.6% for clinical and environmental <i>A. fumigatus</i> isolates, respectively), a 34 bp tandem repeat (TR<sub>34</sub>) sequence was observed in the CYP51A promoter region in combination with a L98H substitution. The estimated rates were lower than the values reported in European countries and higher than the rates in Asian countries. Recently, van der Linden et al. (2013) described a novel CYP51A-mediated resistance mechanism, consisting of two amino acid substitutions and a 46 bp tandem repeat in the TR<sub>46</sub>/Y121F/T289A gene promoter region. This mechanism could majordy reduce susceptibility to voriconazole (MIC > 16 mg l<sup>-1</sup>), while reducing resistance to itraconazole and posaconazole (MIC: 0.25–0.2 mg l<sup>-1</sup>). They revealed that 20.6% of patients harboured azole-resistant strains due to TR<sub>46</sub>/Y121F/T289A mutations. Moreover, this resistant mechanism has been reported in Belgium (Vermeulen et al., 2012), India (Chowdhary et al., 2013), Denmark (Astvad et al., 2014) and Germany (Fischer et al., 2014). However, in the present study, none of the isolates harboured TR<sub>46</sub>/Y121F/T289A, G54, M220, G138C or G432C mutations. As discussed earlier, the TR<sub>34</sub>/L98H resistance mechanism has been shown to have a vast geographical spread. Such isolates can be found in the environment, as well as azole-susceptible patients (Bader et al., 2013; Chowdhary et al., 2011; Mortensen et al., 2010). Interestingly, individuals with no prior use of azole antifungals may develop infections from the environment through frequent exposure of the fungus to azole fungicides used in agriculture. Lavergne et al. (2015) described the first azole-resistant <i>A. fumigatus</i> with TR<sub>46</sub>/Y121F/T289A mutations in a patient with cystic fibrosis in France where this mutation had not been previously reported. The patient had travelled to the Netherlands and regularly received advertising postal packages from Dutch flower companies. The researchers believed that inhalation and colonization of spores harbouring TR<sub>46</sub>/Y121F/T289A mutations were responsible for the patient’s poor condition. Also, flower packages from the Netherlands and the patient’s surroundings were speculated as the possible causes. In this case, French resistant strains were genetically distinguishable from Dutch isolates; it should be mentioned that the route of acquisition in this patient remained unknown (Lavergne et al., 2015). Notably, Chowdhary et al. (2012) reported an Indian azole-resistant <i>A. fumigatus</i> genotype, which was an extremely adaptive recombinant progeny. The significant phylogenetic incompatibility was consistent with the mating of natural populations of this species in India (Chowdhary et al., 2012); it seems that the spores followed an airborne migration. Medical application of azole antifungals seems to contribute to the widespreadazole resistance in the environment. In addition, shampoos, supplemented with 2% ketoconazole and fluconazole for the treatment of pityriasis versicolor or candidiasis, are unlikely to be effective, as the two compounds have shown negligible activities against <i>Aspergillus</i> species (Bowyer & Denning, 2014).

The present epidemiological study demonstrated that some isolates with TR<sub>34</sub>/L98H mutations were highly resistant to itraconazole, while showing cross-resistance to voriconazole and posaconazole (5 out of 10, 50%). Nevertheless, we found a significant number of isolates for which no mutation in the CYP51A gene could be identified (2 out of 10, 20%). Therefore, other unrelated mechanisms such as increased production of drug target CYP51A protein (Albarrag et al., 2011; Arendrup, et al., 2010; Camps et al., 2012) or overexpression of efflux pumps (Bowyer et al., 2012; Cannon et al., 2009; Manavathu et al., 1999; Slaven et al., 2002) should be considered. In conclusion, although the number of patients affected by azole-resistant <i>A. fumigatus</i> isolates was limited, strict supervision of clinical azole-resistant <i>A. fumigatus</i> isolates and persistent environmental screening of azole resistance are vital to the development of approaches for the management of azole resistance in human pathogenic fungi.

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