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Quantitative Analysis of Single-Nucleotide Polymorphism for Rapid Detection of TR_{34}/L98H- and TR_{46}/Y121F/T289A-Positive *Aspergillus fumigatus* Isolates Obtained from Patients in Iran from 2010 to 2014

Faezeh Mohammadi,[a] Seyed Jamal Hashemi,[a] Jan Zoll,[b] Willem J. G. Melchers,[b] Haleh Rafati,[c] Parvin Dehghan,[d] Sasan Rezaie,[a] Ali Tolooe,[e] Yalda Tamadon,[e] Henrich A. van der Lee,[b] Paul E. Verweij,[b] Seyedmojtaba Seyedmousavi[b,f,g]

Department of Medical Mycology and Parasitology, School of Hygiene & Institute of Public Health Research, Tehran University of Medical Sciences, Tehran, Iran[a]; Department of Medical Microbiology, Radboud University Medical Centre, Nijmegen, the Netherlands[b]; Department of Biochemistry, Erasmus University Medical Center, Rotterdam, the Netherlands[c]; Department of Medical Mycology and Parasitology, Isfahan University of Medical Sciences, Isfahan, Iran[d]; Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran[e]; Invasive Fungi Research Center, Mazandaran University of Medical Sciences, Sari, Iran[f]; Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Center, Rotterdam, the Netherlands[g]

We employed an endpoint genotyping method to update the prevalence rate of positivity for the TR_{34}/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 TR_{46}/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 TR_{34}/L98H mutation in the *cyp51A* genes of six isolates. No isolates harboring the TR_{46}/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 TR_{34}/L98H controls. In conclusion, there was not a significant increase in the prevalence of azole-resistant *A. fumigatus* isolates harboring the TR_{34}/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 TR_{34}/L98H- and TR_{46}/Y121F/T289A-positive isolates and can easily be incorporated into clinical mycology algorithms.

Azole resistance in *Aspergillus fumigatus* is a global and evolving public health threat which translates into treatment failure (1). Surveillance studies indicate that the incidence of azole resistance is increasing (2–6), with the TR_{34}/L98H mutation (a 34-bp tandem repeat mutation in the promoter region of the *cyp51A* gene in combination with a substitution at codon L98) emerging in multiple European countries and in the Middle East, Asia, and Africa and with a new resistance mechanism, the TR_{46}/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), emerging more recently in Europe and India (2–6). We also previously reported the occurrence of the TR_{34}/L98H mutation in 3.2% of clinical *Aspergillus fumigatus* isolates obtained from patients in Iran to the end of 2009 (5).

The trend of increases in the rates of azole resistance among *A. fumigatus* isolates in different regions and patient groups exemplifies the fact that knowledge of the (local) epidemiology of azole-resistant *Aspergillus* diseases is important for clinical mycology/microbiology reference laboratories (7–9). Moreover, rapid and specific molecular methods for the identification of the recently identified azole-resistant *A. fumigatus* strains can significantly influence a timely decision on patient management (10).

In our search for a novel, rapid, sensitive, accurate, and high-throughput method for detection and screening of azole resistance in *A. fumigatus*, we found that endpoint genotyping targeting a single-nucleotide polymorphism (SNP) in the *cyp51A* gene could provide an option. The quantitative analysis of SNPs has been a reliable method in diagnostic microbiology for identification of a single nucleotide in the genomes of humans (11–15), viruses (16–20), and bacteria (18). In this assay, an extension probe can be simply designed to anneal to the template in a posi-

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Address correspondence to Seyedmojtaba Seyedmousavi, S.Seyedmousavi@gmail.com.

TABLE 1 Distribution of azole-resistant and azole-susceptible (wild-type) *A. fumigatus* isolates examined in this study according to year of isolation

Yr of isolation	No. of isolates with each phenotype and resistance mechanism		
	Wild type	Resistant TR_{34}/L98H mutant	Resistant TR_{46}/Y121F/T289A mutant
2010	24	1	
2011	35	1	
2012	37	1	
2013	38	2	
2014	32	1	
Total	166	6	0

tion that places the mutation site immediately adjacent to the 3′ end of the probe, and the use of dideoxynucleoside triphosphates (ddNTPs) allows the extension of only 1 nucleotide from the 3′ end of the probe. Labeling of each ddNTP with a different fluorescent dye allows the differentiation of the genotype at the SNP by the color of the extended probes (11–20).

In the current study, we therefore evaluated the prevalence of TR_{34}/L98H- and TR_{46}/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). All isolates were plated onto a four-well agar plate containing one well each with 4 mg/liter of itraconazole, 1 mg/liter of voriconazole, and 0.5 mg/liter of posaconazole and a growth control well (23). The ability to grow on each well was assessed after 48 h. Any isolate that was able to grow on one of the azole-containing media was further investigated by antifungal susceptibility testing, PCR sequencing of the *cyp51A* gene, and the novel SNP endpoint genotyping technique.

In addition, a collection of wild-type and azole-resistant *A. fumigatus* strains (10 wild-type strains, 8 strains positive for the TR_{34}/L98H mutation, and 6 strains positive for the TR_{46}/Y121F/T289A mutation) were obtained from the culture collection of Radboud University Medical Centre, Nijmegen, the Netherlands. The genomic DNAs of these isolates were used as negative and positive controls for amplification and detection of the L98H and Y121F mutations by the novel quantitative PCR assays that we developed.

***In vitro* antifungal susceptibility testing.** *In vitro* antifungal susceptibility testing was performed using a broth microdilution method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (24). Itraconazole, voriconazole, posaconazole, and amphotericin B were assayed over a 2-fold concentration range of from 16 to 0.016 mg/liter. Visual readings were performed with a reading mirror, and the MIC was defined as the lowest antifungal concentration that inhibited growth by 100% after 48 h compared with the growth of the drug-free well. Susceptibility tests were performed three times with each strain on different days. *Paecilomyces variotii* (ATCC 22319), *Candida parapsilosis* (ATCC 22019), and *Candida krusei* (ATCC 6258) were used for quality control in all experiments. The EUCAST breakpoints and epidemiological cutoff (ECOFF) values were used for the interpretation of the *in vitro* drug susceptibility testing results (25).

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[a]

Azole-resistant *Aspergillus fumigatus* isolate	Underlying disease[b]	MIC (mg/liter)			
		Amphothericin B	Itraconazole	Voriconazole	Posaconazole
T-IR-AF 1002	CPA	0.5	≥16	4.0	0.25
T-IR-AF 1088	CPA	0.5	≥16	2.0	0.5
T-IR-AF 1143	CPA	0.5	≥16	8.0	0.5
T-IR-AF 1416	CPA	0.5	≥16	8.0	0.5
T-IR-AF 1499	ABPA	0.5	≥16	4.0	0.5
T-IR-AF 1521	CPA	0.5	≥16	8.0	0.5

[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.

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

Assay	Primer or probe	Sequence (5′–3′)[a]
cyp51A amplification	Forward primer	GGCGTTCAGGGGAACGAG
cyp51A amplification	Reverse primer	CTTGATGAACTTTTTCTGCTCCATCAG
cyp51A L98 detection	L98 probe	6FAM-AACGGCAAG+C+T+CAAGGATGTC-BBQ
cyp51A L98H detection	L98H probe	Cy5-CAACGGCAAG+C+A+CAAGGATGTCA-BBQ
cyp51A Y121 detection	Y121 probe	LC610-TTGGGACAATC+A+T+ACACCACGTCCG-BBQ
cyp51A Y121F detection	Y121F probe	6HEX-TTGGGACAATC+A+A+ACACCACGTCCG-BBQ

[a] The following dyes were used as 5′ fluorophores: 6-carboxyfluorescein (6FAM), LightCycler Red 610 dye (LC610), cyanine 5 (Cy5), and 3′-quencher BlackBerry quencher (BBQ). +X (where X indicates any nucleotide), an LNA residue.

previously (26–28). To detect mutations, the sequences were compared with the *cyp51A* gene sequence with GenBank accession number AF338659 (29).

Endpoint genotyping. Using a LightCycler 480 real-time PCR system, a novel endpoint genotyping analysis method was evaluated to detect the L98H and Y121F mutations in all of the 172 clinical *A. fumigatus* isolates, as described previously (J. Zoll, S. Seyedmousavi, W. J. Melchers, and P. E. Verweij, submitted for publication). The assay is based on the competition during annealing between probes detecting the wild type and the mutants. The use of locked nucleic acid (LNA) residues at the SNP and adjacent positions increases the discriminative properties of the probes. A fragment of the *A. fumigatus cyp51A* gene covering both the L98 and Y121 codons was amplified in the presence of TaqMan probes detecting L98, L98H, Y121, and Y121F. The primer and probe sequences used in the current study are shown in Table 3.

The real-time PCR was performed using a Roche LC480 instrument II. The PCR mixture formulation was 350 nM either forward or reverse primer, 250 nM the TaqMan probe, and 1 μl sample DNA in the Roche LC480 probe master mix according the manufacturer's protocol. Thermal cycling was performed with an initial decontamination program for 10 min at 40°C, followed by hot-start activation and initial DNA denaturation for 10 min at 94°C. Template DNA was amplified in a two-step cycling program of 45 cycles consisting of denaturation for 10 s at 94°C and annealing and extension for 1 min at 60°C. A series of control samples was analyzed in parallel. Control samples consisted of DNA extracted from *A. fumigatus* with wild-type, TR_{34}-L98H, and TR_{46}-Y121F/T289A *cyp51A*. The *cyp51A* genotype was determined on the basis of the fluorescence ratios of the discriminative probes.

Microsatellite genotyping. Genotyping was performed on all *A. fumigatus* isolates for which the MIC of itraconazole was ≥16 mg/liter, using an *A. fumigatus* short tandem repeat (STR) assay, as described previously (29–31). Briefly, six loci consisting of three trinucleotide repeat fragments (*A. fumigatus* STRs 3A, 3B, and 3C) and three tetranucleotide repeat fragments (*A. fumigatus* STRs 4A, 4B, and 4C) were amplified by using fluorescently labeled primers (29–31). The sizes of the fragments were determined by addition of the GeneScan LIZ500 marker and subsequent analysis of the fragments on an Applied Biosystems 3730 DNA analyzer. Assignment of repeat numbers in each marker was determined from the GeneScan data by using Peak Scanner (version 1.0) software (Applied Biosystems). The sizes of the fragments were determined on the basis of the size of the LIZ500 marker, and the repeat numbers of these isolates were compared to those of a collection of 20 Dutch TR_{34}/L98H-positive isolates. Allele-sharing distance matrices were generated from the tandem repeat numbers and were used as input into the Neighbor program of the PHYLIP (version 3.6) software package to produce dendrograms (32–34).

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 ITS_1, ITS_2, and β-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%.

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 TR_{34}/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 TR_{46}/Y121F/T289A mutation were detected. Sequence analysis of the *cyp51A* gene confirmed the presence of the TR_{34}/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 TR_{34}/L98H control isolates and those previously isolated in Iran between 2003 and 2009 (5).

DISCUSSION

In the present study, we found a 3.5% prevalence of resistance to triazoles resulting from the TR_{34}/L98H mutation in *A. fumigatus* isolates obtained from patients with underlying *Aspergillus* disease in Iran over a recent 5-year period (2010 to 2014). There was not a significant increase in the prevalence of azole-resistant *A. fumigatus* harboring the TR_{34}/L98H mutation. Of note, five out of six azole-resistant isolates were recovered from CPA patients. The significant predilection to CPA is in agreement with the findings of recent studies in Iran which demonstrated that CPA is the most common clinical presentation of aspergillosis in individuals with healed tuberculosis (35). Importantly, patients with CPA require long-term maintenance antifungal therapy to improve symptoms and prevent the recurrence of infection (36, 37).

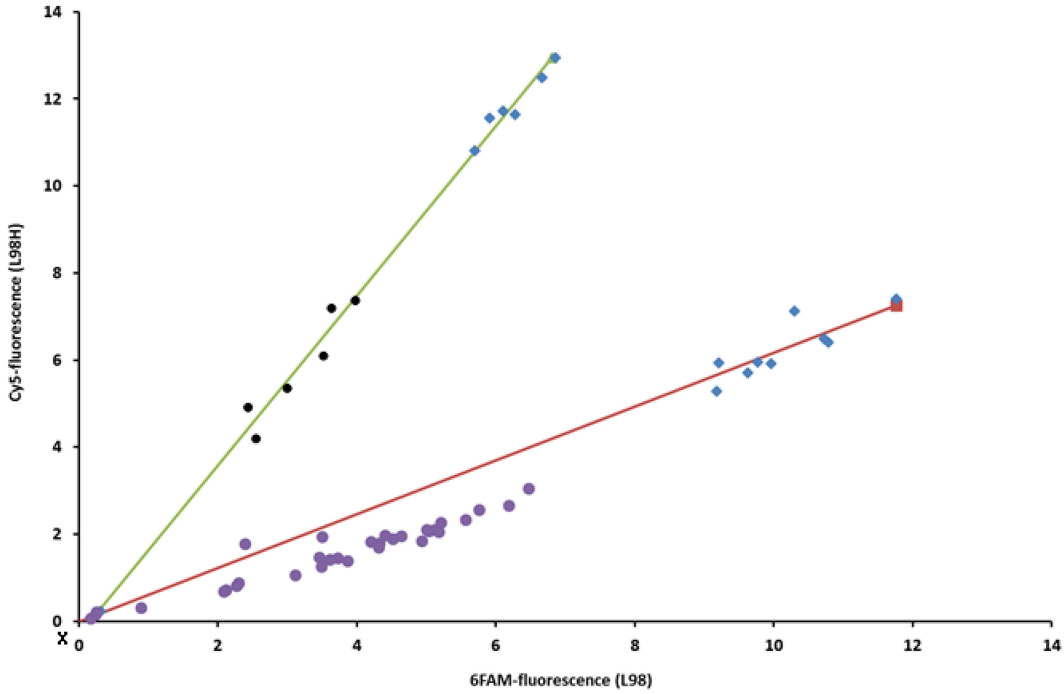

FIG 1 Endpoint fluorescence plot of single-nucleotide variance for detection of the L98H mutation in clinical *Aspergillus fumigatus* isolates using a quantitative PCR assay. Relative L98 (6-carboxyfluorescein [6FAM]) and L98H (cyanine 5 [Cy5]) fluorescence levels are plotted on the *y* and *x* axes, 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.

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 TR_{34}/L98H mutation and the TR_{46}/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 TR_{46}/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 TR_{34}/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-

ical relevance to determine whether *A. fumigatus* isolates have acquired the TR_{34}/L98H and or TR_{46}/Y121F/T289A mutations and can easily be incorporated into clinical mycology algorithms.

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