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Simple, Low-Cost Molecular Assays for TR_{34}/L98H Mutations in the *cyp51A* Gene for Rapid Detection of Triazole-Resistant *Aspergillus fumigatus* Isolates

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Simple, low-cost PCR/PCR-restriction fragment length polymorphism (RFLP) assays targeting *cyp51A* promoter and codon 98 regions were developed for the detection of triazole-resistant *Aspergillus fumigatus* strains carrying TR_{34}/L98H mutations. The assays were evaluated using 40 itraconazole-susceptible isolates and 35 itraconazole-resistant isolates. The prevalence of TR_{34}/L98H mutations in clinical/environmental *A. fumigatus* isolates may now be determined easily from resource-poor settings.

Aspergillus fumigatus is the principal etiological agent of invasive aspergillosis (IA), a life-threatening infection in immunocompromised patients. Treatment options are limited due to the low efficacies of some antifungal drugs against clinical *A. fumigatus* isolates. Oral triazoles (itraconazole, voriconazole, and posaconazole) exhibit excellent activities against *A. fumigatus* isolates *in vitro* and are currently being used as first-line therapies in the management and prophylaxis of IA (1). Ten years ago, acquired triazole resistance among clinical *A. fumigatus* isolates was rare. Clinical failures are now reported frequently, and the frequency of isolation of triazole-resistant clinical *A. fumigatus* isolates has increased in several countries (2–6). Rapid emergence of triazole-resistant *A. fumigatus* isolates has been attributed to the exposure of environmental fungi to 14α-demethylase inhibitors (DMIs) which are structurally and functionally related to clinically licensed triazoles. The DMIs are widely used to control fungal growth for crop plant/ornamental flower protection (7). The occurrence of triazole-resistant *A. fumigatus* strains has been documented in environmental samples from some countries, with isolation frequencies ranging from 5% to 12% (7, 8).

The molecular basis of resistance to triazoles in clinical *A. fumigatus* isolates involves point mutations at several codons in the *cyp51A* gene, which encodes 14α-sterol demethylase. However, a dominant mechanism involving a 34-bp tandem repeat (TR_{34}) in the promoter region together with an L98H substitution (TR_{34}/L98H) in *cyp51A* has been observed in triazole-resistant isolates recovered from environmental sources, treatment-naive subjects, and patients under treatment (6, 9, 10). These studies have largely been carried out at few specialized centers, possibly because these mutations have been detected by sophisticated techniques and expensive instruments typically involving PCR or real-time PCR together with specific probes/molecular beacons or DNA sequencing (6, 10–13). In this report, we describe simple PCR/PCR-restriction fragment length polymorphism (PCR-RFLP) assays for rapid detection of TR_{34}/L98H mutations in the *cyp51A* gene.

The study was approved by the ethical committee of the Faculty of Medicine, Kuwait University.

Reference *A. fumigatus* strains CBS 113.26 (carrying wild-type sequences in the promoter region and codon 98 in *cyp51A* [*cyp51A98*]) and VPCI1042/09 (carrying mutant [TR_{34}/L98H] sequences in the promoter region and codon 98) were used for the establishment of PCR assays. A total of 75 clinical and environmental *A. fumigatus* isolates were used for the evaluation of the developed methods. The background information regarding country of origin, source of isolation, susceptibility to itraconazole (including MICs), and presence or absence of TR_{34}/L98H mutations in the *cyp51A* gene of *A. fumigatus* isolates tested for the performance of the molecular assays is presented in Table 1. The details of clinical/environmental *A. fumigatus* isolates from France, The Netherlands, and India that were used in this study have also been published previously (4, 14, 15). Drug susceptibility testing (DST) of *A. fumigatus* isolates with itraconazole was carried out by Etest as described elsewhere (16). Isolates with reduced susceptibility to itraconazole (MIC of ≥2 μg/ml) were also tested for voriconazole by a broth microdilution (M38-A2) method (4). Isolates with MICs of ≥2 μg/ml were considered resistant (4).

DNA from the isolates was prepared and the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) was amplified with AFUF2 and AFUR2 primers for the identification of *A. fumigatus* isolates as described previously (17). The presence or absence of TR_{34} in the promoter region was determined by PCR amplification by using AFCYPPF (5′-AATAATCGCAGCACCAC TTC-3′) and AFCYPPR (5′-TGGTATGCTGGAACTACACCTT-3′) primers. PCR was carried out in a total volume of 50 μl containing 1× AmpliTaq PCR buffer I, 1 U AmpliTaq DNA polymerase, 4 pmol (each) of AFCYPPF and AFCYPPR primers, 2 μl of DNA, and 0.1 mM each deoxynucleoside triphosphate (dNTP). PCR cycling (total, 35 cycles) included denaturation at 95°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 1 min. An initial denaturation step at 95°C for 5 min and a final extension step at 72°C for 10 min were also included, and the

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TABLE 1 Country of origin, source of isolation, susceptibility to itraconazole with MICs, and presence or absence of TR_{34}/L98H mutations in the *cyp51A* gene of *A. fumigatus* isolates

Serial no.	Isolate no.	Country of origin	Source of isolation	Susceptibility to ITR,[d] MIC (μg/ml)	TR_{34}/L98H mutation
1	E-296	Kuwait	Outdoor air	Susceptible, 0.5	No/no
2	E-149	Kuwait	Outdoor air	Susceptible, 0.38	No/no
3	E-288	Kuwait	Hospital indoor air	Susceptible, 0.75	No/no
4	E-298	Kuwait	Hospital indoor air	Susceptible, 0.38	No/no
5	E-256	Kuwait	Hospital floor swab	Susceptible, 0.75	No/no
6	E-262	Kuwait	Hospital indoor air	Susceptible, 0.38	No/no
7	E-267	Kuwait	Hospital indoor air	Susceptible, 0.38	No/no
8	E-142	Kuwait	Hospital floor swab	Susceptible, 0.38	No/no
9	Kw2287/09	Kuwait	Clinical, sputum	Susceptible, 0.75	No/no
10	Kw3468/10	Kuwait	Clinical, sputum	Susceptible, 0.047	No/no
11	Kw2893/10	Kuwait	Clinical, sputum	Susceptible, 0.75	No/no
12	Kw3068/10	Kuwait	Clinical, sputum	Susceptible, 0.75	No/no
13	Kw1830/08	Kuwait	Clinical, wound swab	Susceptible, 0.5	No/no
14	Kw3916/10	Kuwait	Clinical, sputum	Susceptible, 0.5	No/no
15	E-307	Kuwait	Hospital indoor air	Susceptible, 0.38	No/no
16	Kw2351/11	Kuwait	Clinical, bronchoalveolar lavage specimen	Susceptible, 0.38	No/no
17	Kw3328/10	Kuwait	Clinical, sputum	Susceptible, 0.75	No/no
18	Kw2349/11	Kuwait	Clinical, bronchoalveolar lavage specimen	Susceptible, 0.125	No/no
19	R-1	Kuwait	Outdoor, soil sample	Susceptible, 0.75	No/no
20	R-2	Kuwait	Outdoor, soil sample	Susceptible, 0.38	No/no
21	R-3	Kuwait	Outdoor, soil sample	Susceptible, 0.25	No/no
22	R-4	Kuwait	Outdoor, soil sample	Susceptible, 0.25	No/no
23	R-5	Kuwait	Outdoor, soil sample	Susceptible, 0.75	No/no
24	R-6	Kuwait	Outdoor, soil sample	Susceptible, 0.5	No/no
25	R-7	Kuwait	Outdoor, soil sample	Susceptible, 0.19	No/no
26	R-8	Kuwait	Outdoor, soil sample	Susceptible, 0.38	No/no
27	E-1	Kuwait	Outdoor air	Susceptible, 0.5	No/no
28	E-143	Kuwait	Outdoor air	Susceptible, 0.75	No/no
29	E-290	Kuwait	Outdoor air	Susceptible, 0.38	No/no
30	E-320	Kuwait	Hospital indoor air	Susceptible, 0.38	No/no
31	E-335	Kuwait	Outdoor air	Susceptible, 0.75	No/no
32	E-286	Kuwait	Hospital indoor air	Susceptible, 0.75	No/no
33	E-106	Kuwait	Hospital indoor air	Susceptible, 0.5	No/no
34	Kw1431/10	Kuwait	Clinical, sputum	Susceptible, 0.5	No/no
35	Kw2285/09	Kuwait	Clinical, endotracheal aspirate	Susceptible, 0.19	No/no
36	Kw2941/11	Kuwait	Clinical, sputum	Susceptible, 0.012	No/no
37	Kw3862/10	Kuwait	Clinical, sputum	Susceptible, 0.5	No/no
38	Kw1724/09	Kuwait	Clinical, ear swab	Susceptible, 0.25	No/no
39	Kw1787/10	Kuwait	Clinical, skin swab	Susceptible, 0.75	No/no
40	Kw2881/11	Kuwait	Clinical, bronchoalveolar lavage specimen	Susceptible, 0.38	No/no
41	E-76	Kuwait	Hospital indoor air	Resistant, >16	Yes/yes
42	E-119	Kuwait	Hospital floor swab	Resistant, >16	Yes/yes
43	E-218	Kuwait	Outdoor air	Resistant, >16	Yes/yes
44	E-454	Kuwait	Hospital floor swab	Resistant, >16	Yes/yes
45	R-15	Kuwait	Soil sample	Resistant, >16	Yes/yes
46	R-18	Kuwait	Soil sample	Resistant, >16	Yes/yes
47	R-44	Kuwait	Soil sample	Resistant, >16	Yes/yes
48	10-03-18-79	India	Soil sample	Resistant, >16	Yes/yes
49	10-03-18-83	India	Soil sample	Resistant, >16	Yes/yes
50	10-03-19-75	India	Soil sample	Resistant, >16	Yes/yes
51	10-03-19-73	India	Soil sample	Resistant, >16	Yes/yes
52	10-03-18-81	India	Soil sample	Resistant, >16	Yes/yes
53	10-03-15-27	France	Clinical, sputum	Resistant, >16	No/no[a]
54	10-03-18-82	India	Soil sample	Resistant, >16	Yes/yes

(Continued on following page)

TABLE 1 (Continued)

Serial no.	Isolate no.	Country of origin	Source of isolation	Susceptibility to ITR,[d] MIC (μg/ml)	TR_{34}/L98H mutation
55	10-03-15-38	India	Clinical, sputum	Resistant, >16	Yes/yes
56	10-03-19-72	India	Soil sample	Resistant, >16	Yes/yes
57	10-03-18-80	India	Soil sample	Resistant, >16	Yes/yes
58	10-03-19-74	India	Soil sample	Resistant, >16	Yes/yes
59	10-03-15-19	France	Clinical, sputum	Resistant, >16	No/no[b]
60	10-03-19-70	India	Soil sample	Resistant, >16	Yes/yes
61	10-03-19-71	India	Soil sample	Resistant, >16	Yes/yes
62	10-04-15-05	The Netherlands	Clinical, sputum	Resistant, >16	Yes/yes
63	10-01-02-62	The Netherlands	Clinical, sputum	Resistant, >16	Yes/yes
64	10-01-02-27	The Netherlands	Clinical, sputum	Resistant, >16	Yes/yes
65	10-04-15-16	The Netherlands	Clinical, sputum	Resistant, >16	Yes/yes
66	10-01-13-76	The Netherlands	Clinical, sputum	Resistant, >16	No/no[c]
67	10-01-12-86	The Netherlands	Clinical, sputum	Resistant, >16	Yes/yes
68	10-01-13-15	The Netherlands	Clinical, sputum	Resistant, >16	Yes/yes
69	10-04-15-12	The Netherlands	Clinical, sputum	Resistant, >16	Yes/yes
70	10-04-15-03	The Netherlands	Clinical, sputum	Resistant, >16	Yes/yes
71	10-01-04-26	The Netherlands	Clinical, sputum	Resistant, >16	Yes/yes
72	10-01-04-22	The Netherlands	Clinical, sputum	Resistant, >16	Yes/yes
73	10-03-16-61	India	Soil sample	Resistant, >16	Yes/yes
74	10-01-13-23	The Netherlands	Clinical, sputum	Resistant, >16	Yes/yes
75	10-03-16-63	India	Clinical, sputum	Resistant, >16	Yes/yes

[a] This isolate contained an M220R mutation in *cyp51A*.
[b] This isolate contained a G54E mutation in *cyp51A*.
[c] This isolate contained a G54W mutation in *cyp51A*.
[d] ITR, itraconazole.

amplicons were detected by use of 2% agarose gels (16). *A. fumigatus* isolates containing TR_{34} in the *cyp51A* promoter region should yield an amplicon of 139 bp, while isolates containing the wild-type sequence (no tandem repeat) should yield an amplicon of 105 bp. For the detection of the wild-type sequence or the L98H mutation at *cyp51A98* in *A. fumigatus* isolates, DNA was amplified by using AFCYP98F (5′-CAAGTTCTTCTTTGCGTGCAGA-3′) and AFCYP98R (5′-ATAAGTGGCACATGAGACTCT-3′) primers and the reaction and cycling conditions described above. A portion (5 μl) of PCR products was run on 2% agarose gels. Amplicons in the remaining sample (45 μl) were purified with a PCR product purification kit (Qiagen) according to instructions supplied with the kit. Purified DNA (5 μl) was digested with 5 units of AluI (New England Bio-Labs) in a final volume of 25 μl at 37°C for 5 h, and digested products were separated by 2% agarose gels to generate PCR-restriction fragment length polymorphism (PCR-RFLP) patterns. The 350-bp amplicon from *A. fumigatus* isolates containing the wild-type (L98) sequence (CTC, encoding Leu) in *cyp51A98* should yield three DNA fragments of 189 bp, 90 bp, and 71 bp (with the 189-bp fragment serving as the diagnostic fragment), while isolates containing the L98H mutation (CAC, encoding His) in *cyp51A98* should yield two DNA fragments of 279 bp and 71 bp (with the 279-bp fragment serving as the diagnostic fragment). PCR amplicons from all *A. fumigatus* isolates were also sequenced to confirm the results. Sequencing reactions were carried out and data were analyzed as described previously (16).

In agarose gels, PCR amplification of the promoter region yielded distinct 105-bp and 139-bp amplicons from reference *A. fumigatus* isolates CBS 113.26 (containing the wild-type sequence) and VPCI1042/09 (containing TR_{34} in the promoter region), respectively. Similarly, PCR-RFLP assay of reference *A. fumigatus* isolates CBS 113.26 (containing the wild-type sequence) and VPCI1042/09 (containing the L98H mutation at *cyp51A98*) yielded three (189-bp, 90-bp, and 71-bp) and two (279-bp and 71-bp) DNA fragments, respectively, as expected. Etest data showed that 40 *A. fumigatus* isolates were susceptible to itraconazole, while the remaining 35 isolates were resistant. All itraconazole-resistant isolates were also resistant to voriconazole according to the broth microdilution assay.

PCR amplification of the *cyp51A98* region from all 40 itraconazole-susceptible and 35 itraconazole-resistant *A. fumigatus* isolates yielded an amplicon of ~350 bp (data from 7 isolates are shown in Fig. 1a). When the amplicons were digested with AluI, 32 itraconazole-resistant isolates yielded two (279-bp and 71-bp) fragments (data from 8 isolates are shown in Fig. 1b, lanes 1 to 8), indicating the L98H mutation, while 3 isolates yielded a wild-type pattern in *cyp51A98*. The latter 3 isolates contained a mutation at either codon 54 (two isolates) or codon 220 (one isolate) (Table 1). The PCR-RFLP assay with all 40 itraconazole-susceptible isolates yielded three (189-bp, 90-bp, and 71-bp) fragments (data from 6 isolates are shown in Fig. 1b, lanes 9 to 14) indicating the wild-type sequence in *cyp51A98*. All 32 itraconazole-resistant isolates with the L98H mutation in *cyp51A98* also yielded a 139-bp amplicon (data from 4 isolates are shown in Fig. 2, lanes 1, 2, 5, and 6) for the promoter region, indicating the presence of TR_{34}, while the remaining 3 itraconazole-resistant isolates and all 40 itraconazole-susceptible isolates yielded 105-bp amplicons (data from 4 isolates are shown in Fig. 2, lanes 3, 4, 7, and 8), indicating the absence of the tandem-repeat sequence in the promoter region. The DNA sequencing data confirmed the results of PCR/

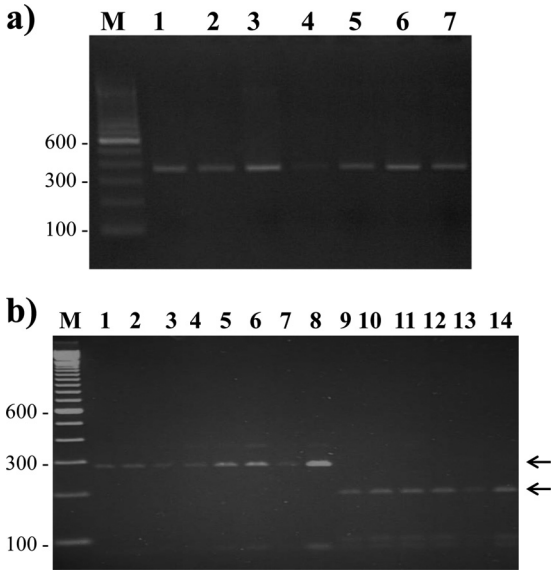

FIG 1 Agarose gels of PCR amplicons with AFCYP98F and AFCYP98R primers from 7 *A. fumigatus* isolates (lanes 1 to 7) (a) and RFLP patterns obtained from 8 triazole-resistant (lanes 1 to 8) and 6 triazole-susceptible (lanes 9 to 14) *A. fumigatus* isolates (b). The positions of migration of diagnostic fragments of 289 bp (for the L98H mutation) and 189 bp (for the wild-type codon at *cyp51A98*) are indicated by arrows. The minor band at 350 bp in some lanes represents the undigested full-length amplicon. In both panels, lane M is a 100-bp DNA ladder, and the positions of migration of 100-bp, 300-bp, and 600-bp fragments are marked.

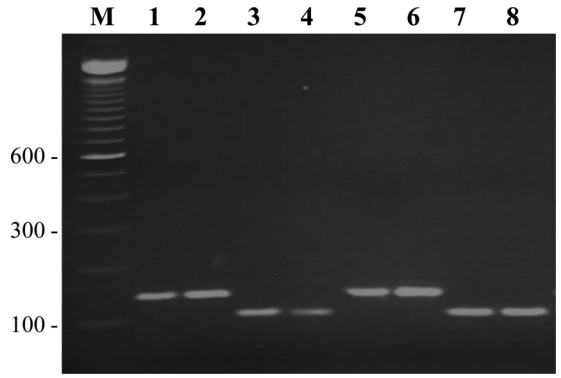

FIG 2 Agarose gel of PCR products obtained with AFCYPPF and AFCYPPR primers from 8 *A. fumigatus* isolates. The amplicon of ~139 bp in lanes 1, 2, 5, and 6 indicates the presence of TR_{34}, while the amplicon of ~105 bp in lanes 3, 4, 7, and 8 indicates the absence of TR_{34} (wild-type sequence) in the promoter region of *cyp51A*. Lane M is a 100-bp DNA ladder, and the positions of migration of 100-bp, 300-bp, and 600-bp fragments are marked.

PCR-RFLP assays for all the isolates analyzed in this study. None of the *A. fumigatus* isolates lacking TR_{34}/L98H mutations yielded false-positive results by PCR/PCR-RFLP assays. It is important to emphasize here that the PCR-RFLP assay described in this study is designed for the detection of only the L98H mutation within the *cyp51A* gene. Since other *cyp51A* mutations are known to occur in triazole-resistant *A. fumigatus* isolates, the lack of detection of the L98H mutation by the PCR-RFLP assay does not rule out triazole resistance (4, 6, 18). Thus, there is a possibility of some triazole-resistant *A. fumigatus* isolates yielding false-negative results by exhibiting wild-type patterns for the promoter region and codon 98 of the *cyp51A* gene.

Previously, the L98H mutation has been detected by other rapid molecular techniques, such as two (L98-specific and L98H-specific) PCR assays (10), PCR amplification of the *cyp51A98* region followed by DNA sequencing (6, 13, 18), real-time PCR with molecular beacons (12), and melting-curve analysis of specific probe primers with real-time PCR amplicons (11). TR_{34} has also been detected by a PCR or nested PCR assay followed by DNA sequencing (6, 13, 18), real-time PCR with molecular beacons (12), and melting-curve analysis of PCR amplicons (11). Most of these methods either are technically demanding or require expensive and sophisticated instruments/probes. A simple PCR-agarose gel assay similar to our protocol has been described previously (10); it generated amplicons of 188 bp and 222 bp from *A. fumigatus* isolates containing wild-type and TR_{34} sequences in the promoter region, respectively. However, the amplicons of 105 bp and 139 bp obtained in this study resolve better in 2% agarose gels than the amplicons of 222 bp and 188 bp, since the distance traveled by a DNA fragment in agarose gels is inversely proportional to the log of its molecular weight (19).

The PCR-based methods developed in this study for rapid identification of TR_{34}/L98H mutations are simple to perform, use basic PCR and gel electrophoresis equipment that is readily available in most mycology laboratories, can be completed within 1 to 2 days, and cost ~US$5 per sample (excluding the cost of culture and personnel time). The methods will help in determining the prevalence of TR_{34}/L98H mutations in the *cyp51A* gene in clinical and environmental *A. fumigatus* isolates in resource-poor settings. The application of these methods will also help in identifying another mechanism (TR_{46}/Y121F/T289A mutations) conferring resistance to triazoles in clinical and environmental *A. fumigatus* isolates described recently (20). This is because the L98H mutation without the TR_{34} mutation has not been described previously. Thus, the detection of *A. fumigatus* isolates containing a tandem repeat in the promoter region but lacking the L98H mutation would indicate the presence of Y121F/T289A in the *cyp51A* gene, which could be subsequently confirmed by other methods (20). Furthermore, the simple molecular assays developed in this study may also help in the rapid identification of TR_{34}/L98H mutations in the *cyp51A* gene among clinical *A. fumigatus* isolates for proper management of patients with IA in developing countries. However, it should be emphasized here that the simple molecular assays described in this study are designed only for the detection of TR_{34}/L98H mutations, and triazole-resistant *A. fumigatus* isolates harboring other mutations in the *cyp51A* gene will yield false-negative results.

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