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Aspergillus fumigatus Strains with Mutations in the *cyp51A* Gene Do Not Always Show Phenotypic Resistance to Itraconazole, Voriconazole, or Posaconazole▽

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We performed molecular identification of 98 *Aspergillus fumigatus* complex isolates with MICs of 1 to 4 μg/ml for itraconazole and searched for the presence of mutations in *cyp51A*. Most of the isolates (91%) belonged to *A. fumigatus* sensu stricto. We found 14 different mutations in nine isolates at codons different from G54, M220, G138, G448, and L98. We report new mutations at positions 165, 262, 479, and 497 (silent). The role of these mutations should be analyzed.

Most *Aspergillus fumigatus* strains are susceptible to azoles (8, 14–16, 21). The primary mechanism of azole resistance involves specific point mutations in the *cyp51A* gene at codons G54, M220, G138, and G448 and at codon L98, in which it is combined with a tandem duplication of a 34-bp sequence in the promoter (2, 7, 11, 13, 17, 19, 21).

The *cyp51A* gene sequence has been studied mainly in strains classified as resistant (MIC, ≥4 μg/ml) or susceptible (MIC, <1 μg/ml) to itraconazole (17–19, 22). We previously reported the antifungal susceptibility to azoles of 601 *A. fumigatus* complex isolates collected from 1999 to 2007 (9, 10). To investigate the presence of mutations in the *cyp51A* gene, we performed molecular identification of 98 (16%) of these isolates that had an MIC of 1 to 4 μg/ml for itraconazole.

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The isolates used were collected from air or from samples from 42 patients (one isolate per patient). According to the European Organization for Research and Treatment of Cancer criteria, 10 of the patients had invasive aspergillosis (5) (Table 1). The antifungal drugs used were itraconazole (Janssen Pharmaceutical, Madrid, Spain), posaconazole (Merck Research Laboratories, Rahway, NJ), and voriconazole (Pfizer Pharmaceutical Group, New York, NY). Antifungal susceptibility was obtained according to the CLSI M38-A2 procedure (4). For each drug-strain combination, the MIC was obtained in 3 to 6 independent experiments; for analysis, the modal MIC was chosen. All isolates were classified according to the proposed breakpoints and epidemiological cutoff values (ECVs) (Table 2) (21).

Internal transcribed spacer (ITS) regions 1 and 2 and the β-tubulin gene were amplified and sequenced (1, 20, 23). A BLAST search of all of the sequences was performed to determine whether the sequences matched those of *A. fumigatus* sensu stricto (here, *A. fumigatus*) and to rule out cryptic species. We amplified and sequenced both strands of the *cyp51A* gene and promoter region of those isolates identified as *A. fumigatus* (3, 12). Each strain showing mutations in the *cyp51A* sequence was fully analyzed twice independently. A panel of nine short tandem repeats for exact and high-resolution fingerprinting of *A. fumigatus* isolates (STR*Af*) was used to study the genetic relationship between the *A. fumigatus* isolates harboring mutations (6). In patients with isolates harboring mutations, all available isolates were revived and studied to search for additional mutations and perform genotyping. Double-stranded DNA sequencing of the PCR products (ITS regions and β-tubulin and *cyp51A* genes) and determination of the sizes of the fragments were done with a 3130xl analyzer (Applied Biosystems, Inc.).

Molecular identification and antifungal susceptibility. Of the 98 *A. fumigatus* complex isolates, 89 (91%) were *A. fumigatus* (42 from clinical samples and 47 from environmental samples). All of the strains we isolated from clinical samples belonged to *A. fumigatus*. The remaining isolates were identified as *Neosartorya fisheri* ($n = 6$), *Neosartorya udagawae* ($n = 2$), and *Aspergillus fumigatiaffinis* ($n = 1$).

The antifungal susceptibility of the isolates is shown in Table 2. Although few isolates belonged to the cryptic species, the MIC_{90} of the three azoles was 1-fold dilution greater than the MIC_{90} found for the *A. fumigatus* isolates. Most *A. fumigatus* isolates were susceptible to itraconazole (82%) or to voriconazole (97.8%). In contrast, most isolates were intermediate to posaconazole (87.6%), and the remaining isolates were resistant. Our results suggest that the prevalence of azole-resistant *A. fumigatus* isolates in Spain is still low. However, we did find that 12.4% of the 89 isolates were resistant to posaconazole. While this percentage is clearly higher than those reported by other authors (8, 14, 16), it may be due to the selection of isolates; in fact, posaconazole-resistant isolates represented less than 2% of the isolates studied in our previous reports (9, 10).

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TABLE 1. Descriptions of the 10 patients with proven or probable invasive aspergillosis showing the site involved, underlying condition, antifungal treatment received, and outcome

Yr	Clinical form	Underlying condition	Diagnosis	Antifungal treatment	Outcome	Patient with mutant isolates[b]
2001	Pericarditis and adenopathies	HIV	Proven	Amphotericin B	Poor	D
2001	Pulmonary	Hematological cancer	Probable	Amphotericin B	Poor	E
2002	Postsurgical	Surgery	Probable	Itraconazole	Survival	
2002	Pulmonary	COPD[a]	Probable	Amphotericin B	Poor	
2003	Postsurgical	Surgery	Probable	Itraconazole	Survival	
2003	Pulmonary	COPD	Probable	Caspofungin	Poor	
2006	Postsurgical mediastinitis	Surgery	Proven	Voriconazole + caspofungin	Poor	B
2006	Pulmonary	COPD	Probable	Voriconazole	Survival	
2007	Pulmonary	COPD	Probable	Voriconazole	Survival	
2007	Pulmonary	COPD	Probable	Voriconazole	Survival	

[a] COPD, chronic obstructive pulmonary disease.
[b] Patients infected with isolates harboring mutations in the *cyp51A* gene are identified by an alphabetical code.

Description of mutations in *cyp51A* and genotyping. In only nine (10%) of the 89 *A. fumigatus* isolates studied, at least one mutation in the *cyp51A* gene was found. Mutant strains were isolated from the environment ($n = 4$) or from the samples of five patients (designated A to E) (Table 3). Patients B, D, and E had invasive aspergillosis and died, whereas the isolation of *A. fumigatus* in patients A and C was considered nonsignificant (Table 1).

We identified 14 different nucleotide substitutions involving or not involving amino acid changes ($n = 9$). Although most of these substitutions have been reported elsewhere (11, 16, 18), we also found new mutations at positions 165, 262, and 479 and a silent substitution at position 497 (Table 3). The presence of these mutations was not associated with itraconazole resistance phenotypes as previously described (11, 16, 18).

Interestingly, the two strains from patients D and E had a combination of 8 different mutations at the same positions and were both isolated in 2001. Hypothetically, both strains could belong to the same genotype. However, the nine mutant isolates were found to be genetically unique (Table 3). Isolates showing the same combination of these eight mutations have recently been reported in the United Kingdom and Netherlands (11, 18). These results suggest that the presence of mutations is independent of the genotype and may be common, thus demonstrating some degree of polymorphism in the gene.

The significance of the mutations with changes in amino acids found in the present study is unknown. Overall, the clinical isolates had a higher number of point mutations (ranging from 1 to 8) than the environmental isolates. To discern whether the mutations in *cyp51A* are a consequence of the adaptation of *A. fumigatus* during colonization or infection, we revived and studied another 18 available strains from patients B, C, and D. We did not find isolates with different mutation profiles or a mixture of genotypes in patients C and D. In patient B, however, 10 of the 11 isolates had the same mutation profile and belonged to the same genotype (Table 3). The remaining isolate presented two additional mutations (F46Y and E427K) and belonged to a genotype related to the predominant genotype (variations only in 3A [28 no. of repeats] and 3B [14 no. of repeats]). Interestingly, this genotype was isolated from the sternum biopsy specimen, whereas the predominant genotype was isolated from the mediastinal wound.

We conclude that 10% of *A. fumigatus* sensu stricto isolates can harbor one or more mutations in *cyp51A*, although they do

TABLE 2. Cumulative percentages of MICs of azoles against the 98 *A. fumigatus* complex isolates obtained by the CLSI M-38A2 procedure; percentages of strains classified as intermediate or resistant to azoles according to recently proposed breakpoints, and percentages of strains with MICs above the ECVs

Organism(s) (no. of strains) and antibiotic	% of strains within MIC (μg/ml) of:					% of strains within each susceptibility category[a]		
	0.25	0.5	1	2	4	Intermediate	Resistant	MICs > ECV[b]
A. fumigatus sensu stricto ($n = 89$)[c]								
Itraconazole			82	**100**[d]		18	0	18
Voriconazole	1.1	25.8	**97.8**	100		2.2	0	2.2
Posaconazole		87.6	**100**			87.6	12.4	12.4
Other cryptic species ($n = 9$)[e]								
Itraconazole			11.1	77.8	**100**			
Voriconazole		11.1		**100**				
Posaconazole			88.9	**100**				

[a] In terms of antifungal susceptibility, isolates were classified according to the breakpoints recently proposed by Verweij et al. (21) for itraconazole and voriconazole (<2 μg/ml, susceptible; 2 μg/ml, intermediate; >2 μg/ml, resistant) and posaconazole (<0.5 μg/ml, susceptible; 0.5 μg/ml, intermediate; >0.5 μg/ml, resistant).
[b] Percentages of isolates with MICs above the ECVs proposed by Espinel-Ingroff et al. and Rodríguez-Tudela et al. (8, 16): itraconazole and voriconazole, ≤1 μg/ml; posaconazole, ≤0.5 μg/ml.
[c] In isolates belonging to *A. fumigatus* sensu stricto, the MIC for each antifungal-strain combination was obtained in 3 to 6 independent experiments.
[d] Values in bold are MIC_{90}s.
[e] For isolates belonging to species of the *A. fumigatus* complex other than *A. fumigatus*, the MIC for each antifungal-strain combination was calculated only once.

TABLE 3. Description of the mutations found in the *cyp51A* gene of the 9 *A. fumigatus* isolates and allelic composition of the strains by STR*Af*

Isolate with mutation(s) and source (no. of isolates)	Mutation(s) in *cyp51A*		Antifungal susceptibility[a]			STR*Af*[b]								
	Change in amino acid composition	Silent	ITC	VRC	POSA	2A	2B	2C	3A	3B	3C	4A	4B	4C
1, outdoor air		D245D	1	1	1	13	20	10	10	10	10	8	9	20
2, outdoor air	**N479D**[c]		1	1	1	22	22	24	53	11	27	15.3	10	8
3, outdoor air	**F165L**		1	1	1	22	30	9	37	9	7	18	9	11
4, outdoor air	N248K		1	0.5	0.5	18	24	21	31	11	19	26.3	10	5
5, patient A	**D262Y**		1	0.25	0.5	18	20	9	10	10	11	8	10	5
6, patient B (10)[d]	M172V	G89G, L358L, C454C, **G497G**	2	1	1	10	14	14	22	17	5	7	5	5
7, patient C (5)[d]	F46Y, M172V, E427K	G89G, L358L, C454C, **G497G**	1	1	1	10	16	11	33	14	9	7	5	5
8, patient D (3)[d]	F46Y, M172V, N248T, D255E, E427K	G89G, L358L, C454C	2	2	1	21	16	16	42	19	10	12	10	10
9, patient E	F46Y, M172V, N248T, D255E, E427K	G89G, L358L, C454C	2	2	1	15	21	17	64	13	16	15.3	5	5

[a] ITC, itraconazole; VRC, voriconazole; POSA, posaconazole. Units are micrograms per milliliter.
[b] Allelic composition of the nine loci for the nine strains harboring mutations in the *cyp51A* sequence.
[c] Newly described mutations are in bold.
[d] Additional isolates that were revived and further analyzed for *cyp51A* sequencing, STR*Af* genotyping, and antifungal susceptibility testing.

not confer resistance to azoles. Further studies should focus on the relationship between these mutations and other processes such as adaptation to tissue or even the acquisition of new mutations conferring resistance.

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