

This is the peer reviewed version of the following article:

A Point Mutation in the 14 $\alpha$ -Sterol Demethylase Gene *cyp51A* Contributes to Itraconazole Resistance in *Aspergillus fumigatus*.

T. M. Diaz-Guerra, E. Mellado, M. Cuenca-Estrella, and J. L. Rodriguez-Tudela

*Antimicrob Agents Chemother.* 2003 Mar;47(3):1120-4.

which has been published in final form at

<https://doi.org/10.1128/AAC.47.3.1120-1124.2003>

**A point mutation in the 14 $\alpha$ -sterol demethylase gene *cyp51A* contributes to the resistance to itraconazole of *Aspergillus fumigatus*.**

**T. M. Diaz-Guerra , E. Mellado\*, M. Cuenca-Estrella, and J.L. Rodriguez-Tudela.**

Unidad de Micología. Centro Nacional de Microbiología. Instituto de Salud Carlos III.  
Majadahonda, Madrid, Spain.

Key words: *Aspergillus fumigatus*, resistance mechanisms, itraconazole, Cyp51.

Running title: Mutated *cyp51A* from *A. fumigatus* resistant to itraconazole.

\*Corresponding address: Dr. E. Mellado. Unidad de Micología. Centro Nacional de  
Microbiología. Instituto de Salud Carlos III.

Carretera Majadahonda-Pozuelo Km2. (28220) Madrid (Spain).

e-mail: [emellado@isciii.es](mailto:emellado@isciii.es) Telephone: -34-91-5097961 and Fax: -34-91-5097966.

Dr. T.M. Díaz-Guerra. Unidad de Micología. Centro Nacional de Microbiología. Instituto de  
Salud Carlos III. Carretera Majadahonda-Pozuelo Km2. (28220) Madrid (Spain).

e-mail [tmartinez@isciii.es](mailto:tmartinez@isciii.es) Phone: -34-91-5097961. Fax: -34-91-5097966.

Dr. Manuel Cuenca-Estrella. Unidad de Micología. Centro Nacional de Microbiología. Instituto  
de Salud Carlos III. Carretera Majadahonda-Pozuelo Km2. (28220) Madrid (Spain).

e-mail [mcuenca-estrella@isciii.es](mailto:mcuenca-estrella@isciii.es) Phone: -34-91-5097961. Fax: -34-91-5097966.

Dr. Juan L. Rodríguez-Tudela. Unidad de Micología. Centro Nacional de Microbiología. Instituto  
de Salud Carlos III. Carretera Majadahonda-Pozuelo Km2. (28220) Madrid (Spain).

e-mail [juanl.rodriquez-tudela@isciii.es](mailto:juanl.rodriquez-tudela@isciii.es) Phone: -34-91-5097961. Fax: -34-91-5097966.

## **ABSTRACT**

The genes encoding 14 $\alpha$ -sterol demethylases (*cyp51A* and *cyp51B*) were analysed in twelve ITC-resistant and three ITC-susceptible clinical isolates of *Aspergillus fumigatus*. Six ITC-resistant strains exhibited a substitution of glycine 54 per another amino acid, located at a very conserved region of the Cyp51A protein. The *cyp51A* gene from the *A. fumigatus* wild type (CM-237) strain was replaced for the mutated *cyp51A* gene copy of one ITC-resistant strain (AF-72). Two transformants exhibited resistance to itraconazole and both had incorporated the *cyp51A* gene mutated copy.

*Aspergillus* infections are an important cause of mortality and morbidity in the immunocompromised host, and *Aspergillus fumigatus* is one of the most prevalent airborne fungal pathogens causing infection worldwide (13). *A. fumigatus* is intrinsically resistant to fluconazole, unlike itraconazole, which has been shown to have good *in vitro* and *in vivo* activity against this species (3). However, a number of *A. fumigatus* isolates with *in vitro* itraconazole resistance have been described (1, 6, 9). In some instances, the resistance detected *in vitro* has been confirmed *in vivo* with animal models (5, 10).

The azole-derived antifungal agents inhibit the ergosterol biosynthesis pathway via the inhibition of 14 $\alpha$ -demethylase, the enzyme that removes the methyl group at position C-14 of precursor sterols. The emergence of resistance to azoles in yeast has accelerated studies of the mechanisms implicated in this resistance, and several alterations of the gene encoding the 14 $\alpha$ -demethylase (*ERG11/cyp51*) had been reported in fluconazole-resistant clinical isolates of *Candida albicans* (12, 14, 16, 21, 24). The mechanisms of resistance to azoles have been studied at some extent for phytopathogenic fungi, but the information about human pathogenic filamentous fungi is very meager. The occurrence of a phenylalanine residue at the position 136 of the *cyp51* sequence instead of a tyrosine residue has been reported for field isolates of *Uncinula necator* (8) and *Erysiphe graminis* (7). In these fungi the Y136F mutation is related to resistance to demethylation inhibitors (DMIs). To date, two molecular mechanisms of resistance to itraconazole has been proposed in *A. fumigatus*: one is the reduced intracellular accumulation of itraconazole, due to an efflux pump (9, 25) and also, a reduced penetration of the drug has been suggested (15). The other one is related to a possible modification of the target enzyme 14 $\alpha$ -sterol demethylase or its overexpression (9, 20).

Recently, we have described the existence of two genes coding two different 14 $\alpha$ -demethylases in *A. fumigatus*, *cyp51A* and *cyp51B*. Also, it has been shown that both genes are expressed (17). The *cyp51A* sequence did exactly match with one previously described sequence of *A. fumigatus* (11). The objective of this work was to compare the sequences of these two genes in itraconazole-susceptible and itraconazole-resistant isolates of *A. fumigatus* looking for specific residues that could account, at least in part, for the resistance of *A. fumigatus* to itraconazole.

A total of fifteen clinical strains of *A. fumigatus* were selected on the basis of their susceptibilities to itraconazole (ITC): i) resistant isolates (R) AF-72, AF-90, AF-91, AF-1422, F/6929, F/7075, Br130, Br181, AF-1237, CM-796, CM1910 and SO/3829 had minimal inhibitory concentrations (MICs) to ITC > 8.0 µg/ml (4, 9, 18). ii) susceptible isolates (S) AF-1119, CM-1369, CM-237 had ITC MICs of 0.5 µg/ml. AF-1119 was isolated four months before AF-1237 and before itraconazole treatment (4). Isolates CM-1369 and CM-796 were obtained from two BAL samples, separated eleven months, from one HIV-positive patient. Strain CM-1910 was obtained from the sputum of a patient. *A. fumigatus* CM-237 was used as control strain. This isolate was utilized for describing the sequence of the genes *cyp51A* and *cyp51B* (17). *Paecilomyces variotii* ATCC22319 and *A. fumigatus* ATCC9197 were used as quality control strains for susceptibility testing.

**Antifungal susceptibility testing.** Despite most of the strains had been previously checked for ITC susceptibility, antifungal susceptibility testing was repeated. A broth microdilution test was performed by following the NCCLS reference method (19), with minor modifications (3). Itraconazole (ITC) (Janssen Pharmaceutica, Madrid, Spain), voriconazole (VCZ) (Pfizer S.A., Madrid, Spain), fluconazole (FCZ) (Pfizer S.A., Madrid, Spain), and ketoconazole (KTZ) (Janssen Pharmaceutica, Madrid, Spain) were obtained as standard powder from their respective manufacturers. Drugs were dissolved in dimethyl sulfoxide (DMSO) (Sigma, Madrid, Spain) to obtain stock solutions of 1600 µg/ml that were conserved at -70°C. For ITC, VCZ and KTZ the final concentration range assayed was 8.0-0.015 µg/ml. FCZ was assayed using concentration ranges from 6,400-0.03 µg/ml. Visual readings were performed with a microtiter reading mirror. MICs were defined as the lowest concentration of the antifungal agent that completely inhibits fungal growth at 48 hours of incubation at 35°C. Susceptibility tests were performed at least twice with each strain at different days.

Three isolates, CM-1369, AF-1119 and CM-237 had ITC MICs of ≤ 0.5 µg/ml. The rest of the isolates was confirmed to be resistant in vitro to ITC, with MICs values > 8.0 µg/ml (Table 1). Although there are no defined breakpoints for ITC, we considered that those isolates with ITC MICs ≤ 0.5 µg/ml were susceptible and the strains with ITC MICs > 8.0 µg/ml were resistant. This putative breakpoints were chosen on the basis of previous works that correlated an ITC

MIC of higher than 8 µg/ml with the lack of clinical response to the treatment with ITC and the lack of response in animal models (5, 10). MICs values for other azole drugs are shown in Table 1.

**PCR-amplification and sequencing of *A. fumigatus* *cyp51A* and *cyp51B* genes** Conidia from each strain were inoculated into 3 ml GYEP broth (2% glucose, 0.3% yeast extract, 1% peptone) and they were grown overnight at 37°C. Mycelia mats were recovered and subject to a DNA extraction protocol described previously (26). The full coding sequences of *cyp51A* and *cyp51B* were amplified by using specific primer sets: P450-A1 (5'-ATGGTGCCGATGCTATGG-3') and P450-A2 (5'-CTGTC-TCACTTGGATGTG-3') for the *cyp51A* gene and P450-B1 (5'-ATGGGTCTCATCGCGTTC-3') and P450-B2 (5'-TCAGGCTTTGGTAGCGG-3') for the gene *cyp51B*. The amplifications were performed in a 50 µl volume as previously described (17). Negative controls including all PCR constituents but without DNA were included in each amplification run. The PCR products were analyzed by electrophoresis on agarose gels and stained with ethidium bromide. Sequence analysis of *cyp51A* and *cyp51B* genes showed some point mutations. To verify that these point mutations were not due to errors in the PCR amplification of the genes, the two genes *cyp51A* and *cyp51B* from all isolates were newly amplified and sequenced a second time. Exactly the same point mutations were found again, indicating that they were not artificially introduced during the PCR.

The target of the azole antifungals is the 14 $\alpha$ -demethylase, and alterations in the sequence of the gene encoding this enzyme (*ERG11/cyp51*) has been demonstrated in *C. albicans* resistant to azoles (12, 14, 16, 21, 24) and in some phytopatogenic fungi resistant to DMIs (7, 8). Thus, in this work, alteration of the *cyp51* genes of *A. fumigatus* is proposed as one of several potential mechanisms for resistance to ITC. The detection of amino acids substitutions in the Cyp51A and/or Cyp51B proteins of those isolates with phenotypic resistance to ITC when compared with the same proteins of ITC-susceptible strains would allow to associate the specific alteration with resistance to ITC. PCR amplification and sequence analysis of *cyp51A* genes showed some point mutations: six ITC-resistant strains presented a single base change (CM-796, AF-72, AF-1237, F/7075, Br130 and Br181) (Table 1). These single base mutations lead to changes at triplet encoding amino acid 54 (glycine). This residue is located at a very conserved region of

the Cyp51 proteins. These mutations were the change of G to T at position 161 (g161t) (in CM-796, and Br181 strains), the change of g161a (in AF-72, F/7075 and Br130 strains) and g160a in the AF-1237 strain. In addition, five other strains (AF-90, AF-1, AF-1422, F/6929 and SO/3829) showed a different mutation which yielded an amino acid substitution of methionine (M) to valine (V), lysine (K) or threonine (T) at codon 236 (Table 1). This methionine 236 is not a conserved amino acid between different yeast and moulds Cyp51s. Regarding *cyp51B* gene, five additional point mutations were observed in the *cyp51B* sequence of some of the ITC-resistant strains (Table 1). Only two of these changes lead to amino acid substitutions: in AF-72 and Br130 strains the a125t change lead to amino acid substitution of glutamine (Q) to leucine (L) at codon 42. (these two strains harboured another mutation c664t which is not responsible for an amino acid substitution). One strain (SO/3829) had a t1264a change at codon 387 that would be responsible for aspartic acid (D) to glutamic acid (E) substitution. Three ITC-resistant isolates, Br-181, F/6919 and AF-1237, did not show any change in their sequence of *cyp51B* gene with respect to the reference strain CM-237. The strains CM-796, AF-90, AF-91, CM-1910 and AF-1422 showed a base change at the position 105 (t105c) in the *cyp51B* sequences when compared to the reference stain (CM-237), but this change was not associated with an amino acid alteration. In total, four different point mutations were detected among twelve ITC-resistant isolates that yield amino acid substitutions. The strain AF-1237, which harboured the amino acid substitution G54R at Cyp51A, it was matched with the initially ITC-susceptible strain AF-1119. Both were obtained from the same patient and they were shown to have identical DNA patterns by different molecular typing methods (4). These two strains only differed in the amino acid at the position 54 of Cyp51A sequence. These findings demonstrate that the mutation at glycine 54 is responsible, at least in part, of the ITC-resistance of strain AF-1237. The fact that another five ITC-resistant strains, harbored a mutation at the same position of Cyp51A suggests that there is a high correlation of this specific substitution with resistance to ITC. In addition, fragments containing the area with the mutation (160/161 position) were PCR amplified (using primer A7 and A5, see below) and sequenced from 22 *A. fumigatus* ITC- susceptible strains from our mould collection of clinical strains. All *A. fumigatus* ITC-S strains have the triplet ggg at 160-162 position (G54).

Regarding the other two mutations resulting in amino acid substitution, previous work, has suggested an altered membrane transporter to be the mechanism of resistance operating in the strain AF-72, as a reduced intracellular concentration of azoles was found in this strain (9). Strain AF-72 harbored one mutation in each of the two 14 $\alpha$ -sterol demethylases, the substitutions G54E in Cyp51A and Q42L in Cyp51B. The mutation Q42L detected in this strain was only present in another resistant strain. This fact together with a G54E mutation from AF-72 resulting in ITC-resistance in the wild type CM-237 recipient strain (which does not bear the Q42L mutation), suggests that there is not a direct involvement in of that amino acid change (Q42L) in the resistance of *A. fumigatus* to ITC. On the other hand, the resistance to ITC of strains AF-90 and AF-91 was previously reported to be mediated by either increased expression of the 14 $\alpha$ -sterol demethylase, or altered enzyme affinity for azoles (9). Both strains were from the same patient and they are resistant to ITC. We found the mutation a729t leading to M236V substitution in a variable region of the Cyp51A of strains: AF-91, AF-90, AF1422, F/6919 and SO/3829. Moreover, M236V and Q42L substitutions are both situated in protein areas which are not conserved in other Cyp51 enzymes. It seems therefore unlikely that neither M236V, nor Q42L would be related to *A. fumigatus* itraconazole resistance. Nevertheless, the appearance of M236 substitution in five strains with similar pattern of azole drugs resistance deserves consideration. Sequencing of *Cyp51A* and *Cyp51B* genes of more ITC-resistant strains is needed before further conclusions can be drawn.

**Replacement of the 237 wild type *cyp51A* gene for the mutated AF-72 *cyp51A* gene copy by DNA-mediated transformation using electroporation.** The *cyp51A* gene from AF-72 strain was PCR amplified as previously described and introduced into the 237 wild type strain. Transformations of *A. fumigatus* 237 strain was achieved by electroporation using a protocol previously described for *A. nidulans* (23) and that it was adapted for *A. fumigatus* (27). In the last step the protocol was slightly modified as follows: After electroporation and incubation at 30°C for 90 min on a rotary shaker, transformation mixtures were cultured on 20 ml minimal medium (2) agar plates containing 1% glucose and 5mM ammonium tartrate and incubated overnight at room temperature. The day after, 10 ml of melted minimal medium (0.6 % agarose) containing 200  $\mu$ g of ITC, were poured plated over the medium to make a final concentration of

8 µg/ml. The plates were incubated at 37°C for a week. Itraconazole resistant transformants were appearing at variable times ranging from 2–7 days. Mutants were named by a letter (T) followed by a number. DNA from ITC-resistant transformants were digested with two different restriction enzymes (*SalI* and *EcoRI*) (Amersham-Pharmacia Biothech, Madrid, Spain) and fractionated by electrophoresis through 0.8% agarose gels in TAE buffer and subjected to Southern blot analysis using a fragment of *cyp51A* gene as a probe (17, 22).

The *cyp51A* gene from AF-72 strain was introduced into the 237 wild type strain by electroporation. After 48 hours of incubation two transformant strains (T-21 and T-23) resistant to itraconazole were obtained. Both transformants had only one copy of the *cyp51A* gene (as shown by the fact that only a single hybridization band appeared after digestions with two different restriction enzymes) (results not shown). In order to ensure that no errors occurred during gene replacement (such a DNA deletions or insertions that could have affected *cyp51A* gene transcription), PCR amplification of the 5' and 3' *cyp51A* ends from both ITC-resistant transformants was used. Two primer sets (A7 + A5, and A9 + A6), previously described (17) were used to PCR amplify DNA fragments of 600 bp and 500 bp respectively. Those fragments included the non coding sequences surrounding the 5' and 3' *cyp51A* ends. The ITC resistance (MIC > 8.0 µg/ml) of T-21 and T-23 was confirmed by the microdilution method (3, 19). MICs for the rest of azole drugs showed similar pattern that of the six strains carrying the G54 substitution (Table 1). PCR amplification of *cyp51A* and *cyp51B* genes from these two transformants and full sequencing of the genes confirmed that both of them have incorporated the *cyp51A* gene mutated copy (g161a change leading to a glycine/glutamic acid substitution at codon 54). None of the mutants have any other base change present in either the rest of the *cyp51A* gene or in the *cyp51B* gene.

The replacement of the *cyp51A* gene from *A. fumigatus* wild type (CM-237) strain with the mutated *cyp51A* gene of AF-72, changed the susceptibility pattern of CM-237 to ITC-resistant. Because we have used a direct itraconazole resistance selection for identifying the gene replacement event incorporating the mutation, we can not rule out the possibility of an spontaneous mutation by drug selective pressure. Although, two facts should be consider: (i) no transformants ITC-resistant were obtained when the wild type spores were electroporated using distilled water without DNA, ruling out in part the possibility of an spontaneous mutation and (ii)

the six strains with G54 substitution harboured three different base changes (gag, gtg, or agg) so that, the existence of two independent transformants harbouring exactly the same mutated 54 codon (gag) will speak in favour of the gene replacement event incorporating the cyp51A mutated copy of AF-72. In conclusion, results strongly suggest that a point mutation leading to G54 substitution for other amino acids is related to *A. fumigatus* ITC-resistance.

It is noteworthy that all strains (but CM-796) carrying the G54 substitution presented a 4X decrease of the MICs of FCZ and KTC while they have no change in MICs of VCZ (Table 1). Also mutants T-21 and T-23 had similar susceptibility patterns. Contrarily, the other six strains presented MICs > 1600µg/ml (at least 2.5 X increase) of FCZ and a moderate elevation for VCZ and KTZ MICs (Table 1). In ITC resistant strains it seems remarkable the 20 X fluctuation of MICs for FCZ between strains with or without the G54 substitution. Whatever the nature of the change due to the G54 substitution, it seems to produce a better molecular environment for FCZ and KCZ access and/or interaction. Strain CM-796 exhibited no variation in its susceptibility against the three antifungal tested. However, coexistence of different mechanism of resistant can not currently be discarded. At least two different azole susceptibility patterns has been already described for some of these strains (18). The results presented here could match one of these patterns with the G54 substitution.

The surveillance of ITC-resistant strains of *A. fumigatus* and the study of the resistance mechanisms operating in them could help to understand the mechanisms of drug resistance and to develop newer and more active molecules. Further investigations on the functional analysis of the Cyp51A and Cyp51B proteins of *A. fumigatus* are straight forward.

This work was supported in part by grants MPY1078/99 and MPY1281/01 from Instituto de Salud Carlos III. E. M. held a contract Ramón y Cajal from Ministry of Science and Technology. T.M.D.-G. is a post-doctoral fellow of the Instituto de Salud Carlos III (grant 99/4149). We thanks D.W. Denning and J. Mosquera, University of Manchester (UK), for providing strains AF-72, AF-90 and AF-91, AF-1422, F/6929, F/7075, Br130, Br181 and SO/3829. We also thank E. Dannaoui, Universite Claude Bernard Lyon I (FR) for the gift of the strains AF-1119 and AF-1237. We are grateful to Daniel Lopez, Instituto de Salud Carlos III (Spain), for critical reading of the manuscript. We thanks Gema del Rio for invaluable technical assistance.

## REFERENCES

1. **Chryssanthou, E.** 1997. In Vitro Susceptibility of Respiratory Isolates of *Aspergillus* species to Itraconazole and Amphotericin B. Acquired Resistance to Itraconazole. *Scand. J. Infect. Dis.* **29**: 509-512.
2. **Cove, D.J.** 1966. The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. *Biochem. Biophys. Acta* **113**: 51-56.
3. **Cuenca-Estrella M., J.L. Rodriguez-Tudela, E. Mellado, J.V. Martinez-Suarez, and A. Monzon.** 1998. Comparison of the in vitro activity of voriconazole (UK-109,496), itraconazole and amphotericin B against clinical isolates of *Aspergillus fumigatus*. *J. Antimicrob. Chemother.* **42**: 531-533.
4. **Dannaoui, E., E. Borel, M.F. Monier, M.A. Piens, S. Picot, and F. Persat.** 2001. Acquired itraconazole resistance in *Aspergillus fumigatus*. *J. Antimicrob. Chemother.* **47**:333-340.
5. **Dannaoui, E., E. Borel, F. Persat, M.F. Monier, and M.A. Piens.** 1999. In-vivo itraconazole resistance of *Aspergillus fumigatus* in systemic murine aspergillosis. EGBA Network. European research group on Biotypes and Genotypes of *Aspergillus fumigatus*. *J. Med. Microbiol.* **48**: 1087-1093.
6. **Dannaoui, E., F. Persat, M.F. Monier, E. Borel, M.A. Piens and S. Picot.** 1999. In-vitro susceptibilities of *Aspergillus* spp. isolates to amphotericin B and itraconazole. *J. Antimicrob. Chemother.* **44**: 553-555.
7. **Délye C., L. Bousset, and M.F. Corio-Costet.** 1998. PCR cloning and detection of point mutations in the eburicol 14 $\alpha$ -demethylase (CYP51) gene from *Erysiphe graminis* f. sp. *hordei*, a "recalcitrant" fungus. *Current Genetics* **34**: 399-403.
8. **Délye C., F. Laigret, and M.F. Corio-Costet.** 1997. A mutation in the 14 $\alpha$ -demethylase gene of *Uncinula necator* that correlates with resistance to a sterol biosynthesis inhibitor. *Appl. Environ. Microbiol.* **63**:2966-2970.
9. **Denning D.W., K. Venkateswarlu, K. L. Oakley, M.J. Anderson, N.J. Manning, D. A. Stevens, D. W. Warnock, and S. L. Kelly.** 1997. Itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **41**: 1364-1368.

10. Denning D.W., S.A. Radford, K. L. Oakley, L. Hall, E.M. Johnson, and D. W. Warnock. 1997. Correlation between in-vitro susceptibility testing to traconazole and in-vivo outcome of *Aspergillus fumigatus* infection. J. Antimicrob. Chemother. **40**: 401-414.
11. Edlind, T.D., K.W. Henry, K.A. Metera, and S.K. Katiyar. 2001. *Aspergillus fumigatus* CYP51 sequence: potential basis for fluconazole resistance. Med. Mycol. **39**: 299-302.
12. Favre, B., M. Didmon and N.S. Ryder. 1999. Multiple amino acid substitutions in lanosterol 14 $\alpha$ -demethylase contribute to azole resistance in *Candida albicans*. Microbiology **145**: 2715-2725.
13. Latgé J.-P. 1999. *Aspergillus fumigatus* and Aspergillosis. Clinical Microbiol. Rev. **12**: 310-350.
14. Loffler J., S.L. Kelly, H. Hebart, U. Schumacher, C. Lass-Flörl, and H. Einsele. 1997. Molecular analysis of cyp51 from fluconazole-resistant *Candida albicans* strains. FEMS Microbiol. Lett. **151**:263-8.
15. Manavathu, E.K., J.A. Vazquez, and P.H. Chandrasekar. 1999. Reduced susceptibility in laboratory-selected mutants of *Aspergillus fumigatus* to itraconazole due to decreased intracellular accumulation of the antifungal agent. Inter. J. Antimicrob. Agents. **12**: 213-219.
16. Marichal, P., L. Koymas, S. Willemsens, D. Bellens, P. Verhasselt, W. Luyten, M. Borgers, F.C.S. Ramaekers, F.C. Odds, and H. Vanden Bossche. 1999. Contribution of mutations in the cytochrome P450 14 $\alpha$ -demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. Microbiology **145**: 2701-2713.
17. Mellado, E., T.M. Diaz-Guerra, M. Cuenca-Estrella, and J.L. Rodriguez-Tudela. 2001. Identification of two different 14- $\alpha$  sterol demethylase-related genes (*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species. J. Clin. Microbiol. **39**: 2431-2438.
18. Mosquera, J., and D.W Denning. 2002. Azole cross-resistance in *Aspergillus fumigatus*. Antimicrob. Agents Chemother. **46**: 556-557.
19. National Committee for Clinical Laboratory Standards. 1998. Reference method for broth dilution antifungal susceptibility. Testing of filamentous fungi: proposed standard. Document M38-P. NCCLS. Villanova, PA.
20. Osherov, N., D.P. Kontoyannis, A. Romans, and G.S. May. 2001. Resistant to itraconazole in *Aspergillus nidulans* and *Aspergillus fumigatus* is conferred by extra copies

of the *A. nidulans* P-450 14 $\alpha$ -demethylase gene, *pdmA*. J. Antimicrob. Chemother. **48**:75-81.

- 21. Perea S., J.L. Lopez-Ribot, W.R. Kirkpatrick, R.K. McAtee, R.A. Santillan, M. Martinez, D. Calabrese, D. Sanglard, and T.F. Patterson.** 2001. Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. Antimicrob. Agents Chemother. **45**:2676-84
- 22. Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- 23. Sanchez O. and J. Aguirre.** 1996. Efficient transformation of *Aspergillus nidulans* by electroporation of germinated conidia. Fungal Genetics Neslett. **43**:48-51.
- 24. Sanglard, D., F. Ischer, K. Koymans, and J. Bille.** 1998. Amino acid substitutions in the cytochrome P450 14 $\alpha$ -demethylase (CYP51A1) from azole resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. Antimicrob. Agents Chemother. **42**: 241-253.
- 25. Slaven J.W., M.J. Anderson, D. Sanglard, G.K. Dixon, J. Bille, I.S. Roberts, D.W. Denning.** 2002. Increased expression of a novel *Aspergillus fumigatus* ABC transporter gene, *atrF*, in the presence of itraconazole in an itraconazole resistant clinical isolate. Fungal Genet Biol. **36**:199-206.
- 26. Tang C.M., J. Cohen, D.W. Holden.** 1992. An *Aspergillus fumigatus* alkaline protease mutant constructed by gene disruption is deficient in extracellular elastase activity. Mol Microbiol. **6**:1663-71.
- 27. Weidner G., C. d'Enfert, A. Koch, P.C. Mol, and A.A. Brakhage.** 1998. Development of a homologous transformation system for the human pathogenic fungus *Aspergillus fumigatus* based on the *pyrG* gene encoding orotidine 5'-monophosphate decarboxylase. Curr. Genet. **33**:378-385.