Primer aislamiento clínico en Sudamérica de una cepa de *Aspergillus fumigatus* resistente a itraconazol con la sustitución G54E en Cyp51Ap.

First itraconazole resistant *Aspergillus fumigatus* clinical isolate harboring a G54E substitution in Cyp51Ap in South America.

Título breve: *Aspergillus fumigatus* resistente a Itraconazol.

Running title: Itraconazole resistant *Aspergillus fumigatus*.

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Resumen

Antecedentes: un trabajador rural de 27 años de edad fue hospitalizado con una queratitis fúngica debido a un traumatismo con un resto vegetal.

Materiales y métodos: se tomaron las muestras para los examenes de miscroscopía y cultivo. El aislamiento se identificó mediante criterios morfológicos y moleculares. Se realizaron pruebas de sensibilidad a los antifúngicos siguiendo el documento del CLSI. Se amplificó y secuenció el gen *CYP51A* de la cepa.

Resultados: se aisló una cepa de *Aspergillus fumigatus* resistente a Itraconazol (CIM > 8 mg/ml). El aislamiento resultó sensible a anfotericina B, posaconazol, voriconazol y caspofungina. La secuenciación del gen *CYP51* reveló dos mutaciones que generan la sustitución G54E. El paciente fue tratado con natamicina oftálmica.

Conclusiones: este es el primer caso informado en Sudamérica de una cepa clínica de *A. fumigatus* con la sustitución G54E en el Cyp51Ap, asociada con resistencia a itraconazol. Teniendo en cuenta que el paciente no había recibido nunca antes tratamientos con azoles, podría haber adquirido esta cepa resistente del ambiente.

Abstract

Background: A 27-year-old male rural worker was hospitalized with a fungal keratitis due to a traumatism with a vegetal detritus.

Materials and methods: Specimens for microscopy examination and culture were collected. The isolate was identified by morphological and molecular criteria. Susceptibility testing was done according to CLSI methods. *CYP51A* gene was PCR amplified and sequenced.

Results: An *Aspergillus fumigatus* strain resistant to itraconazole (MIC > 8.0 µg/ml) was isolated. The isolate was susceptible to amphotericin B, posaconazole, voriconazole and caspofungin. *CYP51A* sequencing showed two mutations leading on the G54E substitution. The patient received natamycin as treatment.

Conclusions: This is the first report in South-America of a clinical *A. fumigatus* strain carrying the substitution G54E at Cyp51Ap linked for itraconazole resistance. Considering the patient was azole-naive, he might have acquired this resistant isolate from the environment.

Text

A 27-year-old male rural worker was admitted to the "Dr. José María Cullen" Hospital (Santa Fe - Argentina) in November 2013 with a corneal ulcer. The patient had suffered an ocular traumatism with a vegetal detritus while he was working in harvesting vegetables. At patient arrival, ocular samples were obtained at the Ophthalmology Department of the Hospital. Conjunctival mucopurulent exudate and corneal surface samples were obtained by swabbing and by scrapping with a Kimura spatula, respectively. Samples were immediately sent to the Microbiology Laboratory for study. Samples were Giemsa stained and cultured in Sabouraud Dextrose Agar (SDA) with Chloramphenicol (Britania, Argentina) and in Thiogycolate broth. Meanwhile, the patient received empirical treatment with oral fluconazole (400 mg/day) and ophthalmic natamycin (5% solution) two drops every 30 minutes during the first 24 hours, together with intravenous ceftazidime and vancomycin. During patient´s first day of hospitalization the laboratory returned the Giemsa stain results where septate fungal hyphae were seen, leading to the diagnosis of a keratitis due to a hialohyphomycete. With these data, fluconazole was suspended and natamycin and antibacterials were maintained. Natamycin dosage intervals were changed after the second hospitalization day to two drops every hour during 7 days.

Then, it was slowly tapered following the good clinical response until the complete resolution of the infection. After the third hospitalization day, colonies of *Aspergillus fumigatus* grew in all the SDA slants.

The strain was identified as *Aspergillus fumigatus* by morphological criteria and by PCR amplification and sequencing of the β-tubulin gene as previously described [1, 2]. Antifungal susceptibility testing was performed using the broth microdilution method according to the CLSI M38A2 document for Itraconazole (ITC), Voriconazole (VRC), Posaconazole (PSC), Amphotericin B (AMB) and Caspofungin (CSF) [3]. *CYP51A* gene (encoding the 14-α-sterol demethylase, target of azole drugs) was PCR amplified including the promotor (5´UTR), ORF and terminator (3´UTR) regions. The obtained amplification fragment was sequenced as described before [4, 5].

The *A. fumigatus* strain MICs and MEC were ITC >8.0 µg/ml, VRC 0.5 µg/ml, PSC 0.12 µg/ml, AMB 0.5 µg/ml and CSF 0.5 µg/ml. According to the ECV values published by Espinel-Ingroff et al., the strain was considered as ITC-resistant and susceptible to all the other tested antifungal agents [6, 7]. *CYP51A* sequencing showed two nucleotide mutations (G161A and G162A) when compared with the one published under the GenBank sequence AF338659.1. These mutations lead to a substitution at codon 54 (G54E) (Figure 1) which was already described and associated to ITC-resistant phenotypes, but

was never seen in South America before [4, 8-11]. Thus, to the best of our knowledge, this is the first ITC-resistant *A. fumigatus* strain reported in this part of the world.

Azole-resistant *A. fumigatus* isolation frequency is increasing. These resistant strains were isolated with or without previous azole exposure [8, 9, 12-14]. Our patient, who has never previously being treated with azole drugs, suffered an infection with an ITC-resistant *A. fumigatus* strain due to an accidental inoculation with vegetal detritus. This fact suggests an environmental origin of the strain. In Argentina there were no reports demonstrating the existence of environmental azole-resistant *A. fumigatus* strains. However, these strains might be being selected as azole antifungal agents are widely used in agriculture for plant protection. Such environmental route of resistance development in *A. fumigatus* was firstly proposed in 2001 and molecularly confirmed later in the Netherlands for a common azole resistance mechanism involving L98H substitution at Cyp51Ap coupled with a promotor modification [15-17].

The emergence and spread of the resistance mechanism described here in *A. fumigatus* is of major concern because ITC is a highly used azole drug in developing countries. It would be useful to analyze environmental sources to detect these strains.

Conflict of interest: None to declare.

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Bibliografy

1. Balajee SA, Marr KA. Phenotypic and genotypic identification of human pathogenic aspergilli. Future Microbiol 2006; 1: 435-45.

2. Balajee SA, Kano R, Baddley JW, et al. Molecular identification of *Aspergillus* species collected for the Transplant-Associated Infection Surveillance Network. J Clin Microbiol 2009; 47:3138-41.

3. Clinical and Laboratory Standards Institute. Reference method for broth diution antifungal susceptibility Testing of Filamentous Fungi, Approved Standard Second Edition. Document M38-A2. 28[16]. 2008.

4. Diaz-Guerra TM, Mellado E, Cuenca-Estrella M, Rodriguez-Tudela JL. A point mutation in the 14alpha-sterol demethylase gene *cyp51A* contributes to itraconazole resistance in *Aspergillus fumigatus*. Antimicrob Agents Chemother 2003; 47:1120-4.

5. Mellado E, Diaz-Guerra TM, Cuenca-Estrella M, Rodriguez-Tudela JL. Identification of two different 14-alpha sterol demethylase-related genes (*cyp51A* and *cyp51B*) in Aspergillus fumigatus and other *Aspergillus* species. J Clin Microbiol 2001; 39:2431-8.

6. Espinel-Ingroff A, Fothergill A, Fuller J, Johnson E, Pelaez T, Turnidge J. Wild-type MIC distributions and epidemiological cutoff values for caspofungin and *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). Antimicrob Agents Chemother 2011; 55: 2855-9.

7. Espinel-Ingroff A, Cuenca-Estrella JM, Cantón E. EUCAST and CLSI: Worcking Together Towards a Harmonized Method for Antifungal Susceptibility Testing. Current Management of Fungal Infections; 15-1-2013, 59-67.

8. Bader O, Weig M, Reichard U, et al. cyp51A-Based mechanisms of *Aspergillus fumigatus* azole drug resistance present in clinical samples from Germany. Antimicrob Agents Chemother 2013; 57:3513-7.

9. Howard SJ, Cerar D, Anderson MJ, et al. Frequency and evolution of Azole resistance in *Aspergillus fumigatus* associated with treatment failure. Emerg Infect Dis 2009; 15: 1068-76.

10. Mann PA, Parmegiani RM, Wei SQ, et al. Mutations in *Aspergillus fumigatus* resulting in reduced susceptibility to posaconazole appear to be restricted to a single amino acid in the cytochrome P450 14alpha-demethylase. Antimicrob Agents Chemother 2003; 47: 577-81.

11. Mosquera J, Denning DW. Azole cross-resistance in *Aspergillus fumigatus*. Antimicrob Agents Chemother 2002; 46: 556-7.

12. Dannaoui E, Borel E, Monier MF, Piens MA, Picot S, Persat F. Acquired itraconazole resistance in *Aspergillus fumigatus*. J Antimicrob Chemother 2001; 47: 333-40.

13. van der Linden JW, Snelders E, Kampinga GA, et al. Clinical implications of azole resistance in *Aspergillus fumigatus*, The Netherlands, 2007-2009. Emerg Infect Dis 2011; 17: 1846-54.

14. Vermeulen E, Lagrou K, Verweij PE. Azole resistance in *Aspergillus fumigatus*: a growing public health concern. Curr Opin Infect Dis 2013; 26: 493-500.

15. Hof H. Critical annotations to the use of azole antifungals for plant protection. Antimicrob Agents Chemother 2001; 45: 2987-90.

16. Snelders E, van der Lee HA, Kuijpers J, et al. Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. PLoS Med 2008 11; 5: e219.

17. Verweij PE, Snelders E, Kema GH, Mellado E, Melchers WJ. Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? Lancet Infect Dis 2009 Dec;9(12):789-95.

Figure 1. *CYP51A* DNA sequencing chromatograms and Cyp51Ap amino acid sequence for the ITC-resistant *A. fumigatus* strains. Upper line: Segment of the wild type *A. fumigatus* Cyp51Ap (GenBank accession no. AAK73659.1) between the amino acid residues 48 and 65. Lower line: the same Cyp51Ap segment of the ITC-resistant strain, showing the amino acid substitution (G54E). The red box in the DNA sequencing chromatograms shows the mutated codon 54.