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Original article

Azole resistance in a clinical isolate of *Aspergillus fumigatus* from Chile

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ABSTRACT

Background: *Aspergillus fumigatus* is a ubiquitous opportunistic pathogen. This fungus can acquire resistance to azole antifungals due to different mutations in the cyp51A gene. Azole resistance has been observed in several continents and appears to be a globally distributed phenomenon. Specific mutations in cyp51A that lead to azole resistance, such as the TR34/L98H modification, have been reported.
Aims: To evaluate the azole resistance in clinically isolated *A. fumigatus* strains.
Methods: As a result of our passive surveillance strategy, a total of 23 *A. fumigatus* isolates from clinical origins were identified through a phylogenetic analysis using the ITS region and β-tubulin gene fragments, and typed with the CSP microsatellite. Azole susceptibility profiles were performed by disk diffusion and microdilution broth methodologies according to CLSI guidelines.
Results: Here we describe, for the first time, the detection of azole-resistant *A. fumigatus* isolates from clinical origins in Chile with mutations in the cyp51A gene. In addition to the TR34/L98H mutation, one isolate exhibited an F46Y/M172V/E427K-type mutation. Furthermore, microsatellite typing based on cell surface protein (CSP) was performed, showing the t02 (TR34/L98H), t15 (F46Y/M172V/E427K) and t01 (susceptible clinical isolates) genotypes.
Conclusions: Our study demonstrates the presence of mutations related to azole resistance in *A. fumigatus* strains isolated from clinical samples in Chile. In order to obtain information that may help to tackle the spread of antifungal resistance among *A. fumigatus* populations, and to ensure the efficacy of future treatments against aspergillosis, a further research is necessary.

Primer aislamiento clínico de *Aspergillus fumigatus* resistente a azoles en Chile

Palabras clave:
Aspergillus fumigatus
Resistencia antifúngica
Mecanismos de resistencia a los antifúngicos
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cyp51A

RESUMEN

Antecedentes: *Aspergillus fumigatus* es un patógeno oportunista ubicuo. Este hongo puede adquirir resistencia a los antifúngicos azólicos gracias a diferentes mutaciones en el gen cyp51A. La resistencia a los azoles se ha observado en varios continentes y parece ser un fenómeno de distribución mundial. Se han descrito mutaciones específicas en cyp51A para adquirir resistencia a los azoles, como la modificación TR34/L98H.
Objetivos: Evaluar la resistencia a los azoles en aislamientos de *A. fumigatus* obtenidos de muestras clínicas.
Métodos: Gracias a la vigilancia pasiva se identificó un total de 23 aislamientos de *A. fumigatus* de origen clínico a través de un análisis filogenético mediante el estudio de la región ITS y de fragmentos del gen de la β-tubulina, que se tipificaron con el microsatélite CSP. Los perfiles de sensibilidad a los azoles se realizaron mediante los métodos de difusión en disco y microdilución.

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Resultados: En el presente trabajo describimos, por primera vez en Chile, la detección de aislamientos clínicos de *A. fumigatus* en este país resistentes a azoles, con mutaciones en el gen cyp51A. Además de la mutación TR34/L98H, un aislamiento presentó una mutación de tipo F46Y/M172V/E427K. Se realizó la tipificación por microsatélites basada en la proteína de superficie celular, que mostró los genotipos t02 (TR34/L98H), t15 (F46Y/M172V/E427K) y t01 (cepas sensibles).
Conclusiones: Nuestro estudio demuestra la presencia de mutaciones relacionadas con la resistencia a los azoles en cepas de *A. fumigatus* aisladas de muestras clínicas en Chile. Para obtener información que pueda ayudar a atajar la propagación de la resistencia a los antifúngicos entre las poblaciones de *A. fumigatus*, y garantizar así la eficacia de futuros tratamientos contra la aspergilosis, es necesario seguir investigando.

In recent years, the number of pathogenic fungi and the incidence of mycoses have increased considerably. Among the causes that may explain this situation, the increase in the population at risk of contracting an invasive fungal disease (IFD), i.e. immunocompromised patients, those with oncohematological problems, or those with prolonged corticosteroid treatment must be mentioned.[39] Another cause is the improvement in both the diagnosis of mycoses and the identification of the etiological agents. Nevertheless, IFDs still have a high morbidity and mortality rate, which can reach 90% in some groups of patients.[4,7,35,36]

Among the different fungi capable of producing IFD, we can find two large groups of etiological agents: yeasts, with *Candida albicans* being the most representative, and filamentous fungi, with *Aspergillus fumigatus* as the most isolated microorganism.

The genus *Aspergillus* is a diverse group of fungi comprising many species capable of producing a wide variety of pathologies. Classically, the identification of the different species has been based on the phenotypic characterization of the isolates. However, due to the improvement and development of new molecular tools in the last decades, these advanced methodologies are fundamental in the identification of the *Aspergillus* species. Currently, this genus contains about 446 species. However, only some of them are pathogenic for humans, being the species belonging to the sections Fumigati, Flavi, Nidulantes, Nigri, Terrei and Usti the most commonly isolated from clinical cases.[29]

We daily inhale a large number of *Aspergillus* spores, most of which get trapped in the epithelium of the lower respiratory tract and are eliminated by the ciliary clearance mechanism. Only the smaller spores (between 2 and 5 μm) are able to reach the pulmonary alveoli, where they will be removed by the alveolar macrophages, responsible for eliminating the conidia, and the neutrophils, responsible for destroying the few hyphae produced from the germination of the spores.[27] However, in immunocompromised patients, the presence of the spores can lead to colonization of the host, enabling the development of IFD.[9,33]

Diseases caused by species of the genus *Aspergillus* are generally referred to as aspergillosis. This genus is responsible for more than 200,000 cases of invasive aspergillosis (IA) per year. Besides, this genus causes more than 1.2 million additional cases of chronic pulmonary aspergillosis (CPA) and about 4.8 million cases of allergic bronchopulmonary aspergillosis (ABPA).[17,18] In Chile, due to the absence of official data about IFD, cases of IA, CPA and ABPA are estimated to occur in 1.7/100,000; 6.9/100,000 and 97.9/100,000, respectively.[2]

One of the threats related to aspergillosis is the resistance to antifungals, mainly azoles, which are the treatment of choice in these mycoses. Such resistance seems to be caused by various mutations in the Cyp51A gene, which codes for 14-α-demethylase, the target enzyme of azole drugs,[34,45,46] and which would be related to high rates of therapeutic failure. The first reports of this phenomenon date back to the 1990s,[16] being an emerging problem widely reported in many countries around the world.

The present work reports the isolation for the first time in Chile of azole resistant strains of *A. fumigatus*. We also prove the mutations in the Cyp51A gene.

Material and methods

Fungal isolates and morphological identification

Twenty-three clinical isolates obtained from March 2017 to March 2021, classified as *Aspergillus* spp., were received to perform the species identification. The isolates were obtained by culturing bronchoalveolar lavage samples from patients diagnosed with aspergillosis. Firstly, the isolates were identified by a phenotypical approach, evaluating their macro- and microscopic features and their ability to grow in the dark at 10 °C, 25 °C, 37 ± 1 °C, and 50 °C on potato dextrose agar (PDA).

Molecular analysis

According to the manufacturer's instructions, the DNA was extracted using an E.Z.N.A.® Fungal DNA Mini Kit (Omega Biotek Store, USA). The universal fungal primers ITS1 and ITS4 for ITS1-5.8S-ITS2 region,[47] and Bt2a and Bt2b[24] for a partial β-tubulin gene sequence, were used. A 30 μl mixture containing 17 μl of BioMixTM Red (New England Biolabs), 10 pM primers, and 10 ng DNA was subjected to a DNA amplification technique, following a protocol of initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min for the ITS region, and 55 °C for β-tubulin; extension at 72 °C for 2 min, and final extension at 72 °C for 7 min. The PCR product was checked on 2% agarose gel. Purification was performed following manufacturer guidelines with FavorPrep™ Gel/PCR purification Mini Kit (Favorgene, Taiwan). Sequencing was carried out in Macrogen (Macrogen, Korea). An NCBI Blastn search for each locus region was performed.[1] Sequences from type and reference strains were retrieved from the GenBank database. The sequences were aligned in MUSCLE software[20] followed by manual adjustments with a text editor. ITS sequences were used to identify isolates to the *Aspergillus*-section level, and partial β-tubulin sequences were used to identify to the species level.

The entire sequences of the Cyp51A gene and promoter regions were amplified as previously described[42] in any isolate that exhibited azole resistance with the purpose of finding mutations that might explain that condition. Sequencing and analyses were carried out as formerly mentioned. The DNA sequences were compared with the wild-type susceptible *A. fumigatus* reference strain (GenBank AF338659).

Finally, the cell surface protein (CSP) encoding gene was partially sequenced[8] in all the isolates to perform the CSP typing according to the nomenclature reported by Klaassen et al.[32]

Table 1
Antifungal susceptibility profiles obtained with the microdilution broth method and the disk diffusion method.

Genotype (*n*)/reference strain	Microdilution broth MIC (mg/L)			Disk diffusion Diameter (mm)		
	ITZ	VRZ	PCZ	ITC	VRZ	PCZ
t15 (1)	1	0.5	0.25	17	27	22
t02 (1)	16	8	1	10	13	16
t01 (21)	0.25–1	0.25–1	0.25–1	18–21	25–28	19–23
Candida parapsilosis ATCC 22019	0.12	0.06	0.12	21	31	26
Aspergillus fumigatus ATCC MYA-3626	0.25	0.25	0.25	17	31	31
Hamigera insecticola ATCC MYA-3630	0.06	0.06	0.12	30	35	37

ITZ: itraconazole; VRZ: voriconazole; PCZ: posaconazole; MIC: minimal inhibitory concentration.

Antifungal analysis

The in vitro susceptibility to itraconazole (ITZ), posaconazole (PCZ) and voriconazole (VRZ) was evaluated by the broth microdilution method performed according to the CLSI document M38M51S.[13] The antifungal concentration of ITZ, PCZ and VRZ ranged from 0.0156 to 8 μg/mL. The inoculated plates were incubated at 35 °C and observed after 24 h. The lack of visual growth in each well was defined as the MIC value. Due to the absence of breakpoints for filamentous fungi in the CLSI method (except for voriconazole and *A. fumigatus*), the epidemiological cut-off values (ECVs) were used (1 mg/L for ITZ and VRZ, 0.5 mg/L for PCZ).[14,10,22] In order to confirm the obtained results, the susceptibility test was performed twice. *Candida parapsilosis* ATCC 22019, *A. fumigatus* ATCC MYA-3626 and *Hamigera insecticola* (ex. *Paecilomyces variotii*) ATCC MYA-3630 were used as controls.

In addition, an antifungal susceptibility test by the disk diffusion method was performed according to the CLSI M38M51S document.[13] Isolates were subcultured on PDA at 25 °C for 5–7 days before testing. Then, the surface of a Mueller Hinton agar (MHA) plate dish was inoculated with a sterile cotton swab with the undiluted mold stock inoculum suspension. Antifungal disks (Rosco Diagnostica, Denmark) including ITZ (10 μg), PCZ (5 μg), and VRZ (1 μg) were applied onto the surface of the inoculated media. The plates were incubated at 35 °C and read after 24–48 h. Zone diameters were interpreted according to CLSI M38M51S. A slight trailing or hyphal element extending into the inhibition zone was ignored.

Results

The twenty-three strains analyzed in the present study were identified as *Aspergillus* section *Fumigati* based on their morphological and physiological characteristics: blue-green colored velutinous colonies, and abundant sporulation on PDA. The isolates showed subclavate vesicles (13–26 μm), uniseriate columnar heads and flexuous conidiophore. No strain grew at 10 °C, but did it at 50 °C on PDA, as expected according to the literature.[28,40] The analysis of the nucleotide sequences of the β-tubulin gene showed that two strains had a percent identity equal to or higher than 99% with those sequences of *A. fumigatus sensu stricto* available in the GenBank/NCBI database (99.6% sequence of the strain >EF669851.1 NRRL 5587).

Antifungal susceptibility

Table 1 shows the antifungal susceptibility profiles obtained by means of the microdilution method and the disk diffusion method. Voriconazole and posaconazole showed the highest activity. One isolate (ChFC 132) was resistant to voriconazole with a MIC of 8 μg/mL. Also, and due to a lack of clinical breakpoints, ChFC 132 was considered as non-wild type (NWT) based on the proposed ECV, exhibiting MIC values of 16 μg/mL for ITZ and 1 μg/mL for PCZ. The remaining 22 isolates showed MIC values of 1 μg/mL, 0.5 μg/mL and 0.25 μg/mL for ITZ, VRZ and PCZ, respectively. Based on these results, these isolates were considered susceptible to VRZ and wild-type for ITZ and PCZ.

The results obtained with the disk diffusion method were consistent with those observed with the microdilution broth methodology. In brief, isolate ChFC 132 showed an inhibition zone ≤17 mm for all triazoles tested. According to these values, ChFC 132 was considered NWT. In the remaining 22 strains, the inhibition halos observed were greater than 17 mm for VRZ, PCZ and ITZ, being considered as susceptible to all the antifungals tested. However, one of these strains (ChFC 104) exhibited inhibition halos ≥17 mm for VRZ and PCZ, but ≤17 mm for ITZ. Interestingly, in the halo around the ITZ disk, a trailing zone was observed, which is why it was decided to sequence the CYP51A gene in order to elucidate putative mutations.

Cyp51A gene sequencing and CSP typing

Sequencing of the Cyp51A gene and its promoter showed the presence of the 34 bp tandem repeat (TR34) and L98H mutation in the strain ChFC 132. In addition, this analysis revealed that the second isolate (ChFC 104) harboured the F46Y/M172V/E427K mutations (Fig. 1). In the remaining isolates, no mutations were observed.

The characterization of the CSP showed that our strains corresponded to types previously described in the literature. The strain ChFC 132 belongs to type t02, while the ChFC 104 was identified as type t15. The other 21 strains were classified into type t01.

Discussion

Invasive aspergillosis is a very important challenge in medical mycology. An early diagnosis may be difficult to achieve, and the treatment of the disease is complex, so antifungal agents are used at the slightest suspicion of infection. In this context, the development of secondary resistance to commonly used antifungals gets facilitated. This fact is becoming a problem that is aggravated by the existence of cross-resistance between clinical triazoles and other azoles of the same family used as antifungals in agriculture.[23]

Likewise, azole resistance is now being reported in countries worldwide.[5,19,44] In Latin America, several countries have reported the presence of azole resistance in *A. fumigatus*, such as Colombia,[3] Argentina[30] and Peru,[11] among others. To our knowledge, this study describes for the first time in Chile the presence of azole resistance in *A. fumigatus*. Our two isolates with the mutations already mentioned were recovered from the bronchoalveolar lavage of two patients with aspergillosis. The samples were sent to our laboratory in order to check the presence of fungal organisms. Due to our passive surveillance strategy, we performed a disk diffusion test to evaluate their susceptibility patterns to some azoles. Based on

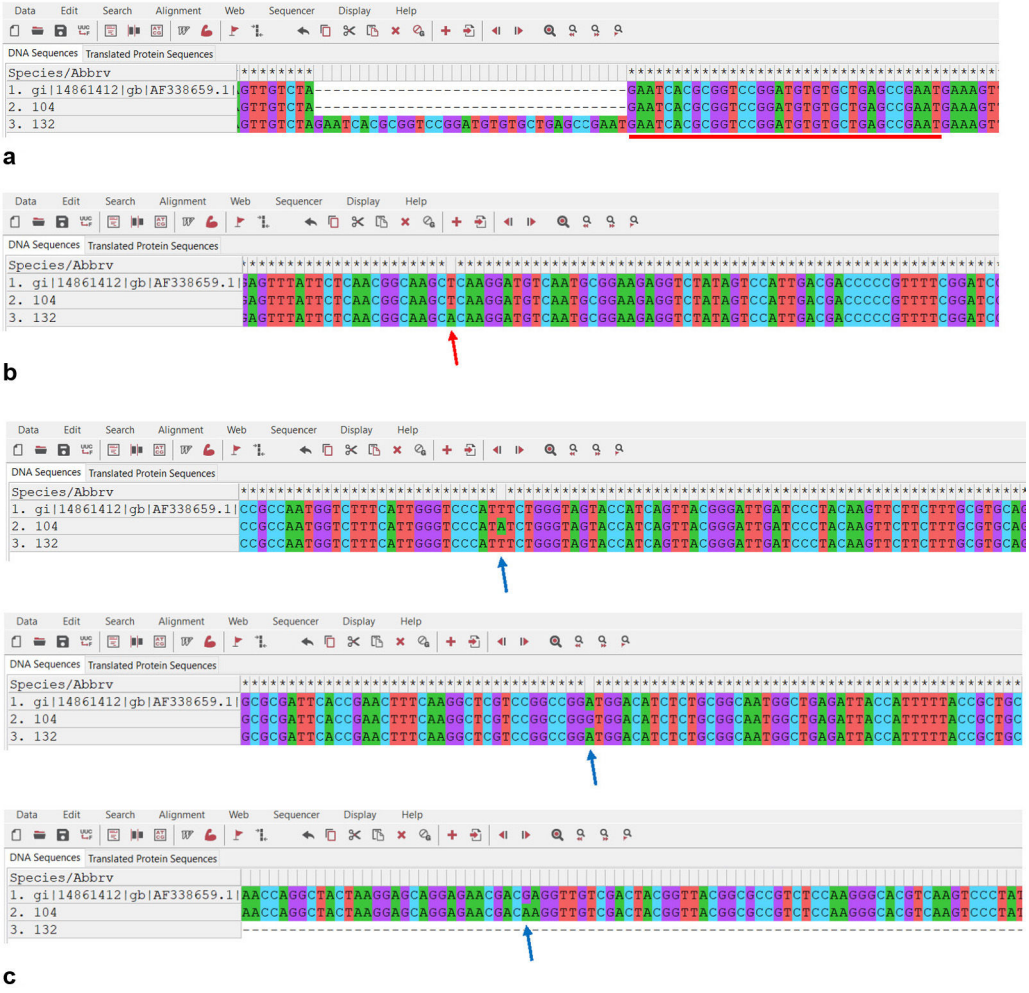

Fig. 1. Mutations observed in the Cyp51A sequences obtained from the isolates ChFC 132 and ChFC 104. (a) TR34 (red bar), (b) L98H (red arrow), and (c) F46Y/M172V/E427K (blue arrows).

the presence of halos ≤17 mm (ChFC 132), as well as the presence of what appeared to be trailing (ChFC 104), the isolates were subjected to the broth microdilution method, and the sequencing of the Cyp51A gene. The first strain was harbouring the TR34/L98H mutations. These substitutions and tandem repeat sequences in the promoter region are some of the most commonly reported azole-resistance mutations.[37,43] They were first described in Dutch *A. fumigatus* isolates, but are now spread worldwide[19] due to the extensive use of azole fungicides in agriculture and animals. These mutations have been related to high MIC values to azoles in several global studies, especially to itraconazole and voriconazole. Our results show MICs over 1 mg/L for VRZ and ITZ, and 0.5 mg/L for PCZ, allowing us to classify this isolate as resistant for voriconazole, and NWT for itraconazole/posaconazole, respectively. This finding agrees with other reports demonstrating the pan-azole resistance that confers the presence of TR34/L98H.[25,48]

The F46Y/M172V/E427K substitutions were observed in the second isolate. Curiously, these mutations had been identified in both azole-susceptible and resistant *A. fumigatus* isolates.[21,31] The F46Y/M172V/E427K mutations seem to be located in a protein region that does not interact with azole compounds or, at least, does not affect the structure of the protein.[42] The above suggests that the mutations found might not be the molecular mechanism related to the observed resistance to azoles. In the same way, other situations, such as mutations of the HapE or Hmg1 gene, or efflux pumps, among others, could explain azole resistance in some isolates.[12,26,38]

In our study, three previously observed genotypes were identified. Most of the clinical strains analyzed in the present study were susceptible to all the antifungal assayed. Moreover, they were identified as the t01 genotype. Worldwide, the t01 genotype has been mostly associated with susceptible strains; however, resistant isolates have also been reported within that genotype. It seems that both susceptible and resistant isolates can be found in all genotypes. In fact, in a study carried out in the United Kingdom, no susceptible isolates were associated with the t01 genotype but with genotypes t03, t04A, t05, and t08.[41] With respect to the other genotypes analyzed, isolate ChFC 132 was linked to t02 genotype, commonly associated with the TR34/L98H mutations.[6] Likewise, the strain ChFC 104 was characterized as t15, a genotype reported in Europe and Australia. Interestingly, strains belonging to genotype t15 harboring F46Y/M172V/E427K mutations seem to be susceptible to all azoles.[15] As we can observe, these genotypes have been recovered in several countries around the world, exhibiting a low genetic variation compared with wild-type strains. The detection of identical genotypes in distant countries suggests the clonal expansion, presumably airborne dispersal.[41]

Conclusions

We report the finding of two clinical strains from Chile with mutations in the Cyp51A gene. The results obtained with both the disk diffusion method and the microdilution broth methodology were consistent. This demonstrates the usefulness and advantages

of disk diffusion: its low cost and easy implementation in low complexity laboratories are the most remarkable features. Likewise, this methodology seems to predict azole resistance efficiently. Future studies involving more clinical and environmental strains are necessary to elucidate Chile's accurate picture of azole resistance.

Ethical approval

Not applicable.

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Authors' contributions

Conceptualization, E.A.D; Methodology, E.A.D., N.C., J.M.; Investigation, E.A.D., N.C., J.M.; Writing – original draft preparation, E.A.D., N.C.; Writing – review and editing, E.A.D. All authors have read and agreed to the published version of the manuscript.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403–10, http://dx.doi.org/10.1016/s0022-2836(05)80360-2.
2. Alvarez Duarte E, Denning DW. Serious fungal infections in Chile. Eur J Clin Microbiol Infect Dis. 2017;36:983–6, http://dx.doi.org/10.1007/s10096-017-2925-8.
3. Alvarez-Moreno C, Lavergne RA, Hagen F, Morio F, Meis JF, Le Pape P. Azole-resistant *Aspergillus fumigatus* harboring TR34/L98H, TR46/Y121F/T289A and TR53 mutations related to flower fields in Colombia. Sci Rep. 2017;7, http://dx.doi.org/10.1038/srep45631.
4. Baddley JW. Clinical risk factors for invasive aspergillosis. Med Mycol. 2011;49:S7–12, http://dx.doi.org/10.3109/13693786.2010.505204.
5. Bader O, Tünnermann J, Dudakova A, Tangwattanachuleeporn M, Weig M, Groß U. Environmental isolates of azole-resistant *Aspergillus fumigatus* in Germany. Antimicrob Agents Chemother. 2015;59:4356–9, http://dx.doi.org/10.1128/aac.00100-15.
6. Bader O, Tünnermann J, Dudakova A, Tangwattanachuleeporn M, Weig M, Groß U. Environmental isolates of azole-resistant *Aspergillus fumigatus* in Germany. Antimicrob Agents Chemother. 2015;59:4356–9, http://dx.doi.org/10.1128/AAC.00100-15.
7. Badiee P, Hashemizadeh Z. Opportunistic invasive fungal infections: diagnosis & clinical management. Indian J Med Res. 2014;139:195.
8. Balajee SA, Tay ST, Lasker BA, Hurst SF, Rooney AP. Characterization of a novel gene for strain typing reveals substructuring of *Aspergillus fumigatus* across North America. Eukaryot Cell. 2007;6:1392–9, http://dx.doi.org/10.1128/EC.00164-07.
9. Barnes PD, Marr KA. Aspergillosis: spectrum of disease, diagnosis, and treatment. Infect Dis Clin North A. 2006;20:545–61, http://dx.doi.org/10.1016/j.idc.2006.06.001, vi.
10. Buil J, Hagen F, Chowdhary A, Verweij P, Meis J. Itraconazole, voriconazole, and posaconazole CLSI MIC distributions for wild-type and azole-resistant *Aspergillus fumigatus* isolates. J Fungi. 2018;4:103, http://dx.doi.org/10.3390/jof4030103.
11. Bustamante B, Illescas LR, Posadas A, Campos PE. Azole resistance among clinical isolates of *Aspergillus fumigatus* in Lima-Peru. Med Mycol. 2019;58:54–60, http://dx.doi.org/10.1093/mmy/myz032.
12. Camps SMT, Dutilh BE, Arendrup MC, Rijs AJMM, Snelders E, Huynen MA, et al. Discovery of a hapE mutation that causes azole resistance in *Aspergillus fumigatus* through whole genome sequencing and sexual crossing. PLoS One. 2012;7:e50034, http://dx.doi.org/10.1371/journal.pone.0050034.
13. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antifungal susceptibility testing of filamentous fungi. CLSI supplement M38M51S. 3rd ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2022.
14. Clinical and Laboratory Standards Institute (CLSI). Epidemiological cutoff values for antifungal susceptibility testing. CLSI supplement M57S. 4th ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2022.
15. Dauchy C, Bautin N, Nseir S, Reboux G, Wintjens R, Le Rouzic O, et al. Emergence of *Aspergillus fumigatus* azole resistance in azole-naïve patients with chronic obstructive pulmonary disease and their homes. Indoor Air. 2018;28:298–306, http://dx.doi.org/10.1111/ina.12436.
16. Denning DW, Venkateswarlu K, Oakley KL, Anderson MJ, Manning NJ, Stevens DA, et al. Itraconazole resistance in *Aspergillus fumigatus*. Antimicrob Agents Chemother. 1997;41:1364–413, http://dx.doi.org/10.1128/AAC.41.6.1364.
17. Denning DW, Pleuvry A, Cole DC. Global burden of chronic pulmonary aspergillosis as a sequel to pulmonary tuberculosis. Bull World Health Organ. 2011;89:864–72.
18. Denning DW, Pleuvry A, Cole DC. Global burden of allergic bronchopulmonary aspergillosis with asthma and its complication chronic pulmonary aspergillosis in adults. Med Mycol. 2013;51:361–70, http://dx.doi.org/10.3109/13693786.2012.738312.
19. Dudakova A, Spiess B, Tangwattanachuleeporn M, Sasse C, Buchheidt D, Weig M, et al. Molecular tools for the detection and deduction of azole antifungal drug resistance phenotypes in *Aspergillus* species. Clin Microbiol Rev. 2017;30:1065–91, http://dx.doi.org/10.1128/CMR.00095-16.
20. Edgar RC. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinform. 2004;5:113, http://dx.doi.org/10.1186/1471-2105-5-113.
21. Escribano P, Recio S, Peláez T, Bouza E, Guinea J. *Aspergillus fumigatus* strains with mutations in the cyp51A gene do not always show phenotypic resistance to itraconazole, voriconazole, or posaconazole. Antimicrob Agents Chemother. 2011;55:2460–2, http://dx.doi.org/10.1128/aac.01358-10.
22. Espinel-Ingroff A, Diekema DJ, Fothergill A, Johnson E, Pelaez T, Pfaller MA, et al. Wild-type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). J Clin Microbiol. 2010;48:3251–7, http://dx.doi.org/10.1128/jcm.00536-10.
23. Georgiadou SP, Kontoyiannis DP. The impact of azole resistance on aspergillosis guidelines. Ann N Y Acad Sci. 2012;1272:15–22, http://dx.doi.org/10.1111/j.1749-6632.2012.06795.x.
24. Glass NL, Donaldson GC. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microbiol. 1995;61:1323–413, http://dx.doi.org/10.1128/aem.61.4.1323-1330.1995.
25. Gonçalves P, Melo A, Dias M, Almeida B, Caetano LA, Veríssimo C, et al. Azole-resistant *Aspergillus fumigatus* harboring the TR34/L98H mutation: first report in Portugal in environmental samples. Microorganisms. 2020;9:57, http://dx.doi.org/10.3390/microorganisms9010057.
26. Hagiwara D, Arai T, Takahashi H, Kusuya Y, Watanabe A, Kamei K. Non-cyp51A azole-resistant *Aspergillus fumigatus* isolates with mutation in HMG-CoA reductase. Emerg Infect Dis. 2018;24:1889–97, http://dx.doi.org/10.3201/eid2410.180730.
27. Hohl TM, Feldmesser M. *Aspergillus fumigatus*: principles of pathogenesis and host defense. Eukaryotic Cell. 2007;6:1953–63, http://dx.doi.org/10.1128/EC.00274-07.
28. Hong SB, Go SJ, Shin HD, Frisvad JC, Samson RA. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. Mycologia. 2005;97:1316–29, http://dx.doi.org/10.1080/15572536.2006.11832738.
29. Houbraken J, Kocsubé S, Visagie CM, Yilmaz N, Wang XC, Meijer M, et al. Classification of *Aspergillus*, *Penicillium*, *Talaromyces* and related genera (Eurotiales): an overview of families, genera, subgenera, sections, series and species. Stud Mycol. 2020;95:5–169, http://dx.doi.org/10.1016/j.simyco.2020.05.002.
30. Isla G, Leonardelli F, Tiraboschi IN, Refojo N, Hevia A, Vivot W, et al. First clinical isolation of an azole-resistant *Aspergillus fumigatus* isolate harboring a TR46/Y121F/T289A mutation in south America. Antimicrob Agents Chemother. 2018;62, http://dx.doi.org/10.1128/aac.00872-18.
31. Kidd SE, Goeman E, Meis JF, Slavin MA, Verweij PE. Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia. Mycoses. 2015;58:350–5, http://dx.doi.org/10.1111/myc.12324.
32. Klaassen CHW, de Valk HA, Balajee SA, Meis JFGM. Utility of CSP typing to sub-type clinical *Aspergillus fumigatus* isolates and proposal for a new CSP type nomenclature. J Microbiol Methods. 2009;77:292–6, http://dx.doi.org/10.1016/j.mimet.2009.03.004.
33. Latgé JP. *Aspergillus fumigatus* and aspergillosis. Clin Microbiol Rev. 1999;12:310–50, http://dx.doi.org/10.1128/cmr.12.2.310.
34. Lestrade PPA, Meis JF, Melchers WJG, Verweij PE. Triazole resistance in *Aspergillus fumigatus*: recent insights and challenges for patient management. Clin Microbiol Infect. 2019;25:799–806, http://dx.doi.org/10.1016/j.cmi.2018.11.027.

35. Lin SJ, Schranz J, Teutsch SM. Aspergillosis case-fatality rate: systematic review of the literature. Clin Infect Dis. 2001;32:358–66, http://dx.doi.org/10.1086/318483.
36. Pagano L, Akova M, Dimopoulos G, Herbrecht R, Drgona L, Blijlevens N. Risk assessment and prognostic factors for mould-related diseases in immunocompromised patients. J Antimicrob Chemother. 2010;66:i5–14, http://dx.doi.org/10.1093/jac/dkq437.
37. Pérez-Cantero A, López-Fernández L, Guarro J, Capilla J. Azole resistance mechanisms in *Aspergillus*: update and recent advances. Int J Antimicrob Agents. 2020;55:105807, http://dx.doi.org/10.1016/j.ijantimicag.2019.09.011.
38. Rajendran R, Mowat E, McCulloch E, Lappin DF, Jones B, Lang S, et al. Azole resistance of *Aspergillus fumigatus* biofilms is partly associated with efflux pump activity. Antimicrob Agents Chemother. 2011;55:2092–7, http://dx.doi.org/10.1128/aac.01189-10.
39. Rüping MJGT, Vehreschild JJ, Cornely OA. Patients at high risk of invasive fungal infections: when and how to treat. Drugs. 2008;68:1941–62, http://dx.doi.org/10.2165/00003495-200868140-00002.
40. Samson RA, Hong S, Peterson SW, Frisvad JC, Varga J. Polyphasic taxonomy of *Aspergillus* section *fumigati* and its teleomorph *Neosartorya*. Stud Mycol. 2007;59:147–203, http://dx.doi.org/10.3114/sim.2007.59.14.
41. Sewell TR, Zhu J, Rhodes J, Hagen F, Meis JF, Fisher MC, et al. Nonrandom distribution of azole resistance across the global population of *Aspergillus fumigatus*. MBio. 2019;10, http://dx.doi.org/10.1128/mBio.00392-19, e00392-19.
42. Snelders E, Karawajczyk A, Schaftenaar G, Verweij PE, Melchers WJG. Azole resistance profile of amino acid changes in *Aspergillus fumigatus* CYP51A based on protein homology modeling. Antimicrob Agents Chemother. 2010;54:2425–30, http://dx.doi.org/10.1128/aac.01599-09.
43. van der Torre MH, Novak-Frazer L, Rautemaa-Richardson R. Detecting azole-antifungal resistance in *Aspergillus fumigatus* by pyrosequencing. J Fungi. 2020;6:12, http://dx.doi.org/10.3390/jof6010012.
44. van Ingen J, van der Lee HA, Rijs TAJ, Zoll J, Leenstra T, Melchers WJG, et al. Azole, polyene and echinocandin MIC distributions for wild-type, TR34/L98H and TR46/Y121F/T289A *Aspergillus fumigatus* isolates in the Netherlands. J Antimicrob Chemother. 2014;70:178–81, http://dx.doi.org/10.1093/jac/dku364.
45. Verweij PE, Ananda-Rajah M, Andes D, Arendrup MC, Brüggemann RJ, Chowdhary A, et al. International expert opinion on the management of infection caused by azole-resistant *Aspergillus fumigatus*. Drug Resist Updat. 2015;21–22:30–40, http://dx.doi.org/10.1016/j.drup.2015.08.001.
46. Verweij PE, Chowdhary A, Melchers WJG, Meis JF. Azole Resistance in *Aspergillus fumigatus*: can we retain the clinical use of mold-active antifungal azoles? Clin Infect Dis. 2015;62:362–8, http://dx.doi.org/10.1093/cid/civ885.
47. White TJ, Bruns TD, Lee SB, Taylor JW. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and applications. New York, NY: USA, Academic Press; 1990. p. 315–22.
48. Wu C, Wang H, Lee J, Lo H, Dai C, Chou P, et al. Azole-resistant *Aspergillus fumigatus* isolates carrying TR34/L98H mutations in Taiwan. Mycoses. 2015;58:544–9, http://dx.doi.org/10.1111/myc.12354.