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ORIGINAL ARTICLE

Distribution of *Aspergillus* species and prevalence of azole resistance in clinical and environmental samples from a Spanish hospital during a three-year study period

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Abstract

Background: Surveillance studies are crucial for updating trends in *Aspergillus* species and antifungal susceptibility information.

Objectives: Determine the *Aspergillus* species distribution and azole resistance prevalence during this 3-year prospective surveillance study in a Spanish hospital.

Materials and Methods: Three hundred thirty-five *Aspergillus* spp. clinical and environmental isolates were collected during a 3-year study. All isolates were screened for azole resistance using an agar-based screening method and resistance was confirmed by EUCAST antifungal susceptibility testing. The azole resistance mechanism was confirmed by sequencing the *cyp*51A gene and its promoter. All *Aspergillus fumigatus* strains were genotyped using TRESPERG analysis.

Results: *Aspergillus fumigatus* was the predominant species recovered with a total of 174 strains (51.94%). The rest of *Aspergillus* spp. were less frequent: *Aspergillus niger* (14.93%), *Aspergillus terreus* (9.55%), *Aspergillus flavus* (8.36%), *Aspergillus nidulans* (5.37%) and *Aspergillus lentulus* (3.28%), among other *Aspergillus* species (6.57%). TRESPERG analysis showed 99 different genotypes, with 72.73% of the strains being represented as a single genotype. Some genotypes were common among clinical and environmental *A. fumigatus* azole-susceptible strains, even when isolated months apart. We describe the occurrence of two azole-resistant *A. fumigatus* strains, one clinical and another environmental, that were genotypically different and did not share genotypes with any of the azole-susceptible strains.

Conclusions: *Aspergillus fumigatus* strains showed a very diverse population although several genotypes were shared among clinical and environmental strains. The isolation of azole-resistant strains from both settings suggest that an efficient analysis of clinical and environmental sources must be done to detect azole resistance in *A. fumigatus*.

KEYWORDS
Aspergillus spp, azole resistance, hospital environment, surveillance, TRESPERG genotyping

1 | INTRODUCTION

Regarding opportunistic fungal pathogens, *Aspergillus* species stand out as major agents, causing a broad spectrum of clinical manifestation named aspergillosis.[1] *Aspergillus fumigatus* is the most frequently isolated species from this genus and is the cause of, among other pathologies, invasive aspergillosis (IA), a critical clinical manifestation of aspergillosis associated with high mortality rates in immunocompromised hosts.[2,3] Currently, triazole drugs are the antifungal of choice for prophylaxis and first-line treatment of *Aspergillus* infections.[4,5] However, the therapeutical options against *A. fumigatus* infections are being reduced as the reports of azole-resistant *A. fumigatus* strains have increased globally during the last decades.[5,6] The development of azole resistance in *A. fumigatus* is caused by selective pressure associated with the employment of azole drugs and has been classically described by two different routes: a medical route that can occur inside the host, in patients that have been treated with long-term azole therapy; and another route related to the environment, where the acquisition of azole resistance happens in the agricultural scenario, due to the use of demethylation inhibitor fungicides (DMIs) to protect crops against fungal plant pathogens.[7,8] Although there are many DMIs used, they share a similar chemical structure to clinical triazoles, thus generating cross-resistance between both antifungal classes.[9,10] Regardless of the development route of azole resistance or the underlying azole mechanism, azole resistance is deeply associated with treatment failure.[5,11]

The 14-α sterol demethylase (Cyp51) is the target of triazole drugs. The mode of action of these antifungals is based on the inhibition of the Cyp51 activity, an enzyme that plays a crucial role in the ergosterol biosynthesis pathway, encoded by the gene *cyp*51A and its homologue *cyp*51B.[12] The majority of the azole resistance mechanisms described have been associated with different point mutations in the *cyp*51A gene, tandem repeat (TR) insertions in its promoter or the combination of both mechanisms.[7,13–15]

Nowadays, two similar reference methods for antifungal susceptibility testing (AFST) are used globally, EUCAST (European Committee on Antibiotic Susceptibility Testing, https://www.eucast.org/) and CLSI (Clinical and Laboratory Standards Institute, https://clsi.org/). Although these methods are validated and standardized, they present some limitations, such as being available only in specialized centres and being slow methodologies, taking at least 5–7 days to be completed. A rapid detection of azole resistance is crucial for increasing the possibilities of therapeutical success and the recovery of the patients. New screening methods, such as four-well azole agar plates, are very useful for detecting azole resistance in *A. fumigatus*,[16–18] being easier, simpler and faster to perform, and they are not restricted to specialized centres, improving the chances of successful clinical outcomes. Although results require confirmation through microdilution susceptibility testing and *cyp*51A sequencing, this method has been recommended by the EUCAST for screening procedures.[19] An expanded version of this method was described and validated by our group,[20] with two types of four-well agar plates, one supplemented with clinical azoles with antifungal concentrations adapted to the last EUCAST breakpoints against *A. fumigatus*,[21] and four-well agar plates containing DMI antifungals. This method could be easily applied to surveillance studies due to the advantages mentioned before.

Moreover, in the last decade, the ECDC (European Center for Disease Prevention and Control) has recommended epidemiological surveillance studies to update locally *A. fumigatus* azole susceptibility information.[22] Several countries have started these surveillance studies, mainly from clinical samples,[23–28] but also, to a lesser extent, including environmental samples.[29–31]

In addition to azole resistance surveillance studies, *A. fumigatus* genotyping is a useful methodology to determine the population structure of this species and to study the epidemiological association between environmental and clinical strains. Several genotyping methods have been described but only two, STRAf and TRESPERG, have been compared and show a good discriminatory power.[32–34] While STRAf assay had a higher discriminatory power ($D=0.9993$) compared to the TRESPERG typing method ($D=0.9972$), the latter can be readily integrated in any clinical microbiology laboratory since it does not demand specialized equipment or trained staff. Both of them are used to determine and analyze genetic distances and have proven to be powerful instruments for *A. fumigatus* molecular typing.[34]

In this study, we aim to determine the distribution of *Aspergillus* species and prevalence of azole resistance using a 3-year prospective collection of clinical and environmental strains from the Severo Ochoa Universitary Hospital in Madrid. Also, we analyze the genetic relatedness of *A. fumigatus* strains isolated from clinical samples and those that coexist in the hospital environment.

2 | MATERIALS AND METHODS

2.1 | *Aspergillus* spp. strains

A total of 335 *Aspergillus* spp. strains were analyzed: 283 clinical and 52 environmental isolates. All *Aspergillus* isolates were cultured using standardized mycological procedures and identified at the section or species level based on local routine procedures (i.e. phenotypic identification and/or sequencing). To extract *Aspergillus* DNA, conidia from every strain were cultured in liquid glucose–yeast extract–peptone (GYEP) medium (0.3% yeast extract, 1% peptone; Difco, Soria Melguizo, Madrid, Spain) containing 2% glucose (Sigma-Aldrich Química, Madrid, Spain) at 37°C for 24 h. After disrupting the mycelium mechanically through vortex-mixing with glass beads, the genomic DNA of the isolates was extracted using the phenol–chloroform method.[35] All *Aspergillus* spp. strains identified from the Severo Ochoa Universitary Hospital were sent to the National Centre for Microbiology to screen for azole resistance and to genotype *A. fumigatus* strains.

2.2 | Environmental surveillance

Environmental air samples were obtained using an air sampler AESAP1075 (Sampl'air Lite, AES Laboratories). Two samples of 1 m^3 of air were captured per day of testing, one at the entrance

of a hospital room and another at the centre of the room selected. Sabouraud/Gentamicin (28 µg/mL)/Chloramphenicol (240 µg/mL) agar plates, irradiated with the air sample, were sealed and incubated at 35°C for 5 days. The environmental surveillance was performed by the preventive medicine department at the hospital. The air sampling procedure was sometimes altered by repetitions due to internal cleaning protocols, COVID-19 impact and construction work inside the hospital. Due to this, the number of air samples was irregular during the environmental surveillance: 230 air samples in 2019, 222 air samples in 2020 and 317 air samples in 2021.

2.3 | Agar-based screening plates

A method consisting of two sets of four-well agar plates was used to screen for azole resistance in all strains of the study.[20] The strains were considered resistant to every specific antifungal if the growth observed in the drug-containing wells was like that of the growth control. All isolates that showed growth in the agar plates were considered possible azole-resistant strains and were evaluated for AFST using the EUCAST.

2.4 | Microdilution antifungal drugs susceptibility testing

Antifungal susceptibility testing was performed following the EUCAST broth microdilution reference method 9.4.[36] Antifungals used were the azoles ITC, VRC, POS and isavuconazole (ISV) (all from Sigma-Aldrich Química). MICs were performed at least twice for each isolate. Clinical breakpoints for interpreting AFST results established by EUCAST[37] were used for classifying the *A. fumigatus* strains as susceptible or resistant. *Aspergillus* isolates with an MIC above the usual epidemiological cut off values for at least one of the mould-active triazoles (VRC, POS and ITC) were submitted to sequencing of the entire *cyp*51A gene and promoter region for detection of mutations.

2.5 | PCR conditions for *cyp*51A amplification and sequencing

The full coding sequence of *cyp51A* including its promoter was amplified and sequenced, using primers and PCR conditions previously described.[12] To exclude the possibility that any change identified in the sequences was due to PCR-induced errors, each isolate was independently analyzed twice. The amplified products were purified using Illustra ExoProStar 1–step (GE Healthcare Life Science, Buckinghamshire, UK) and both strands were sequenced with the Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions. All gene sequences were edited and assembled using Lasergene software package (DNAStar Inc., Madison, WI, USA). DNA sequences were compared against the *cyp*51A sequence of *A. fumigatus* reference strain CBS 144.89 (NCBI accession number AFUB_063960). The GenBank accession numbers for the *cyp*51A DNA sequences from both resistant strains are H100 PP392543 and H208 PP392544.

2.6 | Strains genotyping and genotypic diversity analysis

All *A. fumigatus* strains included in this study were genotyped following the previously described typing method TRESPERG.[34] The combination of the genotypes obtained with each marker has a discriminatory value (*D*) of 0.9972 using the Simpson index.[38] The genotypic diversity analysis was performed as described previously[33] and was represented graphically using a minimum spanning tree (MST) generated with the combination of TRESPERG typing data analyzed by BioNumerics (version 6.0.1) software (Applied Maths, Belgium). The date of isolation, source and genotype of all the strains of the study are displayed in Table S1. The GenBank accession numbers for all four TRESPERG loci have been added in Table S2. The year of isolation, geographical origin and genotypes of the *A. fumigatus* azole-resistant strains, all harbouring TR_{34}/L98H mutation, isolated in Spain from 2012 to 2023, were included for comparison (Table S3).

3 | RESULTS

3.1 | *Aspergillus* spp. strains

During the 3-year study period, 335 *Aspergillus* spp. isolates from 283 clinical samples and 52 environmental samples were included. *A. fumigatus* was the predominant species recovered (174 isolates, 51.94%), followed by *Aspergillus niger* (50 isolates, 14.93%), *Aspergillus terreus* (32 isolates, 9.55%) and *Aspergillus flavus* (28 isolates, 8.36%). Several other less frequent species were identified, including *Aspergillus nidulans* (18 isolates, 5.37%) and *Aspergillus lentulus* (11 isolates, 3.28%). Other species identified to a lesser extent were *Aspergillus calidoustus* (3 isolates, 0.89%), *Aspergillus carneus* (1 isolate, 0.3%), *Aspergillus unguis* (5 isolates, 1.49%), *Aspergillus candidus* (2 isolates, 0.6%), *Aspergillus versicolor* (3 isolates, 0.89%), *Aspergillus fumigatiaffinis* (1 isolate, 0.3%), *Neosartorya udagawae* (3 isolates, 0.89%), *Aspergillus ochraceus* (3 isolates, 0.89%) and *Aspergillus sydowii* (1 isolate, 0.3%) (Table 1).

3.2 | Agar-based screening plates

All strains tested grew in the control well without azole drug. Only two *A. fumigatus* strains grew in all wells supplemented with clinical azole drugs (ITZ, VCZ and POS) and in the agar wells supplemented with the DMIs, MET and EPZ, a pattern that we have previously reported as a possible underlying azole resistance mechanism *Cyp*51A dependant

TABLE 1 *Aspergillus* species, number and percentages (%) of strains isolated by the 3 years study period.

	No. of strains isolated (%)							
	2019		2020		2021		Total	
Aspergillus species	C	E	C	E	C	E	C	E
A. fumigatus	39 (41.05)	5 (5.3)	34 (35.8)	5 (5.3)	66 (45.5)	25 (17.2)	139 (41.5)	35 (10.45)
A. niger	13 (13.7)	1 (1.05)	13 (13.7)	0	14 (9.65)	9 (6.2)	40 (11.9)	10 (3)
A. terreus	8 (8.4)	0	13 (13.7)	1 (1.05)	10 (6.9)	0	31 (9.25)	1 (0.3)
A. flavus	12 (12.6)	1 (1.05)	12 (12.6)	0	3 (2.1)	0	27 (8.1)	1 (0.3)
A. nidulans	3 (3.2)	0	5 (5.3)	1 (1.05)	7 (4.8)	2 (1.4)	15 (4.5)	3 (0.9)
A. lentulus	6 (6.3)	0	5 (5.3)	0	0	0	11 (3.3)	0
A. calidoustus	2 (2.1)	0	0	0	1 (0.7)	0	3 (0.9)	0
A. carneus	1 (1.05)	0	0	0	0	0	1 (0.3)	0
A. unguis	1 (1.05)	0	2 (2.1)	0	0	2 (1.4)	3 (0.9)	2 (0.6)
A. candidus	2 (2.1)	0	0	0	0	0	2 (0.6)	0
A. versicolor	1 (1.05)	0	1 (1.05)	0	1 (0.7)	0	3 (0.9)	0
A. fumigatiaffinis	0	0	1 (1.05)	0	0	0	1 (0.3)	0
N. udagawae	0	0	2 (2.1)	0	1 (0.7)	0	3 (0.9)	0
A. ochraceus	0	0	0	0	3 (2.1)	0	3 (0.9)	0
A. sydowii	0	0	0	0	1 (0.7)	0	1 (0.3)	0
Total	88 (92.6)	7 (7.4)	88 (92.6)	7 (7.4)	107 (73.8)	38 (26.2)	283 (84.5)	52 (15.5)

Abbreviations: C, Clinical origin, E, Environmental origin;

(TR_{34}/L98H or TR_{53}). Although this screening method was explicitly designed to screen for azole resistance in *A. fumigatus*, we considered that it could be useful for the surveillance to use it with the rest of non-*fumigatus Aspergillus* species isolated in this study.

3.3 | Antifungal drugs susceptibility testing

Strains that were positive in the four-well screaning assay were subjected to azole drugs AFST (EUCAST). Two strains of *A. fumigatus* showed azole-resistant MICs, consisting of >8 mg/L to itraconazole, 4 mg/L to voriconazole, 0.5 mg/L to posaconazole and 8 mg/L to ISV. The rest of the strains tested were all azole-susceptible. Thus, the prevalence of azole-resistant strains in this study was 0.6% (2 of 335 strains) and the prevalence of azole-resistant *A. fumigatus* was 1.15% (2 of 174 *A. fumi*gatus strains).

3.4 | Amplification and Sequence Analysis of *cyp*51A

The two *A. fumigatus* azole-resistant strains (H-100 and H-208) were subjected to amplification and sequencing of the complete *cyp*51A gene. Sequence analysis revealed the same azole resistance mechanism in both strains, consisting of a 34-bp TR insertion in the promoter region of *cyp*51A together with a L98H substitution in the coding sequence of the gene (TR_{34}/L98H).

3.5 | Genotypic variability in environmental and clinical samples

The 174 *A. fumigatus* strains were genotyped, although seven of them could not be amplified in one of the TRESPERG markers and were therefore excluded from the genotipic analysis. The TRESPERG genotypes of these 167 strains can be found in Table S1. Out of the 167 strains, 31 were excluded from genotypic analysis because they share the same genotype as other strains from the same patient or were from the same day environmental search. Finally, 136 strains were included in the genotypic analysis. A total of 99 different genotypes were identified according to the TRESPERG typing assay. The TRESPERG results showed a very diverse population with 72.73% of the total genotypes being represented as a single genotype. The *A. fumigatus* clinical strains showed less diversity than the ones from environmental origin (Table 2).

The genotypic diversity of the *A. fumigatus* strains from clinical and environmental origin was graphically represented using a MST (Figure 1).

The strains were distributed in different clusters regardless of their origin, including strains from clinical and environmental origins in each cluster defined. Among the remaining 27 genotypes that were not unique, 15 genotypes were common among clinical *A. fumigatus* strains and two genotypes were shared among environmental *A. fumigatus* strains, some of them being isolated several times, even months apart. Ten of these 27 common genotypes

TABLE 2 *Aspergillus fumigatus* genotypes found in air and in clinical samples collected during the 3 years of the study.

Sample source	No. of strains	No. of single genotypes	Diversity (%)
Clinical	102	60	58.82
Environmental	34	26	76.47

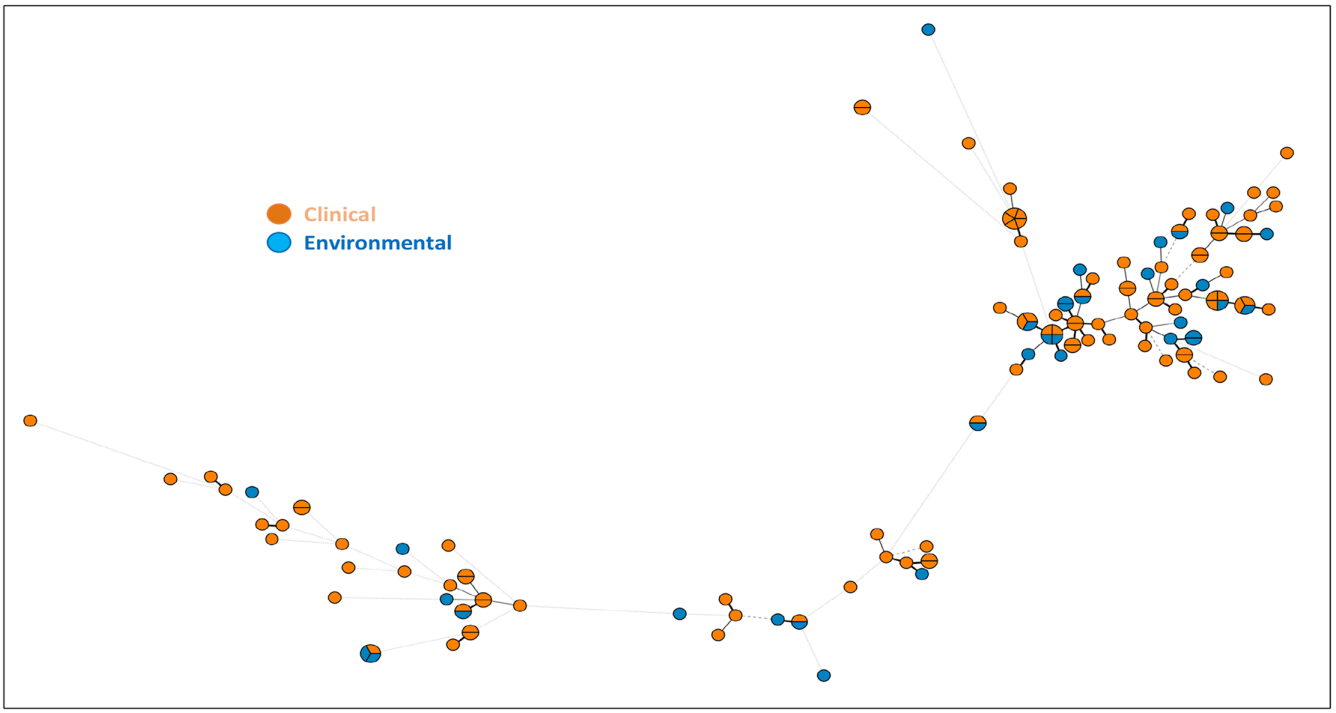

FIGURE 1 Minimum spanning tree showing the genotypic diversity *Aspergillus fumigatus* strains from clinical (in orange) and environmental (in blue) origin. Each circle shows a unique genotype, and its size represents the number of strains belonging to the same genotype. Connecting lines between circles show the similarity between genotypes: solid and bold (shaded in black) indicate only one marker difference, a solid line indicates differences in two markers, and dashed lines for differences in three or more markers.

were shared between clinical and environmental strains (Table 3). Also, some of these genotypes were common between clinical strains from diferent patients and environmental surveillance collected on different days.

The two azole-resistant *A. fumigatus* strains were not isogenic, having different genotypes: (1) the strain from the environment (t04Bm1.2c22be07) and (2) the patient strain (t02Am1.1c09e11). None of the genotypes were coincident with any of the genotypes found in the azole-susceptible *A. fumigatus* strains of this study. The genotypic diversity of azole-susceptible and azole-resistant *A. fumigatus* strains from this study was evaluated using a collection of azole-resistant *A. fumigatus* strains, harbouring the TR_{34}/L98H azole resistance mechanism, from different locations in Spain that were isolated between 2012 and 2023 (Table S3) and represented with a MST (Figure 2). The genotypes of the two azole-resistant strains from this study shared genotypes with azole-resistant *A. fumigatus* strains previously isolated in some locations in Spain. The azole-susceptible strains were widely distributed across the MST and all the azole-resistant strains were highly related and most of them grouped together in close clusters.

4 | DISCUSSION

The spectrum of pathologies caused by *Aspergillus* species is named aspergillosis with IA as one of the most critical diseases due to its high mortality rates among immunocompromised hosts.[1,39,40] *Aspergillus fumigatus* is the most frequently isolated species among the *Aspergillus* genus in different parts of the world.[23,24,26,28,31] Similarly, in our 3-year surveillance results, out of a total of 335 *Aspergillus* spp. isolates, more than half (51.94%) of the strains were identified as *A. fumigatus*. This result was similarly found in other Spanish surveillance studies, although the prevalence order of the rest of *Aspergillus* species differs.[23,24] In our study, the number of *A. fumigatus* isolates was followed by *A. niger*, *A. terreus* and *A. flavus*.

Nowadays, the rise of *A. fumigatus* azole-resistant strains has become globally alarming,[7] representing a severe threat to a successful clinical outcome, because azole resistance is closely associated to treatment failure and a higher mortality rate.[5,11,41,42] In response to this urgent issue, the ECDC has declared that epidemiological surveillance studies are a useful tool to provide local information regarding *A. fumigatus* azole susceptibility

TABLE 3 Genotypes shared between clinical and environmental *Aspergillus fumigatus* strains.

			TRESPERG typing			
Strain ID	**Date of isolation**	**Source**	**CSP**	**MP2**	**CFEM**	**ERG**
H122	2020/03/06	Clinical	t01	m5.3	c08B	e07
H287	2021/07/21	Environmental	t01	m5.3	c08B	e07
H285	2021/07/19	Environmental	t03	m1.1	c05A	e07
H293	2021/08/02	Clinical	t03	m1.1	c05A	e07
H296	2021/08/11	Clinical	t03	m1.1	c05A	e07
H33	2019/05/15	Clinical	t03	m1.1	c08A	e07
H76	2019/10/28	Clinical	t03	m1.1	c08A	e07
H203	2021/02/16	Environmental	t03	m1.1	c08A	e07
H333	2021/12/13	Environmental	t03	m1.1	c08A	e07
H225	2021/04/01	Environmental	t03	m1.3	c08A	e09
H232	2021/04/13	Clinical	t03	m1.3	c08A	e09
H213	2021/03/02	Clinical	t03	m2.3	c07	e06
H283	2021/07/19	Environmental	t03	m2.3	c07	e06
H137	2020/06/23	Environmental	t04A	m1.1	c12	e15
H140	2020/07/06	Clinical	t04A	m1.1	c12	e15
H20	2019/03/02	Clinical	t04A	m1.3	c08A	e07
H108	2020/02/12	Clinical	t04A	m1.3	c08A	e07
H192	2021/01/25	Environmental	t04A	m1.3	c08A	e07
H141	2020/07/15	Clinical	t04A	m1.3	c08B	e07
H217	2021/03/19	Clinical	t04A	m1.3	c08B	e07
H230	2021/04/08	Clinical	t04A	m1.3	c08B	e07
H274	2021/07/09	Environmental	t04A	m1.3	c08B	e07
H96	2020/01/07	Environmental	t04A	m3.4	c20	e11
H97	2020/01/03	Clinical	t04A	m3.4	c20	e11
H278	2021/07/12	Environmental	t09	m5.3	c10	e07
H279	2021/06/24	Clinical	t09	m5.3	c10	e07
H282	2021/07/19	Environmental	t09	m5.3	c10	e07

Note: Date of isolation: yyyy/mm/dd.

The shades are to make more relevant *Aspergillus* types that are identical between clinical and enviromental strains.

levels.[22] In Spain, multiple studies have evaluated the distribution of *Aspergillus* species and the prevalence of azole resistance from clinical samples, but most of these studies were limited due to a lack of environmental samples,[23,24,27] although a few studies have included these type of isolates.[29,31] The inclusion of environmental isolates has been reinforced by the finding of the hospital setting as a hypothetical source of dissemination of azole-resistant *A. fumigatus*.[43]

Broth microdilution reference methodologies can constitute a considerable laborious and time-consuming way to perform surveillance studies. The employment of four-well screening methods is affordable and simple to perform in any mycology laboratory and can easily detect azole-resistant *A. fumigatus* strains.[19] Moreover, they can presumably identify the resistance mechanism involved,[20] despite the fact that those strains considered as resistant have to be confirmed by AFST and *cyp51A* sequencing. The four-well agar expanded method[20] has been used in this study and it permits for screening the entire collection of strains included, and it detected two *A. fumigatus* azole-resistant strains from clinical and environmental origin. Both strains were pan-azole-resistant and harboured a TR_{34}/L98H azole resistance mechanism, the most frequent azole resistance mechanism described in *A. fumigatus*.[7,13,14] The prevalence of azole-resistant *A. fumigatus* in this study was 1.15% (2 of 174 *A. fumigatus* strains), a low rate compared to other locations in Europe,[28,44–46] although it fits within previous studies in Spain which range from the rare occurrence under 1% in 2010–2011[23] and 1.2% in 2016[24] to 5.5% in the most recent study in 2021.[31]

In surveillance studies, genotyping assays are a very useful tool to understand the distribution and dynamics of *A. fumigatus* in both clinical and environmental settings.[47–50] TRESPERG has been employed in this study to genotype *A. fumigatus*, taking advantage of its good discriminatory power and simplicity.[33,34] The results of

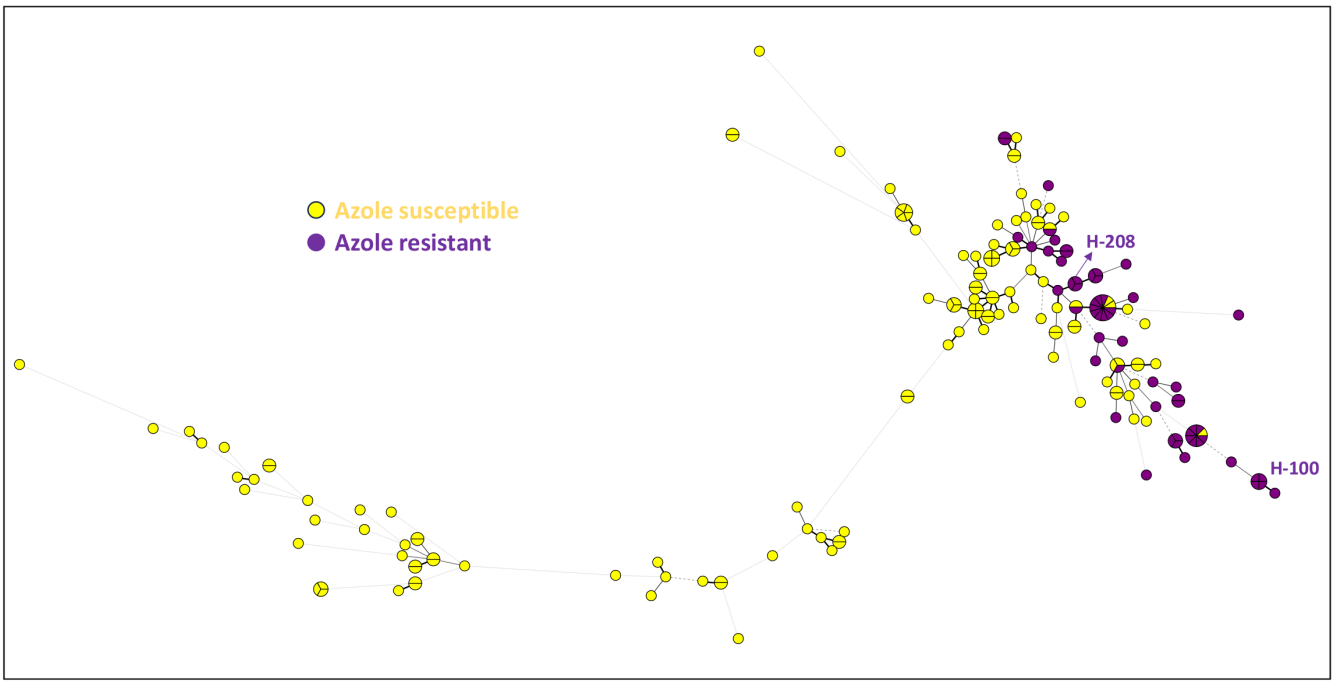

FIGURE 2 Minimum spanning tree showing the genotypic diversity of azole-susceptible (yellow) and azole-resistant (purple) *A. fumigatus* strains. Each circle shows a unique genotype, and its size shows the number of strains belonging to the same genotype. Connecting lines between circles show the similarity between genotypes: solid and bold (shaded in black) indicate only one marker difference, a solid line indicates differences in two markers, and dashed lines for differences in three or more markers. The two azole-resistant strains obtained in this work are indicated: H-100 and H-208.

genotyping showed that the genotypes of the strains included in this study are very diverse, with 72.73% being a single genotype. Although the diversity of the genotypes was high in both settings, we found that it was higher among strains with an environmental origin, in consonance with the findings in other studies.[49,51,52] However, this result should be taken cautiously because there is a considerable difference in the numbers of clinical/environmental strains included in the study.

According to the genotyping results, the two azole-resistant *A. fumigatus* strains had different genotypes and did not share their genotype with any azole-susceptible *A. fumigatus* strains found in this study. Furthermore, the azole-resistant *A. fumigatus* strain isolated from a clinical source comes from an azole-naïve patient, so the development of the azole resistance mechanism could not possibly happen inside the host since there was not selective pressure. Recently, azole-resistant *A. fumigatus* isogenic strains have been found in a patient and in their bathroom, which suggests two hypotheses: that the environmental setting could be contaminated with azole-resistant *A. fumigatus* that could colonise the patient; or that the patient was the source of the environmental contamination.[43] Other studies have found similar situations to these two hypotheses.[53–55] A very interesting finding in this study is that several genotypes were shared between clinical and environmental strains, in alignment with the different hypotheses proposed before. Moreover, if we had tracked the different locations of the environmental captures and the locations of the patients, we could have determined if patients hospitalized in different parts of the same hospital were infected with the same spore population as other studies have found.[49]

The two azole-resistant strains were genotypically different and with no genetic relation with the rest of the azole-susceptible strains included in the study. However, the genotypic comparison that include the collection of azole-resistant *A. fumigatus* strains from different locations in Spain showed that all azole-resistant strains grouped in very close clusters, as has been previously described in other studies.[56–58] Although the reason why these strains that harbour TR_{34}/L98H azole resistance mechanism are so genetically related remains unclear, a better adaptation to persist in the environment or a relation with *A. fumigatus* genetic instability have both been suggested.[59,60]

5 | CONCLUSIONS

Resistance of the human pathogenic fungus *A. fumigatus* to azole drugs is rising. However, the link between patient infections and their potential acquisition from hospital environmental sources remains vague. In this work, we used two recent methodological techniques that for their simplicity allow for easy integration into any clinical microbiology laboratory, fulfilling all the needs of surveillance for azole resistance, combined with a suitable typing assay. In this study, we found that *A. fumigatus* genotypes were highly diverse in both settings, emphasizing the highly mixed nature of *A. fumigatus* populations. However, identical clonal genotypes were found to occur both in the clinical strains and in the

environmental sampling, suggesting that patients hospitalized in different parts of the same hospital can be infected with the same strain as every patient might inhale the same spore population. The isolation of azole-resistant strains from a patient and from the hospital environment is an interesting finding, encouraging more analysis of clinical and environmental sources to detect azole resistance in *A. fumigatus*.

AUTHOR CONTRIBUTIONS

Jose Lucio: Methodology; validation; investigation; writing – original draft; data curation; writing – review and editing. **Laura Alcazar-Fuoli:** Writing – review and editing; data curation; formal analysis; methodology. **Horacio Gil:** Methodology; formal analysis; data curation; writing – review and editing. **Samuel Cano-Pascual:** Methodology; investigation. **Sara Hernandez-Egido:** Methodology; investigation. **Maria Soledad Cuetara:** Writing – review and editing; validation; investigation; formal analysis. **Emilia Mellado:** Conceptualization; writing – review and editing; writing – original draft; funding acquisition; project administration; resources; supervision; validation.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The GenBank accession numbers for all four TRESPERG loci have been added in Table S2. The GenBank accession numbers for the *cyp*51A DNA sequences from both resistant strains are H100 PP392543 and H208 PP392544.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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