

An expanded agar-based screening method for azole-resistant *Aspergillus fumigatus*

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Abstract

Antifungal susceptibility testing is an essential tool for guiding antifungal therapy. Reference methods are complex and usually only available in specialised laboratories. We have designed an expanded agar-based screening method for the detection of azole-resistant *Aspergillus fumigatus* isolates. Normally, identification of resistance mechanisms is obtained only after sequencing the *cyp51A* gene and promoter. However, our screening method provides azole resistance detection and presumptive resistance mechanisms identification. A previous agar-based method consisting of four wells containing voriconazole, itraconazole, posaconazole and a growth control, detected azole resistance to clinical azoles. Here, we have modified the concentrations of voriconazole and posaconazole to adapt to the updated EUCAST breakpoints against *A. fumigatus*. We have also expanded the method to include environmental azoles to assess azole resistance and the azole resistance mechanism involved. We used a collection of *A. fumigatus* including 54 azole-resistant isolates with Cyp51A modifications (G54, M220, G448S, TR₅₃, TR₃₄/L98H, TR₄₆/Y121F/T289A, TR₃₄/L98H/S297T/F495I), and 50 azole susceptible isolates with wild-type Cyp51A. The screening method detects azole-resistant *A. fumigatus* isolates when there is growth in any of the azole-containing wells after 48h. The growth pattern in the seven azoles tested helps determine the underlying azole resistance mechanism. This approach is designed for surveillance screening of *A. fumigatus* azole-resistant isolates and can be useful for the clinical management of patients prior to antifungal susceptibility testing confirmation.

KEYWORDS

Aspergillus fumigatus, azole resistance, Cyp51A mutations, screening method, surveillance studies

1 | INTRODUCTION

Aspergillus fumigatus is the most representative species among the genus *Aspergillus*. This ubiquitous mould is the cause of different diseases named aspergillosis, among which invasive aspergillosis (IA) stands out as of the most critical clinical manifestation due to its high mortality rates in immunocompromised hosts.^{1–4} Triazoles are the primary clinical choice for prophylaxis and treatment of these

pathologies.⁵ Currently, four triazole drugs are used: itraconazole (ITC), voriconazole (VRC), posaconazole (POS) and more recently isavuconazole (ISV). *A. fumigatus* strains resistant to all of them have been reported, and there are different cross-resistance patterns depending on the resistance mechanism involved. Nowadays, the treatment of *Aspergillus* infections is being compromised in some geographical areas as a consequence of the worldwide resistance spread.⁶ Classically, the development of azole resistance in *A. fumigatus* has

been attributed to two different routes: (i) a medical route, involving in vivo or in-host acquisition, that can happen after a prolonged period of azole therapy and (ii) an environmental route, regarding the acquisition of resistance in the agricultural setting due to the use of demethylation inhibitor fungicides (DMIs) for crop protection.⁷ In the agricultural setting, different DMIs are used to combat fungal plant pathogens; however, due to the similar chemical structure to clinical azoles used as therapy, cross-resistance between both types of azole drugs is the rule.^{8–10}

Azole drugs target the 14- α sterol demethylase (Cyp51), inhibiting the activity of an enzyme that plays a key role in the ergosterol biosynthesis pathway.¹¹ In *A. fumigatus*, this enzyme is encoded by two paralogous genes (*cyp51A* and *cyp51B*).¹² Despite *cyp51B*, nowadays most of the azole resistance mechanisms described have been associated with several point mutations in the *cyp51A* gene, tandem repeat (TR) insertions in the promoter of *cyp51A* or a combination of both point mutations and TRs.^{6,7} The different Cyp51A modifications are responsible for different azole susceptibility profiles. In the case of G54 mutations, elevated minimum inhibitory concentrations (MICs) to ITC, POS and unaffected MICs to VRC are the rule.^{13–15} Point mutations at M220 show a susceptibility pattern of resistance to ITC and reduced susceptibility to VRC and POS.¹⁶ Strains harbouring the point mutation G448S show resistance to VRC and reduced susceptibility to ITC and POS.¹⁷ However, those *A. fumigatus* strains with TR insertions in the promoter of *cyp51A* with a combination of different point mutations in the coding sequence (TR₃₄/L98H, TR₃₄/L98H/S297T/F495I, TR₄₆/Y121F/T289A and TR₅₃) commonly share a resistance pattern to all clinical azole drugs.^{18–22} However, different DMI susceptibility profiles have been found in the different *A. fumigatus* population groups based on their azole resistance mechanism and a previous whole genome sequencing (WGS) analysis, which suggests that the different resistance mechanisms have different origins and are specifically associated with the local use of a particular DMI.²³ MICs profiles to both classes of DMIs, imidazoles and triazoles, are generally high, excluding strains harbouring G54 mutations, but the pattern of cross-resistance depend on the different Cyp51A modifications. Strains harbouring G448S mutations are specifically resistant to triazoles DMIs and strains with TR₄₆/Y121F/T289A and TR₃₄/L98H/S297T/F495I showed high MICs to imidazoles.²³

There are two reference methods for antifungal susceptibility testing (EUCAST and CLSI),^{24,25} but they are only available in specialised centres. More than a decade ago, a method consisting of 4-well azole agar plates containing ITC, VRC, POS, and a growth control was designed as a screening test to detect *A. fumigatus* azole-resistant strains.²⁶ Since then, it has been adopted for clinical and environmental testing studies to evaluate azole resistance in *A. fumigatus*.^{27–29} Even the European Committee on Antimicrobial Susceptibility Testing (EUCAST) has recently published recommendations for the screening procedure.³⁰ However, since the method was developed using concentrations of drugs according to the breakpoints in use at that moment, a number of major errors have occurred with voriconazole and posaconazole.^{31,32} These errors are probably due to the concentration of the drug in the agar plate are

higher than the revised EUCAST voriconazole and posaconazole breakpoints (1 mg/L and 0.25 mg/L, respectively).³³

In this study, we designed an expanded agar-based screening method including the previous three clinical azole drugs, with lower concentrations for voriconazole and posaconazole to adapt to the updated EUCAST breakpoints against *A. fumigatus*.³³ In addition, we have included four DMIs used in crop protection (prochloraz (PCZ), imazalil (IMZ), metconazole (MET) and epoxiconazole (EPZ)) to determine the *A. fumigatus* azole resistance profile and to presumptively identify the responsible azole resistance mechanism. We validated the proposed method using a collection of well-characterised *A. fumigatus* isolates with known azole resistance mechanisms.

2 | MATERIALS AND METHODS

2.1 | *Aspergillus fumigatus* strain collection

A total of 104 unrelated *A. fumigatus* strains with environmental and clinical origin were used in this study. All isolates were identified to the species level by PCR amplification and sequencing of ITS and β -tubulin genes.³⁴ Fifty isolates had a susceptible azole phenotype and a wild-type (WT) Cyp51A sequence. The other 54 isolates harboured known azole resistance mechanisms consisting of Cyp51A mutations G54 (11 isolates), M220 (6 isolates), G448S (7 isolates), TR₃₄/L98H (22 isolates), TR₅₃ (2 isolates), TR₃₄/L98H/S297T/F495I (2 isolates) and TR₄₆/Y121F/T289A (4 isolates).

2.2 | Characterisation of azole resistance molecular mechanisms in *A. fumigatus* strains

Mechanisms of azole resistance were characterised by sequencing the main *A. fumigatus* azole target, the *cyp51A* gene. Conidia from each strain were cultured in 3 ml of GYEP liquid medium (0.3% yeast extract, 1% peptone; Difco, Soria Melguizo, Madrid, Spain) with 2% glucose (Sigma-Aldrich Química) and grown overnight at 37°C, after which mycelium mats were harvested and DNA was extracted.³⁵ The full *cyp51A* sequence, including its promoter, was amplified and sequenced for every isolate using the PCR conditions described before.²⁰ DNA sequences were compared against the *cyp51A* sequence of *A. fumigatus* reference strain CBS 144.89 (NCBI accession number AFUB_063960). Several representative strains for each known azole resistance mechanism were included in this work. The geographical origin and genotype of all the strains are displayed in Table S1.

2.3 | Microdilution antifungal susceptibility testing

2.3.1 | Clinical azoles

The minimal inhibitory concentrations (MICs) were determined using a broth micro-dilution method following EUCAST

reference method 9.3.2.²⁴ The clinical azoles used were ITC (Janssen Pharmaceutica), VRC (Pfizer SA) and POS (Merck Sharp & Dohme Corp.). The final concentrations tested ranged from 0.015 to 8 mg/L. *A. flavus* ATCC 204304 and *A. fumigatus* ATCC 204305 were used as quality control strains. MICs were visually read after 24 and 48 h of incubation at 37°C in a humid atmosphere. Clinical breakpoints for interpreting microdilution antifungal susceptibility testing (AFST) results have been established by EUCAST for *A. fumigatus* to ITC, VRC and POS. These breakpoints were used for classifying each isolate as susceptible (S) or resistant (R) to each antifungal, in this case, ITC and VRC ($S \leq 1$, $R > 1$) or POS ($S \leq 0.125$, $R > 0.25$). These clinical breakpoints were updated in February 2020 after the introduction of the concept of area of technical uncertainty (ATU). This new classification was designed as a warning to those problematic MICs overlapping the susceptible and resistant category. The uncertainty of this category gives an instruction to categorise and report the susceptibility based on the MICs to the other azoles tested.³³ [<https://www.eucast.org/astoffungi/clinicalbreakpointsforantifungals/>].

2.3.2 | Agricultural azoles (DMIs)

Antifungal DMIs used were two imidazole drugs, prochloraz (PCZ) and imazalil (IMZ), and two triazole compounds, metconazole (MET) and epoxiconazole (EPZ), all purchased at Sigma-Aldrich. All antifungal drugs were dissolved in DMSO and autosterilised for 30 min at room temperature, as stated in the EUCAST guidelines (<https://eucast.org/>). The final concentrations tested ranged from 0.06 to 32 mg/L. Clinical breakpoints for interpreting AFST results have not been established for these drugs, so isolates were considered susceptible or resistant based on the MICs shown by the group of *A. fumigatus* azole-susceptible strains.

2.4 | Agar-based screening plates

Four-well plates (Nunc™ Non-Treated Multidishes, Thermo Scientific) were prepared containing 1 mL of RPMI 1640%–2% glucose agar. One set of plates (Figure 1A) was supplemented with clinical antifungals—4 mg/L ITC, 1 mg/L VRC, 0.25 mg/L POS and no azole drug as a growth control. The other set of plates (Figure 1B) was supplemented with DMI antifungals—16 mg/L IMZ, 16 mg/L PCZ, 1 mg/L MET and 32 mg/L EPZ. The azole concentrations in the plates for VRC (1 mg/L) and POS (0.25 mg/L) have been reduced one dilution from what was previously recommended VRC (2 mg/L) and POS (0.5 mg/L).³⁰ This modification was done based on the new concept of ATU (Area of Technical Uncertainty) to ensure that strains growing at concentrations compatible with this new concept were not missed.³³ Detailed media and antifungal drug preparation are described in Appendix A.

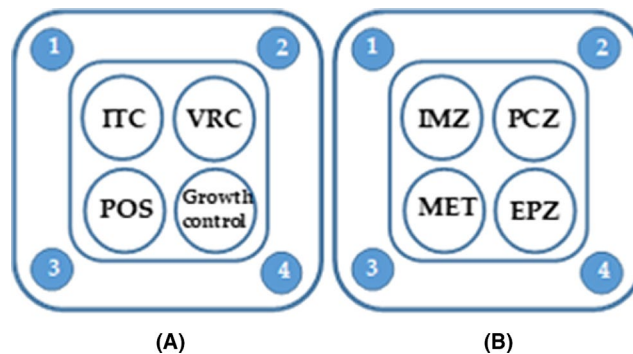


FIGURE 1 Four-well plates containing RPMI-agar media and (A) clinical antifungals: itraconazole (ITC, well 1), voriconazole (VRC, well 2), posaconazole (POS, well 3) and a drug-free control growth well (well 4), and (B) DMI antifungals: imazalil (IMZ, well 1), prochloraz (PCZ, well 2), metconazole (MET, well 3) and epoxiconazole (EPZ, well 4)

2.5 | Inoculation of screening plates

The *A. fumigatus* strains were subcultured on potato dextrose agar (PDA) plates (Oxoid, Madrid, Spain) and incubated at 37°C until the colonies were visibly sporulated (24–48 h). Screening plates were inoculated using a swab soaked in H₂O supplemented with Tween at 0.1% by rubbing colonies from PDA plates and streaking the swab over the four wells. Plates were incubated at 37°C for 48 h and visually read using the growth control well as a positive control. The strains were considered resistant to every specific antifungal if the growth observed in the drug-containing wells was similar to that of the growth control.

2.6 | Ethics

No ethics approval was required.

3 | RESULTS

3.1 | Antifungal susceptibility testing: clinical azoles

Attending to the EUCAST methodology, *A. fumigatus* strains showed a broad range of MIC values to the clinical azoles tested (Table 1). All *cyp51A*-WT strains were susceptible to the clinical azoles tested. Among the azole-resistant strains with point mutations, those *A. fumigatus* isolates harbouring the G54 mutation were resistant to ITC and POS but susceptible to VRC; azole-resistant isolates with M220 were resistant to ITC, showing variable MICs to VRC and POS, and strains harbouring G448S mutations were resistant to VRC, with variable MICs to ITC and POS. Strains with TR insertions in the *cyp51A* promoter showed multiresistance to all clinical azoles tested, as expected.

TABLE 1 Minimal inhibitory concentration ranges of *Aspergillus fumigatus* isolates to clinical antifungal drugs and to demethylation inhibitor drugs (DMIs). *A. fumigatus* isolates are grouped based on their azole susceptibility profile and their Cyp51A azole resistance mechanisms

Azole Susceptibility/Cyp51A modifications (#)	MICs ranges to clinical antifungals (mg/L)			MICs to DMIs antifungals (mg/L)			
	ITC	VRC	POS	Imidazole		Triazole	
				IMZ	PCZ	MET	EPZ
Azole-Susceptible							
WT (50)	0.25-0.5	0.125-0.5	0-06-0.125	0.125-0.5	0.125-0.5	0.125-0.5	2-4
Azole-Resistant: Point mutations							
G54 (11)	>8	0.25-0.5	1- >8	0.06-0.125	0.125-0.25	0.06-0.125	0.5-2
M220 (6)	>8	0.25-1	0.25-2	0.25-2	0.25-1	0.25-1	4-16
G448S (7)	1-2	>8	0.25-1	0.5-2	1-2	1-32	8-32
Azole-Resistant: TR integrations							
TR ₃₄ /L98H (22)	>8	4-8	0.5-1	1-8	2- 8	1-2	>32
TR ₃₄ /L98H/S297T/F495I (2)	>8	4-8	0.5-1	8	> 32	4-16	>32
TR ₄₆ /Y121F/T289A (4)	2-4	4 - > 8	0.5	32 - >32	16- > 32	8-16	>32
TR ₅₃ (2)	>8	2 - 4	0.5-1	2-8	2- 8	2	>32

Note: #Number of isolates.

Abbreviations: AZL, azole; EPZ, epoxiconazole; IMZ, Imazalil; ITC, Itraconazole; MET, metconazole; MIC, minimal inhibitory concentration; PCZ, prochloraz; POS, posaconazole; TR, Tandem Repeat; VRC: Voriconazole.

TABLE 2 Screening plates results of *Aspergillus fumigatus* isolates to clinical azole drugs and imidazole and triazole demethylation inhibitor antifungals

Azole Susceptibility/Cyp51A modifications (#)	Screening results ^a						
	Clinical azole drugs			DMI antifungals			
	ITC	VRC	POS	Imidazoles		Triazoles	
	ITC	VRC	POS	IMZ	PCZ	MET	EPZ
AZL-Susceptible							
WT (50)	-	-	-	-	-	-	-
AZL-R: Point mutations							
G54 (11)	+	-	+	-	-	-	-
M220 (6)	+	v	v	-	-	-	-
G448S (7)	v	+	v	-	-	+	-
AZL-R: TR integrations							
TR ₃₄ /L98H (22)	+	+	+	-	-	+	+
TR ₃₄ /L98H/S297T/F495I (2)	+	+	+	-	+	+	+
TR ₄₆ /Y121F/T289A (4)	v	+	+	+	+	+	+
TR ₅₃ (2)	+	+	+	-	-	+	+

Note: #Number of isolates. ^a -, no growth; + growth; v, variable growth depending on amino acid substitutions or background strains.

Abbreviations: AZL, azole; DMI, demethylation inhibitor fungicides; EPZ, epoxiconazole; IMZ, Imazalil; ITC, Itraconazole; MET, metconazole; PCZ, prochloraz; POS, posaconazole; TR, Tandem Repeat; VRC, Voriconazole.

3.2 | Antifungal susceptibility testing: DMIs

The susceptibility of *A. fumigatus* strains to each DMI antifungal drug is shown in Table 1. The majority of resistant strains with Cyp51A TRs integrations showed higher MICs to all DMIs tested compared to the WT isolates. Although this was a general phenotype among these isolates, some differences were associated

with individual azole resistance mechanisms. Strains harbouring G54 mutations showed hyper-susceptibility to all the DMI antifungals tested, both imidazoles and triazoles. Strains with G448S mutation displayed higher susceptibility to imidazoles than to triazoles. Within the TR group, the specific susceptibility phenotype observed led to a particular resistance mechanism: strains that harboured TR₃₄/L98H/S297T/F495I showed high resistance to

triazoles and to imidazole PCZ; strains with TR₄₆/Y121F/T289A were highly resistant to all imidazole and triazole drugs tested; whereas strains with TR₃₄/L98H or TR₅₃ were highly resistant only to EPZ.

3.3 | Pattern of growth for 4-well screening plate visualisation

All *A. fumigatus* strains tested grew in the control well. Azole susceptible isolates only grew in the no-drug control well while the *A. fumigatus* azole-resistant strains—based on the phenotype of azole resistance—showed different growth patterns in the different wells with azole drugs. This could be associated with their specific azole resistance mechanism. Growth in the 4-well screening plates matched the microdilution antifungal susceptibility testing (Table 2). Only two azole-resistant groups presented some peculiarities due to their susceptibility profile to clinical azoles. Strains with M220 substitutions showed variable growth on VRC and POS supplemented wells whereas those with G448S mutation showed variable growth on ITC and POS supplemented wells, which is in agreement with the variable MICs obtained in the susceptibility testing. The growth pattern obtained for each *A. fumigatus* Cyp51A resistance mechanism to clinical azoles and DMIs is shown in Figure 2. One of the limitations of this study is that isolates with resistance mechanisms TR₃₄/L98H and TR₅₃ showed similar MIC profiles to the DMIs evaluated so that the final confirmation would always be by molecular methods, sequencing the *cyp51A* gene.

4 | DISCUSSION

Azole resistance in *A. fumigatus* is a worldwide emerging problem that is compromising IA clinical treatment.^{1,6,36} The outcome of IA depends on the early initiation of an effective therapy, which relies on a rapid detection of antifungal resistance. Several factors are involved in treatment failure, but knowledge of azole resistance and the associated resistance mechanism is vital for the therapeutic response and patient survival.^{36,37} Identification of azole-resistant *A. fumigatus* strains by broth microdilution reference procedures can represent a major problem for most routine laboratories as well-trained personnel is required. Furthermore, standardised in vitro susceptibility testing takes at least 5–7 days to be fully completed so it is a time-consuming procedure. The 4-well screening method assessed in this study is an easier and faster way to detect azole-resistant *A. fumigatus* strains in the clinical setting. Also, and due to its simplicity, it can be implemented in any mycology laboratory.

This expanded method includes a modification on the VRC and POS concentrations in the screening plates, which is an improvement in the detection of azole resistance since these new plates were modified according to the introduction of the concept of ATU.³³ Thus, incorporating this new category, and the implication of those *A. fumigatus* isolates that grow at bordering MICs, it is possible to detect those isolates that show a susceptibility profile that matches the new clinical breakpoints and the recently incorporated ATU concept. This can avoid false susceptibility errors that could have happened in the past due to the higher clinical breakpoints established, and the wrong classification of isolates as susceptible. As this methodology has been modified to be updated

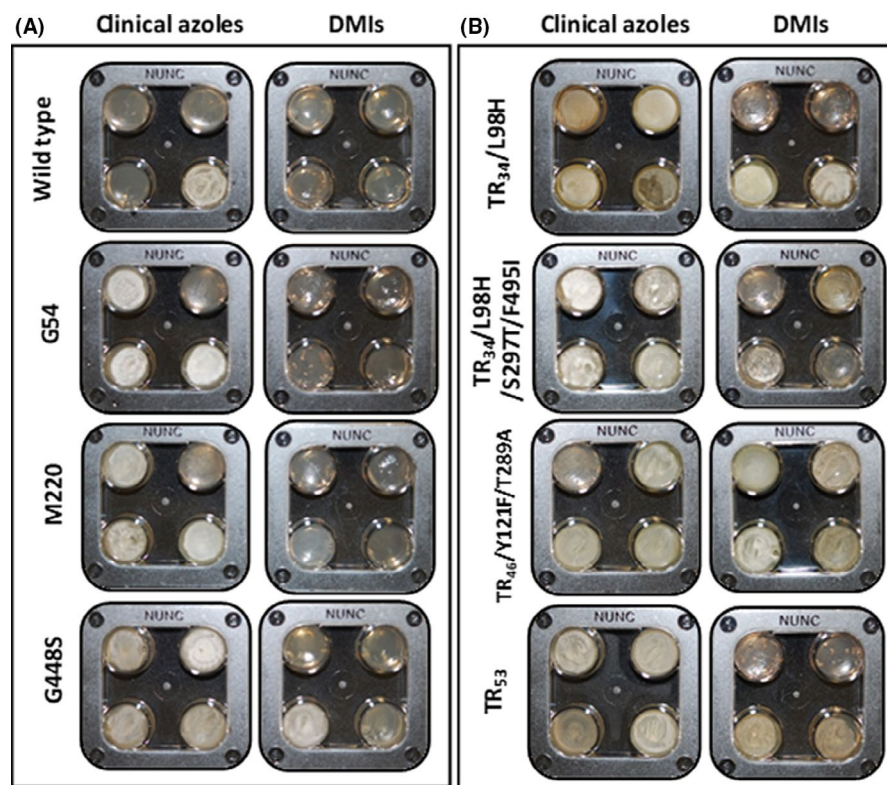


FIGURE 2 Screening plates (clinical azoles and DMIs) inoculated with strains carrying the different Cyp51A azole resistance mechanisms. Growth pattern for *Aspergillus fumigatus* strains with Cyp51A point mutations (A) or TR-based mutations in Cyp51A (B)

to the new EUCAST clinical breakpoints, our susceptibility growth pattern obtained for each *A. fumigatus* Cyp51A azole resistance mechanism could be very useful as an easier and quicker initial susceptibility test for mycology laboratories, and in surveillance studies to catch isolates growing in this ATU concept that should be selected to send to reference laboratory for CYP51A sequencing and confirmation of MICs.

Aspergillus fumigatus azole resistance is commonly found as a cross-resistant phenotype. In a previous work, we analysed *A. fumigatus* cross-resistance between clinical azoles and nine DMI drugs used for crop protection.²³ We were able to confirm different patterns of cross-resistance based on the azole resistance mechanism of each strain.²³ Specific differences detected in DMI susceptibility profiles were used to design and validate the proposed screening method. This work describes an improved screening method using four DMIs used for crop protection in addition to three clinical azoles. Identification of the specific mechanism of resistance is possible based on the particular growth pattern detected. Our results showed a strong correlation between the susceptibility profiles observed in our agar-based screening method and the expected AFST (EUCAST) of each strain based on their Cyp51A alteration.¹⁴⁻²³

Azole susceptibility of an isolate is assumed when there is growth only in the no-drug control well, which is ideal for surveillance studies. The necessity of epidemiological surveillance studies was started in 2013 by the European Center for Disease Prevention and Control (ECDC)³⁸ as updated information of azole susceptibility status of *A. fumigatus* is needed at a local level.^{8,39-41} The European Society of Clinical Microbiology and Infectious Diseases (ESCMID) recommended that antifungal susceptibility testing of *Aspergillus* isolates causing invasive aspergillosis disease should be performed in patients from regions with high percentages of resistance.⁴² Surveillance studies could benefit from this easy, low-cost and sensitive screening method to detect azole-resistant strains among the local *A. fumigatus* epidemiology.

One limitation of this study is that is not possible to establish a DMIs pattern for azole-resistant *A. fumigatus* strains with non-cyp51A mutations, as they usually present a wide MIC range to azoles. However, any azole-resistant strain will be studied by sequencing of *cyp51A* and in case that the strains are wild type for *cyp51A* they will be included in a different study, but they will be registered as azole resistant and this information would be helpful to the clinical case. Even if results need to be confirmed through microdilution susceptibility testing and *cyp51A* sequencing, this method could be very useful to reduce the response time for clinical diagnosis of azole resistance in patients colonised/infected with *A. fumigatus* azole-resistant strains, as resistance is quickly detected, facilitating an appropriate antifungal therapy, and therefore improving the clinical outcome of the patient. This methodology used could also contribute to isolate unidentified *cyp51A* mutations conferring unknown/new profiles of azole cross-resistance in *A. fumigatus*.

5 | CONCLUSIONS

Due to the selective pressure of azole drugs used in medicine and in the environment, an increased isolation of azole-resistant *A. fumigatus* strains has been encountered. In this scenario, the active surveillance of *A. fumigatus*, both in clinical and environmental setting, is essential for the control of azole resistance. The screening method described and validated in this study provides an easy way to detect azole-resistant *A. fumigatus* strains. The proposed method could be used in any mycology laboratory and have a dual use in hospital laboratories as a prompt indicative of the *A. fumigatus* azole susceptibility, as well as in surveillance studies to increase the knowledge of the local epidemiology of *A. fumigatus* azole resistance.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Jose Lucio: Data curation (equal); Investigation (lead); Methodology (lead); Validation (equal); Writing – original draft (lead); Writing – review & editing (equal). **Irene Gonzalez-Jimenez:** Investigation (supporting); Writing – original draft (supporting); Writing – review & editing (supporting). **Rocio Garcia-Rubio:** Validation (supporting); Writing – review & editing (supporting). **Maria Soledad Cuetera:** Validation (supporting); Writing – review & editing (supporting). **Emilia Mellado:** Conceptualization (lead); Data curation (equal); Formal analysis (lead); Funding acquisition (lead); Project administration (lead); Resources (lead); Supervision (lead); Visualization (lead); Writing – review & editing (equal).

DATA AVAILABILITY STATEMENT

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

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