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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 specialized 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, TR_{53}, TR_{34}/L98H, TR_{46}/Y121F/T289A, TR_{34}/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, screening method, Cyp51A mutations, surveillance studies

1. Introduction

Aspergillus fumigatus is the most representative species among the genus *Aspergillus*. This ubiquitous mold 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 harboring 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 (TR_{34}/L98H, TR_{34}/L98H/S297T/F495I, TR_{46}/Y121F/T289A, and TR_{53}) 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 to the local use of a particular DMI [23]. MICs profiles to both classes of DMIs, imidazoles and triazoles , are generally high, excluding strains harboring G54 mutations, but the pattern of cross-resistance depend on the different Cyp51A modifications. Strains harboring G448S mutations are specifically resistant to triazoles DMIs and strains with TR_{46}/Y121F/T289A and TR_{34}/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 specialized centers. 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-characterized *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 harbored known azole resistance mechanisms consisting of Cyp51A mutations- G54 (11 isolates), M220 (6 isolates), G448S (7 isolates), TR_{34}/L98H (22 isolates), TR_{53} (2 isolates), TR_{34}/L98H/S297T/F495I (2 isolates) and TR_{46}/Y121F/T289A (4 isolates).

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

Mechanisms of azole resistance were characterized 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, Madrid, Spain) 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 display in Supplementary Table 1.

2.3. Microdilution antifungal susceptibility testing (AFST)

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, Madrid, Spain), VRC (Pfizer SA, Madrid, Spain), and POS (Schering-Plough Research Institute, Kenilworth, NJ). 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 hours of incubation at 37°C in a humid atmosphere. Clinical breakpoints for interpreting 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≤1, R>1) or POS (S≤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 problematics MICs overlaping the susceptible and resistant category. The uncertainty of this category gives an instruction to categorize 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 (PCZ and IMZ), and two triazole compounds (MET and EPZ), all purchased at Sigma Aldrich (Madrid, Spain). All antifungal drugs were dissolved in DMSO and autosterilized for 30 minutes 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, Waltham, MA) were prepared containing 1mL of RPMI 1640-2% glucose agar. One set of plates (Fig. 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 (Fig. 1B) was supplemented with DMI antifungals - 16 mg/L imazalil (IMZ), 16 mg/L prochloraz (PCZ), 1 mg/L metconazole (MET) and 32 mg/L epoxiconazole (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 is described in Appendix A.

[Figure 1 position]

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 (24-48h). Screening plates were inoculated using a swab soaked in H_2O 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 hours 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 ethical 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 harboring 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 harboring 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 position]

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 harboring 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 harbored TR_{34}/L98H/S297T/F495I showed high resistance to triazoles and to imidazole PCZ; strains with TR_{46}/Y121F/T289A were highly resistant to all imidazole and triazole drugs tested; whereas strains with TR_{34}/L98H or TR_{53} were highly resistant only to EPZ.

3.3. Pattern of growth for 4-well screening plate visualization

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 to 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 Fig. 2. One of the limitations of this study is that isolates with resistance mechanisms TR_{34}/L98H and TR_{53} showed similar MIC profiles to the DMIs evaluated so that the final confirmation would always be by molecular methods, sequencing the *cyp51A* gene.

[Table 2 position]

[Figure 2 position]

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, standardized *in vitro* susceptibility testing takes at least 5 to 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 stablished, and the wrong classification of isolates as susceptible. As this methodology has been modified to be updated 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 up isolates growing in this ATU concept that should be selected to send to reference laboratory for CYP51A sequencing and confirmation of MICs.

A. fumigatus azole resistance is commonly found as a cross-resistant phenotype. In a previous work, we analyzed *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 stated 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 stablish 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 register 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 colonized/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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TABLES

Table 1. Minimal inhibitory concentration ranges of *A. 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)			
				Imidazole		Triazole	
	ITC	VRC	POS	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

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

Table 2 Screening plates results of *A. 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			
				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)	+	+	+	-	-	+	+

Number of isolates. [a] -, no growth; + growth; v, variable growth depending on amino acid substitutions or backrgound strains. Abbreviations: DMI, demethylation inhibitor fungicides; AZL, azole; TR, Tandem Repeat; ITC: Itraconazole; VRC: Voriconazole; POS: posaconazole; IMZ, Imazalil; PCZ, prochloraz; MET, metconazole; EPZ, epoxiconazole.

FIGURE LEGENDS

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).

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

Appendix A

Medium and antifungal drugs preparation:

RPMI preparation:

1. Add 10.4 g RPMI 1640 Medium powder, 34.53 g MOPS, 18 g glucose, all purchased at Sigma Aldrich (Madrid, Spain) and 400 mL distilled water to a 600 mL beaker.
2. Mix on a magnetic stirrer for 10 min using a magnetic stir bar.
3. While stirring, adjust the pH to 7 using 1M NaOH, and add additional distilled water to bring the medium to 500 mL.
4. Sterilize the medium by filtration, using a bottle top filter so the final medium is dispensed into a sterile 500 mL glass bottle.
5. Store the RPMI medium at 2-8°.

Screening plates preparation:

The volumes stated in the following instructions produce ~45 screening plates.

1. Add 0.75 g Agar Bacteriological, Agar No. 1 (Oxoid, Madrid, Spain) and 25 mL of distilled water to 8 different 50 mL glass bottles (4 bottles for clinical antifungals screening plates and 4 bottles for DMI antifungals screening plates).
2. Mix on a magnetic stirrer for 5 min using a magnetic stir bar.
3. Autoclave the bottles to sterilize the agar medium.
4. Leave the RPMI medium at room temperature to temper before mixing with the agar.
5. While the agar is sterilizing prepare the antifungal stocks. 1600 mg/L stock solution of clinical antifungals and 1000 mg/L stock solution of DMI antifungals.
6. Once sterilization has finished, allow the agar to cool down to about 60°C.
7. Transfer 25 mL RPMI medium and the corresponding volume of clinical azole drug or DMI to each bottle:
    a. The volumes of clinical antifungals are 125 µL of ITC stock, 32 µL of VRC stock and 8 µL of POS stock.
    b. The volumes of DMI antifungals are 800 µL of IMZ and PCZ stocks, 50 µL of MET stock and 1600 µL of EPZ stock.
8. Mix it and transfer 1 mL into its corresponding well.

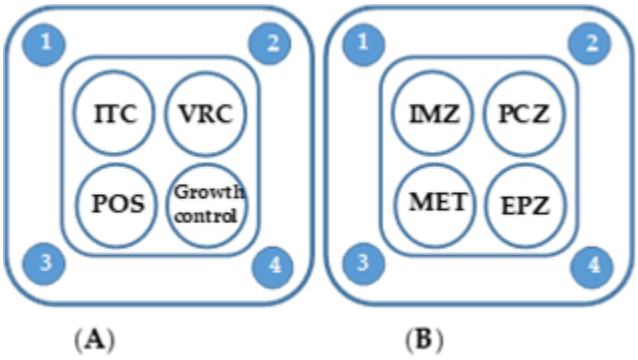

Figure 1. Four-well plates containing RPMI-agar media and (A) clinical antifungals: itraconazole (ITC, well 1), voriconazole (VRC, well 2), posaconazole (POS, well 3) and a drug-free control growth well (well 4), and (B) DMI antifungals: imazalil (IMZ, well 1), prochloraz (PCZ, well 2), metconazole (MET,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

87x48mm (96 x 96 DPI)

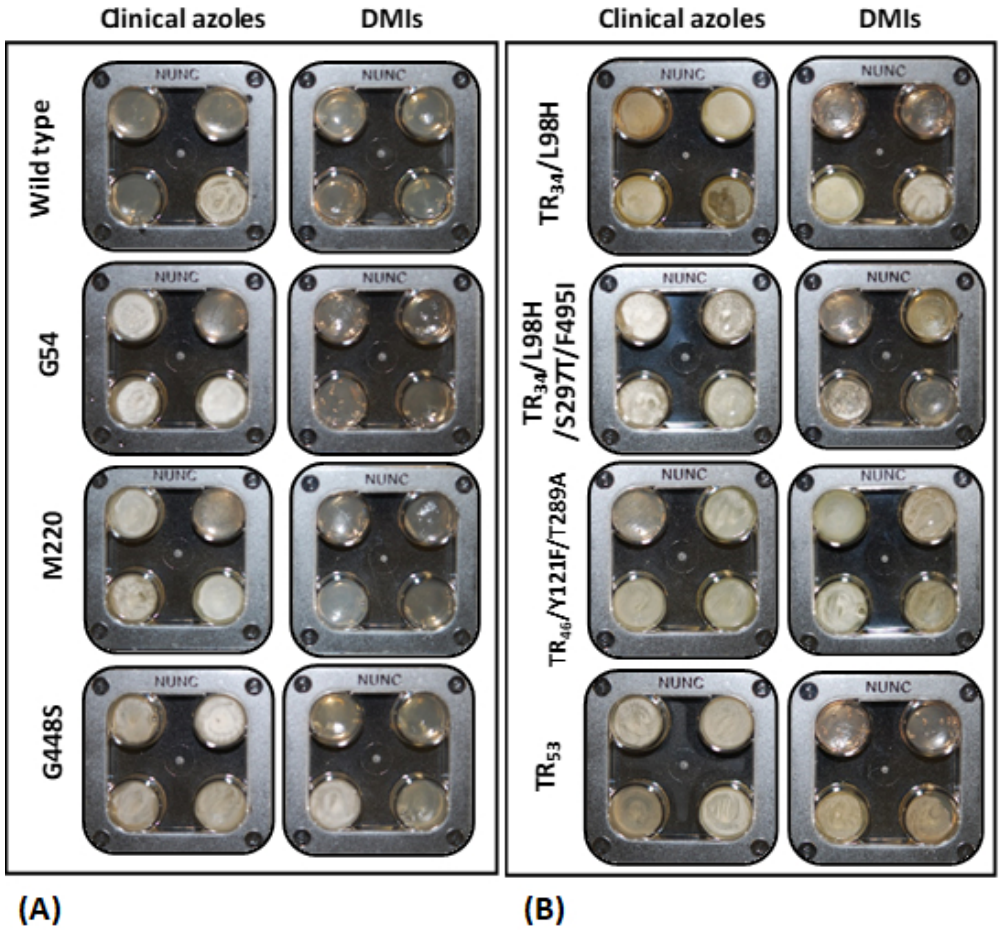

Figure 2. Screening plates (clinical azoles and DMIs) inoculated with strains carrying the different Cyp51A azole resistance mechanisms. Growth pattern for A. fumigatus strains with Cyp51A point mutations (A) or TR-based mu-tations in Cyp51A (B).

144x136mm (96 x 96 DPI)

Appendix A

Medium and antifungal drugs preparation:

RPMI preparation:

1. Add 10.4 g RPMI 1640 Medium powder, 34.53 g MOPS, 18 g glucose, all purchased at Sigma Aldrich (Madrid, Spain) and 400 mL distilled water to a 600 mL beaker.

2. Mix on a magnetic stirrer for 10 min using a magnetic stir bar.

3. While stirring, adjust the pH to 7 using 1M NaOH, and add additional distilled water to bring the medium to 500 mL.

4. Sterilize the medium by filtration, using a bottle top filter so the final medium is dispensed into a sterile 500 mL glass bottle.

5. Store the RPMI medium at 2-8°.

Screening plates preparation:

The volumes stated in the following instructions produce ~45 screening plates.

1. Add 0.75 g Agar Bacteriological, Agar No. 1 (Oxoid, Madrid, Spain) and 25 mL of distilled water to 8 different 50 mL glass bottles (4 bottles for clinical antifungals screening plates and 4 bottles for DMI antifungals screening plates).

2. Mix on a magnetic stirrer for 5 min using a magnetic stir bar.

3. Autoclave the bottles to sterilize the agar medium.

4. Leave the RPMI medium at room temperature to temper before mixing with the agar.

5. While the agar is sterilizing prepare the antifungal stocks. 1600 mg/L stock solution of clinical antifungals and 1000 mg/L stock solution of DMI antifungals.

6. Once sterilization has finished, allow the agar to cool down to about 60°C.

7. Transfer 25 mL RPMI medium and the corresponding volume of clinical azole drug or DMI to each bottle:

    a. The volumes of clinical antifungals are 125 μL of ITC stock, 32 μL of VCZ stock and 8 μL of POS stock.

    b. The volumes of DMI antifungals are 800 μL of IMZ and PCZ stocks, 50 μL of MET stock and 1600 μL of EPZ stock.

8. Mix it and transfer 1 mL into its corresponding well.

Supplementary Table 1. Geographical origin and genotype of the *A. fumigatus* isolates.

Strain	Origin (*)	Cyp51A modification	TRESPERG			
			CSP	MP2	CFEM	ERG
TP-3	Spain (E)	TR34/L98H	t10	m1.1	c08A	e05
TP-367	Spain (C)	TR34/L98H	t02	m1.1	c09	e11
TP-369	Spain (C)	TR34/L98H	t02	m1.1	c09	e05
TP-391	Spain (C)	TR34/L98H	t02	m1.1	c09	e05
TP-436	Spain (C)	TR34/L98H	t02	m1.1	c09	e05
TP-494	Spain (C)	TR34/L98H	t02	m1.1	c09	e05
TP-579	Spain (C)	TR34/L98H	t02	m1.1	c09	e11
TP-1003	Spain (C)	TR34/L98H	t10	m1.1	c08A	e05
TP-1003E	Spain (C)	TR34/L98H	t10	m1.1	c08A	e05
TP-1004	Spain (C)	TR34/L98H	t10	m1.1	c08A	e05
TP-1004E	Spain (C)	TR34/L98H	t10	m1.1	c08A	e05
TP-1005_	Spain (C)	TR34/L98H	t2	m1.1	C09	e16
H100	Spain (C)	TR34/L98H	t04B	m1.2	c22	e07
TP-14988	Spain (C)	TR34/L98H	t4	m1.1	c8B	e7
CNM CM-7582	Spain (C)	TR34/L98H	t02	m1.1	c09	e16
CNM CM-7609	Spain (C)	TR34/L98H	t02	m1.4	c09	e16
CNM CM-8535	Spain (C)	TR34/L98H	t02	m1.1	c09	e13
CNM CM-8714	Spain (C)	TR34/L98H	t02	m1.1	c09	e16
CNM CM-9339	Spain (C)	TR34/L98H	t02	m1.1	c09	e05
CNM CM-9399	Spain (C)	TR34/L98H	t02	m1.1	c09	e05
CNM CM-9670	Spain (C)	TR34/L98H	ND	ND	ND	ND
CNM CM-9701	Spain (C)	TR34/L98H	t28	m12.1	c08A	E11
CNM CM-9886	Spain (C)	TR34/L98H	t01	m1.1	c12	e07
M10731	The Netherlands (C)	TR34/L98H/S297T/F495I	t02	m1.1	c09	e05
CNM CM-3271	The Netherlands (C)	TR34/L98H/S297T/F495I	t02	m1.5	c09	e16
TP-90812	Spain (C)	TR46/Y121F/T289A	t01	m1.1	c09	e05
CNM CM-8057	Spain (C)	TR46/Y121F/T289A	t01	m1.8	c08A	e09
CNM CM-9103	Spain (C)	TR46/Y121F/T289A	t01	m1.1	c09	e15
CNM CM-9396	Spain (C)	TR46/Y121F/T289A	t01	m1.1	c09	e15
CR051	Spain (LM)	TR53	t01	m5.6	c08.1	e07
CNM CM-4599	The Netherlands (C)	TR53	t04A	m1.1	c09	e13
CNM CM-1244	United Kingdom (C)	G54	t01	m5.3	c08A	e07
CNM CM-2160	United Kingdom (C)	G54	t02	m1.1	c09	e16
CNM CM-2161	United Kingdom (C)	G54	t01	m5.3	c07	e05
CNM CM-2162	United Kingdom (C)	G54	t04A	m1.1	c08A	e07
CNM CM-2266	Spain (C)	G54	t03	m1.1	c08B	e02
CNM CM-3500	Spain (C)	G54	t03	m1.1	c08B	e02
CNM CM-8940	Spain (C)	G54	t04A	m1.3	c08A	e07
CNM CM-9114	Spain (C)	G54	t02	m1.1	c22	e16
CNM CM-9501	Spain (C)	G54	t02	m1.8	c22	e20
CNM CM-9551	Spain (C)	G54	t02	m1.8	c22	e20
CR061	Spain (LM)	G54	t01	m5.6	c08.1	e07
CNM CM-1245	United Kingdom (C)	M220	t04.1	m5.3	c08.1	e07
CNM CM-1252	United Kingdom (C)	M220	t04.1	m5.3	c08.1	e07
CNM CM-2159	United Kingdom (C)	M220	t02	m1.1	c09	e16
CNM CM-2164	United Kingdom (C)	M220	t02	m1.2	c04	e05
CNM CM-4593	The Netherlands (C)	M220	t01	m5.1	c09	e06
CR 060	Spain (LM)	M220	t01	m5.6	c08.1	e07
TP-1362	Spain (C)	G448S	t01	m3.4	c08A	e07
CNM CM-9702	Spain (C)	G448S	t01	m5.3	c06	e07
CNM CM-9820	Spain (C)	G448S	ND	ND	ND	ND

CNM CM-9821	Spain (C)	G448S	ND	ND	ND	ND
TP-1	Spain (E)	G448S	t04A	m1.3	c05A	e07
TP-2	Spain (E)	G448S	t04A	m1.3	c05A	e07
TP-4	Spain (E)	G448S	t04A	m1.3	c05A	e07
AF237	Reference strain	WT	t03	m1.1	c08.1	e07
ATCC46645	Reference strain	WT	t02	m1.1	c23	e05
CBS	Reference strain	WT	t01	m5.6	c08.1	e07
CNM CM-2120	Spain (C)	WT	t03	m1.1	c09	e09
CNM CM-2123	Spain (C)	WT	t01	m1.2	c08.1	e07
CNM CM-2126	Spain (C)	WT	t01	m3.4	c22	e11
CNM CM-2141	Spain (C)	WT	t02	m6.5	c08.1	e16
CNM CM-2142	Spain (C)	WT	t03	m1.3	c05.1	e07
CNM CM-2157	Spain (C)	WT	t04.1	m5.5	c03	e11
CNM CM-2167	Spain (C)	WT	t01	m1.1	c08.1	e07
CNM CM-2182	Spain (C)	WT	t04.1	m1.3	c08.2	e11
CNM CM-2195	Spain (C)	WT	t01	m5.3	c10	e07
CNM CM-2200	Spain (C)	WT	t04.1	m1.8	c08.1	e06
CNM CM-2202	Spain (C)	WT	t03	m1.1	c07	e09
CNM CM-2580	Spain (C)	WT	t01	m5.5	c03	e11
CNM CM-4210	Spain (C)	WT	t10	m1.1	c05.1	e07
CNM CM-4601	Spain (C)	WT	t01	m1.1	c19	e07
CNM CM-4862	Spain (C)	WT	t02	m1.1	c09	e05
CNM CM-4876	Spain (C)	WT	t04.1	m1.1	c08.1	e15
CNM CM-4896	Spain (C)	WT	t02	m1.1	c09	e05
CNM CM-4982	Spain (C)	WT	t26	m1.1	c08.1	e16
CNM CM-5052	Spain (C)	WT	t01	m5.7	c08.1	e07
CNM CM-5143	Spain (C)	WT	t01	m1.1	c05.1	e07
CNM CM-5163	Spain (C)	WT	t01	m1.7	c08.1	e11
CNM CM-5178	Spain (C)	WT	t03	m5.4	c08.1	e07
CNM CM-5297	Spain (C)	WT	t04.1	m1.1	c02	e04
CNM CM-5325	Spain (C)	WT	t03	m5.3	c09	e07
CNM CM-5360	Spain (C)	WT	t01	m1.1	c12	e07
CNM CM-5390	Spain (C)	WT	t02	m5.4	c09	e07
CNM CM-5392	Spain (C)	WT	t25	m1.1	c08.1	e11
CNM CM-5393	Spain (C)	WT	t01	m3.1	c08.1	e07
CNM CM-5403	Spain (C)	WT	t03	m1.1	c12	e09
CNM CM-5409	Spain (C)	WT	t04.1	m1.1	c03	e07
CNM CM-5410	Spain (C)	WT	t04.1	m1.1	c12	e15
CNM CM-5411	Spain (C)	WT	t04.1	m3.4	c09	e06
CNM CM-5417	Spain (C)	WT	t03	m1.1	c05.1	e06
CNM CM-5419	Spain (C)	WT	t06.2	m6.2	c08.1	e11
CNM CM-5485	Spain (C)	WT	t01	m3.4	c20	e05
CNM CM-5489	Spain (C)	WT	t04.1	m3.4	c08.1	e06
CNM CM-5536	Spain (C)	WT	t02	m1.2	c09	e05
CNM CM-5590	Spain (C)	WT	t04.1	m1.1	c08.1	e15
CNM CM-5621	Spain (C)	WT	t18.1	m1.1	c05.1	e06
CNM CM-5635	Spain (C)	WT	t01	m5.1	c09	e06
CNM CM-5703	Spain (C)	WT	t02	m1.2	c09	e05
CNM CM-5725	Spain (C)	WT	t06.2	m3.4	c08.1	e07
CNM CM-5736	Spain (C)	WT	t06.2	m3.4	c08.1	e07
CNM CM-5756	Spain (C)	WT	t04.1	m1.3	c08.1	e07
CNM CM-5757	Spain (C)	WT	t04.1	m5.3	c16	e07
CNM CM-5836	Spain (C)	WT	t02	m1.1	c08.1	e13
CNM CM-5888	Spain (C)	WT	t01	m1.1	c08.1	e07

* C, clinical; E, enviromental; LM, laboratory mutant. ND: Not determined.