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Screening method for the detection of azole and echinocandin resistance in *Aspergillus* using antifungal-containing agar plates, exemplified by *A. fumigatus*

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INTRODUCTION

The emergence of drug resistance in *Aspergillus fumigatus* isolates is a matter of significant concern. Most important is the rise in azole resistance with rates typically ranging from a few percent to up 30% in hospitalised patients in some countries [1-7]. Consequently, the treatment guidelines in the Netherlands, the country with the highest documented prevalence of azole-resistant *A. fumigatus*, now recommend initial combination therapy until the susceptibility pattern is known. Therefore, susceptibility testing of clinically relevant isolates has been strongly recommended in the ESCMID-EFISG aspergillosis guidelines [8]. Azole resistance in *A. fumigatus* has been ascribed to various molecular resistance mechanisms. Most often, the isolates carry *cyp51A* target gene mutations that lead to unique patterns of reduced susceptibility to itraconazole, voriconazole, posaconazole, and isavuconazole depending on the codon and specific amino acid alteration involved [3, 8, 9]. Less often, other mechanisms cause azole resistance in *A. fumigatus* strains, such as over-expression of efflux pumps in phenotypically resistant isolates with wild type (WT) *cyp51A* gene [10-14]. Importantly, resistance has been found in environmental isolates explaining the occurrence of azole-resistant infections in azole naïve patients [12, 15-18]. Furthermore, mixed azole-susceptible and azole-resistant (isogenic as well as non-isogenic) infections have been reported to occur which implies that colonies of clinical cultures may harbour various phenotypes of azole susceptibility [12, 19, 20].

The first *A. fumigatus* isolate clinically resistant to echinocandins was reported in 2008 from a patient failing itraconazole and caspofungin treatment and was linked to up-regulated target enzyme production [21]. The second clinically resistant *A. fumigatus* isolate was recently recovered from a chronic pulmonary aspergillosis patient who failed azole and polyene therapy and subsequently failed micafungin treatment and this time was linked to a target gene alteration [22]. Notably, reduced susceptibility to echinocandins may be under-diagnosed as the breakthrough isolates are often not recovered and the gold standard method for echinocandin susceptibility testing of *Aspergillus* suffers from technical difficulties, making it inconvenient and not easily incorporated into daily laboratory practice. Moreover clinical breakpoints for the echinocandins against *Aspergillus* spp. have not been established.

Microdilution reference procedures developed by CLSI (M38 3rd edition) or EUCAST (E.Def 9.4) are available for antifungal susceptibility testing of azoles against moulds [23, 24]. Alternative approved commercial tests are not available. Broth microdilution susceptibility testing is time consuming, requires well-trained personnel, and resistant isolates may be overlooked in cases of mixed infections if only a single colony is tested. Although molecular detection of *cyp51A* target gene mutations is valuable in confirming azole resistance, limitations are such that tests can confirm resistance but not susceptibility, and that the technique is not widely available for moulds in routine clinical microbiology laboratories. Moreover, and owing to their mode of action, echinocandins do not induce complete growth inhibition and consequently significant trailing

growth most often complicates a visual endpoint determination. Therefore, the recommended endpoint for determining echinocandin activity against moulds is the minimal effective concentration (MEC), defined as the minimum concentration that leads to formation of altered aberrant multibranched hyphal rosette morphology. MEC reading is labour intensive, subjective, and requires expertise, which may lead to suboptimal reproducibility. Therefore, the development of easy-to-use screening procedures for the detection of clinical *Aspergillus* isolates with potential azole or echinocandin resistance are needed.

The first version of the 10.1 standard method was released as the definitive document for the screening of azole resistance in *A. fumigatus* isolates in September 2018 and subsequently published [25]. This second version 10.2 includes a corresponding agar-based screening method used to detect echinocandin non-wild-type (NWT) *Aspergillus* spp. isolates.

SCOPE

The standard method here described is intended for the phenotypic detection of *A. fumigatus* isolates potentially resistant to itraconazole, voriconazole, or posaconazole and of *Aspergillus* spp. isolates non-wild type (NWT) to echinocandins in routine laboratories of clinical mycology.

The azole test plate was originally proposed by van der Linden *et al* [26] and has been used in several environmental and clinical studies [26-28]. It has been validated in a multicentre study using a molecularly characterised *A. fumigatus* culture collection, and in-house as well as commercially available azole-containing plates [29]. The test principle is based on the use of plates containing agars supplemented with itraconazole (4 mg/L), voriconazole (2 mg/L) and posaconazole (0.5 mg/L, optional), and a drug-free agar control well for screening of resistance in *A. fumigatus* (Figure 1). Posaconazole is optional because *A. fumigatus* isolates displaying mono-azole resistance to posaconazole have not been found so far, and because studies indicate that all posaconazole resistant isolates would have been detected due to growth on the itraconazole agar [29, 30]. Moreover, since posaconazole MIC distributions for susceptible and resistant isolates are less well separated than those for itraconazole and consequently the posaconazole concentration in the agar is closer to the ECOFF, there is a risk of misclassification on the posaconazole agar. The echinocandin test plate was originally proposed by Meletiadis *et al* [31], has been recently validated in a multicentre study using 4-well plates, and proved useful for screening of echinocandin resistance in *Aspergillus* spp. The test principle is based on the use of plates containing agars supplemented with anidulafungin (0.25 mg/L), micafungin (0.125 mg/L), and a drug-free agar control well (Figure 2). In the original study, a lower cost principle was adopted (lower amount of agar [0.5 *vs* 1 mL]), higher inoculum concentration (1-2 *vs* 0.5 MacFarland), lower inoculum volume (20 *vs* 25 µL), medium supplementation (without vs with chloramphenicol) and medium sterilization (filtration

vs autoclave) [31]. For simplicity, the azoles and echinocandin methods were subsequently harmonised upon validation in a multicentre study with *A. fumigatus* confirming equal performance.

TERMS AND DEFINITIONS

1. **Antifungal agent**: substance of biological, semi-synthetic or synthetic origin that inhibits the growth of fungi or is lethal to them. Disinfectants, antiseptics and preservatives are not included in this definition.
2. **Properties of antifungal agents**
    a. **Potency**. Antimicrobially active fraction of a test substance. The potency is expressed as mass fraction in milligrams per gram (mg/g), or as activity content in International Units (IU) per gram, or as a volume fraction or mass fraction in percent, or as an amount-of-substance concentration (mass fraction) in mole per litre of ingredients in the test substance.
    b. **Concentration**. Amount of an antimicrobial agent in a defined volume of liquid. The concentration is expressed in SI units as mg/L.
3. **Stock solution**. Initial solution used for additional dilutions.
4. **Agar:** The term 'agar' refers to the solid medium used for the *in vitro* growth of microorganisms.
5. **Wild type (WT) and non-wild type (NWT) isolates**. An isolate is defined as WT if it is devoid of phenotypically detectable acquired resistance mechanisms to a specified antimicrobial agent. An isolate is defined as NWT for a species by the presence of phenotypically detectable acquired resistance mechanisms to the agent in question.
6. **Agar dilution:** Agar dilution is a susceptibility testing technique in which serial dilutions (usually two-fold) of an antifungal agent are made in an agar medium that is inoculated with a standardised suspension of organisms and incubated for a defined period of time. The purpose is the screening for resistance, or in other words separation of phenotypically NWT isolates from the WT population (a given threshold concentration is used at which growth of WT isolates is inhibited but allowed for mutant strains).
7. **Reference strain for quality control**. Catalogued, characterized strains with stable, defined antifungal susceptibility phenotypes and/or genotypes. They are obtainable from culture collections and used for quality control.
8. **Inoculum:** The number of conidia (colony-forming units) produced by a given isolate in a defined volume of solution. The inoculum is expressed as colony-forming units per millilitre (CFU/mL).

TEST PROCEDURES

General

The test is performed in 4-well plates (volume per well: 1 mL) although other sizes of plates may also be applicable.

The test principle of the screening of either azole resistance or echinocandin resistance in *A. fumigatus* isolates is based on the preparation of plates containing agars supplemented with anti-mould active azoles (itraconazole and voriconazole with or without posaconazole) or echinocandins (anidulafungin, and micafungin) as well as a drug-free agar control well. Once solidified the plates are inoculated with an inoculum containing *A. fumigatus* in a volume of 25 µL on the surface of each of the agars. Growth is visually assessed after 48 h of incubation at 34° - 37°C.

Antifungal agents

General

All antifungal drug solutions should be prepared in accordance with Good Manufacturing Practice. Antifungal pure powders must be obtained directly from the drug manufacturer or from reliable commercial sources. Clinical preparations must not be used because they may contain excipients or be prodrugs that are not appropriate for antifungal susceptibility testing. Powders must be supplied with generic name of the drug, a lot number, potency, expiry date and recommended storage conditions. Store powders in sealed containers at -20°C or below with a desiccant unless otherwise recommended by the manufacturers. Ideally, hygroscopic agents should be dispensed into aliquots before freezing, one of which is then used on each occasion. Allow containers to warm to room temperature before opening them to avoid condensation of water on the powder.

Preparation of stock solutions

Antifungal drug solutions must be prepared taking into account the potency of the lot of antifungal drug powder that is being used. The amount of powder or diluent required to prepare a standard solution may be calculated as shown below.

$$\text{Weight (g)} = \frac{\text{Volume (L)} \times \text{Concentration (mg/L)}}{\text{Potency (mg/g)}}$$

$$\text{Volume (L)} = \frac{\text{Weight (g)} \times \text{Assay potency (mg/g)}}{\text{Concentration (mg/L)}}$$

The antifungal powder should be weighed on an analytic balance that has been calibrated by approved reference weights from a certified metrology organisation. The precision of the balance should be taken into

account when determining the minimum amount of powder that can be weighed at a time to ensure correct concentration of the stock solutions.

Prepare antifungal drug stock solutions in DMSO at concentrations at least 200 times higher than the concentration to be tested in the agar plate (see example below). Information on solubility of antifungal compounds should be provided with the drug by the supplier. From the stock solutions, prepare antifungal drug working solutions in DMSO at concentrations 100 times higher than the final concentrations needed (itraconazole 400 mg/L, voriconazole 200 mg/L, posaconazole 50 mg/L, anidulafungin 25 mg/L, and micafungin 12.5 mg/L) (Table 1). It is essential to ensure the drug is fully dissolved before freezing. Several antifungals can be difficult to dissolve resulting in artificially elevated MICs. Placing the stock tube on a rocking table for an hour or more before continuing overcomes this problem. Sterilisation of stock solutions is not normally necessary. However, if required the sterilisation procedure shall be validated by appropriate means (e.g. samples obtained before and after filtration must be assayed) to ensure that drugs are not adsorbed (for example to a sterile filter) or degraded during the process.

Unless otherwise indicated by the drug manufacturer, store drug solutions in small volumes in sterile polypropylene or polyethylene vials at -70°C or below. Drugs may be stored at -70°C for at least six months without significant loss of activity [29].

Remove vials from -70°C and use them the same day that they are defrosted. Discard any drug not used on that day. Significant deterioration of an antifungal drug will be reflected in the results of testing the susceptibility of quality control strains [32]. If necessary, the drug can be assayed to determine the potency.

Medium

RPMI 1640 (with L-glutamine and a pH indicator but without bicarbonate) supplemented with glucose to a final concentration of 2% (RPMI 2% G) is recommended. 3-(N-morpholino) propanesulfonic acid (MOPS) at a final concentration of 0.165 mol/L, pH 7.0, is the recommended buffer to be used for RPMI 1640 media.

Preparation of the azole- or echinocandin-containing plates

Use sterile disposable 4-well untreated pyrogen free polystyrene plates with flat-bottom wells (nominal capacity of approximately 1 mL per well) and lids (avoid “low evaporation lids"). The drug-containing agar plates (supplemented with chloramphenicol for azole assay) are prepared as follows (Table 2) (250 plates approximately):

1. Dissolve the RPMI powder (10.4 g), glucose (18 g, final concentration 2 %), Bacto™ Agar (20 g), MOPS (34.53 g) and chloramphenicol base form (at a final concentration of 0.2 mg/mL) in 900 mL of sterile water.
2. Stir until components are completely dissolved.

3. Adjust the pH to 7.0 with a solution of NaOH (10M), and then fill up to 990 mL with sterile water.
4. Autoclave the solution at 121°C for 15 minutes.
5. Pour the solution into four Erlenmeyer flasks (247.5 mL in each flask).
6. Cool the agar down to 45°C.
7. Add 2.5 mL of each antifungal working solution (100x final concentration, prepared as outlined in Table 1) to each of three flasks. Ensure proper mixing (homogenous haziness without visible agar blocks within the solution).
8. The remaining fourth antifungal-free flask will be used for the growth control well.
9. Aliquot the agar from each flask (1 mL per well) to the respective wells of the multi-dish plate. If 4-well plates are used it could be as follows:

   for azoles (Figure 1a):

   - Well I: itraconazole (final concentration = 4.0 mg/L) (Fig 1a well 1 upper left)

   - Well V: voriconazole (final concentration = 2.0 mg/L) (Fig 1a well 2 upper right)

   - Well P: posaconazole (final concentration = 0.5 mg/L) (Fig 1a well 3 lower left)

   - Well GC: growth control (antifungal-free well) (Fig 1a well 4 lower right)

   for echinocandins (Figure 1b):

   - Well A: anidulafungin (final concentration = 0.25 mg/L) (Fig 1b well 2 upper right)

   - Well M: micafungin (final concentration = 0.125 mg/L) (Fig 1b well 3 lower left)

   - Well GC: growth control (antifungal-free well) (Fig 1b well 4 lower right)

   However, alternative plates and plate-design can also be used, for example 6 well plates with two sets of itraconazole, voriconazole and a drug-free control well allowing testing of two *A. fumigatus* isolates, or combinations of azoles and echinocandins in the same plate (e.g. itraconazole and voriconazole in order to detect most azole-resistance mechanisms and anidulafungin in order to detect *fks1* mutants).

10. For quality control purposes use one aliquot of the sterilised medium for sterility checks, for retesting the pH (6.9-7.1 is acceptable) and as a growth control with a reference strain [29].

Storage of the azole- and echinocandin-containing plates

The plates prepared, properly sealed, can be stored at 4°C for up to 6 months from the date of preparation if there is no reduction in the height of agar (suggesting evaporation).

242

243 **Preparation of the inoculum**

244 The *Aspergillus* isolate needs to have sporulated before testing (cultures incubated for at least 2-3 days).
245 Single or multiple colonies (up to 5 colonies) can be tested simultaneously in the same drug-containing plate
246 by creating a mixed inoculum (see below). Standardisation of the inoculum concentration is essential for
247 accurate and reproducible antifungal susceptibility tests.

248 The isolates should be cultured on potato dextrose agar or Sabouraud dextrose agar (with or without
249 chloramphenicol) and incubated at 34° - 37°C. Other culture medium where the fungus is able to sporulate
250 sufficiently can also be used. Inoculum suspensions are prepared from fresh, mature (2- to 5-day-old) cultures.
251 In some cases, an extended incubation is required for proper sporulation of the isolate.

252 Clinical samples may contain a mixture of susceptible and resistant isolates. For this reason, several
253 colonies, when present, should be tested. Single sampling using a moist swab of up to five colonies and
254 subsequently seeding into sterile water supplemented with 0.1% Tween 20 has previously been validated for
255 the azole screening method [29] and echinocandin screening method (Figure 1). Alternatively, for pure
256 cultures, cover colonies with approximately 5 mL of sterile water supplemented with 0.1% Tween 20. Then,
257 carefully rub the colony surface with a sterile cotton swab and then transfer the resulting conidia-containing
258 suspension with a pipette to a sterile tube. The suspension is vortexed for 15 seconds with a gyratory vortex
259 mixer at approximately 2,000 rpm. Ensure homogeneity of the conidia suspension preferably by filtering the
260 inoculum using a sterile filter (11 µm pore size), to avoid presence of clumps before adjusting the inoculum
261 concentration to 0.5 McFarland (corresponding to 2×10^6 to 5×10^6 CFU/mL). Filtration ensures a "conidia only"
262 and more standardised suspension.

263

264 **Inoculation of agar plates**

265 The agar plates should be inoculated within 30 min of the preparation of the inoculum suspension to
266 maintain viability of the conidia. Agar plates should be allowed to adjust to room temperature before
267 inoculation. Pipette 25 µL of the inoculum in the centre of each of the wells of the plate without touching the
268 surface and leave it on the bench to be fully absorbed (for 10-15 min) before incubation.

269

270 **Incubation of agar plates**

Incubate agar plates without agitation at 34° – 37°C in ambient air for 48 h (consider reporting the isolate as azole-resistant after 24 h if *Aspergillus* growth is observed in any azole-containing well and NWT to echinocandins if similar growth is observed between the drug-free well and any echinocandin-containing well).

Reading results

The endpoint is read visually as follows:

- In the azole-containing agar, by recording the presence or absence of growth on each well. Any growth on one or more azole-containing agar should be taken into account and lead to confirmatory MIC testing (Figure 1).
- In the echinocandin-containing agar, by recording the morphology of *A. fumigatus* colonies on each well. Isolates with WT phenotype produce aberrant, compact (non-fluffy) colony morphology whereas isolates with NWT phenotype produce fluffy colony morphology similar to the colony morphology in the drug-free control well (Figure 2, 3 and 4).

INTERPRETATION OF RESULTS

Azole-containing agar method.

- **Azole susceptible *A. fumigatus* isolate**: growth on the antifungal-free well and absence of growth on wells 1-3.
- **Potentially azole non-susceptible *A. fumigatus* isolate**: growth on the antifungal-free and on any of the other wells.

Susceptible isolates can be reported as such. A preliminary report of azole non-susceptible isolates may be issued and the finding should be confirmed by reference microbroth dilution testing according to the E.Def 9.4 document [24]. *Cyp51A* gene sequencing may be performed in isolates confirmed as resistant *A. fumigatus* sensu stricto. The overall sensitivity and specificity for the azole agar screening method using itraconazole, posaconazole and voriconazole and drug-free control agar to detect resistant strains harbouring G54E/R/V/W, G54R+N284K, M220I/K/T/V, M220I+V101F, TR_{34}/L98H and TR_{46}/Y121F/T289A *cyp51A* mutations was 99% (range 97%-100%) and 99% (95%-100%), respectively [29]. The performance values were 99% (range 95%-100%) and 99% (range 95%-100%) if omitting the posaconazole agar.

Echinocandin-containing agar method.

- **Echinocandin WT *Aspergillus* isolate**: WT isolates produce colonies with compact appearance on echinocandin-containing agars, but fluffy colonies on the control agar (Figure 2). Ignore production of irregular or smaller than control halos and aerial hyphae usually observed after incubation for 48 h.
- **Potentially echinocandin NWT isolate**: NWT *A. fumigatus* isolates produce fluffy colonies on echinocandin agars (diameter ≥15 mm after 48 h or of similar diameter as on the drug-free control agar) on the 4-well plate for the echinocandins.

WT isolates can be reported as such. A preliminary report of echinocandin NWT isolates may be issued and the finding should be confirmed by reference microbroth dilution testing according to the E.Def 9.4 document or by demonstrating a target gene alteration by of *A. fumigatus* sensu stricto [24]. Of note, prior studies have found batch-to-batch variation of caspofungin complicating reproducibility of *Candida* antifungal susceptibility testing [33, 34]. Batch-to-batch variation of caspofungin has not been evaluated in this study. Hence, it is not known if such variation may impact performance of the caspofungin agar screening and caspofungin has not been included in the method. Anidulafungin produced the clearest endpoints and discrimination. The *A. fumigatus fks1* mutant isolates that have been reported so far are pan-echinocandin resistant; however, a single non-*fks1* mutant only NWT to caspofungin has been described [35]. Future studies are needed to confirm if anidulafungin can be used as the only marker for detection of echinocandin-NWT *Aspergillus*.

QUALITY CONTROL

Control procedures are the means by which the quality of results is assured and are described in detail by the CLSI [23]. All four QC strains should be tested for each batch of plates that are taken into use. Once the performance of a new batch of plates is confirmed the number of QC strains run in parallel with routine testing can be reduced but should include both susceptible/WT and resistant/NWT *A. fumigatus* isolates, each time an isolate is tested or once weekly if incorporated in the daily routine (see below and the QC document available at www.eucast.org) [32]. Figures 1, and 4 show indicative photos of QC strains on azole- and echinocandin containing 4-well agar plates.

Control strains

The recommended control strains and source of these QC strains is found in the most recent version of the QC available on the EUCAST website http://www.eucast.org/ast_of_fungi/qcafsttables/.

Storage of control strains

Fungal isolates may be stored lyophilised or frozen at -60°C or below [36]. Cultures can be stored short-term (less than 2 weeks) on Sabouraud dextrose agar or potato dextrose agar slopes at 2-8°C, with new cultures prepared from frozen stocks, stored in multiple copies, every two weeks. Fresh cultures should be taken from the freezer every month as repeated subculturing of the *Aspergillus* QC strains may lead to contamination/or loss of the resistance mechanism.

Routine use of control strains

For routine use of control strains, fresh cultures must be prepared from agar slopes, frozen or lyophilized cultures by inoculation on non-selective nutritive agar medium (e.g. Sabouraud dextrose agar or potato dextrose agar). At least one azole-resistant (*A. fumigatus* SSI-4524, SSI-5586) and one echinocandin-NWT (*A. fumigatus* SSI-1794 or AFM32458) control strain must be included per test run to ensure the expected fungal growth pattern of susceptibility is seen and preferably also a susceptible/WT strain (*A. fumigatus* ATCC 24305 or MYA-1163) should be run weekly (Figures 1 and 4). If the growth control pattern is incorrect the test should be repeated. If the repeated test does not yield the expected results, then drug degradation (in stored plates) or wrong azole or echinocandin concentrations (in newly prepared plates) is assumed. The plates should be discarded, and new plates prepared (see also the QC document V 6.0 available on the http://www.eucast.org/ast_of_fungi/qcafsttables/). For the echinocandin plates the two *Candida* strains are also shown (Figure 4). These were selected due to the fact that the MICs for these organisms are closer to the echinocandin concentrations in the wells, and thus these strains may potentially (at least in theory) be sensitive indicators of loss of potency. This however, warrants further studies, as potency loss was not observed during the shelf life study over 6 months.

350 **Tables and figures**

351 **Table 1.** Example of preparation of 10 mL of the working solutions of each of the azoles and echinocandins
352 from a stock solution.

353

	Concentration of the stock solution	**Volume of the stock solution**	**DMSO solvent added**	**Concentrations in working solution (100x)**	**Final concentrations in the agar**
	(mg/L)	(µL)	(µL)	(mg/L)	(mg/L)
Itraconazole	1600	2500	7500	400	4
Voriconazole	1600	2500	7500	200	2
Posaconazole	800	625	9375	50	0.5
Anidulafungin	400	625	9375	25	0.25
Micafungin	400	312.5	9687.5	12.5	0.125

354
355

356 **Table 2.** Reagents to prepare antifungal-containing agar plates (1 litre of 1x RPMI).
357

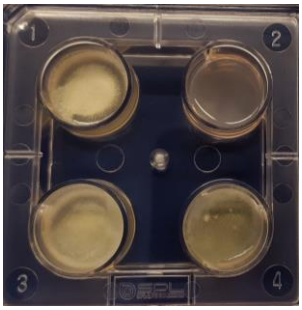
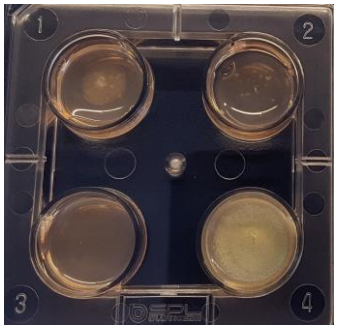
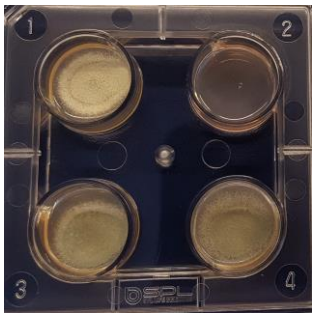
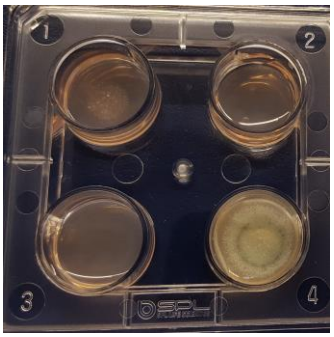
Reagents	Amount
RPMI 1640 powder with L-glutamine, without bicarbonate[1]	10.4 g
D (+) glucose[2]	18 g
Bacto™ Agar[3]	20 g
MOPS[4]	34.53 g (0.165M)
Chloramphenicol base form [5]	10 mL of stock solution 100 mg/mL in ethyl alcohol (Final concentration: 0.2 mg/mL)
Drug	10 mL of 100x working solution (Table 1)

358

359 The following brands and catalogue numbers have been tested and found appropriate:
360 [1]Sigma-Aldrich (R6504)/Applichem, [2]Merck (R 1.08342.1000)/Applichem, [3]Becton
361 Dickinson (214010)/Oxoid Ltd, [4]Sigma-Aldrich (M1254)/Applichem, [5]Sigma-Aldrich
362 (C0378).

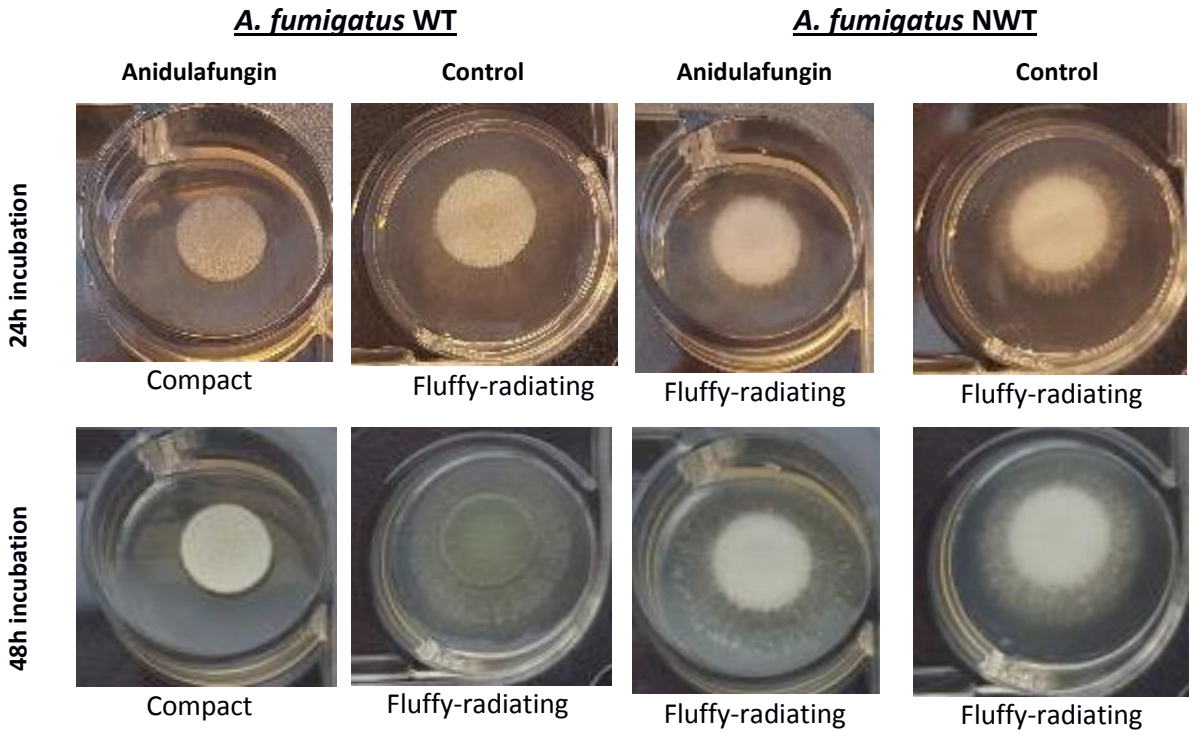
363

Figure 1. Detection of mixed inocula using azole resistant/susceptible isolates (Pictures a) and echinocandin NWT/WT isolates (Pictures b)*. Expected growth patterns for two azole-resistant *A. fumigatus* QC strains harbouring G54W (SSI-5586) and TR_{34}/L98H (SSI-4524) *cyp51A* substitutions, respectively, and one echinocandin-NWT *A. fumigatus* (DPL1035homo/SSI-1794) alone and in mix with the WT *A. fumigatus* ATCC 204305 isolate. Pictures (a and b) show the growth pattern of the mutant isolates alone (top images) and mixed 1 to 5 with a WT isolate (bottom images). Azole susceptible strains, including the QC strain *A. fumigatus* ATCC 204305, show no growth on the azole agars (not shown). Wells of the plate contain itraconazole (4 mg/L; well 1 [upper left]), voriconazole (2 mg/L; well 2 [upper right]), posaconazole (0.5 mg/L; well 3 [lower left]), and azole-free agar (growth control; well 4 [lower right]). Echinocandin-WT *A. fumigatus* isolates show the typical compact appearance in drug containing wells (well 2 [upper right], anidulafungin 0.25 mg/L; well 3 [lower left], micafungin 0.125 mg/L) (Figure 4). The QC strains are available from the CCUG strain collection: www.ccug.se.

Inoculum	a) Azole-agar screening method		b) Echinocandin-agar screening method
Mutant isolates alone	**G54W** 	**TR_{34}/L98H** 	***A. fumigatus* NWT DPL1035homo/SSI-1794**
Mutant isolates together with WT isolate (1:5)	**G54W+WT** 	**TR_{34}/L98H+WT** 	***A. fumigatus* NWT DPL1035homo/SSI-1794+WT**

*Inocula were prepared by mixing one resistant colony with five susceptible colonies.

Figure 2. **Echinocandin screening.** Characteristic colony morphology of an *A. fumigatus* NWT (DPL1035homo/SSI-1794) and an *A. fumigatus* WT strains on anidulafungin-containing agar (0.25 mg/L) and drug-free agar after 24h and 48h of incubation. Note the fluffy (colony diameter ≥15 mm) versus compact appearance. Ignore production of irregular or small halos usually observed after incubation for 48h.



Ignore the following patterns that may be observed in WT isolates only in drug-containing wells

Irregular halos Halos smaller than control Aerial hyphae

Figure 3. Colony morphology of challenging echinocandin-WT isolates with irregular halos (see bottom photos in Figure 2) from different *Aspergillus* species on echinocandin-containing wells (well 2 [upper right], anidulafungin 0.25 mg/L; well 3 [lower left], micafungin 0.125 mg/L with compact appearance) and drug-free agar (well 4 [lower right], no drug with fluffy appearance) after 24h and 48h of incubation. This atypical morphology was observed among the three centers in 0-25% of the 40 echinocandin-WT *Aspergillus* strains tested in the multicenter study.

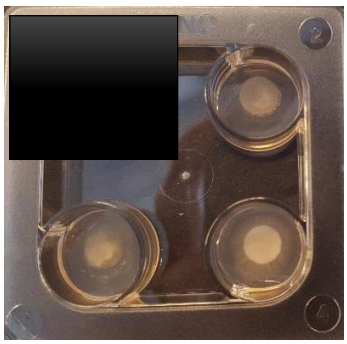
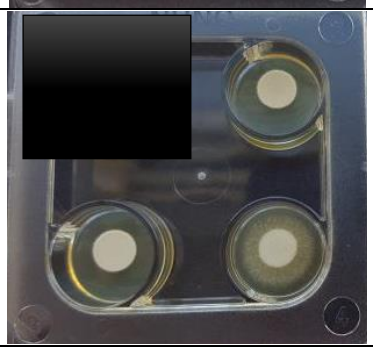
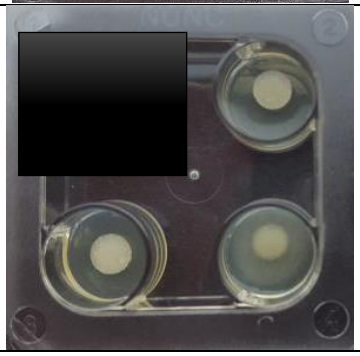
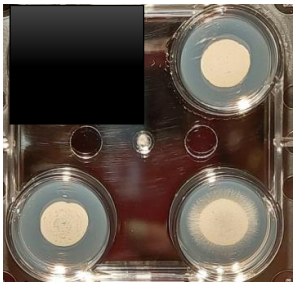
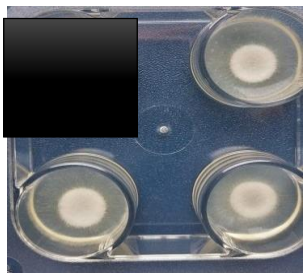
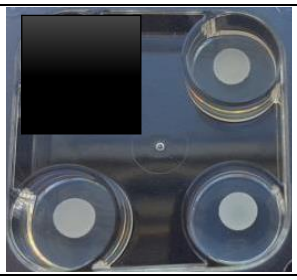
	24h	**48h**
Aspergillus fumigatus		
Aspergillus flavus		
Aspergillus terreus		
Aspergillus niger		

Figure 4. *Aspergillus fumigatus* and *Candida* quality control strains for testing stability of echinocandin-containing 4-well plates (well 2 [upper right], anidulafungin 0.25 mg/L; well 3 [lower left], micafungin 0.125 mg/) and drug-free agar (well 4 [lower right], no drug). *Aspergillus* ATCC 204305 WT strain grows with the typical fluffy radiation halo around the main colony on drug-free agar, but not on echinocandin agars. The echinocandin NWT strain produces the fluffy radiating ring both on drug-free and drug containing agars. *Candida* QC strains grow on drug-free agar forming compact colonies whereas in drug-containing wells, *C. krusei* ATCC6258 but but not *C. parapsilosis* ATCC22019 is completely inhibited. ATCC strains are available from ATCC.org, the DPL1035 homo/SSI-1794 is available from the www.ccug.se.

	24h	48h
A. fumigatus ATCC 204305		
A. fumigatus DPL1035homo/SSI-1794		
C. krusei ATCC6258		
C. parapsilosis ATCC22019		

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