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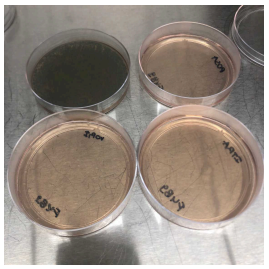
Screening procedure to identify triazole-resistant Aspergillus spp. using agar plates



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We use this protocol and it's working

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Abstract

The methodology of the screening procedure for detecting resistance to azoles in the genus *Aspergillus* spp. consists of an in house technique, with visual analysis of fungal growth on agar plates supplemented with azoles, Itraconazole at a concentration of 4 µg/ml, Voriconazole 2 µg/ml and Posaconazole 0.5 µg/ml. This protocol is based on the E.Def 10.1 from EUCAST

Materials

Reagents:
1L of sterile water: 1000 µL
RPMI 1640 powder – 10.4 g
D-Glucose 2% - 18 g
Bacteriological agar – 20 g
MOPS - 34.53 g (0.165 M)

Materials:
- 4 autoclavable vials (for each antifungal and control)
- 15ml Falcon type tube
- Pasteur pipette
- Pipettes and tips: 1000, 200, 100, 20, 10
- 1L beaker
- Funnel
- Glass stick
- 90 petri plates 60mmx15mm (If you use petri plates divided into 4 the medium will yield more)
- Swabs
- Eppendorfs

Before start

Separate all reagents and identify the plates

Preparation of antifungal solutions:

1 From the antifungal powder prepare stock solutions in DMSO, at concentrations at least 200 times higher than those tested on the agar plate

2 From the stock solution, prepare the working solutions at the following concentrations:
Itraconazole (ITZ) - 400 µg/ml,
Voriconazole (VRZ) - 200 µg/ml
Posaconazole (PSZ) - 50 µg/ml

Storage Temperature: -25 to -18º C (Freezer)

For calculations, use the formula:
Initial concentration x Initial volume = Final concentration x Final volume

	A	B	C	D	E	F
		Stock Solution Concentration	**Stock Solution Volume**	**Volume of DMSO**	**Working Solution Concentration**	**Agar Final Concentration**
		µg/mL	µL	µL	µg/mL	µg/mL
	ITZ	1600	2500	7500	400	4
	VRZ	400	5000	5000	200	2
	PSZ	400	1250	8750	50	0,5

Example: Preparation of 10 mL of working solution of each of the azoles from the stock solution

Preparation of the medium:

5m

3 Weigh all reagents:
RPMI 1640 powder – 10.4 g
D-Glucose 2% - 18 g
Bacteriological agar – 20 g
MOPS - 34.53 g (0.165 M)

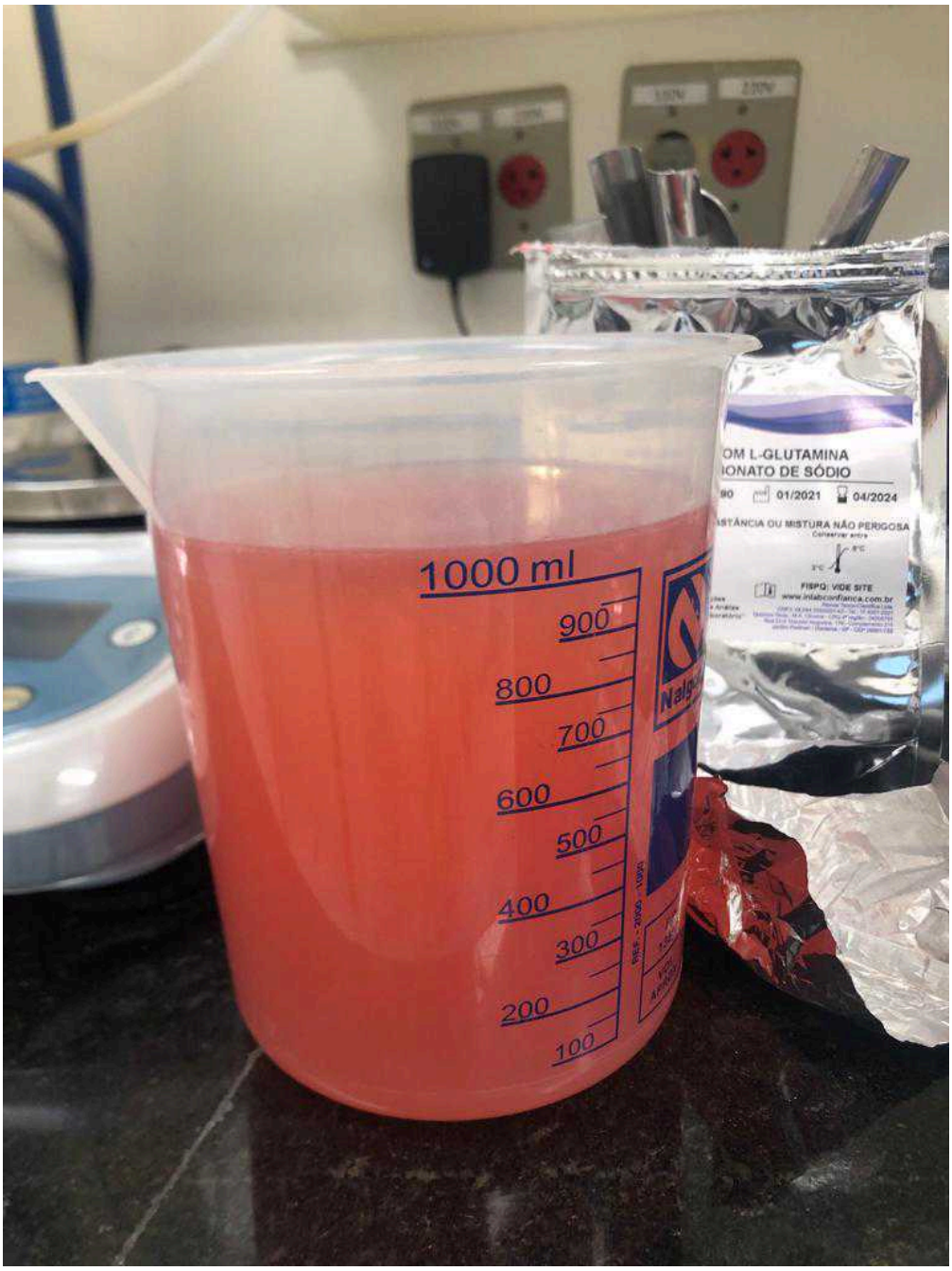
4 In a 1L becker, Add 900 mL of distilled water and RPMI powder 10.4 g , glucose 2% 18 g , Bacto Agar 20 g , MOPS 34.53 g

5 Microwave 00:05:00 , little by little, stirring with a glass rod, until the solutes are completely dissolved 5m

6 Confirm the pH of 7.0

6.1 If necessary adjust with a solution of NaOH (10 M) to 7.0

7 Add more 100 mL of distilled water



Incorrect beaker measurements, it was measured with the test tube.

8 Divide the medium into 4 Erlenmeyer flasks containing 250 mL each

9 Autoclave the medium at 121º for 00:15:00 . 15m

Preparation of the azole-containing plates:

10 Identify all the plates with the azole or "control"

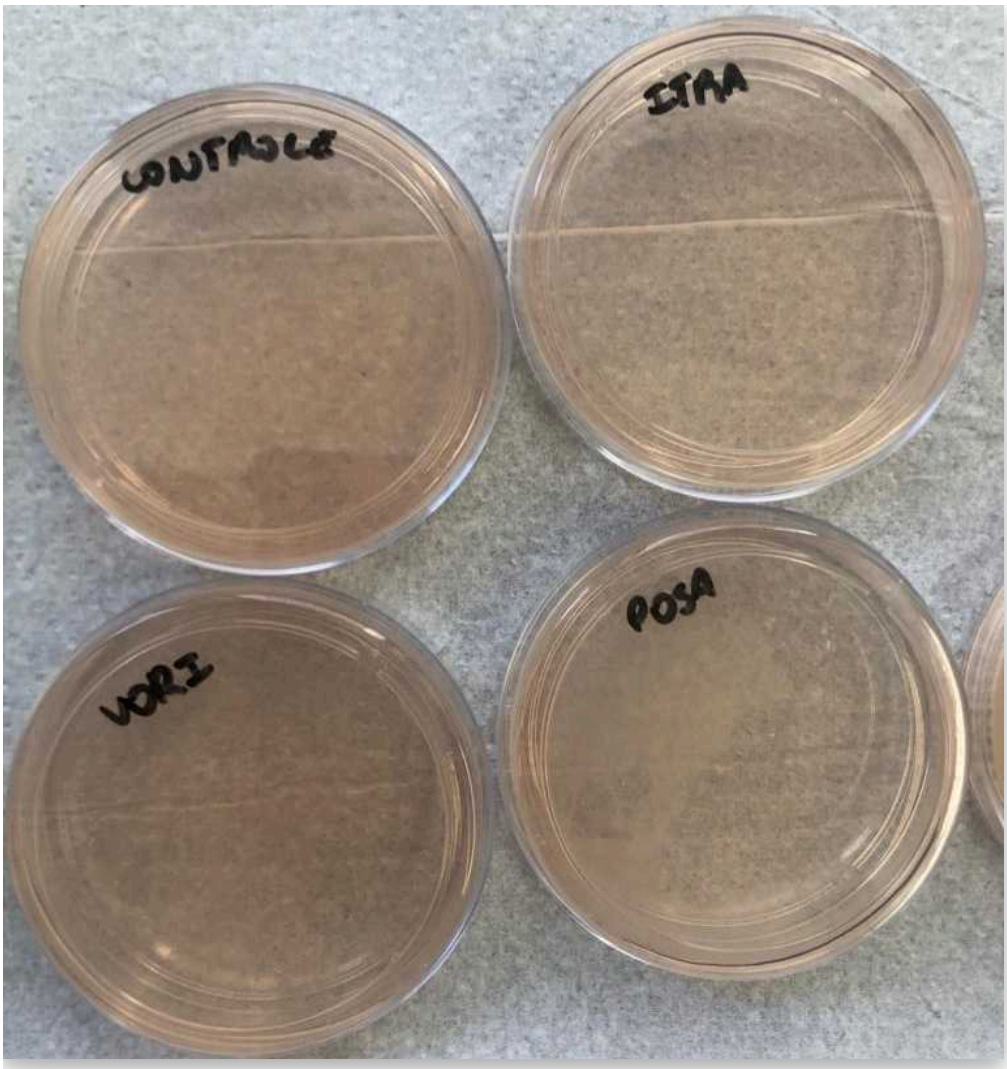
11 Cool the agar down to 45°C

12 Add 2.5 mL of each azole working solution (100% final concentration) to each of the vials and ensure proper mixing

13 The remaining antifungal free vial will be used for the growth control

14 Pour the agar from each vial into the respective plates

15 After solidification of the plates (approximately 00:20:00) Storage the plates at 4°C for up to 6 months from the date of preparation. 20m



Preparation of the inoculum:

16 For inoculum suspensions use fresh and mature cultures incubated for at least 2–7 days

16.1 The isolates should be cultured on Sabouraud dextrose agar (with or without chloramphenicol) or other culture medium where the fungus is able to sporulate sufficiently and incubated at 34–37°C

Aspergillus fumigatus culture ready for the inoculum suspension

17 Make a diluent stock solution by adding 1 ml of Tween 20 to every 50 ml of distilled water

18 Working in the biological safety cabinet, add 3 ml of the water/Tween 20 solution to the 3-7 day slope. Tween 20 will help to put the *Aspergillus* conidia into suspension.

19 If necessary gently detach only the conidia from the culture with a handle until the solution becomes turbid

20 Put 1.5 mL of this conidial suspension in identified eppendorfs

20.1 This conidial suspension can be used in case of retest

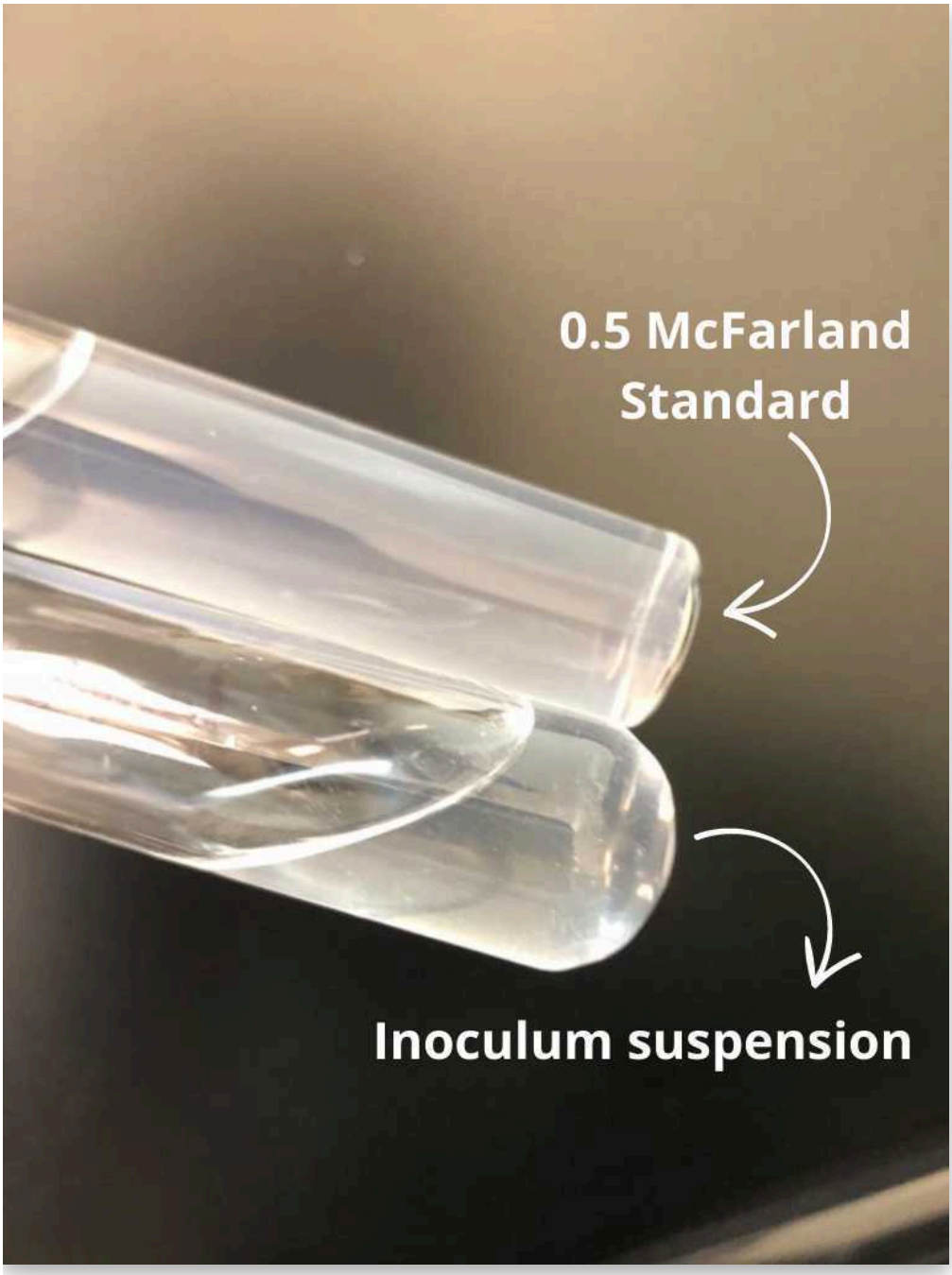
21 Use the residual solution from the tube from steps 17-20 to perform the next steps

22 Add 2 mL of destilled water in a tube and transfer 1-2 drops of the residual solution with conidial

22.1 Or transfer 1-2 drops of the conidial suspension

22.2 In some cases you will need more drops, for *Aspergillus flavus* for example

23 Visually compare the suspension to the 0.5 McFarland Standard and make adjustments as described until the suspension visually matches the standard



23.1 Adjustment is made by adding more destilled water if the suspension is very turbid and more conidia if the turbidity is very low.

Inoculation and incubation of agar plates: 2d

24 Dip a sterile cotton swab in the inoculum suspension and rub the entire surface of the control plate in a back-and-forth motion

25 Re-dip the cotton swab and rub the entire surface in the same way of each of the three remaining plates with the azoles

26 Incubate the plates at 35-37°C for 48:00:00 and observe growth. 2d

26.1 Visual analysis score definition:
0: no visible growth
1: weak/minimal growth (such as >5 tiny colonies or confluent weak growth where isolate was inoculated (covering ≤ half of the plate)
2: clearly visible growth with hyphal extension, but not covering entire plate
3: prominent uninhibited growth covering most of the plate

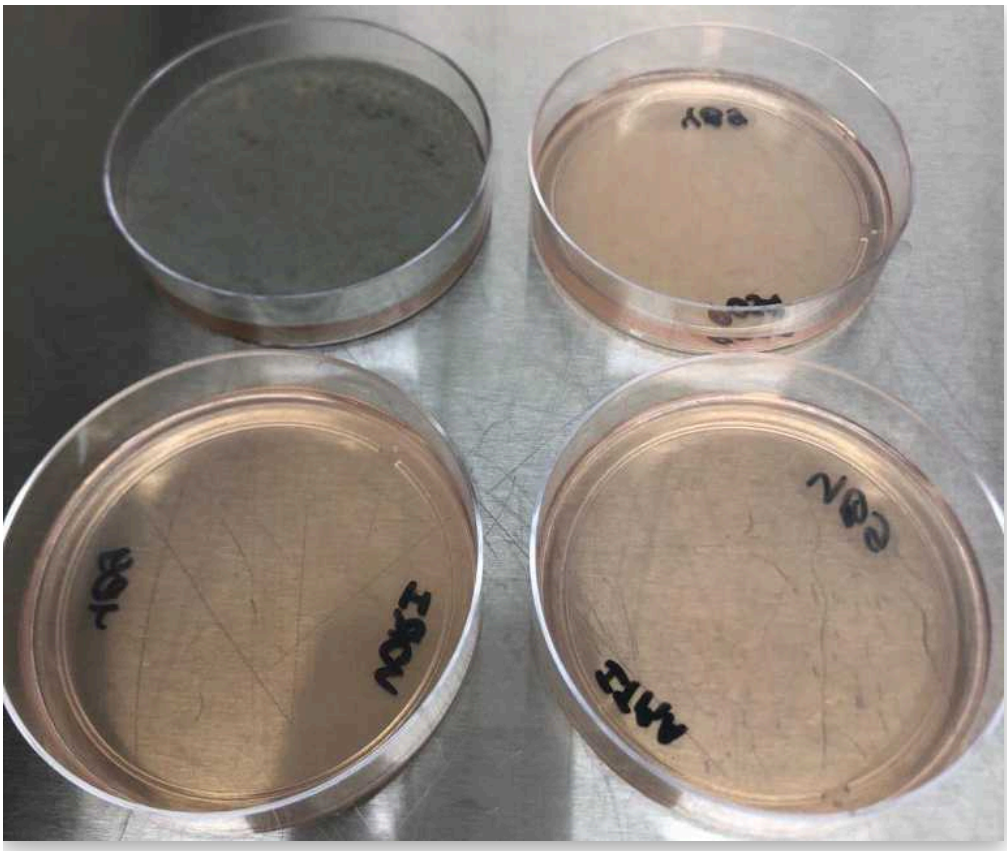
Analyzing the results

27 The presence or absence of fungal growth on the surface of the plates will yield the following preliminary classification:

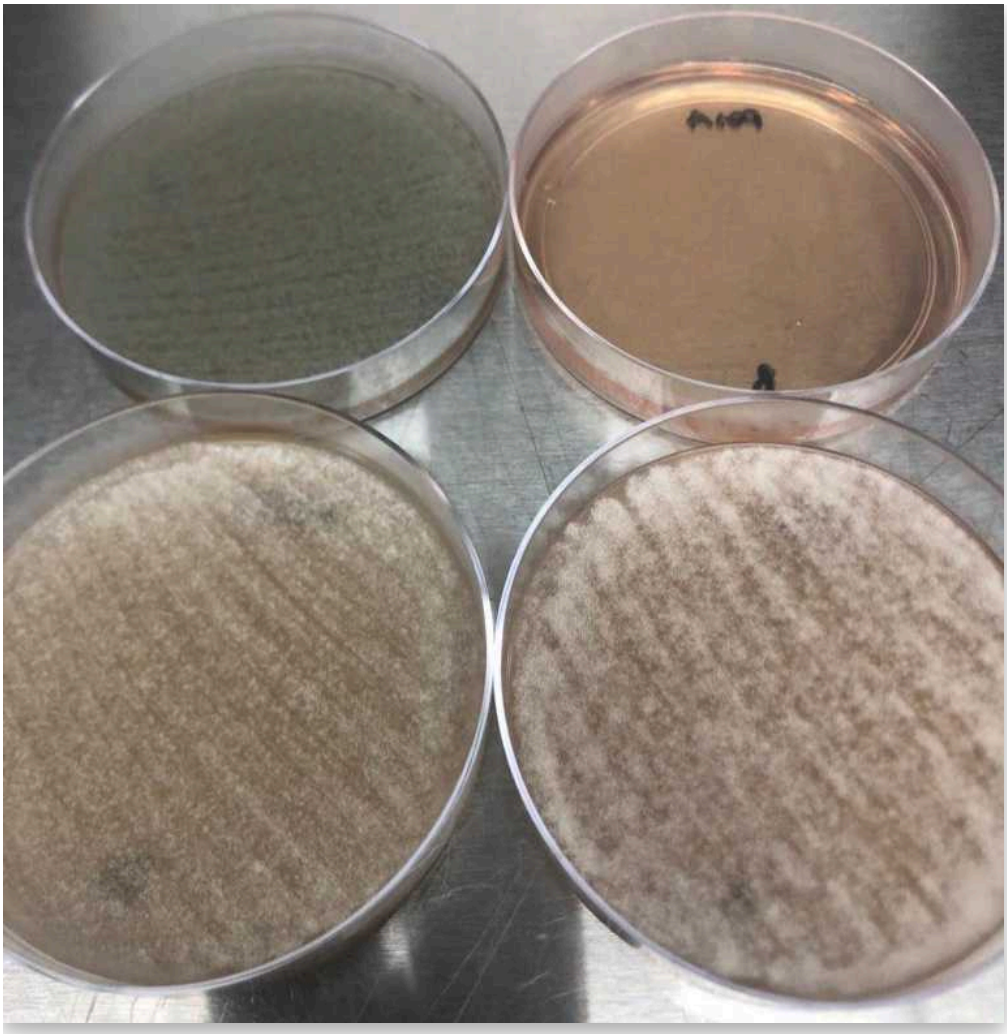
- **Azole susceptible isolate:** growth on the azole-free plate and absence of growth on the others plates
- **Potentially an azole non-susceptible isolate:** growth on the azole-free plate and on any of the other supplemented plates.

Expected result

The control plate should always show growth (score definition = 3)
The azole containing plates will show growth only if the isolate is potentially resistant to the azole.



Azole susceptible isolate - Being the upper left control plate, without azole, upper right PSZ 0.5 µg/mL, lower left VRZ 2 µg/mL and lower right ITZ 4 µg/mL.



Potentially an ITZ and VRZ resistant isolate - Being the upper left control plate, without azole, upper right PSZ 0.5 µg/mL, lower left VRZ 2 µg/mL and lower right ITZ 4 µg/mL.