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Postantifungal Effect Methods

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Summary

Postantifungal effect (PAFE) is the evaluation of antifungal activity after the suppression of fungal growth when the drug is removed from the fungal suspension. In vitro, this effect might simulate the in vivo situation when the concentration of the drug falls to less than the minimum inhibitory concentration values and could be another tool, together with the classic in vitro susceptibility tests, to optimize the interaction of drugs–fungi. In this chapter, two model methods to evaluate the PAFE of yeasts and filamentous fungi are described in which practical advices and tricks are given to help the worker to develop the techniques. The procedures outlined include preparation of stock solutions of the drugs, concentration medium, exposure time colony count determination, and interpretation of the results to quantify the PAFE.

Key Words: Yeasts; filamentous fungi; postantifungal effect; PAFE; *Aspergillus*; *Candida.*

1. Introduction

Several in vitro methods to evaluate the action of different antifungal drugs against filamentous fungi and yeasts currently are used in which constant levels of the drugs are analyzed. However, in vivo, the organism is exposed to fluctuant levels of antimicrobial agents. In vivo, drug concentrations decrease to levels less than the minimum inhibitory concentration (MIC). In vitro, this situation can be simulated by removing the drug from the fungal suspension. The evaluation of whether any effect of the drug persists is called "post-drug exposure effect."

The persistent suppression of micro-organism growth after the removal of antimicrobials was first studied for bacteria. The effect was called postantibiotic effect ***(1)***.

In this chapter, two model methods to evaluate the postdrug exposure effect for yeasts and molds, named the postantifungal effect (PAFE), are described in **refs. *1–3***, based on methods previously described for bacteria, yeasts, and molds.

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Practical advices and tricks are written to help the worker develop the techniques.

The idea to evaluating whether certain drug display postantifungal effects against certain fungi is to have another tool together with the classic in vitro susceptibility tests to optimize the drug–fungi interaction. The in vitro evaluation with animal studies might provide a better understanding to correlate the in vitro tests with the in vivo outcome.

The method for yeasts differs in some steps from the method for molds because of their nonhomogeneous growth. The quantification of PAFE also is described differently for both groups.

The procedures outlined in this chapter are mostly confined to the preparation of stock solution of the drugs, the appropriate dilution in the medium to obtain the desired concentration, exposure time, washing steps for removing of the drug, monitoring of growth, colony count determination, and interpretation of the results to quantify the PAFE.

2. Materials

1. Fresh isolates of yeasts or filamentous fungi.
2. Antifungal powders: amphotericin B (AMB), which is sensitive to light, should be stored at 5°C. However, drugs such as itraconazole, fluconazole, and terbinafine can be stored at room temperature. For the storage of other drugs, follow the manufacturer's instructions.
3. Solvents: dimethylsulfoxide (DMSO), water, or others according to the manufacturer's instructions.
4. Stock solutions of the antifungal agents can be stored at –70°C until the day of use.
5. Medium: standard medium, RPMI-1640 with glutamine, without bicarbonate.
6. Buffer: 0.165 *M* of 3-(*N*-morpholino) propanosulfonic acid (MOPS).
7. Sterile saline solution.
8. Tween-20 or -80.
9. 20–1000-µL; 5, 10, and 25 mL pipet.
10. 100-µL Multichannel pipet.
11. 10-mL Plastic tubes adequate for centrifugation.
12. Centrifuge.
13. Petri dishes.
14. Spectrophotometer.
15. Hemocytometer chambers.
16. 96-Well, flat-bottom microtitration plates.
17. Computerized spectrophotometric reader.
18. McFarland 0.5 scale.

3. Methods

The methods described in this chapter explain how to evaluate the PAFE in yeasts and filamentous fungi. The quantification of PAFE is addressed in each case.

3.1. PAFE in Yeasts

1. Isolates. Pass the isolates by subculturing onto Sabouraud dextrose (SD) for 48 h at 37°C.
2. Determine the MIC according to the National Committee for Laboratory Clinical Standards (NCCLS ***[4]***).
3. Prepare the stock solution of the antifungal agent at convenient starting concentration and storage at –70°C until use (*see* **Note 1**).
4. Dilute the stock solution at least 50 times in RPMI-1640 for nonwater-soluble drugs (*see* **Note 2**).
5. Prepare serial dilution from the above resulting starting drug concentration to obtain the desired concentration of the drug according to the MIC values for PAFE evaluation (*see* **Note 3**).
6. To prepare the yeasts for suspension, transfer some colonies from the 48-h culture in sterile water and adjust to 0.5 McFarland.
7. One milliliter of this suspension should be added to 9 mL of RPMI-1640 containing the desired concentration of the drug. Use plastic tubes adequate for centrifugation.
8. Prepare controls following the same steps without drug.
9. Incubate both, controls and the exposed yeasts at 37°C during the period of time that had been chosen.
10. After the incubation period, remove the drug by three sequential washings in sterile normal saline. Centrifuge for 10 min at 3500*g* each (*see* **Note 4**).
11. After the third washing, resuspend the pellet in sterile normal saline and readjust to 0.5 McFarland.
12. Add 1 mL of the readjusted fungal suspension to 9 mL of warm RPMI-1640.
13. Conduct colony count determination at predetermined time points. For instance, remove and aliquot from the controls and the exposed yeasts at 0, 6, 8, 10, 12, 14, 16, 18, 20, 24, and 26 h, and make 10-fold serial dilutions at each time for each condition. Streak 30 µL onto SD for 24 h (*see* **Note 5**).

3.2. PAFE in Filamentous Fungi

1. Isolates. Pass the isolates by subculturing onto SD or potato dextrose agar for 5–7 d at 28°C.
2. Determine the MIC according to the NCCLS ***(5).***
3. Prepare the stock solution of the antifungal agent at convenient starting concentration and store at –70°C until use (*see* **Note 1**).
4. Dilute the stock solution at least 50 times in RPMI-1640 plus 0.5% Tween-20 for nonwater-soluble drugs (*see* **Note 2**).

5. Prepare serial dilution from the above resulting starting drug concentration to obtain the desire concentration of the drug that is according with the MIC values for PAFE evaluation (*see* **Note 3**).
6. Prepare the fungal suspension in a saline solution with 0.5% Tween-20 (*see* **Note 6**). After the particles are allowed to settle, transfer the supernatant to another tube, vortex, and make 10- and 100-fold dilutions. Establish the concentration of conidia or spores microscopically by hemocytometer chambers. Adjust the concentration to obtain 4×10^5–5×10^5 conidia or spores/mL.
7. Add 1 mL from the adjusted suspension to 9 mL of RPMI-1640 plus 0.5% of Tween-20 with the concentration of the drug that had been chosen, such, 4, 2, or 1× the corresponding MIC value. The final volume should be 10 mL. Use plastic tubes adequate for centrifugation (*see* **Note 7**).
8. Prepare controls with 1 mL of fungal suspension and 9 mL of RPMI-1640 plus Tween-20, without drug.
9. Incubate both, controls and exposed conidia or spores at 37°C with shaking during a period of time that depends on the drug and the strain tested (*see* **Note 9**).
10. After the incubation period, wash the conidia or spores with saline plus Tween-20, centrifuge at 3500*g* for 15 min. Repeat for three wash cycles (*see* **Note 4**).
11. Decant the 98% of supernatant and resuspended the pellet with RPMI-1640 plus 0.05% Tween-20 to a final volume of 10 mL.
12. Make a colony count determination (colony-forming unit [CFU]/milliliter) of the control and the exposed conidia or spores by plating 30 µL of 10- and 100-fold dilution in water or saline onto SD agar plates. Incubate at 37°C and check after 24 and 48 h for the presence of colonies (*see* **Note 9**).
13. Place 200 µL of the control and the exposed conidia or spores in microtitration plates and incubate at 37°C or 48 h in a spectrophotometer reader that is able to monitor the growth automatically.
14. Always microscopically examine the controls and exposed conidia or spores to ensure that there are not differences in morphology between them. Pay attention that in the case of presence of PAFE, the difference is the time at which the germination starts; no changes in morphology should be present during the exposure. In addition, the experiments have to be performed in duplicate or triplicate (*see* **Note 8**).

3.3. Quantification of PAFE for Yeasts

1. PAFE is calculated by taking the difference in time required for the control and the exposed yeasts to grow 1 $\log_{10}$ after drug removal.
2. The formula PAFE = $T - C$ can be applied, for which, T is the time at which the exposed yeasts growth and C is the time at which the corresponding control growth and is expressed in hours (*see* **Note 5**).

3.4. Quantification of PAFE for Filamentous Fungi

1. It is recommended to use the point in the growth curve optical density $(OD)_0$. This point is calculated as the first increase in OD for three consecutive measure-

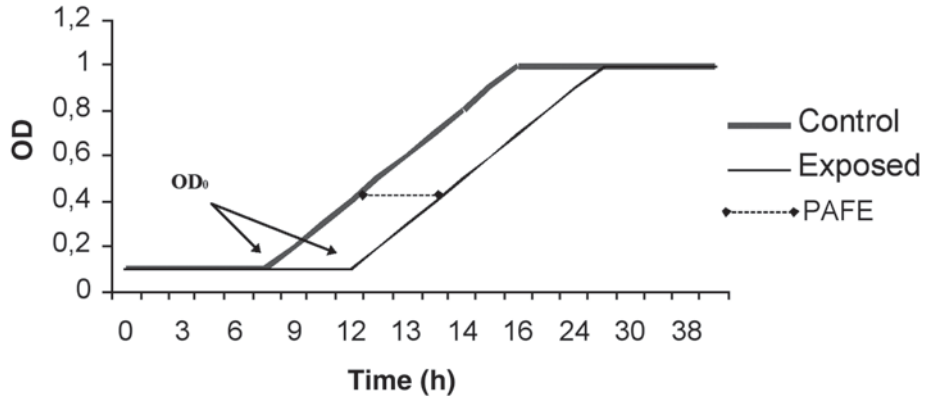

Fig. 1. Interpretive schema of growth curves of control and exposed conidia. Example: $T = 12.5$ h; $C = 6.5$ h. Postantifungal effect = $T - C = 12.5 - 6.5 = 6$ h.

ments for the control and the exposed conidia or spores for each strain at each tested condition.

2. PAFE is calculated using the formula: PAFE = $T - C$, where T is the first increase in OD_0 of the drug exposed conidia or spores and C is the first increase in OD_0 to the corresponding control (*see* **Fig. 1**).
3. Calculate the mean, coefficient of variation, and upper-lower 95% confidence interval for those, control and exposed conidia or spores at each condition for at least 4–8 growth curves for each.
4. PAFE is considered to be present when the growth of the exposed conidia or spores is delayed and the lower 95% confidence interval is delayed until beyond the upper 95% confidence interval of the control. Otherwise, when regrowth of drug-exposure isolates occurred within the upper 95% confidence interval of the corresponding controls, PAFE is considered absent.

4. Notes

1. Drugs that are dissolved in DMSO should be diluted at least 50 times in the medium. If the stock solution is at concentration of 400 µg/mL, then the starting concentration will be 8 µg/mL. Calculate carefully the total volume that is necessary to evaluate all the strains with all the concentrations.
2. RPMI-1640 is the standard medium, but other media also can be used.
3. To evaluate PAFE, it is recommended first to determine the MIC of the strains to plan adequately the concentration at which the PAFE will be evaluated. This concentration might be equal to, greater than, or less than the MIC values, for instance 1, 4, 0.5× the MIC. Always explore the pharmacokinetic profile of the drug.
4. Note that the CFU methodology is described here for the determination of PAFE for yeasts; however, the spectrophotometer procedure also is suitable.

5. Washing and decanting of 98% of the supernatant was demonstrated to reduce the concentration of the drug as much as 10,000-fold ***(1)***. Some technical problems might be appear as precipitation of some drugs, especially in the case of AMB. In such cases other procedure than washing, as filtration, can be employed. Dilution is not recommended for the fact that the amount of conidia or spores might insufficient for the correct monitoring of fungal growth.
6. Tween-80 is recommended for *Zygomycetes*, whereas Tween-20 is recomended for the other filamentous genera, including *Aspergillus*, black fungi, and others.
7. If the drug concentration is four times the MIC with a MIC value of 4 µg/mL, the final concentration will be 16 µg/mL. Therefore, the final volume of 10 mL is: 1 mL of fungal suspension plus the calculated mL that contain 16 µg/mL and the rest to complete the volume is RPMI-1640 alone.
8. At this point, it is important to perform killing studies and microscopic evaluation, for instance, if after 4 h of incubation of *Aspergillus* with AMB or azoles, germination and killing are not observed ***(2)***. However, for *Zygomycetes*, it depends on the genera and the time of incubation ***(6)***.
9. For CFU determination, incubation varies depending on slow- or fast-growing fungi, from 12–24 h for *Zygomycetes* and to 96 h for black fungi. In addition, it is very important that the CFU of controls and exposed fungi remain in a narrow range, such 2×10^4 to 4×10^4 CFU/mL. The inconvenience is that if the range is wide, false-positive results can be present. In other words, the difference in time for growing between the control and exposed fungi can be caused by small amount of conidia or spores and not as result of the presence of PAFE.

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