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Antifungal Combinations

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Summary

The increase in fungal infections and the change in fungal epidemiology is caused by the extensive use of antifungal agents to treat fungal infections that are being diagnosed in severly immunocompromised hosts. In addition, opportunistic fungal infections resistant to antifungal drugs have become increasingly common, and the armamentarium for treatment remains limited. A possible approach to overcoming these problems is to combine antifungal drugs, especially if the mechanisms of action are different. The in vitro test is the first step to evaluate possible antifungal combinations. In this chapter, the three most frequently used metholodologies are described: checkerboard, E-test, and time-kill curves. The description of each technique and intrepretaion of the results are addressed in detail.

Key Words: Yeasts; filamentous fungi; antifungal combinations; FICI; E-test; kill curves;, *Aspergillus*; *Candida.*

1. Introduction

Antimicrobial combinations are used most frequently to provide broad-spectrum empiric coverage in the treatment of infections in patients who are seriously ill. Advantages of antimicrobial combination therapy include the possibility of decreasing the emergence of resistant strains, decreasing dose-related toxicity as a result of reduced dosage and, most importantly, achieving antimicrobial synergism. Less frequently, combination of antimicrobials are chosen because an identified pathogen is resistant to the conventional dose of single antimicrobial.

The mortality rate of invasive fungal infections is high despite antifungal chemotherapy, monotherapy with amphotericin B (AMB), azoles (fluconazole, itraconazole, or voriconazole), or caspofungin. Therefore, new approaches for treating these infections are warranted. Promising results are coming from the field of in vitro combination of antifungal drugs against fungi ***(1–4)***.

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In this chapter, different methodologies to evaluate the in vitro antifungal interactions are described by the most frequent techniques, such as checkerboard, E-test, and the time-kill curves.

2. Materials

2.1. Checkerboard Technique

1. RPMI-3-(*N*-morpholino) propanesulfonic acid (MOPS) medium: dissolve 10.4 g of RPMI-1640 powder (with glutamine and phenol red, but without bicarbonate) in 900 mL of distilled water. Add 34.53 g of MOPS (0.165 mol/L final) and adjust the pH to 7.0 with NaOH. Make up to a final volume of 1 L with distilled water. Filter sterilize and store at 4°C.
2. Sabouraud's dextrose (SD) and potato dextrose agar (PDA) slants.
3. Spectrophotometer.
4. McFarland scale 0.5.
5. Hemocytometer.
6. Tween-20.
7. 96-Well, flat-bottom microtitration plates.
8. 25 cm^2 Polystyrene tissue culture flasks.
9. Antifungal drugs: AMB, fluconazole (FLU), itraconazole (ITZ), voriconazole (VRZ), 5-flucytosine (5FC), caspofungin (CAS), or any other investigational drugs. A powder of known potency should be used.

2.2. E-Test Method

1. RPMI-1640–MOPS medium (2X) dissolve 20.8 g of RPMI-1640 powder (with glutamine and phenol red but without bicarbonate) in 900 mL of distilled water. Add 69.06 g of MOPS and adjust the pH to 7.0 with NaOH. Make up to a final volume of 1 L with distilled water. Sterilize by filtration.
2. Spectrophotometer reader.
3. McFarland scale 0.5.
4. Antifungal drugs: E-test strips provided by the manufactured.
5. Sterilized Petri dishes that are 15 cm in diameter.
6. SD agar.
7. PDA agar.

2.3. Time-Kill Curves

1. RPMI-MOPS medium: dissolve 10.4 g of RPMI-1640 powder (with glutamine and phenol red, but without bicarbonate) in 900 mL of distilled water. Add 34.53 g of MOPS (0.165 mol/L final) and adjust the pH to 7.0 with NaOH. Make up to a final volume of 1 L with distilled water. Filter sterilize and store at 4°C.
2. SD slants.
3. Hemocytometer.
4. SD plates.

5. Sterile spreaders (Hockey sticks).
6. Orbital shaker.
7. 25 cm^2 Polystyrene tissue culture flasks.
8. Antifungal drugs: an antifungal powder of known potency should be used.

3. Methods

3.1. Checkerboard Technique

1. Prepare concentrated antifungal stock solutions and store in aliquots at –80°C. Most of the antifungal drugs can be stored at –80°C for up to 6 mo without loss of activity (*see* **Note 1** ***[5,6]***).
2. For two-dimensional microplate preparation, make a series of 2-fold dilutions of each drug in the corresponding solvents according to the dilution scheme of the National Committee for Clinical Laboratory Standards (NCCLS) and then dilute 100-fold in the medium to obtain four times the final concentration. Add 50-µL aliquots of each drug concentration of the drug A to columns 1 to 11, and then 50-µL aliquots of each concentration of drug B to rows A to H. In the wells of column 11 and in the wells of row H, add 50 µL of the medium containing 1% of the solvent. Thus, column 11 and row H contain the drugs A and B alone, respectively. Column 12 are the drug-free wells that serve as the growth control (**Fig. 1**).
3. Inoculum preparation for yeasts: culture each isolate for 1–2 d on SD agar at 35°C. Take some colonies and suspend in sterile water. After, vortex vigorously, and adjust the turbidity to 75–77% of transmittance at 530 nm or adjust the yeast suspension to 0.5 McFarland scale. Then dilute the suspension 1/1000 in RPMI-1640 medium, which results in a 2X inoculum. Therefore, the final inoculum will range from 0.5×10^2 to 5×10^3 colony-forming units (CFU)/mL.
4. Inoculum preparation for molds: culture each isolate for 7 d on PDA medium for 5–7 d at 35°C. Collect conidia or spores with a swab and suspend in sterile saline plus Tween-20 (0.05%). After the heavy particles are allowed to settle, measure the turbidity of the supernatants spectrophotometrically at 530 nm and adjust the transmittance to 80–82% for *Aspergillus* and *Sporothrix,* 68–72% for *Scedosporium* and *Fusarium* species. In the case of Zygomycetes or other uncommon fungi, count spore suspensions with a hematocytometer and then dilute into RPMI-1640 to a concentration of 2×10^4 spores/mL (*see* **Note 2**). Dilute each suspension 1:50 in RPMI-1640 to obtain two times the final inoculum size, ranging from 0.5×10^4 to 5×10^4 CFU/mL. Verify the inoculum size by plating 100 µL of serial dilutions of each inoculum onto a SD agar plate and incubate until growth became visible.
5. Inoculate each microplate with 100 µL of the inoculum suspension.
6. Incubate the microplates at 35°C for 48–72 h (*see* **Note 3** ***[5–7]***).
7. Minimum inhibitory concentration (MIC) end point determination: can be determined visually and/or spectrophotometrically at 450 nm for yeasts and 405 nm for molds. In general for spectrophotometric reading the optical density (OD) of

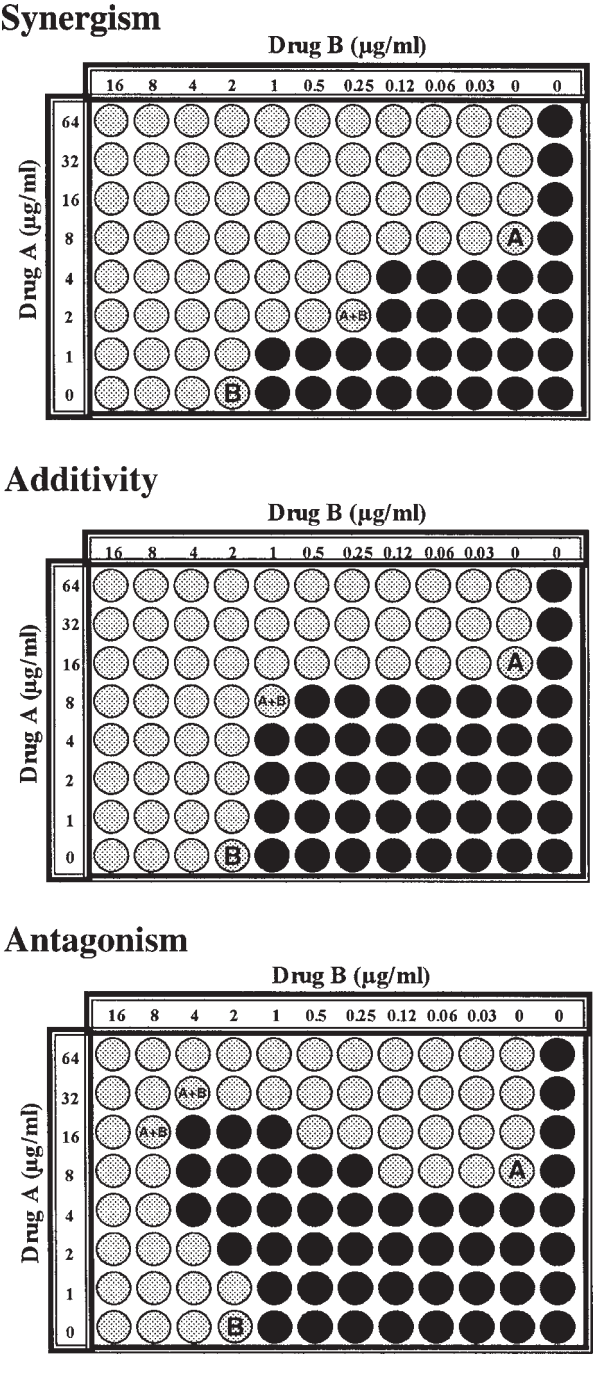

Fig. 1. Checkerboard technique: interpretation of the results.

the blank, a microtitration plate that is incubated with a conidium-free inoculum, is subtracted from the OD values. The percentage of growth for each well is calculated by comparing the OD of the well with that of the drug-free control. The MIC for azoles and 5FC is defined as the lowest concentration showing prominent growth inhibition (MIC–2: 50% reduction) against *Candida* and *Cryptococcus.* The MIC for ITZ, voriconazole, posaconazole, and AMB against molds are defined as the lowest concentration showing no growth (MIC–0: 100% reduction) according to NCCLS guidelines ***(5,6)***. The MIC end point for CAS is not yet standardized. Both on-scale and off-scale MICs should be included in the analysis. High off-scale MICs should be converted to the next twofold dilution, whereas low off-scale MICs are left unchanged.

8. To evaluate the interaction of the drugs, the fractional inhibitory concentration index (FICI) has to be calculated for each concentration. The FICI is calculated for each agent by dividing the inhibitory concentration of each drug when used in combination by its MIC. FICI values are calculated as follows: (MIC of Drug A in combination/MIC of Drug A alone) + (MIC of Drug B in combination/MIC of Drug B alone). The interpretation of the FICI is determined as follows: ≤ 0.5, synergistic effect; >0.5 but < 4, no interaction; and ≥ 4, antagonistic effect (*see* **Note 4** ***[8]***). Among all FICIs calculated for each data set, the FICI is determined as the $FICI_{min}$ (the lowest FICI) when the $FICI_{max}$ (the highest FICI) is smaller than four; otherwise, the FICI is determined as the $FICI_{max}$ (*see* **Fig. 1**).

3.2. E-Test Method

1. RPMI-1640–MOPS medium: dissolve 20.8 g of RPMI-1640 powder (2X) (with glutamine and 0.165 mol/L final solution) and adjust the pH to 7.0 with NaOH. Make up to a final volume of 1 L. Dissolve by heating 30 g of agar in 1 L of distilled water autoclave.
2. Heat the RPMI-1640–MOPS until 45°C and let the agar cool until 45°C. Then, mix both preparations in order to obtained 1X final solution and dispense in Petri dishes (25 mL for a 90-mm Petri dish; *see* **Note 5**).
3. Inoculum preparation for yeasts: to adjust the suspension, *see* **Subheading 3.1., step 3**. Dip a sterile swab into the yeast suspension and press out excess fluid. Inoculate the plates by swabbing the entire surface of the agar in three directions to achieve uniform distribution. Allow the moisture to be absorbed (15 min).
4. Inoculum preparation for molds: to adjust the suspension, *see* **Subheading 3.1., step 4**. Inoculate the plates in the same way as for yeasts. For a uniform distribution, after 1–2 min, aspirate the excess moisture and let the plate dry.
5. Place the antifungal strips onto the inoculated agar using sterile forceps.
6. Place the E-test strip (Drug A) onto the agar and remove it after 1 h (*see* **Notes 6** and **7**). Then place the E-test strip (Drug B) onto the agar over demarcation left from previous strip. Apply additional E-test strips for Drug A and B alone (**Fig. 2**).
7. Incubate the plates at 35°C for 48–72 h. The incubation time for *Candida* is 24–48 h; 48 h for *Aspergillus* and 72 h for *Cryptococcus neoformans.*

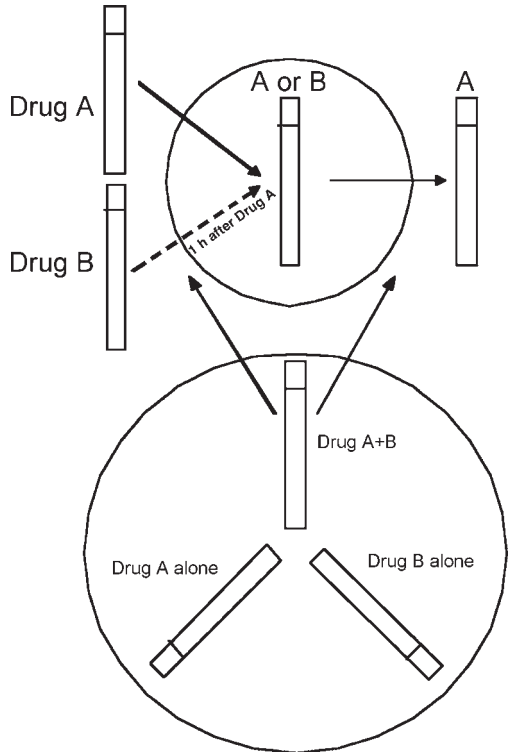

Fig. 2. E-test: scheme to prepare the plate.

8. E-test susceptibility end points: MICs for azoles and 5FC are read at the intersection of the scale of the strip and the first discernible growth-inhibition ellipse according to the recommendations of the manufacturer. The E-test susceptibility end point MIC for AMB is reading at the intersection of the scale of the strip with the first completely clear ellipse.
9. For interpretation of the results and FICI calculation, *see* checkerboard methods in **Subheading 3.1.**, **step 8**.

3.3. Time-Kill Methods

1. *See* **Note 8**.
2. Prepare concentrated antifungal stock solutions and store in aliquots at –80°C. Most of the antifungal drugs can be stored at –80°C for up to 6 mo without a loss of activity (*see* **Note 9**).
3. Culture the strain to be tested on SD agar slant for 24 h at 35°C.
4. Pick up a few colonies and make a suspension in 20 mL of sterile RPMI-1640–MOPS.
5. Count the yeast suspension in a hemocytometer.

6. Adjust the yeast suspension to 1×10^6 CFU/mL by adding the appropriate volume of RPMI-1640–MOPS.
7. Fill two or more tissue culture flasks with 17.8 mL of RPMI (for testing drugs alone) and two or more flasks with 17.6 mL of RPMI (for testing drugs in combination).
8. From stock solutions, prepare antifungal solutions at 100× the final desired concentration in the tests (*see* **Note 10**).
9. Add 200 µL of the 100X antifungal solution to obtain a 1X final concentration (*see* **Note 11**). At least four flasks must be used: two for the drugs alone, one for the drugs in combination, and one without any drug (control).
10. Inoculate each tissue culture flask with 2 mL of the yeast suspension at 1×10^6 CFU/mL to obtain a final inoculum size of 1×10^5 CFU/mL.
11. Incubate the flasks at 35°C with shaking for 48 h (*see* **Note 12**).
12. At predetermined time points (0, 1, 2, 4, 8, 24, and 48 h) aseptically remove 50-µL samples from each test solution, and dilute 1/10 and 1/100 in sterile NaCl 0.85% (*see* **Notes 13** and **14**). Plate 50 µL of each dilution (including the neat dilution) with a hockey stick on SD agar plates in duplicate. Incubate the plates at 35°C for 48 h.
13. Count CFU on each plate and calculate the mean CFU/mL (*see* **Note 15**).
14. Transform the CFU data to $\log_{10}$ and plot the mean $\log_{10}$CFU/mL vs time (*see* **Fig. 3**).
15. Analyze the results by comparing the activity of the drugs alone with that of the drugs in combination as follows: an increase in killing of ≥ 2 $\log_{10}$CFU/mL for the combination compared to the most active drug alone defined synergy. Antagonism is defined as ≥ 2 $\log_{10}$CFU/mL decrease in killing with the combination, as compared with the most active single drug alone. Indifference is defined when the killing effect with the two agents in combination is within 2 $\log_{10}$CFU/mL of either agent alone (*see* **Fig. 3** and **Note 16**).

4. Notes

1. Some antifungal agents can be dissolved in water (e.g., FLU, 5FC, CAS) but some others in dimethylsulfoxide (DMSO) (e.g., AMB, ITZ, voriconazole, posaconazole, and ravuconazole ***[5,6]***).
2. For preparation of the inocula for *Aspergillus* species, Zygomycetes, and *Scedosporium* species, using a hemocytometer could be a better approach.
3. Generally, an incubation time of 24–48 h is used for *Candida* spp., 48 h for *Aspergillus*, 24 h for *Zygomycetes*, and up to 72 h for *C. neoformans* and *Scedosporium* species ***(5–7)***.
4. In practice, synergism or antagonism calculated as was pointed above is equivalent to a reduction or increase of at least two dilution steps in the MICs of both drugs when they are combined compared to the MICs for the drugs alone. However, there are no NCCLS guidelines for performing synergy studies and no standardization for analysis of the results. Then, criteria used to define synergism, additivity (or indifference), and antagonism may vary from one publication to another. According to the Loewe theory of drug interaction, in order to calculate

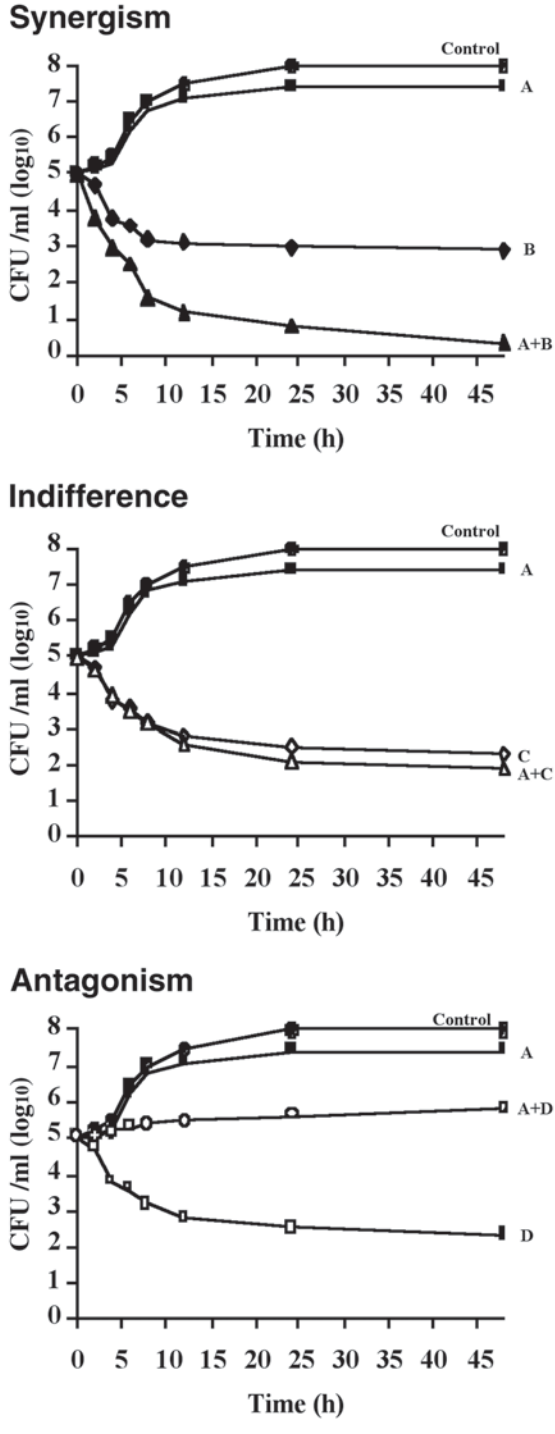

Fig. 3. Time-kill curves for assessment of in vitro antifungal combinations: interpretation of the results. An antifungal A with no activity against the tested strain is combined with antifungal B, C, or D. By comparison with the activity of the drugs alone, synergism, indifference, or antagonism is concluded for the combination. CFU, colony-forming units.

the FICI, the end point determination (MIC) for the drugs alone or in combination have to be the same; this is specially important when drugs with different end points, such as FLU and AMB against *Candida*, are combined.

5. To preserve the L-glutamine activity, is not possible to sterilize by autoclave the RPMI-1640 media. For that reason, to solve this problem, prepare the media and the agar at twice the final concentration and mix afterward. To facilitate the growth of the yeasts, addition of glucose, 2% final concentration onto the RPMI media is recommended.
6. According to the manufacturer's instructions, 1 h is enough to achieve an effective release of the drugs from the carrier (E-test strip) into the agar matrix .
7. The E-test diffusion technique also can be set up differently when assessing in vitro antifungal combinations. For example, an E-test strip containing an antifungal A is placed on the agar plate containing an antifungal B at a concentration below the MIC against the tested strain. If the ellipse of inhibition decreases (corresponding to an increase of the MIC) antagonism is concluded and if the ellipse of inhibition increases (corresponding to an decrease of the MIC), synergy can be concluded.
8. It has to be noted that most of the fungi for which antifungal combination is of interest are classified as BSL (BioSafety Level)-2 organisms (e.g., *C. albicans* and several other *Candida* spp., yeast form of *C. neoformans*). Therefore, it is recommended that one manipulate the cultures and inoculated media in a safety cabinet.
9. Some antifungal agents can be dissolved in water (e.g., FLU, 5FC, CAS) and some must be dissolved in DMSO (e.g.. AMB, ITZ, voriconazole ***[6]***).
10. With nonwater-soluble drugs that are dissolved in DMSO, the final concentration of solvent in the test will be 1%. This concentration of DMSO is assumed to have no activity against most of the fungi.
11. The final desired concentration in the test is variable depending on the susceptibility of the strains, the achievable drug level in serum in humans, and the chemical nature of the antifungal (fungistatic or fungicidal). Generally tests are carried out with final concentrations ranging between 0.25–16X MIC.
12. Some antifungal drugs are light-sensitive (e.g., AMB) and the flasks must be then incubated in the dark to avoid drug degradation during the incubation period. Incubation time depends on the species that are tested. Generally, an incubation time of 24–48 h is used for *Candida* spp. but can be extended to 72 h for *C. neoformans* ***(7)***.
13. For the controls and for the flasks containing antifungal drugs with no activity or tested at low concentrations a more important dilution factor must be used (e.g., $1/10^3$ to $1/10^5$) before plating.
14. For some antifungals used at high concentration (compared with the MIC of the tested strain) a drug carryover effect may be observed. Preliminary experiments should be performed to assess the presence of a carryover effect, which is defined as more than 25% reduction in CFU/mL in presence of the drug compared with the control value. Different techniques can be used to minimize this effect. For

example it has been shown that dilution followed by filtration of the samples is effective in eliminating the antifungal carryover ***(9)***.

15. The limit of quantitation of the technique also must be determined. In theory, this limit is 20 CFU/mL when undiluted samples of 50 µL are plated. It could be useful to determined experimentally the lower limit of accurately detectable CFU/mL ***(9)***.
16. At the present time, there are no NCCLS guidelines for performing synergy studies and no standardization for analysis of the results. Then, criteria used to define synergism, additivity (or indifference), and antagonism may vary from one publication to another.

References

1. Afeltra, J. Dannaoui, E., Meis, J. F. G. M., Rodriguez-Tudela, J. L., and Verweij, P. E. (2002) In vitro synergistic interaction between amphotericin B and pentamidine against *Scedosporium prolificans*. *Antimicrob. Agents Chemother*. **46,** 3323–3326.
2. Barchiesi, F., Di Francesco, L. F., Compagnucci, P., Arzeni, D., Giacometti, A., and Scalise, G. (1998) In-vitro interaction of terbinafine with amphotericin B, fluconazole and itraconazole against clinical isolates of *Candida albicans*. *J. Antimicrob. Chemother.* **41,** 59–65.
3. Dannaoui, E., Afeltra, J., Meis, J. F. G. M., and Verweij, P. E. (2002) In vitro susceptibilities of Zygomycetes to combinations of antimicrobial agents. *Antimicrob. Agents Chemother.* **46,** 2708–2711.
4. Vitale, R. G., Afeltra, J., De Hoog, G. S., Rijs, A. J., and Verweij, P. E. (2003) In vitro activity of amphotericin B and itraconazole in combination with flucytosine, sulfadiazine and quinolones against *Exophiala spinifera*. *J. Antimicrob. Chemother.* **51,** 1297–1300.
5. National Committee for Clinical Laboratory Standards. (2003) *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Conidium-Forming Filamentous Fungi; Approved Standard*. Document M-38A. National Committee for Clinical Laboratory Standards, Wayne, PA.
6. National Committee for Clinical Laboratory Standards. (2002) *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts*. M-27A2. National Committee for Clinical Laboratory Standards, Wayne, PA.
7. Rodero, L., Cordoba, S., Cahn, P., et al. (2000) In vitro susceptibility studies of *Cryptococcus neoformans* isolated from patients with no clinical response to amphotericin B therapy. *J. Antimicrob. Chemother.* **45,** 239–242.
8. Eliopoulos, G. M., and Moellering, R. C. (1996) Antimicrobial combinations, in *Antibiotics in Laboratory Medicine* (V. Lorain., ed.), The Williams & Wilkings Co., Baltimore, pp. 330–396.
9. Klepser, M. E., Ernst, E. J., Lewis, R. E., Ernst, M. E., and Pfaller, M. A. (1998) Influence of test conditions on antifungal time-kill curve results: proposal for standardized methods. *Antimicrob. Agents Chemother*. **42,** 1207–1212