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In Vitro Pharmacodynamic Characteristics of Amphotericin B, Caspofungin, Fluconazole, and Voriconazole against Bloodstream Isolates of Infrequent *Candida* Species from Patients with Hematologic Malignancies

Giovanni Di Bonaventura,[1,2] Ilaria Spedicato,[1,2] Carla Picciani,[1,2] Domenico D'Antonio,[3] and Raffaele Piccolomini[1,2]*

Department of Biomedical Sciences, Laboratory of Clinical Microbiology, "G. d'Annunzio" University,[1] and Aging Research Center, Ce.S.I.,[2] "G. D'Annunzio" University Foundation, Chieti-Pescara, and Clinical Microbiology Service, Department of Hematology and Oncology, "Spirito Santo" Hospital, Pescara,[3] Italy

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Time-kill and postantifungal effect (PAFE) of amphotericin B, caspofungin, fluconazole, and voriconazole were determined against clinical isolates of *Candida guilliermondii*, *Candida kefyr*, and *Candida lusitaniae*. Azoles displayed fungistatic activity and no measurable PAFE, regardless of the concentration tested. Amphotericin B and caspofungin demonstrated concentration-dependent fungicidal activity, although amphotericin B only produced a significant dose-dependent PAFE against all isolates tested.

Invasive fungal infections are important causes of morbidity and mortality in immunosuppressed patients (10). Although *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* account for the majority of *Candida* bloodstream infections, recent epidemiologic trends indicate a shift toward infections by the less frequently isolated non-*albicans Candida* (NAC) species (12). Among NAC species, *C. kefyr*, *C. guilliermondii*, and *C. lusitaniae* are rare causes of invasive infections but are increasingly encountered among severely immunosuppressed patients occurring in nosocomial clusters and/or exhibiting innate or acquired resistance to one or more established antifungal agents, often related to intravascular catheters and breaks in infection control precautions (3–5, 11, 12, 14).

Currently, knowledge of the in vitro pharmacodynamic characteristics of *C. kefyr*, *C. guilliermondii*, and *C. lusitaniae* is poor and limited to amphotericin B (AMB) and voriconazole (VRC) only (7, 15). Therefore, we conducted time-kill and postantifungal effect (PAFE) studies with AMB, caspofungin, fluconazole (FLC), and VRC against bloodstream isolates of *C. guilliermondii*, *C. kefyr*, and *C. lusitaniae* from neutropenic patients.

Antifungal agents. Stock solutions of AMB (Sigma-Aldrich SRL, Milan, Italy), caspofungin (Merck Sharp & Dohme Italia SpA, Rome, Italy), FLC (Pfizer Inc., New York, N.Y.), and VRC (Pfizer) were prepared in RPMI 1640 medium (Sigma) buffered to a pH of 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma) and stored at −80°C until use. Antifungals were solubilized in sterile water, except AMB in dimethyl sulfoxide (Sigma).

Test isolates. Six *Candida* isolates were obtained from the Clinical Microbiology Service, Department of Hematology and Oncology, "Spirito Santo" Hospital, Pescara, Italy, for use in this study: two strains each of *C. guilliermondii* (337 and 555), *C. kefyr* (240 and 270), and *C. lusitaniae* (325 and 447) were selected for testing.

Antifungal susceptibility testing. The MIC for each isolate was determined, in triplicate, by broth microdilution techniques as outlined by the National Committee for Clinical Laboratory Standards (17). The endpoint was defined as 50% inhibition of visible growth for azoles and complete inhibition of visible growth for AMB and caspofungin.

Time-kill. Before the time-kill studies were initiated, antifungal carryover effects were examined. Briefly, 100 μl of a standardized suspension (1×10^3 CFU/ml) of each isolate were added to either 900 μl of sterile water with (sample) or without (control) antifungal at 4 and 8 times the MIC. Immediately following the addition of the antifungal, 100 μl was removed and streaked across Sabouraud dextrose agar (SDA) for colony count determination. Antifungal carryover was defined as a reduction in colony counts in samples by >25% compared to control.

Time-kill experiments, conducted in duplicate, began by suspending colonies from a 24-h SDA growth in RPMI 1640 with MOPS (RPMI 1640-MOPS) to approximately 10^6 CFU/ml. One milliliter of this fungal suspension was then added to 9 ml of RPMI 1640-MOPS without (growth control) or with (test) antifungal at concentrations ranging from 0.125 to 8 times the MIC. The culture was then incubated at 37°C with shaking. At predetermined time points (0, 1, 2, 4, 8, 24, and 48 h), 100 μl samples were removed and serially diluted in cold sterile distilled water, and 100 μl was plated onto SDA for colony counting.

PAFE. Growth control and test vials were prepared as for time-kill experiments. Following an incubation period of 1 h, antifungal was removed by three sequential centrifugations (3,000 × *g*, 10 min). The fungal pellet was resuspended in normal saline after the first two centrifugation periods and in 9 ml of warm RPMI 1640-MOPS after the final centrifugation. The resuspended samples were then incubated at 35°C with agitation,

* Corresponding author. Mailing address: Center of Excellence on Aging, Room 27, 5th level, "G. D'Annunzio" University, Via Colle dell'Ara, 66100 Chieti, Italy. Phone: (39) 0871 54 15 19. Fax: (39) 0871 355 52 82. E-mail: r.piccolomini@dsb.unich.it.

TABLE 1. Microdilution broth results for study isolates

Test isolate	Median MIC (μg/ml) (n = 6)			
	AMB	Caspofungin	FLC	VRC
C. guilliermondii 337	2	128	2	1
C. guilliermondii 555	0.5	128	2	1
C. kefyr 240	4	0.25	0.12	0.12
C. kefyr 270	4	0.25	0.25	0.25
C. lusitaniae 325	4	0.5	2	0.5
C. lusitaniae 447	4	0.5	2	1

and colony counts were performed at 0, 2, 4, 6, 8, and 24 h after the final wash. PAFE experiments were conducted in duplicate.

Analysis. Fungicidal activity was defined as a ≥3 $\log_{10}$ (99.9%) reduction in CFU per milliliter from the starting inoculum concentration. To quantify the extent of antifungal activity, maximal effect (E_{max}), the concentration producing 50% of E_{max} (EC_{50}), the EC_{90}, and the range of the net change ($\log_{10}$ CFU per milliliter) in fungal density were determined for each time point by a sigmoidal Hill three-parameter model with GraphPad Prism (version 4; GraphPad Software Inc., San Diego, Calif.). PAFE was calculated by taking the difference in time required for control and test isolates to grow 1 $\log_{10}$ following drug removal.

Antifungal susceptibility results. Median MICs ranged from 0.5 to 4.0 μg/ml for AMB, 0.25 to 128 μg/ml for caspofungin, 0.12 to 2.0 μg/ml for FLC, and 0.12 to 1.0 μg/ml for VRC (Table 1).

Time-kill results. No antifungal carryover has been observed with any of the isolates at the concentrations tested. Time-kill plots representative of those noted in this study are showed in Fig. 1. AMB yielded fungicidal activity at concentrations ≥4 times the MIC against *C. guilliermondii* isolates, reaching fungicidal activity between 1.1 and 3.1 h. AMB exhibited fungici-

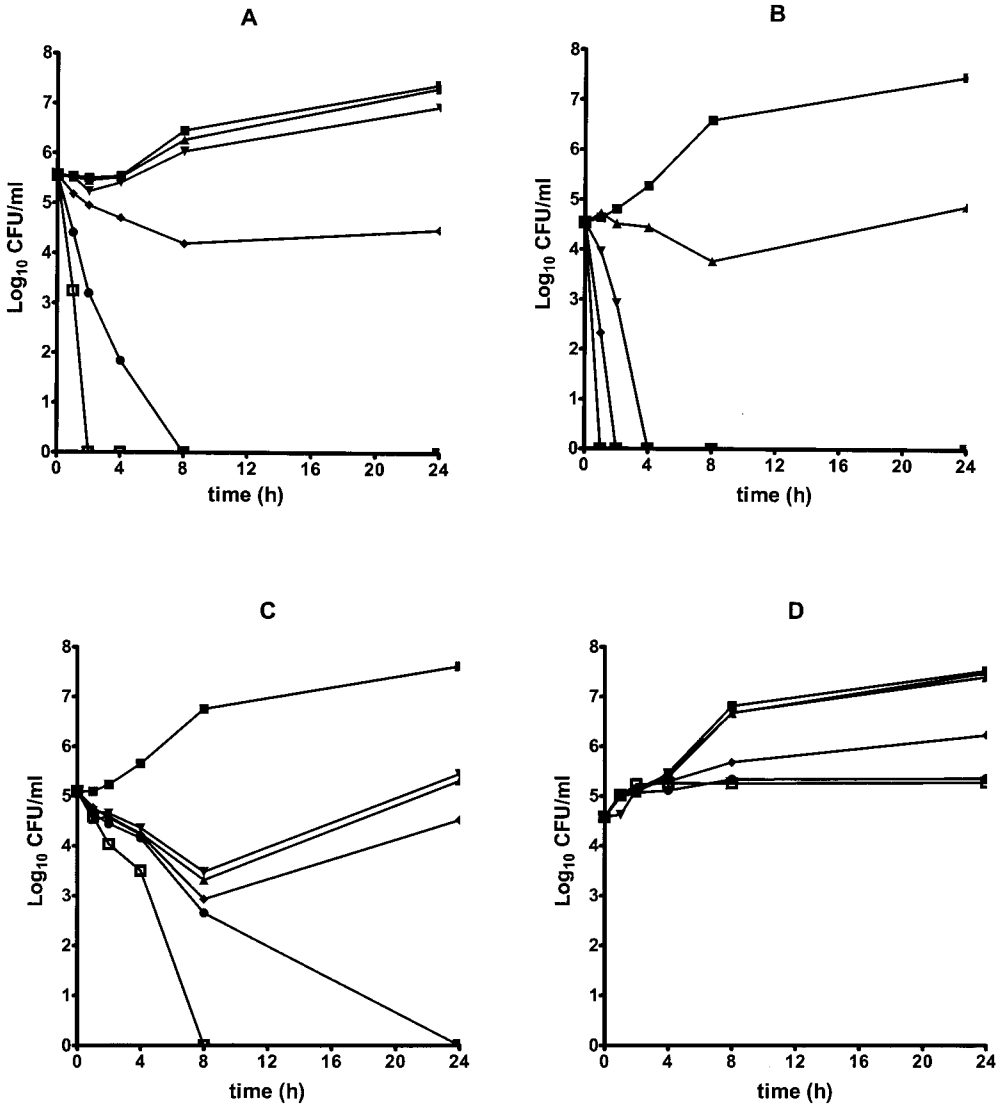

FIG. 1. Representative time-kill plots for the following: AMB against *C. guilliermondii* 337 (A) and *C. lusitaniae* 447 (B); caspofungin against *C. kefyr* 270 (C); and VRC against *C. lusitaniae* 325 (D). Antifungals were tested at the following concentrations: 0.125 times the MIC (▲), 0.25 times the MIC (▼), the MIC (◆), 4 times the MIC (●), and 8 times the MIC (□). ■, control.

TABLE 2. Composite E_{max} model parameters

Antifungal and time point (h)	EC_{50} (multiple of MIC)	EC_{90} (multiple of MIC)	EC_{max} (log_{10} CFU/ ml)	Net inoculum change (log_{10} CFU/ml)
AMB				
1	0.99	1.97	−2.96	−5.28 to 0.25
2	0.80	1.68	−3.65	−5.56 to 0.17
4	0.64	1.78	−4.42	−5.56 to 0.36
8	0.37	1.18	−4.63	−5.56 to 1.00
24	0.70	1.94	−4.55	−5.56 to 2.37
Caspofungin				
1	2.02	2.97	−1.45	−5.47 to −0.14
2	1.40	2.34	−1.91	−5.47 to 0.13
4	1.39	2.32	−2.44	−5.47 to −0.50
8	0.48	1.20	−3.06	−5.47 to −0.33
24	1.05	1.94	−3.62	−5.47 to 0.60
FLC				
4	1.34	2.28	0.33	−0.04 to 1.02
8	1.18	2.10	0.41	0.03 to 2.08
24	1.26	2.18	0.59	0.06 to 2.50
VRC				
4	0.99	1.99	0.31	−0.20 to 1.03
8	0.74	1.60	0.30	−0.34 to 2.09
24	0.88	1.77	0.47	−0.09 to 2.89

dal activity beginning at the MIC against both *C. kefyr* isolates (fungicidal endpoint range, 0.5 to 3.9 h). However, regrowth was observed for strain 240 after 8 h at 1 and 4 times the MIC. AMB showed fungicidal activity against both *C. lusitaniae* isolates beginning at 0.25 times the MIC (fungicidal endpoint range, 0.6 to 6.2 h). Caspofungin exhibited fungicidal activity against both *C. guilliermondii* isolates tested at ≥4 times the MIC (fungicidal endpoint range, 0.5 to 13 h). A comparable trend was observed for *C. kefyr* isolates (fungicidal endpoint range, 2.5 to 11 h), although regrowth was observed for both *C. kefyr* isolates after 8-h exposition at 0.125, 0.25,r and 1 times the MIC. Caspofungin tested against *C. lusitaniae* produced fungistatic activity only, except at 8 times the MIC against *C. lusitaniae* 447 reaching the fungicidal endpoint after 15.6 h. FLC and VRC showed fungistatic activity against all test isolates, regardless of the concentrations tested. Composite EC_{max} model parameters are summarized in Table 2. The EC_{90} for both AMB and caspofungin decreased to about the MIC at 8 h and then increased to about 2 times the MIC at 24 h. EC_{max} increased over time up to 24 h for both antifungals, although AMB yielded fungicidal activity more rapidly than caspofungin did (2 versus 8 h, respectively). The EC_{90} for both FLC and VRC was approximately 2 times the MIC up to 24 h.

PAFE results. Yeasts exposed to FLC and VRC did not produce any measurable PAFE regardless of antifungal concentrations. Caspofungin induced measurable PAFEs for *C. lusitaniae* 325 only, regardless of concentration tested. In contrast, significant PAFEs were induced by AMB against each of the test isolates. PAFE was generally influenced by concentration in a dose-dependent manner ranging from 1.3 to 9.4 h (mean, 5.5 h), 3.6 to 10 h (mean, 7.8 h), and 9.2 to 14.9 h (mean, 11.2 h) at 0.125, 0.25, and 1 times the MIC, respectively. At 4 and 8 times the MIC, AMB yielded a PAFE of about 13 h for *C. lusitaniae* 325 only.

Discussion. According to previous findings (7, 15), AMB was fungicidal against all isolates tested, showing the greatest activity against *C. lusitaniae* isolates despite the fact that *C. lusitaniae* is known for rapid development of AMB resistance (4). Caspofungin displayed fungicidal activity against all isolates but *C. lusitaniae* 325, suggesting that its fungicidal activity is isolate and species specific. Composite EC_{max} model parameters suggested that the rate of killing of caspofungin and AMB was influenced by increases in the drug concentration. In fact, by comparing the relative EC_{90}s over time, we observed decreased values until 8 h, hence increased up to 24 h, suggesting that higher concentrations result in more-rapid expression of activity. AMB proved to be more potent than caspofungin, as suggested by EC_{50} data (mean EC_{50}, 0.8 versus 1.3, respectively) against all isolates tested up to 24 h after antifungal exposure. Further, the EC_{max} of caspofungin and AMB did increase over time, suggesting that maximal activity occurs at the latest time point observed. However, AMB reached the fungicidal endpoint more rapidly than caspofungin did (2 versus 8 h, respectively). The azoles produced fungistatic effects regardless of concentration, supporting previously published data for *C. albicans* and other NAC species (7, 13). EC_{50}s and EC_{90}s calculated for FLC and VRC suggested that fungistatic activity did not improve with increasing concentrations of the azoles. The increase observed in the EC_{50}s and EC_{90}s of antifungals tested at 24 h may represent an amount of regrowth seen at fungistatic concentrations, too small to be appreciated in time-kill plots but enough to modify the EC_{50} and EC_{90}.

The information obtained in this study could be further applied to optimize future dosing regimens for this agent. MIC_{90}s of AMB, caspofungin, FLC, and VRC for the NAC species we tested have been previously reported to range from 0.5 to 8.0, 1 to 16, 0.5 to 16, and 0.06 to 0.5 μg/ml, respectively (1, 8, 9, 16, 18–22). Considering mean EC_{90}s calculated from our E_{max} model (1.4 times the MIC for AMB and 2 times the MIC for caspofungin and both azoles), we predict that close-to-maximal effect (EC_{90}) would be observed if in vivo AMB, caspofungin, FLC, and VRC concentrations of 1 to 16, 1.4 to 22.4, 1 to 32, and 0.12 to 1 μg/ml were achieved, respectively. These values exceed the peak plasma FLC and VRC concentrations (40 and 4 μg/ml, respectively) achieved in human serum following clinically utilized doses (23, 24), suggesting that concentration-independent fungistatic activity is likely to predominate at clinically achieved FLC and VRC concentrations. On the contrary, peak plasma AMB and caspofungin concentrations (2 and 1 μg/ml, respectively) (24) suggest that current dosing strategies for these antifungals should be re-evaluated in order to produce higher peak serum levels.

This is the first published study on the PAFE produced by antifungal agents against *C. guilliermondii*, *C. kefyr*, and *C. lusitaniae*. Although no PAFE was induced by the azoles in any of the strains tested, the method we used for removing drug gets the azole concentrations below the sub-MIC levels that have been previously reported by Turnidge et al. (25) to result in a clinically relevant slower growth of the yeasts. AMB produced a significant dose-dependent PAFE for all isolates tested, as has been previously described for other *Candida* spp. (2, 25). Caspofungin, although it exhibited fungicidal activity as well as AMB, did not induce a measurable PAFE, in contrast

with findings of Ernst et al. (6) concerning *C. albicans* and *Cryptococcus neoformans*.

In conclusion, our results demonstrate that AMB may be useful for the treatment of infections caused by *C. kefyr*, *C. guilliermondii*, and *C. lusitaniae*. Animal and clinical studies are warranted to define the clinical relevance of our data.

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REFERENCES

1. **Cheng, M. F., K. W. Yu, R. B. Tang, Y. H. Fan, Y. L. Yang, K. S. Hsieh, M. Ho, and H. J. Lo.** 2004. Distribution and antifungal susceptibility of *Candida* species causing candidemia from 1996 to 1999. Diagn. Microbiol. Infect. Dis. **48:**33–37.
2. **Chryssanthou, E., O. Cars, and J. Sjölin.** 2002. New automated method for determining postantifungal effect of amphotericin B against *Candida* species: effects of concentration, exposure time, and area under the curve. Antimicrob. Agents Chemother. **46:**4016–4018.
3. **Clancy, C., M. Nguyen, and V. Yu.** 1996. Fungemia caused by *Candida lusitaniae.* Infect. Med. **13:**940, 948–951.
4. **Collin, B., C. J. Clancy, and M. H. Nguyen.** 1999. Antifungal resistance in non-*albicans Candida* species. Drug Resist. Updat. **2:**9–14.
5. **Dick, J. D., R. R. Rosengard, W. G. Merz, R. K. Stuart, G. M. Hutchins, and R. Saval.** 1985. Fatal disseminated candidiasis due to amphotericin B-resistant *Candida guilliermondii*. Ann. Intern. Med. **102:**67–68.
6. **Ernst, E. J., M. E. Klepser, and M. A. Pfaller.** 2000. Postantifungal effects of echinocandin, azole, and polyene antifungal agents against *Candida albicans* and *Cryptococcus neoformans*. Antimicrob. Agents Chemother. **44:**1108–1111.
7. **Ernst, E. J., K. Yodoi, and M. E. Klepser.** 2002. Rates and extent of antifungal activities of amphotericin B, flucytosine, fluconazole, and voriconazole against *Candida lusitaniae* determined by microdilution, Etest, and time-kill methods. Antimicrob. Agents Chemother. **46:**578–581.
8. **Espinel-Ingroff, A., M. Pfaller, S. A. Messer, C. C. Knapp, N. Holliday, and S. B. Killian.** 2004. Multicenter comparison of Sensitre YeastOne colorimetric antifungal panel with the NCCLS M27-A2 reference method for testing new antifungal agents against clinical isolates of *Candida* spp. J. Clin. Microbiol. **42:**718–721.
9. **Favel, A., A. Michel-Nguyen, A. Datry, S. Challier, F. Leclerc, C. Chastin, K. Fallague, and P. Regli.** 2004. Susceptibility of clinical isolates of *Candida lusitaniae* to five systemic antifungal agents. J. Antimicrob. Chemother. **53:** 526–529. (Advance Access published 12 February 2004.)
10. **Groll, A. H., and T. J. Walsh.** 2002. Invasive fungal infections in the neutropenic cancer patient: current approaches and future strategies. Infect. Med. **19:**326–334.
11. **Hadfield, T. L., M. B. Smith, R. E. Winn, M. G. Rinaldi, and C. Guerra.** 1987. Mycoses caused by *Candida lusitaniae*. Rev. Infect. Dis. **9:**1006–1012.
12. **Hazen, K. C.** 1995. New and emerging yeast pathogens. Clin. Microbiol. Rev. **8:**462–478.
13. **Klepser, M. E., E. J. Wolfe, R. N. Jones, C. H. Nightingale, and M. A. Pfaller.** 1997. Antifungal pharmacodynamic characteristics of fluconazole and amphotericin B tested against *Candida albicans*. Antimicrob. Agents Chemother. **41:**1392–1395.
14. **Krcmery, V., and A. J. Barnes.** 2002. Non-*albicans Candida* spp. causing fungaemia: pathogenicity and antifungal resistance. J. Hosp. Infect. **50:**243–260.
15. **Manavathu, E. K., J. L. Cutright, and P. H. Chandrasekar.** 1998. Organism-dependent fungicidal activities of azoles. Antimicrob. Agents Chemother. **42:** 3018–3021.
16. **Maxwell, M. J., S. A. Messer, R. J. Hollis, L. Boyken, S. Tendolkar, D. J. Diekema, M. A. Pfaller, and the International Fungal Surveillance Participant Group.** 2003. Evaluation of Etest method for determining fluconazole and voriconazole MICs for 279 clinical isolates of *Candida* species infrequently isolated from blood. J. Clin. Microbiol. **41:**1087–1090.
17. **National Committee for Clinical Laboratory Standards.** 2002. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A2, 2nd ed. National Committee for Clinical Laboratory Standards, Wayne, Pa.
18. **Ostrosky-Zeichner, L., J. H. Rex, P. G. Pappas, R. J. Hamill, R. A. Larsen, H. W. Horowitz, W. G. Powderly, N. Hyslop, C. A. Kauffman, J. Cleary, J. E. Mangino, and J. Lee.** 2003. Antifungal susceptibility survey of 2,000 bloodstream *Candida* isolates in the United States. Antimicrob. Agents Chemother. **47:**3149–3154.
19. **Pfaller, M. A., D. J. Diekema, S. A. Messer, L. Boyken, R. J. Hollis, R. N. Jones, and the International Fungal Surveillance Participant Group.** 2003. In vitro activities of voriconazole, posaconazole, and four licensed systemic antifungal agents against *Candida* species infrequently isolated from blood. J. Clin. Microbiol. **41:**78–83.
20. **Pfaller, M. A., S. A. Messer, L. Boyken, C. Rice, S. Tendolkar, R. J. Hollis, and D. J. Diekema.** 2003. Caspofungin activity against clinical isolates of fluconazole-resistant *Candida*. J. Clin. Microbiol. **41:**5729–5731.
21. **Pfaller, M. A., D. J. Diekema, S. A. Messer, R. J. Hollis, and R. N. Jones.** 2003. In vitro activities of caspofungin compared with those of fluconazole and itraconazole against 3,959 clinical isolates of *Candida* spp. including 157 fluconazole-resistant isolates. Antimicrob. Agents Chemother. **47:**1068–1071.
22. **Pfaller, M. A., D. J. Diekema, S. A. Messer, L. Boyken, R. J. Hollis, and R. N. Jones.** 2004. In vitro susceptibilities of rare *Candida* bloodstream isolates to ravuconazole and three comparative antifungal agents. Diagn. Microbiol. Infect. Dis. **48:**101–105.
23. **Schwartz, S., D. Milatovic, and E. Thiel.** 1997. Successful treatment of cerebral aspergillosis with a novel triazole (voriconazole) in a patient with acute leukaemia. Br. J. Hematol. **97:**663–665.
24. **Summers, K. K., T. C. Hardin, S. J. Gore, and J. R. Graybill.** 1997. Therapeutic drug monitoring of systemic antifungal therapy. J. Antimicrob. Chemother. **40:**753–764.
25. **Turnidge, J. D., S. Gudmundsson, B. Vogelman, and W. A. Craig.** 1994. The postantibiotic effect of antifungal agents against common pathogenic yeasts. J. Antimicrob. Chemother. **34:**83–92.