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Comparison of Etest, chequerboard dilution and time–kill studies for the detection of synergy or antagonism between antifungal agents tested against *Candida* species

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Currently, there is considerable debate regarding the best *in vitro* method for testing antifungal combinations against *Candida* spp. In this study, we compared the results obtained by chequerboard dilution, time–kill studies and Etest for several antifungal combinations against *Candida* spp. Three *Candida albicans* isolates (fluconazole MICs of 1.0, 32 and >256 mg/L) and three non-*albicans Candida* isolates (*C. glabrata*, *C. tropicalis* and *C. krusei*) were tested in RPMI 1640 medium. By chequerboard testing, the majority of antifungal combinations were found to be indifferent. Notably, antagonism was identified by time–kill studies and by Etest for combinations of amphotericin B–fluconazole, but it was not detected by the chequerboard method. Pre-exposure of isolates to fluconazole did not affect results of the Etest or chequerboard method, but it did increase the frequency of antagonism noted by time–kill methods. This study indicates that chequerboard dilution testing in RPMI medium may not reliably detect the attenuation of amphotericin B activity. Of the three methods, Etest was the simplest to use and yielded reproducible results for testing antifungal combinations.

Introduction

Interest in the use of combination antifungal therapy has grown considerably over the last decade with the emergence of fluconazole (FLU)-resistant *Candida* species and the development of new antifungal agents by the pharmaceutical industry. Currently, the National Committee for Clinical Laboratory Standards (NCCLS) has not proposed standardized methods for *in vitro* testing of antifungal combinations. Although a number of *in vitro* methods have been described, the chequerboard dilution method and time–kill studies have become the most widely accepted techniques. Each method has advantages and limitations.[1] Chequerboard testing is easy to carry out and interpret but provides only a relative measure of potency for the combination (MIC), and it provides little data on the dynamics of the antifungal interaction. Time–kill studies, on the other hand, can help elucidate the pharmacodynamics of an antifungal combination by measuring the effects of the antifungal interaction on the rate and extent of fungal killing. The drawback to time–kill studies, however, is that they are laborious to complete.

Chequerboard testing and time–kill studies are generally carried out under the assumption that the agents tested in combination possess similar dose–response curves and a comparable timecourse of activity.[1] This assumption may be problematic, however, for polyene–azole combinations. Previous *in vitro* studies carried out in our laboratories have demonstrated that the onset of activity for azoles against *Candida* and *Cryptococcus* species is considerably slower than the fungicidal activity exerted by amphotericin B (AMB).[2,3] Additionally, several investigators have documented the importance of FLU pre-exposure with *in vitro* test methodology for the detection of antagonism between AMB and FLU.[4–6] Because the rapid onset of AMB activity may pre-empt the contribution of azole antifungal activity in the test system, traditional techniques of assessing antifungal combinations, which rely on simultaneous administration of drugs, may fail to detect synergic or antagonistic antifungal interactions.

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More recently, the Etest has been investigated as a method for antifungal susceptibility testing and has demonstrated good correlation with broth macrodilution and microdilution testing methods.[7–11] Moreover, Etest has been used successfully to test drug combinations against mycobacterium and aerobic Gram-negative bacilli.[12,13] If an Etest method could be standardized for testing antifungal combinations, it would represent an ideal testing methodology for the clinical microbiology laboratory.

The objectives of this study were three-fold: (i) to examine the utility of Etest for testing antifungal combinations against *Candida* species; (ii) to compare agreement between chequerboard, time–kill and Etest methods for the detection of synergy or antagonism of antifungal combinations against *Candida* species; and (iii) to compare the results of chequerboard, time–kill and Etest methods before and after pre-exposure of test isolates to FLU.

Materials and methods

Test organism

Six isolates obtained from the Clinical Microbiology Laboratory at the University of Iowa College of Medicine were selected. Three *C. albicans* isolates (ATCC CA 90028, CA 1378.33 and CA 20096.0097), one *C. glabrata* isolate (ATCC CG 582), one *C. krusei* isolate (ATCC CK 6258) and one *C. tropicalis* isolate (ATCC CT 2697) were used for all test procedures.

Antifungal agents

FLU (Pfizer, New York, NY), AMB and flucytosine (5FC) (Sigma, St Louis, MO, USA) were used to prepare stock solutions in RPMI 1640 (Sigma) buffered to a pH of 7.0 with 0.165 M morpholinepropanesulphonic acid (MOPS) according to NCCLS recommendations.[14]

Antifungal susceptibility testing

The MIC of each antifungal was determined by broth microdilution and Etest using methods described by the NCCLS and the Etest manufacturer (AB Biodisk, Solna, Sweden).[14] Broth-microdilution MICs were determined in RPMI 1640 medium buffered to a pH of 7.0 with MOPS. The starting inoculum was *c.* 0.5×10^2 to 2.5×10^3 cfu/mL.

Etest MICs were determined with FLU (0.016–256 mg/L), AMB (0.002–32 mg/L) and 5FC (0.002–32 mg/L) strips provided by the manufacturer (AB Biodisk). Solidified (1.5%) 150 mm RPMI + MOPS agar plates (Remel, Lenexa, KS, USA) served as the test medium. A standardized cell suspension (0.5 McFarland) in sterile 0.85% NaCl was prepared by transferring 3–4 colonies from a 24 h culture on potato dextrose agar (PDA) (Remel). Plates were then inoculated by pouring 5 mL of a 1:4 dilution of this standardized cell suspension on to the agar. After allowing 1–2 min for the suspension to achieve a uniform distribution, excess moisture was aspirated with a vacuum pipette and the plate was allowed to dry at room temperature (15 min). Antifungal strips were then placed on to the inoculated agar using sterile technique.

All tests were incubated at 35°C in a dark, moist chamber. MICs were recorded at 24 and 48 h. The susceptibility endpoint for broth-based testing of AMB was defined as the lowest concentration of drug that resulted in complete inhibition of visible growth. Broth-based susceptibility endpoints for FLU and 5FC were defined as the lowest concentration of drug that resulted in an 80% reduction of visual growth compared with the growth of the control. Similarly, Etest susceptibility endpoints for FLU and 5FC were 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 Etest susceptibility endpoint for AMB was read at the intersection of the scale of the strip with the first completely clear ellipse.

Chequerboard microdilution studies

Combinations of AMB + FLU, AMB + 5FC and FLU + 5FC were tested in duplicate using previously described methods.[15] The concentration of each antifungal agent tested ranged from 1/32 to 4 × MIC. The initial inoculum was prepared as described for broth-microdilution susceptibility testing. Readings were determined visually and spectrophotometrically (80% reduction) at 490 nm. MIC endpoints were determined as described for broth-microdilution tests. Both on- and off-scale MICs were included in the analysis. High off-scale MICs were converted to the next two-fold dilution, whereas low off-scale MICs were left unchanged. Trays were incubated at 35°C in a dark, moist chamber. Results were read at 24 and 48 h.

To evaluate the interaction of antifungals, the fractional inhibitory concentration (FIC) was calculated for each combination.[1,16] The FIC was calculated for each agent by dividing the inhibitory concentration of each antifungal when used in combination by its MIC. FIC values were then added together to define the interaction of the combination. Synergy was defined as an FIC of ≤ 0.5, and additivity was defined as an FIC of >0.5 but <1. Indifference was defined as an FIC of ≥ 1 but <4, whereas antagonism was defined as an FIC of ≥ 4.

Time–kill studies

Time–kill studies of each antifungal combination were carried out by a method that we have described previously.[17] Earlier studies in our laboratory have documented a lack of antifungal carryover with these methods with the range of antifungal concentrations tested in this study. The lower limit of accuracy with this method is 50 cfu/mL. Both a fungicidal high concentration (HC) 2.5 mg/L (2–4 ×

MIC) and a fungistatic low concentration (LC) 0.25 mg/L (0.25–0.5 × MIC) of AMB alone and in combination were tested. One millilitre of a standardized cell suspension (0.5 McFarland) was transferred to sterile tubes containing 9 mL of growth medium (RPMI 1640 + MOPS) plus the following antifungal concentrations: (i) control (no drug); (ii) LC-AMB; (iii) HC-AMB; (iv) FLU 20 mg/L; (v) 5FC 50 mg/L; (vi) LC-AMB + FLU; (vii) HC-AMB + FLU; (viii) LC-AMB + 5FC; (ix) HC-AMB + 5FC; (x) FLU + 5FC. Tubes were incubated at 35°C for 24 h on an orbital shaker. At predetermined timepoints (0, 4, 8, 12 and 24 h), samples were aseptically removed and serially diluted, and 30 μL aliquots were plated on to PDA for colony-count determination. All plates were incubated for 48 h at 35°C. All testing was carried out in duplicate.

Antifungal interaction tested by time–kill methods was defined using established criteria. Briefly, a ≥ 2 $\log_{10}$ increase in killing as measured by viable cfu/mL at 24 h between the combination and the most active constituent was defined as synergy, and a <2 but >1 $\log_{10}$ increase was defined as additive. A decrease in killing from the least active agent of <2 $\log_{10}$ cfu/mL was defined as indifference, whereas a decrease in killing of >2 $\log_{10}$ cfu was defined as antagonism.[16]

Etest studies

A modification of previously reported methods for testing antibacterial combinations against *Mycobacterium* spp. with Etest was used to test antifungal combinations (Figure 1).[12,13] Test medium and inoculum preparation were as described previously for susceptibility testing. Antifungal interactions were defined by criteria recommended by the manufacturer (AB Biodisk). Synergy was defined as a decrease of ≥ 3 dilutions in the resultant MIC. Additivity was defined as a decrease of ≥ 2 but <3 and indifference as a decrease of <2 dilutions in the MIC. Antagonism was defined as an increase of ≥ 3 dilutions of the MIC for the antifungal combination. To facilitate comparisons with the chequerboard method, an FIC index was calculated from Etest MIC data that were rounded up to the nearest two-fold dilution. All testing was carried out in duplicate.

FLU pre-exposure

To evaluate the influence of FLU pre-exposure, each test isolate was prepared as follows. One millilitre of a standardized cell suspension (3.0 McFarland) was added to a culture vial containing 9 mL of growth medium with FLU (20 mg/L). The cell suspension was then incubated for 12 h at 35°C on an orbital shaker. Following the incubation period, the cell suspension was pelleted and washed in sterile saline twice by centrifugation (2800 rpm × 10 min × 3) and resuspended in warm, drug-free growth medium. The resultant suspension was then re-standardized to 1×10^5 to 5×10^5 cfu/ml (0.5 McFarland turbidity) and used for chequerboard, time–kill and Etest testing.

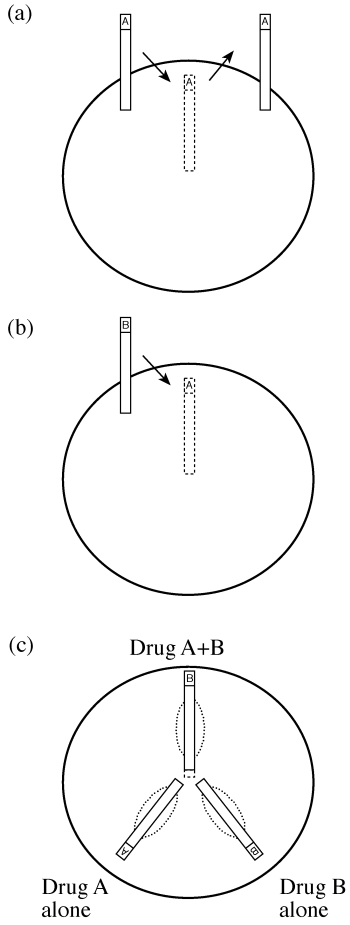

Figure 1. Etest method for testing antifungal combinations. (a) Etest strip (Drug A) is placed on agar and removed after 1 h. (b) Second Etest strip (Drug B) is placed on agar over demarcation left from previous strip. (c) Additional Etest strips are applied (Drugs A and B), the plate is incubated and MICs are read at 24 and 48 h.

Analysis

All plates and trays were read at 24 and 48 h by a single investigator, and results were confirmed by a second investigator. The frequency of synergy, additivity, indifference and antagonism were tabulated and compared for each isolate–drug combination before and after FLU pre-exposure. For purposes of comparison, the time–kill test was considered to be the reference method.

Results

In vitro susceptibility testing

The median MICs determined by broth microdilution and Etest are presented in Table 1. In general, there was good

Table 1. Median MIC (mg/L) results by broth microdilution and Etest ($n = 5$)

	CA 90028[a]		CA 1378.33[b]		CA 20096.0097[c]		CG 582[a]		CK 6258[c]		CT 2697[b]	
Antifungal	broth	Etest	broth	Etest	broth	Etest	broth	Etest	broth	Etest	broth	Etest
AMB	1.0	0.38	1.0	0.38	1.0	0.38	1.0	0.094	2.0	1.5	1.0	2.0
FLU	1.0	0.75	32	48	>256	>256	8.0	24	>256	>256	32	2.0
5FC	8.0	2.0	32	>32	1.0	8.0	0.125	0.125	16	>32	16	0.125

FLU breakpoint designation: [a]susceptible; [b]susceptible dose-dependent; [c]resistant.

agreement between the Etest and broth-microdilution methods.

Antifungal combination testing

Results presented in Tables 2 and 3 demonstrate that agreement between the Etest and the chequerboard methods was poor. Antifungal synergy was detected by the chequerboard method in six of 18 (33%) tests with or without FLU pre-exposure, whereas synergy was not detected by Etest (0 of 18). Combinations that included 5FC were most likely to exhibit synergic activity by chequerboard testing. Antagonism was not detected for any of the antifungal combinations tested by chequerboard dilution, but it was noted in three of 18 (17%) isolates tested by Etest (all isolates susceptible or susceptible dose-dependent to FLU).

Time–kill studies (Figures 2 and 3) identified antagonism between HC-AMB and FLU for three isolates (CA 90028, CA 1378.33 and CT 2697) after FLU pre-exposure. Synergy was only noted by time–kill studies for LC-AMB + 5FC against isolate CA 20096.0097. Overall, agreement between chequerboard testing and time–kill studies (concurrent identification of synergy, antagonism, indifference or additivity) was found in seven of 18 tests (38%) with those isolates without FLU pre-exposure and only two of 18 tests (11%) with FLU pre-exposure.

In contrast to the chequerboard dilution method, the agreement between Etest and time–kill studies was good (Tables 2 and 3 and Figures 2 and 3). Analysis of the results of antifungal combinations (synergy, antagonism, indifference, additivity) concurred with time–kill studies in 15 of 18 tests (83%) using LC-AMB without FLU pre-exposure and 14 of 18 tests (78%) with FLU pre-exposure. When HC-AMB was used in time–kill studies, concordance between Etest results and time–kill studies was 15 of 18 (83%) without FLU pre-exposure and 18 of 18 (100%) with FLU pre-exposure. As with time–kill studies, antagonism was noted by Etest with AMB–FLU combinations against CA 90028, CA 1378.33 and CT 2697 (Tables 2 and 3 and Figure 4). Pre-exposure with FLU had no discernible effect on the determination of synergy or antagonism with the Etest method.

Discussion

Despite the recent proposal of standardized methods for antifungal susceptibility testing, uniform techniques for assessing the activity of antifungals in combination are still lacking.[14,18] Chequerboard testing and time–kill studies have emerged as the methods most frequently employed for testing antifungal combinations. There have been few comparative studies to determine the best method. Petrou & Rogers[6] utilized both chequerboard testing and time–kill studies to evaluate polyene–azole interactions against *Candida* spp. and *Saccharomyces cerevisiae*. Although agreement between the two methods was deemed acceptable by the authors, they noted better sensitivity and reproducibility with time–kill studies, particularly in the ability of time–kill testing to detect azole-mediated antagonism of AMB fungicidal activity. Our findings[4,5] support their observations.

AMB and azole antifungals possess a different time-course of activity; therefore, we speculated that pre-exposure of the *Candida* isolates to FLU (thus ensuring the full antifungal effect of FLU at the time that AMB or 5FC was introduced into the test system) might change the results of the chequerboard tests. Interestingly, FLU pre-exposure did not increase the frequency of antagonism noted by the chequerboard method. This lack of antagonism could be explained in two ways. First, broth-microdilution antifungal susceptibility testing of AMB in RPMI medium frequently demonstrates a 'clustering' (narrow distribution) of MICs.[14,18] Thus, the method is not well suited for discriminating between susceptible and resistant isolates. Therefore, the broth-microdilution method (on which chequerboard testing is based) may not be able to discern a synergic or antagonistic interaction with AMB combinations. Secondly, because AMB–azole antagonism appears to be one-sided (the activity of the azole appears to persist despite the abrogation of AMB fungidal activity), detecting a four-dilution change in the MIC of AMB, which would be necessary to detect antagonism (an FIC index of $\geqslant 4$), seems unlikely. Whether or not antagonism could be detected by chequerboard testing in other media (i.e. antibiotic medium #3) is a question currently under investigation.

Table 2. Chequerboard and Etest results (without FLU pre-exposure)

Isolate	Drug combination	Chequerboard FIC index (interpretation)	Etest FIC index (interpretation)
CA 90028	AMB+ FLU	1.06 (I)	43.10 (A)
	AMB+ 5FC	0.06 (I)	1.13 (I)
	FLU+ 5FC	0.074 (I+)	1.10 (I)
CA 1378.33	AMB+ FLU	1.06 (I)	6.26 (A)
	AMB+ 5FC	1.06 (I)	2.00 (I)
	FLU+ 5FC	2.00 (I)	2.00 (I)
CA 20096.0097	AMB+ FLU	1.06 (I)	1.10 (I)
	AMB+ 5FC	0.45 (S)	2.00 (I)
	FLU+ 5FC	1.06 (I)	1.16 (I)
CG 582	AMB+ FLU	0.04 (S)	2.00 (I)
	AMB+ 5FC	0.06 (S)	2.00 (I)
	FLU+ 5FC	0.04 (S)	2.00 (I)
CK 6258	AMB+ FLU	0.53 (I+)	3.00 (I)
	AMB+ 5FC	1.06 (I)	2.00 (I)
	FLU+ 5FC	1.06 (I)	2.00 (I)
CT 2697	AMB+ FLU	1.06 (I)	17.25 (A)
	AMB+ 5FC	0.09 (S)	1.06 (I)
	FLU+ 5FC	0.18 (S)	2.00 (I)

S, synergy; I, indifference; A, antagonism.

Table 3. Chequerboard and Etest results (with FLU pre-exposure)

Isolate	Drug combination	Chequerboard FIC index (interpretation)	Etest FIC index (interpretation)
CA 90028	AMB+ FLU	1.06 (I)	16.79 (A)
	AMB+ 5FC	0.28 (I)	1.66 (I)
	FLU+ 5FC	0.56 (I+)	1.06 (I)
CA 1378.33	AMB+ FLU	1.06 (I)	6.26 (A)
	AMB+ 5FC	0.52 (I)	2.00 (I)
	FLU+ 5FC	0.01 (I)	2.00 (I)
CA 20096.0097	AMB+ FLU	1.06 (I)	1.76 (I)
	AMB+ 5FC	0.04 (S)	1.76 (I)
	FLU+ 5FC	1.06 (I)	2.00 (I)
CG 582	AMB+ FLU	0.05 (S)	3.00 (I)
	AMB+ 5FC	0.27 (S)	2.00 (I)
	FLU+ 5FC	0.27 (S)	2.00 (I)
CK 6258	AMB+ FLU	0.25 (I+)	3.00 (I)
	AMB+ 5FC	0.06 (I)	2.00 (I)
	FLU+ 5FC	0.03 (I)	2.00 (I)
CT 2697	AMB+ FLU	1.06 (I)	17.25 (A)
	AMB+ 5FC	0.09 (S)	1.06 (I)
	FLU+ 5FC	0.13 (S)	2.00 (I)

S, synergy; I+, additivity; I, indifference; A, antagonism.

Another interesting finding in this study was the influence of FLU susceptibility on the development of AMB–FLU antagonism detected *in vitro*. With the exception of *C. glabrata* (CG 582), antagonism was noted by time–kill and Etest methods for isolates that were FLU susceptible or susceptible dose-dependent (CA 90028, CA 1378.33 and CT 2697). Antagonism, however, was absent or less pronounced for the FLU-resistant *C. albicans*

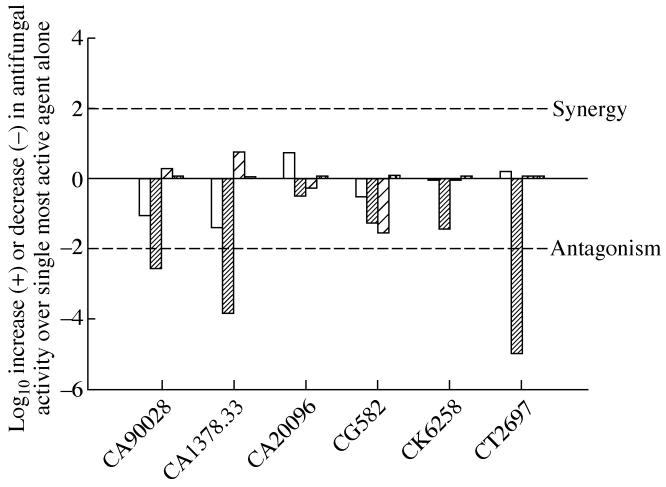

Figure 2. Summary of 24 h time–kill study results for AMB + FLU combinations: □, AMB 0.25 mg/L FLU pre-exposed; ▨, AMB 2.5 mg/L FLU pre-exposed; ◰, AMB 0.25 mg/L FLU simultaneous; ⊠, AMB 2.5 mg/L FLU simultaneous.

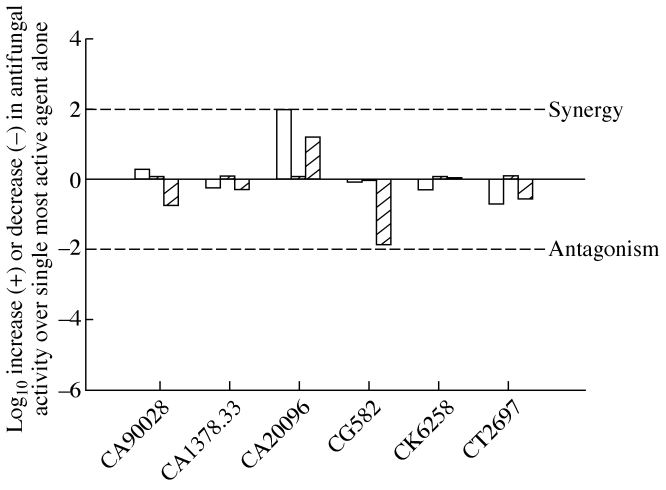

Figure 3. Summary of 24 h time–kill study results for 5FC + AMB and 5FC + FLU combinations: □, AMB 0.25 mg/L + 5FC; ▨, AMB 2.5 mg/L + 5FC; ◰, FLU + 5FC.

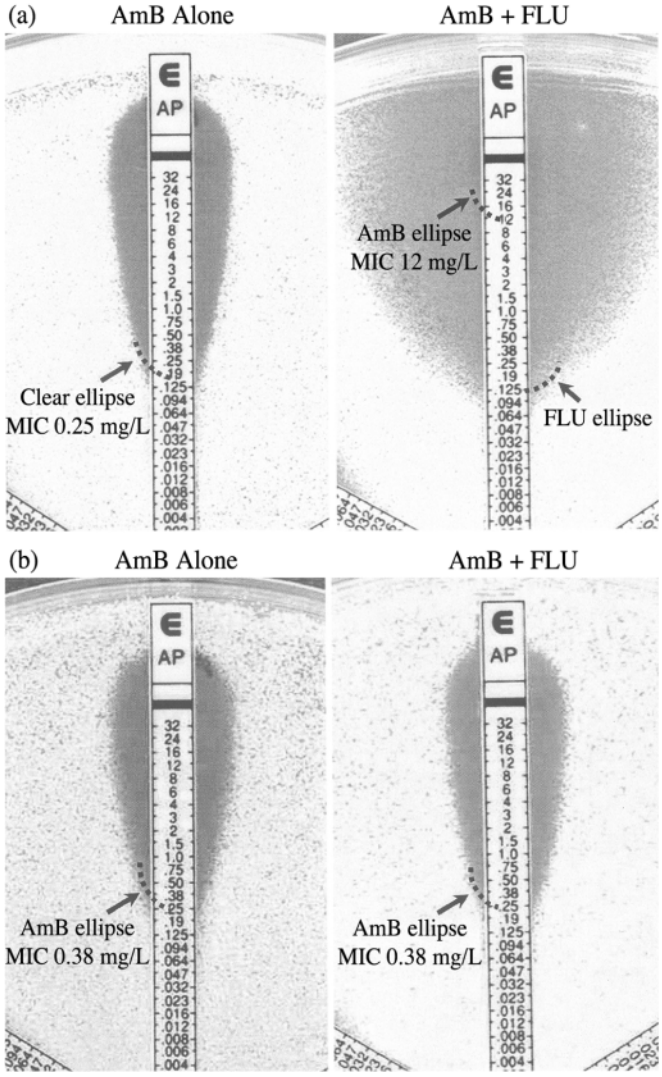

Figure 4. Antifungal antagonism demonstrated by Etest with FLU-susceptible and FLU resistant species. (a) *C. albicans* 90028; (b) *C. albicans* 20096.0097. Arrows represent intersection of ellipse and Etest strip.

isolate (CA 20096.097) or for *C. krusei* (CK 6258). These findings are consistent with the theory that AMB antagonism develops secondary to azole-mediated depletion of ergosterol binding sites. Further studies are required, however, before this finding can be validated.

Antifungal synergy in this study was seen only with 5FC combinations. However, the fact that synergy was largely detected by the chequerboard method and not corroborated by Etest or time–kill studies raises the question of a testing artefact seen only with chequerboard dilution. Broth-microdilution susceptibility testing of 5FC alone is often difficult to interpret because of trailing phenomena seen with 5FC against *Candida* spp. In the presence of a second agent, the trailing seen with 5FC is often absent or decreased. Therefore, it is possible that the visual/spectrophotometric endpoint (80% reduction) used to determine the MIC of 5FC alone is less appropriate when the drug is used in combination.

The results of the Etest studies indicate that this method could be an acceptable alternative to time–kill studies with antifungal agents. The technique employed for testing the antifungal combination was simple to use, time-efficient and yielded reproducible results. Unlike the findings with time–kill studies, FLU pre-exposure produced no discernible effect on the Etest results. It is possible that pre-exposure for 1 h with an azole in agar-based medium is sufficient for detecting polyene–azole antagonism. Unlike previous Etest work carried out with antibacterials, we opted not to place the Etest strips simultaneously on the agar at 90º angles.[12] AMB and FLU exhibit different diffusion characteristics through agar, resulting in dissimilar

ellipse patterns. Moreover, the growth endpoints used to measure the MIC by Etest are different for the two agents. Therefore, we felt interpretation of separate ellipses at 90° angles would be difficult. It may be possible to test antifungal combinations by incorporating azoles or 5FC into agar, but this would be time-consuming and the advantage of testing continuous gradients of antifungal concentrations would be lost.

In summary, our results indicate that Etest and time–kill methods correlate well for the testing of antifungal combinations. Chequerboard testing for antifungals, particularly those involving AMB in RPMI media, should be interpreted with caution, as this technique appears to display poor sensitivity for detecting changes in the activity of AMB. On the basis of our work, Etest appeared to be a good method for the *in vitro* testing of antifungal combinations, although more prolonged pre-exposure studies are required. Further studies utilizing different growth media, testing of novel antifungal combinations, synergy testing of azole-resistant yeast and the evaluation of antifungal combinations against moulds such as *Aspergillus* should be pursued with Etest methodology. Although the concentrations used in our studies reflect antifungal concentrations that can be achieved in human patients, the *in vivo* significance of our findings remains to be determined. Therefore, our *in vitro* results cannot be extrapolated to the clinical setting until better *in vivo* correlative data are available.

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