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Simple Method for Detecting Fluconazole-Resistant Yeasts with Chromogenic Agar

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A method for detecting fluconazole-resistant yeasts was developed that uses chromogenic agar containing fluconazole. Yeasts were plated on media with fluconazole at 0, 8, and 16 μg/ml. On media without fluconazole, normal growth of susceptible yeasts (defined as those having a fluconazole MIC of <8 μg/ml) was detected, while fluconazole-containing media suppressed susceptible strains and normal colonies of resistant yeasts (fluconazole MICs of ≥8 μg/ml) were detected. This method was used to screen for resistance in oropharyngeal candidiasis. Isolates having fluconazole MICs of ≥8 μg/ml and <8 μg/ml were correctly predicted in 43 of 45 cultures and 115 of 116 cultures, respectively. This screening method appears to be rapid and sensitive for detection of fluconazole-resistant yeasts.

Fluconazole resistance is becoming an important clinical concern (3, 4, 7, 8, 11, 13–20). Decreased fluconazole susceptibility has been noted especially in human immunodeficiency virus (HIV)-infected patients with recurrent oropharyngeal candidiasis and in patients infected with yeasts other than *Candida albicans* (4, 16, 20–22). The National Committee for Clinical Laboratory Standards' (NCCLS) proposed standard has made yeast susceptibility testing more reproducible and may correlate with clinical outcome (5, 9). However, the standard broth macrodilution technique is not easily applied for screening and is not used routinely in clinical microbiology laboratories (1).

A newly developed chromogenic medium, CHROMagar Candida (2, 10, 12), allows identification of many *Candida* species, including *C. albicans*, *C.* (*Torulopsis*) *glabrata*, *C. krusei*, and *C. tropicalis*, on the basis of colony color (10). The chromogenic agar establishes presumptive identification but does not determine susceptibility.

An agar dilution method for susceptibility testing using CHROMagar Candida with the addition of fluconazole was developed; this method is aimed at rapid and simple identification of fluconazole-resistant yeasts from clinical samples. The correlation of this method with the standard NCCLS broth macrodilution technique is reported.

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MATERIALS AND METHODS

RPMI 1640. Nutrient medium was prepared at double the desired concentration to give a final concentration of 10 of RPMI 1640 per liter plus L-glutamine without sodium bicarbonate (Mediatech, Washington, D.C.) and with chloramphenicol (0.4 g/liter) (Sigma, St. Louis, Mo.) and gentamicin (0.025 g/liter) (Gibco BRL, Grand Island, N.Y.). The pH was adjusted to 7.0, and the solution was sterile filtered. Agar was prepared at twice the final concentration by adding 40 g of Bacto agar (Difco, Detroit, Mich.) per liter to sterile water, boiled for 15 to 30 s to dissolve the agar, and cooled to 45°C in a water bath. Fluconazole (2 mg/ml) (Pfizer, Roerig, New York, N.Y.) was added to the agar solution for a final fluconazole concentration of 8 and 16 μg/ml. The agar and RPMI 1640 solutions were combined at 45°C and stirred, and approximately 20 ml was poured into sterile 100-mm-diameter petri dishes. Hardened plates were stored at 4°C for up to 1 week prior to use.

CHROMagar Candida. CHROMagar Candida (CHROMagar Company, Paris, France) was prepared from a powdered medium according to the manufacturer's instructions with the addition of fluconazole to give 8- and 16-μg/ml concentrations. The prepared medium, which contains chloramphenicol (0.5 g/liter) and agar (15 g/liter), was dispensed (20 ml) into 100-mm-diameter petri dishes. Hardened plates were stored at 4°C for up to 1 week prior to use.

Organisms. *Candida* species from stock cultures (30 isolates) with fluconazole MICs determined by NCCLS broth macrodilution (9) were tested blindly on both RPMI 1640 with fluconazole and CHROMagar Candida with fluconazole. On chromogenic media, CHROMagar Candida-specific color patterns were used for presumptive identification, which was confirmed by standard techniques: *C. albicans* (green), *C. krusei* (lavender), *C. tropicalis* (blue), and *C.* (*Torulopsis*) *glabrata* (purple) (10). Fluconazole was added to the media to differentiate resistant from susceptible yeasts, but color patterns remained unchanged on media with or without fluconazole.

Clinical samples. HIV-positive patients with oropharyngeal candidiasis were enrolled in a longitudinal study following informed consent. Clinical samples were obtained by swabbing of oral lesions and by having patients swish and spit 10 ml of normal saline. A 100-μl sample of the swish solution was plated on the media described above and incubated at 30°C for 48 h. Three to five colonies from each sample were submitted for fluconazole broth macrodilution MIC determination by NCCLS methods (9).

Results from the fluconazole-containing media were recorded as susceptible or resistant on the basis of growth characteristics. Colonies that demonstrated suppressed growth on media with fluconazole (pinpoint colonies) as compared with growth on media without fluconazole were recorded as susceptible. Colonies that demonstrated growth that was indistinguishable on media with or without fluconazole were recorded as resistant.

Strain identification. Yeast strains were identified by contour-clamped homogeneous electric field (CHEF) analysis of whole-cell yeast DNA (11), and chromosomal patterns were analyzed with DENDRON software (Solltech, Iowa City, Iowa).

RESULTS

In initial experiments, stock yeast isolates were plated on RPMI 1640 with and without fluconazole. Mixtures of the susceptible *C. albicans* (NCCLS fluconazole MIC, 0.25 μg/ml) and resistant *C. albicans* (>64 μg/ml), *C.* (*Torulopsis*) *glabrata* (32 μg/ml), and *C. krusei* (64 μg/ml) at sensitive-to-resistant

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FIG. 1. (A) A 1,000:1:1:1 mixture of susceptible *C. albicans*, resistant *C. albicans*, *C. krusei*, and *C.* (*Torulopsis*) *glabrata* on RPMI 1640 without fluconazole. The resistant *C. albicans* and non-*albicans* yeasts are not distinguishable. (B) A 1,000:1:1:1 mixture of susceptible *C. albicans*, resistant *C. albicans*, *C. krusei*, and *C.* (*Torulopsis*) *glabrata* on RPMI 1640 with fluconazole (8 μg/ml). The resistant strains are easily distinguishable as larger colonies.

yeast ratios of 1,000:1 were plated at a concentration of 10^4 CFU of the susceptible yeast on RPMI 1640 with fluconazole at 0, 8, and 16 μg/ml. On the RPMI 1640 without fluconazole, only normal growth of the susceptible *C. albicans* was detected. In contrast, the fluconazole-containing media suppressed the susceptible strain of *C. albicans* and colonies of resistant yeasts were detected.

Mixed populations of resistant and susceptible yeasts were evaluated by use of combinations of the susceptible *C. albicans* with the resistant *C. albicans*, *C. krusei*, and *C.* (*Torulopsis*) *glabrata* described above at susceptible-to-resistant yeast ratios of 1,000:1:1:1. In these studies, 10^4 CFU of the susceptible *C. albicans* were plated on RPMI 1640 with and without fluconazole. On RPMI 1640 without fluconazole, only the susceptible *C. albicans* was detected (Fig. 1A). In contrast, with the addition of fluconazole to the media, the normal growth of resistant

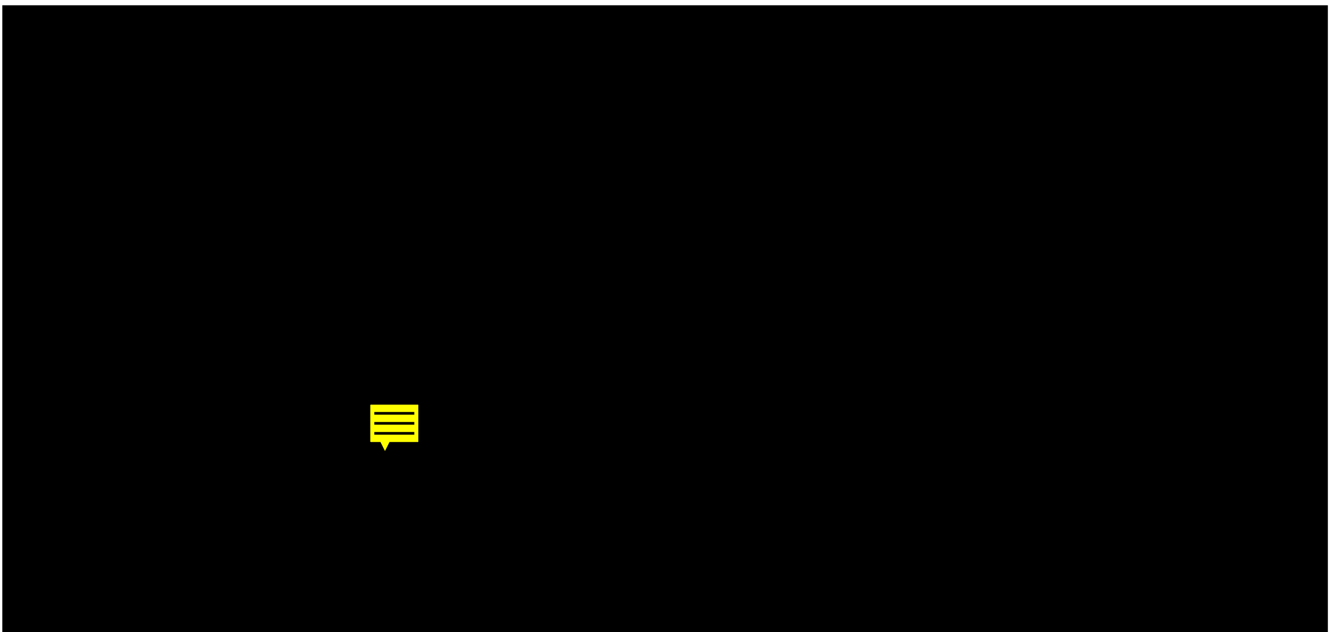

FIG. 2. (A) A 1,000:1:1:1 mixture of susceptible *C. albicans*, resistant *C. albicans*, *C. krusei*, and *C.* (*Torulopsis*) *glabrata* on CHROMagar Candida without fluconazole. The non-*albicans* yeasts are not identifiable but can be minimally detected as darkly colored isolates as compared with *C. albicans* (large green colonies). (B) A 1,000:1:1:1 mixture of susceptible *C. albicans* (pinpoint green), resistant *C. albicans* (large green), *C. krusei* (flat lavender), and *C.* (*Torulopsis*) *glabrata* (purple) on CHROMagar Candida with fluconazole (8 μg/ml). The resistant strains are easily distinguishable as larger colonies, with different species producing a unique color.

TABLE 1. Correlation between cultures with fluconazole susceptibility determined by NCCLS 48-h fluconazole broth macrodilution MICs and predicted susceptibility from cultures grown on CHROMagar Candida with fluconazole

Cultures with fluconazole broth macrodilution MIC of:	No. of cultures with CHROMagar Candida predicted susceptibility of:	
	<8 μg/ml	≥8 μg/ml
<8 μg/ml (*n* = 116)	115 (99%)	1
≥8 μg/ml (*n* = 45)	2	43 (96%)

C. albicans, *C. krusei*, and *C.* (*Torulopsis*) *glabrata* (Fig. 1A) was easily distinguished from the suppressed growth of susceptible yeasts (pinpoint colonies) (Fig. 1B).

The detection of mixed populations of susceptible and resistant yeasts was further investigated with CHROMagar Candida, which would allow for rapid presumptive yeast identification and screening of susceptibility. A mixture of susceptible *C. albicans* and each of the resistant isolates was again plated in a susceptible-to-resistant yeast ratio of 1,000:1:1:1. On CHROMagar Candida without fluconazole, the dark-colored colonies of yeasts other than *C. albicans* were masked by overgrowth of the predominant susceptible *C. albicans* (large green colonies) (Fig. 2A). In contrast, in Fig. 2B, CHROMagar Candida with fluconazole at 8 μg/ml suppressed the susceptible strain (now pinpoint green) and allowed detection and identification of the resistant strains with unique colors: resistant *C. albicans* (large green), *C. krusei* (flat lavender), and *C.* (*Torulopsis*) *glabrata* (purple).

Recovery of the isolates predicted to have fluconazole MICs >8 μg/ml and of the susceptible *C. albicans* isolate with a fluconazole MIC of 0.25 μg/ml was confirmed with NCCLS broth macrodilution testing. Colonies picked from the agar dilution plates were found to have 100% agreement among fluconazole MICs obtained prior to inoculation and those recovered from the agar dilution plates.

To further confirm identification of the original isolates on the agar plates, CHEF analysis of whole-cell DNA was performed from the original stock cultures used for inoculation and from colonies picked from the agar dilution plates. The three strains predicted from the agar plates to be resistant and the susceptible *C. albicans* strain were confirmed to be identical to the original isolates by CHEF analysis of whole-cell DNA with DENDRON analysis. DNA patterns from the pairs were identical before and after inoculation on the RPMI 1640 (data not shown).

In order to evaluate agar dilution in screening for resistant yeasts, 30 stock cultures of *C. albicans* with a range of broth macrodilution NCCLS fluconazole MICs were tested in a blinded fashion on CHROMagar Candida and RPMI 1640 with fluconazole at 0, 8, and 16 μg/ml. NCCLS fluconazole MICs for these strains were as follows: ≥64 μg/ml (*n* = 4), 32 μg/ml (*n* = 1), 16 μg/ml (*n* = 1), 8 μg/ml (*n* = 2), 4 μg/ml (*n* = 5), 2 μg/ml (*n* = 1), 1 μg/ml (*n* = 1), 0.5 μg/ml (*n* = 9), 0.25 μg/ml (*n* = 5), and ≤0.125 μg/ml (*n* = 1). On both RPMI 1640 and CHROMagar Candida, MICs were correctly predicted as ≥8 or 16 μg/ml, respectively, for eight of eight *C. albicans* strains with a broth macrodilution fluconazole MIC of ≥8 or 16 μg/ml as well as the remaining 22 strains with fluconazole MICs of <8 or 16 μg/ml.

Clinical samples were tested in 46 HIV-positive patients serially evaluated for recurrent oropharyngeal candidiasis. Isolates with suppressed growth on CHROMagar Candida plates with 8 or 16 μg of fluconazole per ml were predicted to have a fluconazole MIC of <8 μg/ml, whereas isolates with normal growth on both 8- and 16-μg/ml fluconazole plates were predicted to have fluconazole MICs of ≥8 μg/ml. Isolates with broth macrodilution fluconazole MICs of ≥8 μg/ml were identified from 18 of 46 (39%) patients serially evaluated, with 45 of 161 (28%) cultures containing at least one isolate with a fluconazole MIC of ≥8 μg/ml.

Isolates having fluconazole broth macrodilution MICs of ≥8 μg/ml were correctly predicted by normal growth on CHROMagar Candida media containing fluconazole at 8 or 16 μg/ml in 43 of 45 cultures (96%) (Table 1). Yeasts with fluconazole broth macrodilution MICs of <8 μg/ml were also correctly predicted in 115 of 116 (99%) cultures on the basis of suppressed growth on media containing fluconazole at 8 and 16 μg/ml.

Fluconazole susceptibility of *C. albicans* and other yeasts was successfully predicted as <8 μg/ml or ≥8 μg/ml with the CHROMagar Candida from strains with a wide range of NC CLS fluconazole MICs, as shown in Table 2. The two strains determined to have broth macrodilution MICs of ≥8 μg/ml which were not detected with the CHROMagar Candida screening were single strains of *C. albicans* and *C.* (*Torulopsis*) *glabrata* which had 24- and 48-h fluconazole MICs of 2 and 8 μg/ml and 4 and 32 μg/ml, respectively. The only strain with a fluconazole broth macrodilution MIC of <8 μg/ml which was predicted by CHROMagar Candida screening to have an increased fluconazole MIC was a single strain of *C.* (*Torulopsis*) *glabrata*, which had a 24- and 48-h fluconazole MIC of 0.5 and 2 μg/ml. The overall correlation between NCCLS broth macrodilution testing and detection of resistant yeasts on the

TABLE 2. Distribution of 48-h fluconazole broth macrodilution MICs for yeasts from HIV-positive patients with oropharyngeal candidiasis and correlation with predicted susceptibility from CHROMagar Candida screening

Species (*n*)	No. of isolates with a fluconazole MIC (μg/ml) of:									
	≤0.125	0.25	0.5	1	2	4	8	16	32	≥64
C. albicans (139)	3	39	28	31	11	2	6[a]	10	8	1
C. (*Torulopsis*) *glabrata* (30)				2	9[b]	5	7	1	4[c]	2
C. krusei (9)									2	7
C. parapsilosis (2)			1	1						
Rhodotorula rubra (2)										2

[a] One *C. albicans* isolate with a fluconazole broth macrodilution MIC of 8 μg/ml was predicted on CHROMagar Candida to have a fluconazole MIC of <8 μg/ml.

[b] One *C.* (*Torulopsis*) *glabrata* isolate with a fluconazole broth macrodilution of 2 μg/ml was predicted on CHROMagar Candida to have a fluconazole MIC of ≥8 μg/ml.

[c] One *C.* (*Torulopsis*) *glabrata* isolate with a fluconazole broth macrodilution MIC of 32 μg/ml was predicted on CHROMagar Candida to have a fluconazole MIC of <8 μg/ml.

CHROMagar Candida screening was over 98% (158 of 161 cultures).

DISCUSSION

The addition of fluconazole to either RPMI 1640 or CHROMagar Candida suppressed growth of yeasts with fluconazole MICs of <8 μg/ml and allowed strains with higher fluconazole MICs to be identified. CHROMagar Candida with the addition of fluconazole was particularly useful in that presumptive identification and susceptibility could both be accomplished from the screening culture of the clinical sample. The yeast color patterns observed on the CHROMagar Candida also assisted in the comparison of colony growth on media with and without fluconazole, so that susceptibility could be predicted more readily.

The addition of fluconazole to agar allowed rapid and accurate prediction of fluconazole susceptibility from clinical samples with an agreement between predicted susceptibility (NCCLS fluconazole MICs of <8 μg/ml or ≥8 μg/ml) in 179 of 182 (98%) isolates from 161 cultures. Susceptibility was incorrectly predicted from the CHROMagar Candida screening in only three isolates. Two colonies with fluconazole MICs of ≥8 μg/ml were predicted to be susceptible on the basis of the CHROMagar Candida screening: one *C.* (*Torulopsis*) *glabrata* isolate and one *C. albicans* isolate, which had fluconazole MICs at 24 and 48 h of 4 and 32 μg/ml and 2 and 8 μg/ml, respectively. One *C.* (*Torulopsis*) *glabrata* isolate with a 24- and 48-h fluconazole MIC of 0.5 and 2 μg/ml was predicted to have a fluconazole MIC of ≥8 μg/ml.

This method also detected mixed populations of resistant and susceptible yeasts. Under laboratory conditions, resistant *C. albicans* and other yeasts could be detected at a 1,000:1 ratio of susceptible to resistant yeasts. Louwagie and colleagues reported that the addition of fluconazole to a chromogenic broth did not increase detection of yeasts other than *C. albicans* when compared with detection by chromogenic media alone. However, that method required subculturing for identification and did not attempt to predict susceptibility (6).

This method may be useful in assessing the epidemiology of fluconazole-resistant yeasts. Isolates with fluconazole MICs of ≥8 μg/ml were identified from 18 of 46 (39%) patients serially evaluated, but responses to fluconazole were excellent. The optimal strategy for managing patients with yeasts with increased fluconazole MICs and the role of early detection of resistant yeasts are not known (3, 15, 16).

The use of chromogenic media with fluconazole appears to be a rapid, simple, and sensitive method for detection and identification of fluconazole-resistant yeasts. Additional studies should be conducted to determine the utility of this method in screening clinical samples and in establishing the optimal management of resistant yeast infections.

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