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Article *in* Journal of Medical Sciences(Faisalabad) · March 2007

DOI: 10.3923/jms.2007.374.382 · Source: DOAJ

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J. Med. Sci., 7 (3): 374-382
1st April, 2007

Epsilometer Test-Based Determination of Susceptibility of Clinically Important *Candida* Isolates to Conventional Antifungal Agents

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One hundred thirty yeast clinical isolates comprised of 81 *C. albicans*, 22 *C. tropicalis*, 11 *C. parapsilosis*, 9 *C. glabrata* and 7 *C. krusei* were evaluated for antifungal susceptibility using the E-test. MIC, MIC_{50} and MIC_{90} of amphotericin B (AMB), flucytosine (FC), fluconazole (FL), itraconazole (IT) and voriconazole (VOR) were determined at 24 and 48 h of incubation and compared with data obtained by the broth microdilution method. MIC and MIC_{90} determined at 24 h for *C. albicans* were: AMB ≤0.002-0.064, 0.032, FC 0.023-2.0, 0.75, FL 0.064->256, 64, IT 0.012-2.0, 0.19 and VOR 0.008-0.750, 0.047. Those for *C. glabrata* were: AMB 0.016-4.0, 1.25, FC≤0.06-4.0, ≤0.06, FL 1.0->256.0, 64.0, IT 0.003-4.0, 1.0 and VOR 0.047-2, 0.38. Overall, *Candida* species remain uniformly susceptible to AMB and FL; this not withstanding the finding that *C. glabrata* and *C. krusei* showed significant resistance to FL. MICs after 48 h of incubation were higher than those determined at 24 h of incubation; increased resistance rates and enhanced endpoint trailing particularly with FL and IT were also evident. Overall agreement between the MICs obtained by the E-test and broth microdilution methods was ≥86% within ±2 dilution for AMB, FL and VOR for *C. albicans*, *C. tropicalis* and *C. parapsilosis* and 67% for *C. glabrata* and 52% for *C. krusei* for FL. Based on the 24 h MICs determinations, resistant strains in *Candida* species from Jordan were encountered at a range of 8-22% against azole antifungals tested. These results suggest that the E-test is simple, inexpensive, easy both to read and interpret and has a good correlation to the CLSI microdilution test and can be conveniently incorporated and performed in a hospital-based clinical laboratory.

Key words: Antifungal susceptibility, *Candida* sp., broth microdilution method, E-test

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INTRODUCTION

Invasive opportunistic fungal infections have become important causes of morbidity and mortality in both critically ill and immunocompromised patients (Krcmery and Barnes, 2002; Bodey *et al*., 2002). *Candida* species are the fourth most common class of pathogens isolated from bloodstream in USA, reflecting the increased risk of *Candida* infections in critically ill patients (Pfaller *et al*., 2000a; Safdar *et al*., 2001). *Candida* species also cause a wide spectrum of diseases, including oral and vaginal thrush, severe oropharyngeal candidosis in AIDS patients where *C. albicans*, *C. glabrata* and *C. tropicalis* count for more than 80% of isolates from clinical infections (Akpan and Morgan, 2002). The number of available antifungal drugs was limited to amphotericin B deoxycholate +/- flucytosine. In the late 1980s, several effective and less toxic systemic antifungal agents such as ketoconazole, itraconazole and fluconazole became available (Abu-Elteen and Hamad, 2005). However, the widespread use of fluconazole during the 1990s has resulted in an increased prevalence of azole-resistant strains of *Candida* (Rex *et al*., 1997, 1993).

The Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards [NCCLS]) has proposed a standardized reference broth macro- and microdilution methods for antifungal susceptibility testing of yeast (M 27-p and M 27-T, respectively) (NCCLS, 1997). The initially proposed version (M 27-P) has been revised to the approval level (M 27 A) (NCCLS, 1997). Consequently, a reference method for microdilution antifungal susceptibility testing of filamentous fungi (CLSI M 38-A) has been developed (NCCLS, 2002). Furthermore, modified less cumbersome procedures including a macro- and microbroth dilution method (Cuenca-Estrella *et al*., 2002; Espinel-Ingroff *et al*., 1992), the Epsilometer (E)-test (Chang *et al*., 2001), flow cytometry (Lee and Kwak, 1999) and well diffusion (Magaldi *et al*., 2004) has been proposed as alternatives to the microbroth dilution method proposed by CLSI (NCCLS, 1997). Although flow cytometry is a rapid (2-4 h) and sensitive *in vitro* method for antifungal susceptibility testing (Lee and Kwak, 1999), the need for expensive and sophisticated equipment and specialized personnel to run it makes this approach less appealing than more conventional techniques. Introduction of the E-test, a novel susceptibility testing method, has greatly facilitated the antifungal susceptibility testing studies. The test involves the placement of a plastic strip (AB Biodisk, Solna, Sweden) containing a defined continuous gradient of antifungal drug on the surface of an inoculated agar (Biodisk, 1993). The test has proven to be simple and reliable for antifungal drug susceptibility testing of *Candida* sp. It has been reported that results generated by the E-test very closely correlate and comparable with results obtained by the broth as well as well diffusion methods (Chen *et al*., 1996; Pfaller *et al*., 2003a, b; Pfaller *et al*., 2000b).

Here, we report on the antifungal susceptibility of five clinically important *Candida* species against the most frequently used five conventional antifungal drugs in Jordan for the treatment of *Candida* infection- using the E-test. This is the first report on the application of the E susceptibility test procedure on clinical isolates from Jordan.

MATERIALS AND METHODS

Test organisms and clinical isolates: A total of 130 clinical isolates of *Candida* sp. were selected for testing. The collection included *C. albicans* (n = 81), *C. tropicalis* (n = 22), *C. parapsilosis* (n = 11), *C. glabrata* (n = 9) and *C. krusei* (n = 7). Members of this collection were obtained from oral cavity and vaginal swabs and from sputum specimen taken from diabetic patients, patients with vulvovaginal candidosis and cancer patients. Presumptive identification of yeast isolates to the species level was based on traditional methods and growth morphology, germ tube testing and color characteristics on CHROMagar Candida plates (Abu-Elteen *et al*., 2006) and confirmed by using API20C AUX (Bio-Merieux, Marcy I'Etoile, France). *C. albicans* (ATCC 36082), *C. parapsilosis* (ATCC 22019), *C. glabrata* (ATCC 90030) and *C. krusei* (ATCC 6258) were used as control strains.

Antifungal susceptibility testing: The E-test (AB Biodisk, Solna, Sweden) was performed to determine the Minimal Inhibitory Concentrations (MICs) of antifungal agents according to the manufacturers' instructions (Biodisk, 1993). Test strips used included: amphotericin B (AMB), flucytosine (FC), fluconazole (FL), itraconazole (IT) and voriconazole (VOR). Each strip present a continuous gradient of antifungal concentrations on one side and a reading scale on the opposite side. Briefly, the stored *Candida* sp. was subcultured on Sabouraud dextrose agar (SBA) medium, prior to testing to ensure viability and purity. Inoculums of *Candida* sp. were prepared from 5-6 colonies in sterile phosphate buffer saline (PBS) (pH 7.0). Following mixing, turbidity of the suspension was adjusted to match a 0.5 McFarland

turbidity standard. Swabs from the prepared suspension were streaked over culture plates containing RPMI-agar with 2% glucose, prepared by adding sterile liquid RPMI 1640 and buffered with 0.165 M morpholine-propane sulphonic acid (MOPS) to pH 7.0, solidified with 1.5% Bacto-agar (Difco Laboratories, Detroit, M.I., USA). Plates were allowed to dry for 15 min prior to placing five antifungal E test strips on the surface of media. Plates were incubated at 35°C; MIC values were read at 24 and 48 h after incubation.

Interpretations of MIC values as generated by the E-test were done according to the manufacturer's instructions. Briefly, values were read where the border of the elliptical inhibition zone intersected the scale on the strips or where there was a sharp decline in the amount of growth (≈ 80% inhibition). In the case of growth of small colonies inside the inhibition zone, the limit of the inhibition zone was defined as the border where the colonies started to change size and the density of growth decreased (≈ 80% inhibition).

For comparison of data, broth microdilution test was performed according to CLSI document M 27-A (NCCLS, 1997). An inoculum concentration of 0.5×10^3 cells per mL was standardized spectrophotometrically and by quantitative plate counts. A 100 μL yeast inoculum was added to each well of the microdilution trays. The final concentrations of the antifungal agents were 0.125 to 4 μg mL^{-1} for amphotericin B, 0.007 to 16 μg mL^{-1} for itraconazole and voriconazole, 0.06 to 128 μg mL^{-1} for flucytosine and 0.12 to 128 μg mL^{-1} for fluconazole. The trays were incubated at 35°C and read after 48 h of incubation. MIC endpoints were determined in RPMI medium, pH 7.0, as the lowest concentration that produced a significant decrease in turbidity relative to the control (drug-free) well (NCCLS, 1997; Abu-Elteen *et al*., 2006). The interpretation of antifungal resistance-susceptible category among the *Candida* isolates was based on the CLSI criteria of MICs breakpoints (NCCLS, 1997). The MICs (μg mL^{-1}) breakpoint categories for flucytosine of susceptible (S), intermediate susceptible (I) and resistant (R), are ≤4, 8-16 and ≥32 μg mL^{-1}, respectively. Those for fluconazole of S, susceptibility depending upon dose (S-DD) and R are ≤8, 16-32 and ≥64 μg mL^{-1}, respectively and for itraconazole categories of S, S-DD and R are ≤0.125, 0.25-0.5 and ≥1.0 μg mL^{-1}, respectively. The breakpoint values for amphotericin B of susceptible (S) and resistant (R), are < or = 2 μg mL^{-1}, respectively.

Statistical analysis: Data analysis was carried out by means of one-way analysis of variance (ANOVA) and by multiple comparison using computer statistical analysis software (STATISTICA for Windows (1995), Stat Soft Inc, OK, USA), differences were considered as statistically significant at $p<0.05$.

RESULTS

Table 1 shows the MIC values (MIC_{50} and MIC_{90}) and resistant strain percentages of clinical *Candida* isolates tested against conventional antifungal agents as determined by the E-test at 24 and 48 h incubation are listed in (Table 1). The MICs endpoint readings were easily and clearly determined for AMB, FC and VOR at both 24 and 48 h as very few colonies were noticed to grow within the inhibition zone (Fig. 1). In contrast, MIC inhibition zones for the FL and IT showed significant growth of small colonies within almost all inhibition zones, at both 24 and 48 h, making the endpoint determination of MICs difficult. MIC values determined at 48 h were generally higher than those determined at 24 h for all antifungal agents tested; this although they were less pronounced for AMB and FC. Changes noted in the MIC patterns for both *C. albicans* (n = 81) and *C. tropicalis* (n = 22), at 48 h compared to that at 24 h are shown in Table 2. Such changes involved the category of susceptibility as well as the MIC scale. For instance, at 24 h, resistance was noted for some isolates of *C. albicans* against FL and IT (8%) and *C. tropicalis* against IT (14%). At 48 h however, a substantial increase in resistance was observed for *C. albicans* against FC (10%), FL (23%) and IT (28%). *C. tropicalis* showed increased resistance to FL (24%) and IT (39%) (Table 1 and 2).

For AMB, MIC values determined at both 24 and 48 h were very low (≤0.094 μg mL^{-1}) and MIC_{50} and MIC_{90} for *C. albicans* after 24 h were 0.016 and 0.032 μg mL^{-1}; the number of susceptible strains was 81 out of 81 (100%). The MIC_{50} and MIC_{90} after 48 h were 0.023 and 0.64 μg mL^{-1}. The range of MICs for 9 isolates of *C. glabrata* against AMB after 24 h was 0.016-4 μg mL^{-1} and the MIC_{50} and MIC_{90} were 0.7 and 1.25 μg mL^{-1}; the number of susceptible strains was 8 out of 9 (89%). At 48 h, MIC range was 0.016-8 μg mL^{-1} and the MIC_{50} and MIC_{90} were 1.23 and 2.21 μg mL^{-1} (Table 1). *C. glabrata* isolates showed high resistance to FL (MIC_{50} and MIC_{90} after 48 h were 16 and 256 μg mL^{-1}, respectively) while *C. krusei* isolates showed higher MIC for FL after 48 h (Table 1). Voriconazole demonstrated excellent potency and broad-spectrum against all *Candida* species and 100% of isolates were inhibited by voriconazole at ≤1 μg mL^{-1} (Table 1).

Table 1: MICs of antifungal agents against *Candida* sp. and the percentages of resistant strains

Candida sp. (number tested)	Anti fungal agent[a]	Reading time (h)	Range	MICs (μg mL^{-1})[b] 50%	90%	Resistant strains (%)[c]
C. albicans (81)	AMB	24	≥0.002-0.064	0.016	0.032	0
		48	≥0.002-0.094	0.023	0.64	0
	FC	24	0.023-2	0.19	0.75	0
		48	0.023-≤ 32	0.25	4.00	10
	FL	24	0.064-<256	0.50	64.00	8
		48	0.19-<256	1.00	256.00	23
	IT	24	0.012-2	0.047	0.19	8
		48	0.012-<256	0.125	32.00	28
	VOR	24	0.008-0.750	0.032	0.047	0
		48	0.008-64	0.032	0.032	0
C. tropicalis (22)	AMB	24	>0.002-0.047	0.002	0.016	0
		48	>0.064-0.002	0.006	0.032	0
	FC	24	0.023-0.19	0.047	0.125	0
		48	0.064-1.5	0.094	0.75	0
	FL	24	0.5-2	1.00	2.00	0
		48	3-<256	12.00	64.00	24
	IT	24	0.047-1	0.38	0.50	14
		48	0.125-<32	0.75	12.00	39
	VOR	24	0.016-1.5	0.064	0.19	0
		48	0.019-64	0.19	0.75	0
C. parapsilosis (11)	AMB	24	>0.00`2-0.002	>0.002	>0.002	0
		48	0.002-0.094	0.023	0.64	0
	FC	24	0.003-0.5	0.032	0.047	0
		48	≥0.06-1	0.12	0.25	0
	FL	24	≥0.25-1	0.50	1.00	0
		48	≥0.25-4	1.00	2.00	0
	IT	24	≥0.002-0.004	0.002	0.004	0
		48	≥0.008-0.25	0.03	0.12	0
	VOR	24	0.008-2	0.032	0.094	0
		48	0.008-64	0.032	0.25	0
C. glabrata (9)	AMB	24	0.016-4	0.7	1.25	11
		48	0.016-8	1.23	2.21	11
	FC	24	≥0.06-4	≥0.06	≥0.06	0
		48	≥0.06-4	≥0.06	0.12	0
	FL	24	1-<256	48.00	96.00	100
		48	2-<256	16.00	256.00	100
	IT	24	0.003-4	0.25	1.00	22
		48	0.06-<8	1.00	4.00	22
	VOR	24	0.047-2	0.125	0.38	0
		48	0.094-3	0.38	1.00	0
C. krusei (7)	AMB	24	0.002-0.047	0.002	0.016	0
		48	0.002-0.064	0.006	0.032	0
	FC	24	≥0.06-0.12	≥0.06	≥0.06	0
		48	≥0.06-1	0.12	0.25	0
	FL	24	1.5-<256	48.00	96.00	100
		48	2-512	512.00	512.00	100
	IT	24	≥0.008-0.25	0.03	0.12	0
		48	0.016-0.5	0.03	0.25	0
	VOR	24	0.016-1	0.19	0.38	0
		48	0.016-1	0.75	1.00	0

[a]AMB, amphotericin B; FC, flucytosine; FL, fluconazole; IT, itraconazole; VOR, Voriconazole; [b]50 and 90%; MICs at which 50 and 90% of isolates tested, respectively, are inhibited; [c]Defined as follows: amphotericin B resistance, ≥2 μg mL^{-1} at 24 h and ≥4 μg mL^{-1} at 48 h; flucytosine resistance; ≥32 μg mL^{-1}; itraconazole resistance; ≥1.0 μg mL^{-1} and fluconazole resistance; ≥64 μg mL^{-1}

The MIC range for *Candida* species using the microbroth dilution method and the E-test at 24 and 48 h are listed in Table 3. For the 81 *C. albicans* isolates against AMB using microbroth dilution method, MIC range was 0.125-16 μg mL^{-1} and the MIC_{50} and MIC_{90} were 1.5 and 2.69 μg mL^{-1}; number of susceptible strains was 71 (87.7%) and the agreement between MICs by the two methods (at ±2 dilutions) was 95, 86 and 97% for AMB, FL and VOR, respectively. Moreover, differences between results obtained by the E-test at 24 versus 48 h were statistically insignificant. *C. glabrata* and *C. krusei* were the least susceptible against FL and the agreement between MICs by the two methods were 67 and 52%, respectively. The agreement between microbroth dilution

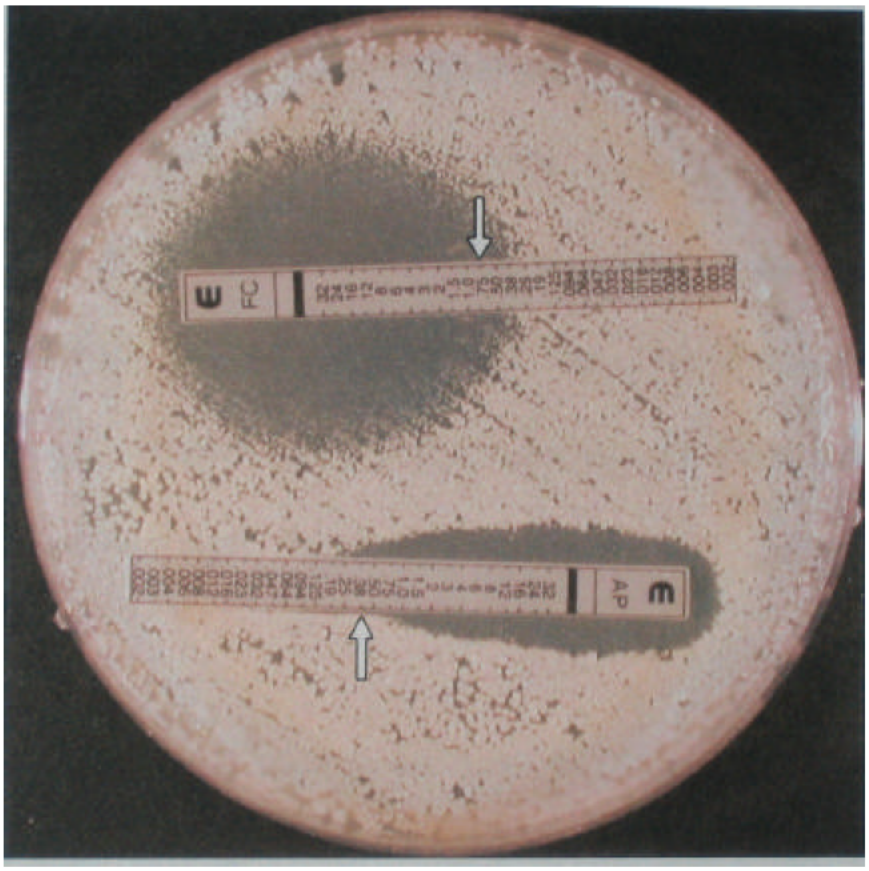

Fig. 1: Amphotericin B (AP) and flucytosine (FC) E-test patterns for *C. albicans*. Clear inhibition zone (ellipse) on RPMI agar. The numbers on the scale correspond to the antifungal concentrations on the strip (in μg mL^{-1})

Table 2: Observed changes in MICs determinations at 48 h compared to 24 h incubation

		Number (%) of isolates showing changes in	
Antifungal drug	Scale changes in MICs[a] at 48 vs. 24 h	*C. albicans* (n = 81)	*C. tropicalis* (n = 22)
Amphotericin B	No or within 2-scales higher	74 (91)	20 (91)
	3-6 higher	4(5)	2(9)
	7-9 higher	3 (4)	-
Flucytosine	No or within 2-scales higher	70 (86)	9 (41)
	3-6 higher	3(4)	11 (50)
	7-9 higher	4 (6)	2 (9)
	From S to R	4 (6)	-
Fluconazole	No or within 2-5 scales higher	55 (68)	9 (41)
	7-9 higher	-	4 (18)
	≤10 higher	26 (32)	9 (41)
	From S to S-DD	-	7 (32)
	From S to R	24 (30)	7 (32)
Itraconazole	No or within 2 scale higher	56 (69)	14 (64)
	3-6 higher	5(6)	-
	7-9 higher	3 (4)	4 (18)
	≤10 higher	17(21)	4 (18)
	From S to S-DD	11 (14)	2 (9)
	From S to R	11 (14)	-
	From S-DD to R	3(4)	6 (27)

[a]S, susceptible; R, resistant; I, intermediate susceptible; S-DD, susceptibility dependent upon dose

Table 3: *In vitro* activity of amphotericin B (AMB), fluconazole (FL) and voriconazole (VOR) against *Candida* species as determined by micro-broth dilution method and E-test method

Candida species (number tested)	Antifungal agents	Method	Range	MIC[a]$_{50}$	MIC[a]$_{90}$	No. of susceptible[b] (%)	% agreement ±2 dilutions
C. albicans (81)	AMB	Microbroth	0.125-16	1.50	2.69	71 (87.7)	95.0
		E-test 24	≤ 0.002-0.064	0.016	0.032	81 (100.0)	
		E-test 48	≤ 0.002-0.094	0.023	0.64	81 (100.0)	
	FL	Microbroth	0.25-64	14.90	26.80	74 (91.3)	86.0
		E-test 24	0.064->256	0.50	64.00	78 (96.3)	
		E-test 48	0.19->256	1.00	256.00	79 (97.5)	
	VOR	Microbroth	0.031-16	0.031	0.031	79 (97.5)	97.0
		E-test 24	0.008-0.750	0.032	0.047	81 (100.0)	
		E-test 48	0.008-64	0.032	0.032	81 (100.0)	
C. tropicalis (22)	AMB	Microbroth	≤ 0.125-2	0.50	2.00	16 (72.7)	90.0
		E-test 24	<0.002-0.047	0.002	0.016	22 (100.0)	
		E-test 48	<0.002-0.064	0.006	0.032	22 (100.0)	
	FL	Microbroth	≤ 0.125-2	0.50	2.00	14 (63.6)	87.0
		E-test 24	0.5-2	1.00	2.00	22 (100.0)	
		E-test 48	3->256	12.00	64.00	20 (90.9)	
	VOR	Microbroth	0.031-32	0.031	0.125	18 (81.8)	88.2
		E-test 24	0.016-1.5	0.064	0.19	22 (100.0)	
		E-test 48	0.019-64	0.19	0.75	22 (100.0)	
C. parapsilosis (11)	AMB	Microbroth	≤ 0.125-2	0.50	1.57	9 (81.8)	96.5
		E-test 24	<0.002-0.002	<0.002	<0.002	11 (100.0)	
		E-test 48	0.002-0.094	0.023	0.64	11 (100.0)	
	FL	Microbroth	0.25-1	0.50	1.00	10 (91.0)	98.1
		E-test 24	≤ 0.25-1	0.50	1.00	11 (100.0)	
		E-test 48	≤ 0.25-4	1.00	2.00	11 (100.0)	
	VOR	Microbroth	0.031-2	0.031	0.125	10 (91.0)	98.1
		E-test 24	0.008-2	0.032	0.094	11 (100.0)	
		E-test 48	0.008-64	0.032	0.25	11 (100.0)	
C. glabrata (9)	AMB	Microbroth	2-4	1.57	2.83	9 (100.0)	98.0
		E-test 24	0.016-4	0.70	1.25	8 (89.0)	
		E-test 48	0.016-8	1.23	2.21	8 (89.0)	
	FL	Microbroth	2-64	8.00	64.00	5 (55.5)	67.0
		E-test 24	1-<256	48.00	96.00	3 (33.3)	
		E-test 48	2->256	16.00	256.00	1(11.1)	
	VOR	Microbroth	0.031-2	0.125	1.00	8 (88.8)	98.2
		E-test 24	0.047-2	0.125	0.38	9 (100.0)	
		E-test 48	0.094-3	0.38	1.00	9 (100.0)	
C. krusei (7)	AMB	Microbroth	0.5-8	1.6	2.87	6 (85.7)	98.1
		E-test 24	0.002-0.047	0.002	0.016	7 (100.0)	
		E-test 48	0.002-0.064	0.006	0.032	7 (100.0)	
	FL	Microbroth	0.125-64	16.00	64.00	4 (57.1)	52.0
		E-test 24	1.5-<256	48.00	96.00	1 (14.3)	
		E-test 48	2-512	512.00	512.00	0 (0.0)	
	VOR	Microbroth	0.031-1	0.25	0.50	6 (85.7)	95.5
		E-test 24	0.016-1.	0.19	0.38	7 (100.0)	
		E-test 48	0.016-1	0.75	1.00	7 (100.0)	

[a] 50 and 90%; MICs at which 50 and 90% of isolates tested, respectively, are inhibited; [b]Defined as follows: amphotericin B resistance; ≥2 μg mL^{-} at 24 h and ≥4 μg mL^{-1} at 48 h and fluconazole resistance, ≥64 μg mL^{-1}

method and the E-test for VOR against *C. glabrata* and *C. krusei* were 98.2 and 95.5%, respectively (Table 3).

DISCUSSION

Based on the observations made by this study, several important points can be concluded. Consistent with previous studies (Araj *et al*., 1998; Birinci *et al*., 2001; Colombo *et al*., 1995; Kronvall and Karlsson, 2001) the endpoint determination of MICs are easier to read for amphotericin B, flucytosine (Fig. 1) and voriconazole than for fluconazole and itraconazole. This is mainly due to the frequent presence of yeast colonies growing within the inhibition zones of azoles. The exact reason as to why the trailing endpoints problem persists in the case of azoles remains obscure. The consistent persistence of trailing endpoints in the case of azoles irrespective of the clinical isolate tested, even those which show no trailing effects with other classes of antifungal, suggests that the problem is unlikely to be related to defective manufacturing of the test kit. It is possible that the apparent less-finely graded effects of azoles may partially explain this phenomenon. This is supported by the observation that introduction of certain modifications to the test media of the E-test has proved to be useful in minimizing azole-associated endpoint trailing

(Pfaller *et al*., 1998). Manufacturers of the E-test have previously suggested the use of one of five different solidified media for antifungal susceptibility testing of yeast. Consistent with previous data (Espinel-Ingroff, 1994; Pfaller *et al*., 1998) the RPMI agar with 2% glucose used in this study seems to yield results that correlate well with those attained the broth microdilution (86-98%, <2 dilutions). This is particularly true in the case of testing for susceptibility against flucytosine and ketoconazole. The rate of agreement between the E-test and CLSI reference method results in tests on RPMI agar was similar to that obtained in other studies (Ruhnke *et al*., 1996; St-Germain *et al*., 2001; Wanger *et al*., 1995).

The duration of incubation prior to reading the MICs is different with antifungal susceptibility tests. The CLSI macro-and microbroth dilution methods are determined at 48 h incubation, compared with the 24 h incubation for E-test agar method. In this study, the E-test MICs were determined at both 24 and 48 h. It is clear that whereas MIC determinations taken at 24 h were easy to read and interpret, determinations taken at 48 h showed more trailing endpoints and higher MICs; this is especially true for itraconazole (Table 2). Significant discrepancies in MICs findings between 24 versus 48 h incubations have been previously reported (Araj *et al*., 1998; Chang *et al*., 2001). Contradictory to these findings, a previous report has suggested that similar MIC values were attained by the E-test at either 24 or 48 h (Ruhnke *et al*., 1996). Yet another study has indicated that a 48 h incubation period prior to reading yields better results (Espinel-Ingroff, 1994). Colombo *et al*. (1995) has suggested that a 24 h incubation period is not always enough for accurately determining E-test MICs in the case of *C. parapsilosis*. Reconciling these conflicting results could mainly rest upon the standardization of the type of media to be used.

The CLSI reference method for *Candida* species has provided a starting point for the development of a standard method for testing of antifungal agents. In agreement with published reports (Fidel *et al*., 1999; Sewell *et al*., 1994; Wanger *et al*., 1995), data obtained by the E-test correlated more nicely with those obtained by the broth reference method for *C. albicans* and other *Candida* species against amphotericin B, fluconazole and voriconazole at 24 h than at 48 h (Table 3). Wanger *et al*. (1995) has demonstrated that the E-test is superior to the broth macrodilution reference method as a means of distinguishing amphotericin B-resistant/susceptible isolates of *Candida* species. Sewell *et al*. (1994) has also reported more consistent MIC readings for *C. albicans* against fluconazole at 24 h (93%) than at 48 h (79%). Determination of the MICs breakpoints at 48 h for fluconazole and itraconazole against *C. albicans* and *C. tropicalis* revealed higher resistance rates than those determined at 24 h (Table 2).

Although resistance of *Candida* species to amphotericin B is considered as uncommon (Abu-Elteen and Hamad, 2005; Fidel *et al*., 1999), it has been previously reported for *C. lusitaniae* (St-Germain *et al*., 2001). The MICs ranges for amphotericin B in this study against *C. albicans*, *C. tropicalis* and *C. parapsilosis* remained very low even at 48 h ($\leq$0.094 μg mL^{-1}), indicating the absence of resistance to amphotericin B in clinical isolates tested. This although, the MIC for a single isolate of *C. glabrata* was $\geq$4 μg mL^{-1}. Earlier studies have indicated that *C. krusei*, *C. glabrata* and *C. lusitaniae* were less susceptible *in vitro* to amphotericin B than other *Candida* species (Majoros *et al*., 2002). While only 8% of *C. albicans* isolates were found to be resistant to fluconazole, it was 22% in the case of *C. glabrata* (Table 1). This is in agreement with the proposition that resistance of *C. albicans* to fluconazole appears to be associated with long-term treatment with this drug (Abu-Elteen, 2001).

ACKNOWLEDGMENT

This study was supported by research grant, deanship of scientific research, The Hashemite University. We thank all the staff at the Clinical Microbiology Division, Jordan University Hospital for their valuable help.

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