J Microbiol Immunol Infect.
2009;42:148-153

Original Article

Disk diffusion test and E-test with enriched Mueller-Hinton agar for determining susceptibility of *Candida* species to voriconazole and fluconazole

Sai-Cheong Lee[1,2], Hsiu-Jung Lo[3], Chang-Phone Fung[4,5], Ning Lee[6], Lai-Chu See[7]

[1]Division of Infectious Diseases, Chang Gung Memorial Hospital, Keelung; [2]Nursing Department, Chang Gung Institute of Technology, Linkou; [3]Division of Clinical Research, National Health Research Institutes, Miaoli; [4]Division of Infectious Diseases, Veterans General Hospital, Taipei; [5]College of Medicine, Yang Ming University, Taipei; [6]Department of Pathology, Chang Gung Memorial Hospital, Keelung; and [7]Department of Public Health, Chang Gung University, Linkou, Taiwan

Received: June 18, 2007 Revised: January 15, 2008 Accepted: April 4, 2008

Background and purpose: A simplified antifungal disk diffusion test using Mueller-Hinton agar containing 2% glucose and methylene blue 5 μg/mL (GM-MH, Clinical and Laboratory Standards Institute [CLSI] M44-A) has proved to correlate well with the standard reference test. A new azole, voriconazole, has recently been approved for clinical therapy in Taiwan. This study investigated the reliability of the disk diffusion test with GM-MH agar and compared the results with those of the E-test using GM-MH agar to determine the voriconazole and fluconazole susceptibility of *Candida* isolates.

Methods: The antimicrobial susceptibility of *Candida* isolates were evaluated by E-test and disk diffusion test in accordance with the guidelines of the CLSI, and compared with the reference antifungal macrodilution susceptibility test (CLSI M27-A).

Results: The antifungal disk diffusion test and the E-test using GM-MH agar plate provided a sufficiently accurate, time-efficient, and cost-effective way to determine the susceptibility of 182 *Candida* spp. to voriconazole and fluconazole. There was a high correlation between the test results of the E-test using the GM-MH agar plate and those obtained by the reference antifungal macrodilution susceptibility test (CLSI M27-A). The results of the E-test and those of the 1-μg voriconazole disk diffusion test on the GM-MH agar plate at 24 h had a high correlation. All the minimal inhibitory concentrations of voriconazole for all *Candida* spp. were <8 μg/mL. The positive predictive value of the susceptible disk test of voriconazole on the GM-MH agar plate was 100% at 24 h for *C. albicans* and other *Candida* spp.

Conclusion: The disk diffusion test and the E-test using the GM-MH agar plate can be performed quickly, simply, and cost-effectively, and are practicable methods for the initial testing of the susceptibility of *Candida* spp. to voriconazole and fluconazole.

Key words: *Candida*; Drug resistance, bacterial; Fluconazole; Voriconazole

Introduction

Fungal infections are still an important cause of mortality and morbidity for immunocompromised patients [1-3]. With effective antifungal therapy and improved medical care, immunocompromised patients can live longer lives [4-6]. Resistance to antifungal agents is emerging, so in vitro susceptibility data are required to guide the selection of antifungal chemotherapy [7,8].

Although a standard reference procedure for evaluating the susceptibility of yeasts to antifungal agents was approved by the Clinical and Laboratory Standards Institute (CLSI) in June 1997, the procedure is a macrodilution technique, which is not cost-effective for use

Corresponding author: Dr. Sai-Cheong Lee, Division of Infectious Diseases, Chang Gung Memorial Hospital, 222, Mai Chin Road, Keelung, Taiwan.
E-mail: lee.sch@msa.hinet.net

in most clinical laboratories [9]. A broth microdilution test derived from the standard reference test and the E-test are simplified tests and are acceptable [10-13]. However, both the microdilution test and the E-test with RPMI (Roswell Park Memorial Institute) agar are not sufficiently cost effective to be performed routinely for every yeast isolate in most clinical laboratories.

A simplified antifungal disk diffusion test using Mueller-Hinton (MH) agar containing 2% glucose and methylene blue 5 μg/mL (GM-MH, CLSI M44-A), which costs US$0.62 per agar in Taiwan, has been found to correlate well with the standard reference test [14-16]. A new azole, voriconazole, has been recently approved for clinical therapy in Taiwan. Thus, the reliability of the disk diffusion test with GM-MH agar was investigated and the results compared with those of the E-test method using GM-MH agar to test the voriconazole and fluconazole susceptibility of *Candida* spp. isolates.

Methods

Design

Initially, this study investigated the correlation of the minimal inhibitory concentrations (MICs) obtained with the E-test using GM-MH agar read at 24 h and the antifungal reference macrodilution test for fluconazole in *Candida* spp. isolates [9]. Then, the MICs of fluconazole obtained with the E-test using GM-MH agar read at 24 h were compared with the MICs of fluconazole obtained with the E-test using GM-MH agar read at 24 h.

Media

MH agar was purchased from Difco Laboratories (Sparks, MA, USA) and was solidified with 2% glucose and methylene blue 5 μg/mL. Both glucose and methylene blue were obtained from Sigma Chemical Company (St. Louis, MO, USA). E-test strips were purchased from AB Biodisk (Solna, Sweden).

Microorganisms

182 isolates of *Candida* spp. collected from the clinical laboratory of Keelung Chang Gung Memorial Hospital, Keelung, Taiwan, between January 1998 and December 1998 for a previous study [16] were selected for this study. The 182 isolates included 131 *Candida albicans*, 14 *Candida glabrata*, 12 *Candida tropicalis*, 9 *Candida parapsilosis*, 13 *Candida guilliermondii*, 1 *Candida sake*, 1 *Candida intermedia*, and 1 *Candida utilis*. The control strains were American Type Culture Collection (ATCC) 90028 *C. albicans*, ATCC 22019 *C. parapsilosis*, and ATCC 6258 *C. krusei*. According to CLSI M44-A [17] and other studies [15,16,18,19], the acceptable range of inhibitory clear zones on GM-MH agar with the 25-μg fluconazole disk for these control strains are: *C. albicans* ATCC 90028, 28 to 39 mm; *C. parapsilosis* ATCC 22019, 22 to 33 mm; and *C. krusei* ATCC 6258, 6 to 17 mm. The acceptable range of inhibitory clear zones on GM-MH agar with the 1-μg voriconazole disk for these control strains are: *C. albicans* ATCC 90028, 31 to 42 mm; *C. parapsilosis* ATCC 22019, 28 to 37 mm; and *C. krusei* ATCC 6258, 16 to 25 mm [17]. The acceptable range of MICs of fluconazole for these control strains, according to the CLSI M44-A, are: *C. albicans* ATCC 90028, 0.25 to 1.0 μg/mL; *C. parapsilosis* ATCC 22019, 2.0 to 8.0 μg/mL; and *C. krusei* ATCC 6258, 16 to 64 μg/mL [9].

Disk diffusion test

The disk diffusion test was performed according to the procedure outlined in the CLSI M44-A document [17]. The *Candida* spp. that had been stored in a refrigerator at –70°C were recultured in Sabouraud dextrose agar by overnight incubation. Five-well isolated colonies from Sabouraud dextrose agar were suspended in 0.85% sterile normal saline 5 mL to achieve 0.5 McFarland turbidity to yield a yeast stock suspension of 1×10^6 to 5×10^6 cells/mL, which should produce semiconfluent growth with most *Candida* spp. isolates. A sterile swab was dipped into the inoculum suspension and excess fluid was pressed out. GM-MH agar was swabbed carefully in 3 directions to achieve even growth on the surface of the GM-MH agar plate. All moisture was absorbed for at least 5 min. One voriconazole 1-μg disk (Pfizer Inc, Chicago, IL, USA) was added to each inoculated plate surface with sterile forceps. The swabbed agar containing the voriconazole disk was incubated at 35°C in a moist incubator until growth and the zone of inhibition could be clearly seen after 24 to 48 h. When growth and the ellipse of inhibition were clearly seen after overnight incubation, the plates were read at 24 and 48 h. Azoles could give a diffuse zone edge. The inhibitory zones on GM-MH agar were measured at the point where there was a sharp decline in the amount of growth (approximately 80% inhibition). Macrocolonies ≥1 mm within the eclipse zone were regarded as significant growth, whereas microcolonies <1 mm could be neglected.

E-test method

The *Candida* spp. stored at –70°C were recultured in Sabouraud dextrose agar by overnight incubation. Five-well isolated colonies from Sabouraud dextrose agar were suspended in 0.85% sterile normal saline 5 mL to achieve 0.5 McFarland turbidity. A sterile swab was dipped into the inoculum suspension and excess fluid was pressed out. GM-MH agar was swabbed carefully in 3 directions to obtain even growth on the surface of the GM-MH agar plate. All moisture was absorbed for at least 5 min. The E-test strip was applied to the agar surface with sterile forceps. The swabbed agar containing the E-test strip was incubated at 35°C in a moist incubator until growth and the ellipse of inhibition could be clearly seen after 24 to 48 h. When growth and the ellipse of inhibition were clearly seen after overnight incubation, the plates were read at 24 and 48 h. The MIC was read where the inhibition ellipse intersected the scale on the strip. Azoles could give a diffuse zone edge. The MIC was read at the first point of significant inhibition or marked decrease in growth intensity to visually select the end point.

Interpretive criteria

For the E-test, MIC breakpoints of ≤8 μg/mL were interpreted as susceptible, 16 to 32 μg/mL were dose-dependent susceptible, and ≥64 μg/mL were resistant according to the criteria of CLSI M27-A [9]. For the disk diffusion test, zone diameters of ≥19 mm were interpreted as susceptible, 15 to 18 mm were dose-dependent susceptible, and ≤14 mm were resistant according to CLSI M44-A [17] and other studies [15,16,18,19].

Results

MICs were determined after 24 and 48 h by the CLSI macrodilution method. Table 1 shows the geometric mean MIC of fluconazole for each species. Table 2 shows the geometric mean MIC of fluconazole with the E-test using GM-MH agar for each species. Table 3 shows the geometric mean MIC of voriconazole with the E-test using GM-MH agar for each species. 177 of the 182 isolates (97.3%) that were susceptible to voriconazole (MIC, ≤8.0 μg/mL) according to the MICs obtained with the E-test method using the GM-MH agar plate at 24 h also demonstrated susceptibility with the voriconazole disk diffusion test on the GM-MH agar plate at 24 h (clear zone, ≥19 mm). Only 5 of the 182 isolates (2.7%) that were susceptible to voriconazole (MIC, ≤8.0 μg/mL) according to the MICs obtained with the E-test method using the GM-MH agar plate at 24 h demonstrated intermediate susceptibility with the voriconazole disk diffusion test on the GM-MH agar plate at 24 h (clear zone, ≥15 to 18 mm). 167 of the 182 isolates (91.7%) that were susceptible to voriconazole (MIC, ≤8.0 μg/mL) according to the MICs obtained with the E-test method using the GM-MH agar plate at 24 h also demonstrated susceptibility with the voriconazole disk diffusion test on the GM-MH agar plate at 48 h (clear zone, ≥19 mm). Fifteen of the 182 isolates (8.3%) that were susceptible to voriconazole (MIC, ≤8.0 μg/mL) according to the MICs obtained with the E-test method using the GM-MH agar plate at 24 h demonstrated intermediate susceptibility with the voriconazole disk diffusion test on the GM-MH agar plate at 48 h (clear zone, ≥15 to 18 mm). Table 4 describes the distribution of differences between the E-test fluconazole MICs at 24 h using GM-MH agar and the reference macrodilution method fluconazole MICs at 48 h for 182 isolates of *Candida* spp. and the essential agreement percentages.

All *Candida* spp. isolates grew well on enriched MH agar at 24 h and 48 h. Trailing phenomena around the zone margin were minimal on enriched MH agar, unlike that on simple MH agar. Due to the absence of the trailing phenomenon, zone diameters on enriched MH agar were more definite, clearer, and easier to read than those on simple MH agar.

The MIC values of the control strains for fluconazole were within the range proposed by CLSI M27-A [9,16]. Fluconazole disk tests on GM-MH agar at 24 h for control strains gave zones within the range proposed by CLSI M27-A [9,16]. Voriconazole disk tests on GM-MH agar at 24 h gave zones within the following ranges proposed by CLSI M44-A for control strains: *C. albicans* ATCC 90028, 31 to 42 mm; *C. parapsilosis* ATCC 22019, 28 to 37 mm; and *C. krusei* ATCC 6258, 16 to 25 mm [17]. The MIC values of voriconazole for the control strains were within the following ranges: *C. albicans* ATCC 90028, 0.25 to 1.0 μg/mL; and *C. krusei* ATCC 6258, 0.5 to 2.0 μg/mL.

Discussion

An antifungal susceptibility test should be time-efficient, inexpensive, and easy to perform for it to be routinely done in a clinical laboratory. According to Table 1 and Table 2, the MIC values of fluconazole for *Candida* spp. with the E-test using GM-MH agar at

Table 1. Minimal inhibitory concentration (MIC) of fluconazole against 182 *Candida* spp. according to the National Committee on Clinical Laboratory Standards macrodilution method M27-A with Roswell Park Memorial Institute 1640 broth.

Species (No. of isolates)[a]	Geometric MIC (μg/mL) Mean (range)	
	24 h	48 h
Candida albicans (131)	0.57 (0.13-32.00)	1.16 (0.13-64.00)
Candida glabrata (14)	1.28 (0.13-64.00)	3.46 (0.13-64.00)
Candida tropicalis (12)	0.94 (0.25-4.00)	2.39 (0.50-16.00)
Candida guilliermondii (13)	18.17 (0.13-64.00)	14.73 (1.00-64.00)
Candida parapsilosis (9)	0.77 (0.25-2.00)	1.68 (0.50-8.00)
Candida intermedia (1)	8.00	8.00
Candida sake (1)	64.00	64.00
Candida utilis (1)	0.13	1.00

[a]Number of isolates with an MIC of ≥8 μg/mL at 48 h: *C. albicans*, 9.9%; *C. glabrata*, 50.0%; *C. tropicalis*, 16.6%; *C. guilliermondii*, 84.6%; *C. parapsilosis*, 11.1%; *C. intermedia*, 100%; *C. sake*, 100%.

Table 2. Minimal inhibitory concentration (MIC) of fluconazole against 182 *Candida* spp. with the E-test using Mueller-Hinton agar.

Species (No. of isolates)[a]	Geometric MIC (μg/mL) Mean (range)	
	24 h	48 h
Candida albicans (131)	1.52 (0.50-32.00)	1.60 (0.50-32.00)
Candida glabrata (14)	9.87 (1.00-24.00)	13.74 (1.00-32.00)
Candida tropicalis (12)	2.10 (0.50-6.00)	2.16 (0.50-6.00)
Candida guilliermondii (13)	14.30 (4.00-32.00)	17.29 (6.00-48.00)
Candida parapsilosis (9)	2.61 (0.75-24.00)	3.00 (0.75-24.00)
Candida intermedia (1)	8.00	12.00
Candida sake (1)	64.00	64.00
Candida utilis (1)	6.00	6.00

[a]Number of isolates with an MIC of ≥8 μg/mL at 48 h: *C. albicans*, 6.9%; *C. glabrata*, 85.7%; *C. tropicalis*, 0%; *C. guilliermondii*, 92.3%; *C. parapsilosis*, 33.3%; *C. intermedia*, 100%; *C. sake*, 100%.

Table 3. Minimal inhibitory concentration (MIC) of voriconazole against 182 *Candida* spp. with the E-test using Mueller-Hinton agar.

Species (No. of isolates)[a]	Geometric MIC (μg/mL) Mean (range)	
	24 h	48 h
Candida albicans (131)	0.05 (0.01-0.75)	0.05 (0.01-1.00)
Candida glabrata (14)	0.45 (0.25-0.75)	0.86 (0.38-2.00)
Candida tropicalis (12)	0.16 (0.01-0.50)	0.21 (0.01-1.00)
Candida guilliermondii (13)	0.19 (0.13-0.38)	0.33 (0.13-0.75)
Candida parapsilosis (9)	0.06 (0.02-0.19)	0.06 (0.02-0.25)
Candida intermedia (1)	0.13	0.25
Candida sake (1)	0.02	0.02
Candida utilis (1)	0.13	0.13

[a]Number of isolates with an MIC of ≥8 μg/mL at 48 h: *C. albicans*, 0%; other *Candida* spp., 0%.

24 h correlate well with the MIC values obtained by the reference macrodilution method of CLSI M27-A. This finding indicates that the MIC values of voriconazole for *Candida* spp. with the E-test using GM-MH agar at 24 h should be accurate, as shown in Table 3. Barry and Brown reported that the subjectiveness of zone size measurements added an important source of variability to the test with RPMI-glucose agar due to the trailing phenomenon around the zone edges [15]. This problem was also noted with the fluconazole disk diffusion test using simple MH agar plate in this study. However, trailing phenomena around the zone margin were

Table 4. Distribution of differences between E-test fluconazole minimal inhibitory concentrations (MICs) at 24 h using GM-MH agar and the reference macrodilution method fluconazole MICs at 48 h and essential agreement percentages for 182 isolates of *Candida* spp.

Species (No. of isolates)	Lower			Same			Higher	Essential agreement percentage[a]
	<2	2	1	0	1	2	>2	
Candida albicans (131)	7	10	10	52	24	19	7	96.1
Candida glabrata (14)	2	4	0	2	1	0	5	50.0
Candida tropicalis (12)	1	0	2	6	2	1	1	84.6
Candida guilliermondii (13)	5	2	2	2	0	1	1	53.8
Candida parapsilosis (9)	0	0	1	3	3	0	2	77.8
Candida intermedia (1)	0	0	0	1	0	0	0	100.0
Candida sake (1)	0	1	0	0	0	0	0	100.0
Candida utilis (1)	0	0	0	0	0	1	0	100.0
Total (182)	15	17	15	66	30	22	16	82.9

[a]Essential agreement percentages are in exact agreement or are within ±1-fold dilution.
Abbreviation: GM-MH = Mueller-Hinton agar containing 2% glucose and methylene blue 5 μg/mL.

infrequent and minimal on the GM-MH agar plate. With this method, the zone edges were frequently definite and clear, facilitating the measurement of zone sizes and minimizing subjective assessment of the zone size measurements [16]. The occurrence of macrocolonies ≥1 mm within the clear zone was also infrequently found with this method. In Barry and Brown's report, some species other than *C. albicans* did not show a good layer of growth on RPMI-glucose after the first 24 h of incubation, especially *C. glabrata,* and 48 h of incubation were needed to produce a good layer of growth [15]. However, in this study, all *Candida* isolates grew well on the GM-MH agar plate at 24 h, with an easily identified layer of growth, enabling the measurement of zone sizes for the E-test and the disk diffusion test at 24 h for all *Candida* spp.

In a previous study, the fluconazole disk diffusion test on the GM-MH agar plate after 48 h of incubation resulted in more false-resistant disk zone sizes than the macrodilution reference method of obtaining MICs [16]. Some isolates were demonstrated to be susceptible to fluconazole with the reference method as they showed susceptible zone sizes on the GM-MH agar plate at 24 h, but manifested resistant zone sizes after 48 h of incubation on the GM-MH agar plate. This may indicate that *Candida* spp. grow sufficiently on the GM-MH agar plate at 24 h but overgrow after 48 h of incubation to give false-resistant zone sizes. Thus, with the GM-MH agar plate, 48 h of incubation is not necessary and may produce false-resistant disk test results [16].

This study indicates that, although there is a difference between the test results obtained by the E-test method using the GM-MH agar plate at 24 h and those obtained by the reference antifungal macrodilution susceptibility test at 48 h, the correlation is generally good (Table 1, Table 2, and Table 4). Although there is some difference between the MICs of fluconazole with the E-test on the GM-MH agar plate at 24 h and at 48 h, the rate of susceptibility of MICs of fluconazole with the E-test on the GM-MH agar plate at 24 h and at 48 h are similar (Table 2). As 177 of the 182 isolates (97.3%) that were susceptible to voriconazole according to the MICs obtained with the E-test method using the GM-MH agar plate at 24 h also demonstrated susceptibility with the voriconazole disk diffusion test, the results of the E-test method for voriconazole and those of the 1-μg voriconazole disk diffusion test on the GM-MH agar plate at 24 h also have a high correlation. All the MICs of voriconazole for all *Candida* spp. were below 8 μg/mL (Table 3). The positive predictive value of the susceptible disk test of voriconazole on the GM-MH agar plate was 100% at 24 h for *C. albicans* and other *Candida* spp. The disk test of voriconazole on GM-MH agar does not adequately separate voriconazole-sensitive strains from voriconazole dose-dependent susceptibilities (Table 4). This problem is not unique to disk tests for antifungal agents. Disk tests for antibacterial agents may also show this problem [20]. Since the positive predictive value of a voriconazole susceptible disk with the GM-MH agar plate at 24 h was 100% in this study, any *Candida* isolate screened to be voriconazole susceptible by this disk test can be reported to be susceptible without further testing. 177 of 182 isolates (97.2%) in this study were in this category. The isolates that were not susceptible in the disk diffusion test should undergo more precise procedures, such as the E-test for MICs. According to the results of this study,

the MICs of fluconazole and voriconazole, ascertained with the E-test using GM-MH agar read at 24 h, correlate well with those using the RPMI 1640 agar plate read at 48 h. Compared with the latter test, the former test can be performed quickly and simply and may be cost-effective for selective screening tests of intermediately susceptible *Candida* isolates.

Acknowledgment

Financial support was provided by the Division of Clinical Research, National Health Research Institutes, Taipei, Taiwan.

References

1. Karlowsky JA, Zhanel GG, Klym KA, Hoban PJ, Kabani AM. Candidemia in a Canadian tertiary care hospital from 1976 to 1996. Diagn Microbiol Infect Dis. 1997;28:5-9.
2. Anaissie EJ, Rex JH, Uzun O, Vartivarian S. Predictors of adverse outcome in cancer patients with candidemia. Am J Med. 1998;104:238-45.
3. Nolla-Salas J, Sitges-Serra A, Leon-Gil C, Martinez-Gonzalez J, Leon-Regidor MA, Ibanez-Lucia P, et al. Candidemia in non-neutropenic critically ill patients: analysis of prognostic factors and assessment of systemic antifungal therapy. Intensive Care Med. 1997;23:23-30.
4. Rex JH, Bennett JE, Sugar AM, Paphas PG, Serody J, Edwards JE, et al. Intravascular catheter exchange and duration of candidemia. Clin Infect Dis. 1995;21:994-6.
5. Dean DA, Burchard KW. Surgical perspective on invasive *Candida* infections. World J Surg. 1998;22:127-34.
6. Solomkin JS. Timing of treatment for nonneutropenic patients colonized with *Candida*. Am J Surg. 1996;172:445-75.
7. Joly V, Carbon C. Editorial response: unfortunate in vitro selection of resistant *Candida albicans* with severe clinical consequences. Clin Infect Dis. 1998;27:692-4.
8. Nguyen MH, Peacock JE, Morris AJ, Tanner DC, Nguyen ML, Snydman DR, et al. The changing face of candidemia, emergence of non-*Candida albicans* species and antifungal resistance. Am J Med. 1996;100:617-23.
9. Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard. Wayne: National Committee for Clinical Laboratory Standards; 1997.
10. Colombo AL, Barchiesi F, McGough DA, Fothergill AW, Rinaldi MG. Evaluation of the E test system versus a microtitre broth method for antifungal susceptibility testing of yeasts against fluconazole and itraconazole. J Antimicrob Chemother. 1995;36:93-100.
11. Rodriguez-Tudela JL, Martinez-Suarez JV. Improved medium for fluconazole-susceptibility testing of *Candida albicans*. Antimicrob Agents Chemother. 1994;8:45-98.
12. Polanco AM, Rodriguez-Tudela JL, Baguer F, Sanchez-Sousa A, Martinez-Suarez JV. Improved method of determining the susceptibility of *Candida albicans* to fluconazole. J Antimicrob Chemother. 1995;35:155-9.
13. Pfaller MA, Grant C, Morthland V, Rhine-Chalberg J. Comparative evaluation of alternative method for broth dilution susceptibilty testing of fluconazole against *Candida albicans*. J Clin Microbiol. 1994;32:506-9.
14. Pfaller MA, Dupont B, Kobayashi GS, Muller J, Rinaldi MG, Espinel-Ingroff A, et al. Standardized susceptibility testing of fluconazole: an international collaborative study. Antimicrob Agents Chemother. 1992;36:1805-9.
15. Barry AL, Brown SD. Fluconazole disk diffusion procedure for determining susceptibility of *Candida* species. J Clin Microbiol. 1996;34:2154-7.
16. Lee SC, Fung CP, Lee N, See LC, Huang JS, Tsai CJ, et al. Fluconazole disk diffusion test with methylene blue- and glucose-enriched Mueller-Hinton agar for determining susceptibility of *Candida* species. J Clin Microbiol. 2001; 39:1615-7.
17. Clinical and Laboratory Standards Institute. Method for antifungal disk diffusion susceptibility testing of yeasts. Proposed guideline. Wayne: National Committee for Clinical Laboratory Standards; 2004.
18. Pfaller MA, Bale M, Buschelman B, Lancaster M, Espinel-Ingroff A, Rex JH, et al. Quality control guidelines for National Committee for Clinical Laboratory Standards recommended broth macrodilution testing of amphotericin B, fluconazole, and flucytosine. J Clin Microbiol. 1995;33:1104-7.
19. Duswald KH, Penk A, Pittrow L. High dose therapy with fluconazole. Mycoses. 1997;40:267-77.
20. Jorgensen JH, Turnidge JD, Washington JA. Antibacterial susceptibility tests: dilution and disk diffusion methods. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH, eds. Manual of clinical microbiology. 7th ed. Washington: ASM Press; 1999:1526-43.

