J Microbiol Immunol Infect
2004;37:335-342

In vitro antifungal susceptibility testing of *Candida* blood isolates and evaluation of the E-test method

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Received: February 23, 2004 Revised: March 31, 2004 Accepted: June 25, 2004

Fungal infections have dramatically increased in recent years, along with the increase of drug-resistant isolates in immunocompromised patients. Ninety eight *Candida* species obtained from blood cultures at the Tri-Service General Hospital, Taiwan, from 1998 to 2000 were studied. These included 50 *Candida albicans*, 13 *Candida glabrata*, 24 *Candida tropicalis* and 11 *Candida parapsilosis* isolates. To investigate their susceptibility to commonly used antifungal drugs, minimum inhibitory concentrations (MIC) of amphotericin B, fluconazole, flucytosine, and ketoconazole were determined. Both the National Committee for Clinical Laboratory Standards reference broth macrodilution method and E-test were used in parallel. Ninety five isolates (95/98, 96.94%) were susceptible to amphotericin B at a concentration ≤1 μg/mL. All isolates (100%, 98/98) were susceptible to flucytosine. Approximately 30% of these *Candida* isolates were resistant to fluconazole. The MIC for 90% of isolates (MIC_{90}) values for both methods for these isolates were 0.5 μg/mL for amphotericin B, 32 μg/mL for fluconazole, 0.25 μg/mL for flucytosine (0.125 μg/mL by E-test method), and 4 μg/mL for ketoconazole. MIC for 50% of isolates (MIC_{50}) values for these agents were 0.25, 2, 0.06, and 0.06 μg/mL, respectively. The essential agreement of MIC values within 2 dilutions for the 2 methods was 99.0% for amphotericin B, 90.8% for ketoconazole, 92.9% for fluconazole, and 91.8% for flucytosine. This study showed that E-test has equivalent performance to the broth macrodilution method and can be used as an alternative MIC technique for antifungal susceptibility testing.

Key words: Antifungal agents, culture media, microbial sensitivity tests, reference standards

In recent years, fungal infections have increased dramatically due to factors such as increased organ transplants, use of immunosuppressive drugs, "over-use" of chemotherapy, dissemination of human immunodeficiency virus, and use of invasive treatments [1,2]. According to reports from the National Nosocomial Infection Surveillance System (NNISS), fungal infections have increased from 2 per 1000 patients in 1980 to 3.8 per 1000 in 1990 [3]. Among pathogens isolated, *Candida albicans* is the most common species, followed by *Candida tropicalis*, *Candida parapsilosis*, and *Candida glabrata*. Bloodstream infections caused by *Candida* spp. have increased significantly and now represents the fourth most common pathogen since 1989 [4-8]. An increase of 400% in invasive infections caused by *Candida* spp. has also been reported in some teaching hospitals [4], with morbidity and mortality of 30-50% being cited [9]. The prophylactic use of any antifungal agent may potentially select resistant organisms.

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Intrinsically fluconazole resistant species, i.e., *C. glabrata* and *Candida krusei*, have become important nosocomial pathogens, especially in institutions with extensive use of fluconazole. Fluconazole-resistant *C. albicans* have been described in late-stage acquired immunodeficiency syndrome patients and immuno-compromised patients [10,11]. Because of the life threatening nature of these infections and reports of resistance to azoles [12-14], susceptibility testing of pathogenic yeast has become very important.

In 1992, the National Committee for Clinical Laboratory Standards (NCCLS) proposed a reference broth macrodilution method for antifungal susceptibility testing of yeast (M27-P) [15]. Although recommendations for a broth dilution format was also developed to the approved level (M27-A, 1997) [16], this method may still have some limitations and further efforts are needed to develop simpler, more reliable and robust and economical methods for use in the clinical laboratory [16,17].

E-test® (AB BIODISK, Solna, Sweden) is a novel agar-based minimum inhibitory concentration (MIC) method that uses a predefined gradient of an antifungal

agent immobilized on a plastic strip, allowing for MIC determinations across 15 dilutions [18]. The purpose of this study was to compare E-test with the NCCLS reference broth macrodilution method by using both methods in parallel to determine the susceptibility of *Candida* spp. isolated from bloodstream infections at our hospital. This work would also provide valuable epidemiology data on recent clinical isolates and further verify empiric therapy choices for physicians.

Materials and Methods

Clinical isolates

Ninety eight clinical yeast isolates from patients with bloodstream infections at Tri-Service General Hospital from May 1998 to May 2000 were studied. Identification of *Candida* spp. isolates was done using the germ tube test and conventional mycological methods [19]. All isolates were stocked in trypticase soy broth containing 20% glycerol at –70°C until susceptibility testing was performed.

Antifungal agents

Reference grade powders of amphotericin B, flucytosine and ketoconazole (all from Sigma Chemical Co., St. Louis, MO, USA) and fluconazole (Pfizer Inc., Sandwich, UK) were used to prepare stock solutions and various dilutions from 0.03 to 32 μg/mL for all drugs except for fluconazole where the range was 0.125 to 64 μg/mL. Stock solutions of amphotericin B and ketoconazole (2560 μg/mL) were prepared in dimethyl sulfoxide and fluconazole and flucytosine in distilled water. All stock solutions were diluted with RPMI 1640 broth (JRH Bioscience Inc., KS, USA) to achieve 10 × final concentrations. E-test had the following MIC ranges: 0.002 to 32 μg/mL for amphotericin B, flucytosine and ketoconazole and 0.016 to 256 μg/mL for fluconazole. E-test reagent strips were stored at –20°C until used. Quality control strains and reference strains of *C. albicans* (ATCC 90028), *C. tropicalis* (ATCC 750), *C. parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258) were included in each test run [20,21].

Susceptibility medium

RPMI 1640 with L-glutamine broth without bicarbonate was buffered with MOPS (3-N-morpholino-propanesulfonic acid) to pH 7.0 and used for the broth macrodilution method [16]. The same batch of RPMI 1640 broth was supplemented with 2% glucose and buffered with MOPS to pH 7.0 and solidified with 1.5% of Bacto-agar (Difco, Detroit, MI, USA) to prepare agar plates for use with E-test.

Susceptibility testing

The broth macrodilution method was performed according to the approved NCCLS document M27-A [16]. In summary, the inoculum was prepared from growth on Sabouraud dextrose agar subcultures incubated at 35°C for 24 to 48 h depending on the species. Colonies were suspended in 0.85% saline and the turbidity of the resulting suspension was adjusted to a calibrated 0.5 McFarland standard. A working suspension was made by diluting the original suspension 1:100 and then 1:20 in RPMI broth. 900 μL of the final inoculum suspension was dispensed into a plastic test tube (13 × 100 mm) containing 0.1 mL of the antifungal dilution of the drug to be tested. The positive growth control tube contained 0.1 mL of drug diluent and 0.9 mL of the inoculum while the negative control had 0.1 mL of drug diluent and 0.9 mL of RPMI broth. The test tubes were incubated at 35°C for 48 h. The MIC endpoint reading criteria were as follows: azoles at the so-called 80% inhibition of growth compared to the growth control, flucytosine at almost complete inhibition ignoring slight hazes and amphotericin B at complete inhibition [16]. E-test was set up and read according to the manufacturer's instructions. As E-test values are based on a continuous gradient of concentrations instead of 2-fold dilutions in the broth macrodilution, between-dilution E-test MIC values were rounded up to the next 2-fold dilution for the purpose of comparison.

Analysis of results

The comparability of the 2 methods was assessed by determining MIC values where 50% (MIC_{50}) and 90% (MIC_{90}) of isolates tested was inhibited, respectively. Essential agreement (EA) was defined as percentage agreement of E-test and macrodilution test values within ± 1 dilution [22].

Results

The distribution of the 98 *Candida* isolates was: *C. albicans* (50), *C. glabrata* (13), *C. tropicalis* (24), and *C. parapsilosis* (11). MIC ranges as determined by macrodilution method and E-test for the 4 drugs are summarized in Table 1. The narrow MIC ranges

for amphotericin B and flucytosine were 0.002 to 2 μg/mL and 0.008 to 0.25 μg/mL, respectively. For amphotericin B, 3 *C. parapsilosis* isolates from 3 patients had an MIC of 2 μg/mL and the other 95 isolates (97%) were susceptible to amphotericin B at a concentration ≤1 μg/mL by macrodilution method

Table 1. Minimum inhibitory concentration (MIC) and MIC for 50% (MIC_{50}) and 90% (MIC_{90}) of clinical *Candida* isolates to the 4 antifungal agents by broth macrodilution method and E-test

Species (no. of strains)	Antifungal agent	MIC (μg/mL)		
		Range	MIC_{50}	MIC_{90}
C. albicans (50)	Amphotericin B			
	Macrodilution	<0.03~0.5	0.25	0.5
	E-test	<0.002~0.5	0.125	0.38
	Fluconazole			
	Macrodilution	0.25~>64	0.5	>64
	E-test	0.25~>256	0.75	256
	Flucytosine			
	Macrodilution	<0.03~0.25	0.06	0.25
	E-test	0.016~0.25	0.064	0.094
	Ketoconazole			
	Macrodilution	<0.03~>32	<0.03	4
	E-test	0.008~>32	0.023	4
C. parapsilosis (11)	Amphotericin B			
	Macrodilution	0.06~2	0.5	2
	E-test	0.032~2	0.5	2
	Fluconazole			
	Macrodilution	0.5~>64	32	>64
	E-test	0.5~>256	64	256
	Flucytosine			
	Macrodilution	<0.03~0.125	<0.03	0.125
	E-test	0.008~0.064	0.016	0.032
	Ketoconazole			
	Macrodilution	0.06~8	1	2
	E-test	0.032~8	0.75	1.5
C. tropicalis (24)	Amphotericin B			
	Macrodilution	<0.03~1	0.5	0.5
	E-test	<0.002~1	0.25	0.5
	Fluconazole			
	Macrodilution	0.5~>64	1	4
	E-test	0.25~>256	1	8
	Flucytosine			
	Macrodilution	<0.03~0.25	0.06	0.125
	E-test	0.008~0.125	0.032	0.094
	Ketoconazole			
	Macrodilution	<0.03~8	0.06	8
	E-test	0.008~16	0.064	8
C. glabrata (13)	Amphotericin B			
	Macrodilution	<0.03~0.5	0.25	0.5
	E-test	<0.002~0.75	0.25	0.38
	Fluconazole			
	Macrodilution	16~>64	32	32
	E-test	16~>256	32	64
	Flucytosine			
	Macrodilution	<0.03~0.125	<0.03	0.06
	E-test	0.008~0.064	0.016	0.047
	Ketoconazole			
	Macrodilution	0.5~16	0.5	1
	E-test	0.5~>32	0.75	2

Table 2. Essential agreement (EA) between National Committee for Clinical Laboratory Standards broth macrodilution method and E-test for *Candida* spp. tested

Organism (no. of strains)	No. of isolates							
Amphotericin B								
Log_2 (E-test/macrodilution)	<–2	–2	–1	0	1	2	>2	EA
C. albicans (50)	0	0	24	25	1	0	0	100.0%
C. parapsilosis (11)	0	1	2	7	1	0	0	90.9%
C. tropicalis (24)	0	0	4	18	2	0	0	100.0%
C. glabrata (13)	0	0	3	9	1	0	0	100.0%
Total	0	1	33	59	5	0	0	99.0%
Fluconazole								
Log_2 (E-test/macrodilution)	<–2	–2	–1	0	1	2	>2	EA
C. albicans (50)	0	0	3	33	10	4	0	92.0%
C. parapsilosis (11)	0	0	1	6	3	1	0	90.9%
C. tropicalis (24)	1	0	1	20	2	0	0	95.8%
C. glabrata (13)	0	1	0	7	5	0	0	92.3%
Total	1	1	5	66	20	5	0	92.9%
Flucytosine								
Log_2 (E-test/macrodilution)	<–2	–2	–1	0	1	2	>2	EA
C. albicans (50)	0	6	21	17	6	0	0	88.0%
C. parapsilosis (11)	0	1	2	8	0	0	0	90.9%
C. tropicalis (24)	0	1	9	12	2	0	0	95.8%
C. glabrata (13)	0	0	2	11	0	0	0	100.0%
Total	0	8	34	48	8	0	0	91.8%
Ketoconazole								
Log_2 (E-test/macrodilution)	<–2	–2	–1	0	1	2	>2	EA
C. albicans (50)	0	0	12	28	5	0	5	90.0%
C. parapsilosis (11)	0	0	2	8	0	1	0	90.9%
C. tropicalis (24)	0	0	7	11	5	1	0	95.8%
C. glabrata (13)	0	0	2	4	5	2	0	84.6%
Total	0	0	23	51	15	4	5	90.8%

as well as E-test method. All isolates were susceptible to flucytosine (at ≤0.25 μg/mL). The MIC distributions for fluconazole and ketoconazole were broader: 0.25 to >256 μg/mL and 0.008 to >32 μg/mL, respectively. Ten isolates of *C. albicans* (10/50, 20%), 5 (5/11, 45.5%) of *C. parapsilosis* and 2 (2/24, 8.3%) of *C. tropicalis* were resistant to fluconazole (MIC ≥64 μg/mL). *C. glabrata* isolates were more resistant to fluconazole with MIC values ranging from 16 to >64 μg/mL. Five *C. albicans* and 1 *C. glabrata* were inhibited by ketoconazole MIC values of ≥16 μg/mL.

The MIC_{90} and MIC_{50} data for the 4 drugs of each *Candida* sp. tested are shown in Table 1. For amphotericin B and flucytosine, the MIC_{90} and MIC_{50} were much lower than those of the other 2 antifungal agents. Differences of MIC_{90} and MIC_{50} determined by the 2 methods were seen in drug-species grouping; however, all of the differences were within 1 dilution if the MIC value was rounded up to the next 2-fold dilution in the E-test for comparison with broth macrodilution method. Overall, MIC_{90} for all isolates by both methods were 0.5 μg/mL for amphotericin B, 32 μg/mL for fluconazole, 0.25 μg/mL for flucytosine (0.125 μg/mL by E-test), and 4 μg/mL for ketoconazole. MIC_{50} values of all isolates for these 4 drugs were 0.25, 2, 0.06 and 0.06 μg/mL, respectively.

EA data for the methods are shown in Table 2. MIC values by E-test tended to be higher for fluconazole and ketoconazole. For all *Candida* spp., EA was 99.0% for amphotericin B, 92.9% for fluconazole, 91.8% for flucytosine, and 90.8% for ketoconazole. The lowest EA was found for *C. glabrata* and ketoconazole (84.6%).

Discussion

Since invasive infections caused by yeasts are associated with high morbidity and mortality and resistance to antifungal agents is increasing, it becomes very important for clinical laboratories to perform susceptibility testing of pathogenic yeasts to support appropriate therapy guidance. In our hospital, susceptibility testing of yeasts had not been performed.

In this study, broth macrodilution and E-test were used to evaluate the susceptibility of the important yeast pathogens isolated from bloodstream and to investigate the capability in performing antifungal susceptibility testing in a clinical routine environment.

Numerous studies have reported good agreement between E-test and the macrodilution method for antifungal susceptibility testing in general [22-25]. However, fewer publications have reported the use of E-test for determining the susceptibility patterns of yeasts isolated from bloodstream infections [26-29]. In this study, excellent agreement was also seen between E-test and broth macrodilution methods on the susceptibility testing of *Candida* blood isolates. Therefore, E-test was found to be a useful alternative MIC method for susceptibility testing of yeast pathogens even for clinical laboratories inexperienced in testing in this area. Furthermore, the endpoint trailing phenomenon for azoles was more often observed in the broth macrodilution method and was also more difficult to read than with E-test.

In the European SENTRY program reported in 1999 [26], resistance to fluconazole (MIC_{90} ≥64 μg/mL) was found among *C. glabrata* and *C. krusei* but was rare among other *Candida* species. In the United States, Pfaller et al showed that fluconazole susceptibility of all *Candida* species (*C. albicans* and other species, including *C. krusei* and *C. glabrata*) isolated from 1992 to 1998 remained stable (MIC_{90} 16 μg/mL) [27]. In Taiwan, Chen and colleagues reported a stable susceptibility of *Candida* blood isolates to fluconazole from 1994 (94.0%) to 2000 (97.9%), despite increased consumption of fluconazole [30]. However, our study showed a resistant rate of 28.6% to fluconazole in all *Candida* species. Most reports recommend that continued resistance surveillance in infections caused by *C. albicans* and other species of *Candida* among hospitalized patients [27-29]. It was also suggested that changing patterns of use of antifungal drugs and broad-spectrum antibiotics, and efforts to improve the rational use of antifungal agents at hospitals should contribute to a stability of antifungal susceptibility [30].

Due to a similar mode of action, cross resistance between the various azoles have been discussed previously [31]. In that case, the clinical isolate with resistance to ketoconazole failed to respond to itraconazole. Another study found that the MICs of itraconazole for fluconazole-resistant isolates were significantly higher than those for fluconazole-susceptible ones [32]. Our results also showed this phenomenon, in that 5 of 10 *C. albicans* (fluconazole MIC >32 μg/mL) had a ketoconazole MIC >8 μg/mL (data not shown). However, only 1 of 13 *C. glabrata* (fluconazole MIC ≥16 μg/mL) gave a higher MIC of ketoconazole (16 μg/mL). This converse observation was also noted in the study by Ruhnke et al [33]. Many cases of candidiasis caused by *C. albicans* that failed to respond to ketoconazole may respond to fluconazole, and vice versa. These results imply that the antifungal mechanisms of azoles might involve novel effects, as discussed in a recent study [34].

Consistent with other reports, our data showed a narrow MIC range for amphotericin B [35-37], making it difficult to set an interpretive MIC breakpoint to define susceptibility for clinical practice since clinical failures are sometimes seen. Despite more than 30 years of clinical use, resistance to amphotericin B is still rarely reported and resistant isolates are often confined to the less common species of *Candida*. Our data, however, only showed 3 *C. parapsilosis* inhibited by an elevated amphotericin B MIC value of 2 μg/mL. It appears prudent to improve the ability of antifungal testing methods to better detect and discriminate amphotericin B resistance since it is the agent of choice for all *Candida* isolates and even for filamentous fungi. A report has shown that E-test identified subpopulations of yeast isolates with high amphotericin B MIC values [38]. This implied that E-test was more powerful in detecting the subpopulation resistant to amphotericin B.

Flucytosine MIC values were low (<0.03 to 0.25 μg/mL) by both methods and all strains were found to be fully susceptible in our study. In other countries, flucytosine resistance has been reported in less than 10% of isolates in 1998 [28,29]. However, flucytosine is a drug with a limited spectrum of action that includes *Candida* spp. and *Cryptococcus neoformans* [39]. The value of monotherapy with flucytosine is limited because of the frequent development of resistance [40]. Recently, it has been combined with newer azole antifungal agents; the drug also plays an important role in a new approach to the treatment of cancer. The severe side effects of flucytosine include hepatotoxicity and bone marrow depression [41,42]. Flucytosine is mainly excreted by the kidneys, and thus potential interactions with nephrotoxic drugs should also be considered [41].

In conclusion, E-test retains the advantages of disk diffusion while providing quantitative MIC values by using a unique predefined gradient technology. The method requires 24 to 48 hours to obtain MIC results compared to 48 to 72 hours with the reference broth

macrodilution method. The precise, stable and continuous gradient in the system makes it possible to obtain reproducible, discrete quantitative MIC values [43]. Overall, E-test was simpler to use and read with the comprehensive and illustrative technical instructions provided by the manufacturer. In addition, improvements of susceptibility testing media and incubation conditions to provide clearer endpoints have been well studied in recent reports [44-46]. Although susceptibility testing of yeast is infrequently done at hospitals and isolates are often sent out to other reference laboratories, our study suggests that blood isolates and isolates from immunocompromised patients can be routinely and reliably tested using E-test.

Acknowledgments

This study was supported by grants TSGH-C89-39 from Tri-Service General Hospital and NSC88-2314-B-016-053 from the National Science Council, Taiwan, ROC. We acknowledge the invaluable assistance of Dr. Anne Bolmström with chart review.

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