

Antifungal Susceptibility testing: New trends

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Abstract:

Development of standardized antifungal susceptibility testing methods has been the focus of intensive research in the last 15 years. Antifungal sensitivity tests were done for selected (46) fungal isolates, (23 dermatophytes and 23 Candida) by E-test and broth microdilution method. E-test gave better sensitivity results on Candida species than dermatophytes for all drugs expect itraconazole. We compared E-test versus broth microdilution method for testing dermatophytes. The sensitive cases obtained by broth microdilution method were (13% for amphotericin B, 21% for fluconazole, 21% for ketoconazole and 69% for itraconazole) higher than those obtained by E-test (8% for amphotericin B, 8% for fluconazole, 4% for ketoconazole and 34% for itraconazole) for all tested drugs. The difference was significantly higher in broth microdilution method than E-test for itraconazole ($P=0.02$). On comparing both methods of antifungal susceptibility for Candida species, there was insignificant lower sensitivity to amphotericin B, fluconazole and itraconazole by E-test than broth microdilution ($p>0.05$) with similar sensitivity to ketoconazole.

In conclusion:

- Broth microdilution method is the preferred method for antifungal susceptibility of dermatophytes.

- E-test is simple method, easy to perform on both dermatophytes and Candida. However it is expensive and difficult in defining the precise borders of the inhibition zones particularly for fluconazole (fungistatic), and on dermatophytes (unequal distribution of inoculum on agar plates).

Introduction and Aim of Work:

Numerous factors have contributed to the increase in fungal infections - most notably, increasing number of immunosuppressed cases e.g AIDS, cancer or diabetes, the use of broad spectrum antibiotics, cytotoxic chemotherapy, and organ transplantation[1] .

The increasing incidence of opportunistic severe fungal infections has greatly enhanced the interest in novel methods for in vitro antifungal susceptibility testing, the standardized methodology recommended by the National Committee for Clinical Laboratory Standards (NCCLS) M27-A have been developed[2].

The establishment of a standardized broth reference method for antifungal susceptibility testing of yeasts has opened the door to a number of interesting and useful developments. Also, the availability of reference methods provides a useful touchstone for the development of commercial products that promise to be more user friendly and to further improvement of test standardization. Incorporation of antifungal susceptibility testing methods into the clinical trials of new antifungal agents will facilitate the establishment of clinical correlates and further enhance the clinical utility of antifungal susceptibility testing[3].

Aim:

To evaluate the commercially introduced E test in comparison to broth microdilution method for antifungal susceptibility testing and the possibility of application of this method for testing dermatophytes.

Subjects and methods:

The study was conducted on 46 fungal isolates: 23 (50%) dermatophytes, 23 (50%) *Candida* species, isolated from culture of different specimens collected from patients with superficial and systemic fungal infections. Cultures were performed on Sabouraud dextrose agar (SDA) with chloramphenicol and SDA with chloramphenicol and cyclohexamide. Isolated colonies were identified by colonial morphology and lactophenol cotton blue stain for dermatophytes. Candifast test (STAGO GROUP, International MICROBIO, France) was used for identification of *Candida* species.

The following antifungal susceptibility procedures were performed:

1) E-test by using the antifungal strips : amphotericin B , fluconazole, flucytosine, ketoconazole , itraconazole and voriconazole .

2) Broth microdilution method by using the following drugs : amphotericin B , fluconazole , ketoconazole and itraconazole .

The 23 isolates of *Candida* species identified by Candifast were: *C. albicans* (10 isolates), *C. guilliermondii* (5 isolates), *C. krusei* (5 isolates), *C. Pseudotropicalis* (2 isolates) and *C. Parapsilosis* (1 isolate).

The 23 dermatophytes species were: *T. mentagroyhytes* (6 isolates), *T. rubrum* (6 isolates), *M. Canis* (6 isolates) and *T. violaceum* (5 isolates).

Inoculum preparation for Candida :

Inocula were prepared from 1-3 days old cultures on SDA. Suspensions were adjusted by using 0.5 McFarland standard .

Inoculum preparation for dermatophyte[4]&[5]:

For each isolate , to induce conidium formation only , the surface of the colony was gently scrapped off and transferred to a sterile flask containing sterile 200 ml of Sabouraud's glucose broth with chloramphenicol and cycloheximide . For isolates that do not readily produce conidia (T. violaceum), a small portion of mycelial growth was gently scrapped with the loop bent , ground with sterile glass rod in the bottom of sterile flask then 50 ml of sterile broth were added . The flask was incubated for 72 hours at room temperature with continuous shaking . After that , 1 ml of the suspension was transferred to sterile tube , the larger hyphal fragments allowed to settle for 10 minutes , while the supernatant was standardized and adjusted to an optical density (OD) that ranged from 0.09-0.11 at wave length 530 nm (80-82% transmittance) [6].

Sabouraud's glucose broth with chloramphenicol and cycloheximide :

Peptone	10g	Glucose	19g	Cycloheximide	0.5g
Bio tryptase	3g	Sodium diphosphate	0.5g	Distilled water	1000ml
Malt extract	1g	Chloramphenicol	0.1g		

1) E-test:

* Medium preparation :

500 ml RPMI 1640 supplemented with L- glutamine (broth) , 65 gm/L Sabouraud Dextrose Agar , 500 ml Distilled water

For agar diffusion E-test , the suspension was applied to the agar surface with a cotton swab. The plates were allowed to dry for 15 min before the E-test strips were applied. The MICs endpoints were read after 48h &72h of incubation for Candida & dermatophytes respectively .

* Test reading:

The MIC was taken as the lowest drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the antifungal strip .

2) Broth microdilution method :

* Drug dilutions :

Antifungals used were dissolved in dimethyl sulfoxide (DMSO) 100% . The drug diluting medium was used to prepare stock solutions and two folds serial dilutions according to[4]&[7]. Stock solutions were stored at - 70 oC until used . Serial dilutions started with 32 ug/ml , 16 , 8 , 4 , 2 , 1 , 0.5 , 0.25 , 0.125 and 0.062 .

* Test procedures :

Sterile microdilution plates (96-u-shaped wells) were used. Rows 1-10 contained the series of drug dilutions in 100 ul volumes starting with the concentration of 32 ug/ml . 100 ul of inoculum suspension were added to each well . The eleventh well was the control well , 100 ul of inoculum suspension and 100 ul of drug free medium were added . The plates were covered , incubated at room temperature and examined after 48 , 72 hours incubation for Candida and dermatophytes respectively.

* Test reading :

The growth in each well was compared with that in the control well visually . The MIC was taken as the lowest drug concentration at which there was no visible turbidity in the wells .

Results:

We have tested each species of dermatophytes and those of Candida for each tested drug. The MICs for each one had been estimated. Collectively the mean MIC \pm SD of tested drugs on all dermatophytes and those on Candida have been calculated.

In (Table 1), among 23 isolates of tested dermatophytes by E-test, 8 (34.8%) isolates were sensitive to itraconazole, 2 (8.7%) isolates to voriconazole, 2 (8.7%) to fluconazole, 2 (8.7%) to amphotericin B, 1 (4.3%) to ketoconazole and all were resistant to flucytosine .

For Candida, 18 (78.3%) isolates were sensitive to amphotericin B , 17 (73.9%) isolates to flucytosine , 7 (30.4%) to ketoconazole , 5 (21.7%) isolates to each fluconazole , voriconazole and itraconazole. The antifungals (amphotericin B, flucytosine and ketoconazole) are more effective on Candida than dermatophytes ($P < 0.05$ for each).

The MICs of E-test for Candida were 0.4 ± 0.3 ug/ml , 0.3 ± 0.2 , 0.2 ± 0.1 , 0.4 ± 0.4 , 0.1 ± 0.2 and 0.02 ± 0.01 for amphotericin B , flucytosine , fluconazole, ketoconazole, voriconazole and itraconazole respectively. Itraconazole had significant lower MIC on Candida than dermatophytes ($P=0.002$) .

Broth microdilution method was performed on dermatophytes and Candida (Table 2) . For tested dermatophytes , 16 (69.6%) isolates were sensitive to itraconazole , 5 (21.7%) isolates to each of fluconazole and ketoconazole and 3 (13%) isolates to amphotericin B .

The MICs readings for dermatophytes were 10.6 ± 4.6 ug/ml for amphotericin B, 9.8 ± 6.3 ug/ml for fluconazole, 14.4 ± 3.5 ug/ml for ketoconazole and 9.8 ± 5.3 ug/ml for itraconazole.

The antifungal susceptibility of 23 Candida isolates was done by broth microdilution method. 19 (82.6%) isolates were sensitive to amphotericin B , 11 (47.8%) isolates to fluconazole, 7 (30.4%) isolates to ketoconazole and 6 (26.1%) isolates to itraconazole.

The MICs readings were 0.77 ± 0.9 ug/ml, 4.2 ± 5.1 ug/ml, 2.1 ± 2.7 ug/ml and 2.9 ± 3.9 ug/ml respectively. They were higher than those obtained by E-test method .

Table (3) shows the comparison of E-test and broth microdilution method for dermatophytes . There was a significant lower sensitivity to itraconazole (P= 0.02) in E-test than broth microdilution . Also there was insignificant lower sensitivity to amphotericin B , fluconazole and ketoconazole in E-test than broth microdilution method .

Table (4) shows the comparison of the E-test and broth microdilution for susceptibility testing of 23 isolates of Candida. There was insignificant lower sensitivity to amphotericin B, fluconazole and itraconazole in E-test than broth microdilution.

Drugs	Dermatophytes (n = 23)		Candida (n = 23)		P1 P*
	S	MIC	S	MIC	
	No(%)	Mean ± SD	No (%)	Mean ± SD	
	Amphotericin B	2 (8.7)	5.0 ± 4.2	18 (78.3)	
Flucytosine	0 (0)	0	17 (73.9)	0.3 ± 0.2	<0.001 --
Fluconazole	2 (8.7)	5.5 ± 3.5	5 (21.7)	0.2 ± 0.1	0.2 0.09
Ketoconazole	1 (4.3)	8	7 (30.4)	0.4 ± 0.4	0.02 0.2
Voriconazole	2 (8.7)	3.5 ± 3.5	5 (21.7)	0.1 ± 0.2	0.2 0.09
Itraconazole	8(34.8)	7.0 ± 4.5	5 (21.7)	0.02 ± 0.01	0.3 0.002

Table (1) E-test for dermatophytes and Candida

P : Can not be calculated

P1 : The sensitive number of dermatophytes versus those of Candida

P* : MICs of dermatophytes versus those of Candida

S : Sensitive cases

Drugs	Dermatophytes (n = 23)		Candida (n = 23)		P1	P*
	S	MIC	S	MIC		
	No (%)	Mean ± SD	No (%)	Mean ± SD		
Amphotericin B	3 (13)	10.66 ± 4.61	19 (82.6)	0.77 ± 0.9	< 0.001	0.005
Fluconazole	5 (21.7)	9.80 ± 6.34	11 (47.8)	4.20 ± 5.11	0.06	0.08
Ketoconazole	5 (21.7)	14.40 ± 3.58	7 (30.4)	2.14 ± 2.66	0.5	0.005
Itraconazole	16 (69.6)	9.87 ± 5.23	6 (26.1)	2.85 ± 3.98	0.003	0.009

Table (2) Broth microdilution method for dermatophytes and Candida

P1: The sensitive number of dermatophytes versus Candida

P* : MICs of dermatophytes versus Candida

S : Sensitive cases

Drugs	E – test		Broth Microdilution		P
	S	R	S	R	
	No (%)	No (%)	No (%)	No (%)	
Amphotericin B	2 (8.7)	21 (91.3)	3 (13)	20 (87)	--
Fluconazole	2 (8.7)	21 (91.3)	5 (21.7)	18 (78.3)	--
Ketoconazole	1 (4.3)	22 (95.7)	5 (21.7)	18 (78.3)	--
Itraconazole	8 (34.8)	15 (65.2)	16 (69.6)	7 (30.4)	0.02

Table (3) E-test versus Broth microdilution method for dermatophytes (n=23).

-- : Can not be calculated

P : E-test versus broth microdilution method for dermatophytes

Drugs	E - test				Broth microdilution				P
	S		R		S		R		
	No	%	No	%	No	%	No	%	
Amphotericin B	18	-78.3	5	-21.7	19	-82.6	4	-17.4	1
Flucytosine	17	-73.9	6	-26.1	*	*	*	*	--
Fluconazole	5	-21.7	18	-78.3	11	-47.8	12	-52.2	0.07
Ketoconazole	7	-30.4	16	-69.6	7	-30.4	16	-69.6	1
Itraconazole	5	-21.7	18	-78.3	6	-26.1	17	-73.9	1

Table (4) E-test versus Broth microdilution for Candida (n = 23)

P : Can not be calculated

P1 : E - test versus broth microdilution for Candida

Discussion

With the rising frequency of fungal infections, as well as increasing reports of resistance to antifungal agents, it is imperative that clinical applicable antifungal susceptibility testing be available. In 1997 the National Committee for Clinical Laboratory Standards published standard guidelines for antifungal susceptibility testing of Candida[8] .

The MIC data are also essential to obtain distribution profiles of MIC values for fungal populations and future correlations of MICs with clinical response[9].

In our study we used commercial E-test for testing dermatophytes and Candida (Table 1). It gave better sensitivity on tested Candida at lower MICs compared to dermatophytes for all drugs except itraconazole.

Pfaller et al. (2000)[10] stated that the testing of susceptibility to antifungal agents had been standardized for yeasts , and additional efforts to adapt NCCLS broth microdilution methodology to test for molds had been advised . And the adaptation of E-test for antifungal susceptibility testing of molds such as Aspergillus had been also described .

Chang et al. (2001)[11] found that the MICs determined by E-test for 138 Candida species by using inoculum concentration at 0.5 McFarland standard were 0.063 - 2 ug/ml for amphotericin B ,

0.016 - 64 ug/ml for flucytosine , 0.063 - 512 ug/ml for fluconazole , 0.016 - 64 ug/ml for ketoconazole and 0.016 - 64 ug/ml for itraconazole .

Performing broth microdilution method on dermatophytes and *Candida* (Table 2), revealed highly significant increased sensitivity of *Candida* to amphotericin B ($P < 0.001$) at lower MIC ($P^* = 0.005$). For itraconazole there was highly significant increased sensitive dermatophyte species than *Candida* ($P = 0.003$). However, the MICs for *Candida* species were lower than those of dermatophytes ($P^* = 0.009$).

Perea et al. (2001)[12] found that the MIC readings of 100 strains of dermatophytes by broth macrodilution method were ranged from 0.5 to 16 ug/ml for fluconazole , 0.25 to 16 ug/ml for ketoconazole and 0.015 to 8 ug/ml for itraconazole (mean 10.71 ug/ml, 1.21ug/ml and 0.09 ug/ml respectively). The mean MICs were lower than those of the present study for ketoconazole and itraconazole and this could be attributed to variations in technical factors such as inoculum size , type of medium , incubation temperature and time .

By comparing E-test method versus broth (Table 3) microdilution method of antifungal susceptibility testing for dermatophytes, the number of sensitive cases obtained by broth microdilution method were higher than those obtained by E- test for all tested drugs. The difference was significantly higher in broth microdilution method than E-test for itraconazole ($P = 0.02$).

This difference could be explained by the antifungal susceptibility testing by E-test for dermatophytes needs special precautions such as mixing by magnetic stirrer for long time to obtain microconidia - contained broth . The long incubation time and slow growth of conidia on solid RPMI agar with unequal distribution of inoculum on the plate could add to this difference . Pfaller et al. (2000) [10] reported slower growth of conidia on RPMI agar relative to improved growth on RPMI broth.

Espinell-Ingroff (2001)[13] evaluated the in vitro activities of itraconazole and amphotericin B by E-test and broth microdilution method against 186 isolates of molds, and reported higher agreement between the itraconazole MICs obtained by both methods .

Comparing E-test versus broth microdilution for *Candida* (Table 4), showed insignificant lower sensitivity to amphotericin B , fluconazole and itraconazole by E-test than broth microdilution ($P > 0.05$ for each) with similar sensitivity to ketoconazole. However, the MICs of broth microdilution method were higher than those of E-test. This could be attributed to good growth of conidia in broth relative to slower growth on agar media as mentioned by Pfaller et al. (2000)[10].

Van Eldere et al. (1996)[14] had performed the frequency distributions of MICs endpoints by E-test versus broth microdilution with amphotericin B and fluconazole on *Candida* species and reported lower MICs endpoints by E-test than that of broth microdilution method . These findings were correlated with that obtained in the current study as shown in tables (1,2,4).

In a complete study for yeasts on broth macrodilution and E-test methods by Vandebossche et al. (2002)[15], they stated that, E-test is an accurate alternative to NCCLS, but experience in determining MICs and careful attention to procedural details are critically important.

Based on the previous results we could conclude that the preferred method for antifungal susceptibility of dermatophytes is broth microdilution and this could be explained on the basis of :

it is simple, cheap, reliable method compared to E-test because of rapid growth of microconidia on fluid media than agar based media . It also generates quantitative MICs. More than one test can be done in the same plate at the same time to conserve time .

E-test is simple method, easy to perform, on both dermatophytes and *Candida* does not require instruments and quantitative MICs could be obtained which are lower than those obtained by broth microdilution. However, it is expensive and difficult in defining the precise borders of the inhibition zones particularly for fluconazole (fungistatic) and in dermatophytes (unequal distribution of inoculum on agar plates).

Recommendations:

Future efforts must be directed towards establishing interpretive break-points for licensed and newly introduced antifungals and correlating them to the clinical outcome .

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