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Original Article

Microbiology Section

Comparative Analysis of Disc Diffusion and E-test with Broth Micro-dilution for Susceptibility Testing of Clinical *Candida* Isolates Against Amphotericin B, Fluconazole, Voriconazole and Caspofungin

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ABSTRACT

Background: Antifungal susceptibility testing remains an area of intense interest because of the increasing number of clinical isolates resistant to antifungal therapy. Clinical and Laboratory Standards Institute has proposed reference broth micro dilution (BMD) method for susceptibility testing. The reference method is time-consuming and poorly suited for the routine clinical laboratory setting. Agar-based susceptibility testing methods, disk diffusion (DD) method and the E-test method can be an easier, reliable and less time consuming alternative for the BMD method.

Aim: To compare the results of Amphotericin B, fluconazole, voriconazole, and Caspofungin susceptibility testing by DD, and the E-test method with the CLSI reference method for clinical *Candida* isolates.

Materials and Methods: Broth Microdilution (BMD), E-test and Disk diffusion testing of the various clinical *Candida* isolates was performed in accordance with CLSI documents. The results obtained were analysed and compared.

Results: The categorical agreement for Amphotericin B, fluconazole, voriconazole, and Caspofungin susceptibility results by E-test and DD method was 65.2%, 67.4%; 100%, 82.6%; 100%, 100%; 100%, 97.8% respectively.

Conclusion: The agar-based E-test and disk diffusion methods are reliable alternatives to the BMD method for *Candida* isolates when test susceptible to fluconazole, voriconazole, and Caspofungin, however the susceptibility testing results must be interpreted with caution in case of Amphotericin B.

Keywords: Antifungal susceptibility testing, Antifungal therapy, CLSI reference method

INTRODUCTION

During the last few decades, *Candida* spp. have become prominent nosocomial pathogens [1-3]. Antifungal susceptibility testing remains an area of intense interest for this pathogen because of the increase in incidence of drug-resistant isolates [4]. An ideal method of susceptibility testing must be easy, reproducible, accurate and cost-effective [5]. The Clinical and Laboratory Standards Institute (CLSI) has developed a reference method for broth micro dilution (BMD) antifungal susceptibility testing of yeasts (M27-A3 document) [6]. Since this reference method is complex, time-consuming and costly, many clinical laboratories do not perform susceptibility testing routinely [7]. Agar-based susceptibility testing methods like the classical disk diffusion (DD) and the newer E-test (ET) methods have been a topic of interest for researchers. Introduction of these alternative tests have made susceptibility testing of yeast easier, reliable and less time consuming than the CLSI M27-A3 [8-14]. The aim of this study was to compare the results of amphotericin B, fluconazole, voriconazole, and caspofungin susceptibility testing by DD, and the E-test method with the CLSI reference method for clinical *Candida* isolates.

MATERIALS AND METHODS

Study design: The set of 46 isolates evaluated included *C. tropicalis* (25 isolates), *C. albicans* (9 isolates), *C. glabrata* (5 isolates), *C. kefyr* (3 isolates), *Candida lusitaniae* (2 isolates), and *C. guilliermondii* (2 isolates). Each isolate originated from a different patient and was received at Department of Microbiology, Gandhi Memorial and Associated Hospitals Lucknow, India. Isolates were maintained at -70°C until testing was performed.

Period of study: The study was carried out over a 12-month period, from August 2011 to July 2012.

Identification of organisms and antifungal susceptibility study: *Candida* isolated from clinical samples was identified to species level according to standard microbiological procedures [15]. Speciation of the isolates were carried out by combination of morphological and biological criteria like germ tube test, cornmeal agar with Tween 20, carbohydrate assimilation and fermentation tests [15,16]. Antifungal susceptibility testing was performed, by the Broth microdilution method, E-test and DD method. The MICs of four antifungal agents: amphotericin B (AMB), fluconazole (FLC), voriconazole (VCZ), and caspofungin (CAS) and categorical agreement were analysed.

Broth Microdilution (BMD) Voriconazole (Pfizer, USA), Amphotericin B (HiMedia, India), Fluconazole (FLC) and Caspofungin (Sigma, India) were obtained as powders. Stock solutions of VCZ and AMB were prepared in dimethyl sulfoxide (DMSO), FLC and CAS were prepared in distilled water, 100 times the highest concentrations tested and further diluted in RPMI-1640 medium, buffered to pH 7.0 with morpholinepropanesulfonic acid (MOPS). The stock solutions were stored at -70^{0}C until used. The fungal suspensions were diluted 1/50 in RPMI (corresponding to 0.4 x10^5–5x10^5/ml) and the diluted suspensions (100 µl) were added to the wells in duplicate. Drug-free growth control wells were included for each isolate E-tested. All broth microdilution plates were incubated at 35^{0}C for 48 hour.

Disk diffusion testing: Disk diffusion testing of amphotericin B, fluconazole, Voriconazole and Caspofungin were performed in accordance with CLSI document M44-A3. Agar plates (90mm in diameter) containing Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg of Methylene blue per ml (GMB) at a depth

of 4.0 mm were used. Inoculum was prepared by picking up five distinct colonies of approximately 1mm from 24 hr old growth on Sabouraud's dextrose agar. Colonies were suspended in 5ml of sterile 0.85% saline. The resulting suspension was vortexed and turbidity adjusted to yield 1 x 10^6 -5 x 10^6 cells/ml (0.5 McFarland standard).

The agar surface was inoculated by using a swab dipped in a cell suspension adjusted to the turbidity of a 0.5 McFarland standard. Amphotericin B (10µg), Fluconazole (25µg), voriconazole (1µg), and caspofungin (5µg), disks (prepared in-house) were placed onto the surfaces of the inoculated plates, and the plates were incubated in air at 35°C to 37°C and read at 18 to 24 hour. Zone diameter is measured to the nearest whole millimeter at the point at which there is prominent reduction of the growth and pinpoint micro colonies at the edge or large colonies within the zone if encountered were ignored [17].

E-test: The E-test gradient strips of amphotericin B, fluconazole, voriconazole and Caspofungin was obtained from Biomerieux. The concentration gradient for Fluconazole ranged from 256 to 0.016 µg/ml while for other drugs was 32 to 0.002 µg/ml. The E-test was performed by following the manufacturer's instructions. Inoculum preparation and media for testing were similar to disc diffusion method. E strips were applied and the plates were incubated at 35°C and read after 48 hour. The MIC was determined from the inhibition ellipse that intersected the scale on the strip. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were included as quality controls. The results were within the control ranges for amphotericin B, fluconazole, voriconazole, and Caspofungin.

RESULTS

The MICs of four antifungal agents: amphotericin B, fluconazole, voriconazole, and caspofungin were analysed. Breakpoints chosen for fluconazole (≤8 µg/ml, susceptible S; 16–32 µg/ml, susceptible-dose dependent SDD; ≥ 64 µg/ml, resistant R), voriconazole (≤1 µg/ml, S; 2 µg/ml, SDD; ≥ 4 µg/ml, R) were as per the Clinical and Laboratory Standards Institute (CLSI). Since no breakpoints have been published for amphotericin B, and caspofungin breakpoints chosen were amphotericin B(≤ 1 µg/ml, S; 2 µg/ml, I; ≥ 4 µg/ml, R), and caspofungin (≤1 µg/ml, S; 2 µg/ml, I; ≥ 4 µg/ml, R).

The zone of inhibition for four antifungal agents: amphotericin B, fluconazole, voriconazole, and caspofungin were analysed. Breakpoints chosen for fluconazole and voriconazole were as per CLSI [6]. The interpretive criteria for the fluconazole disk were: (S), zone diameters ≥ 19mm; SDD, 15 to 18mm; (R), zone diameters ≤ 14mm and for the voriconazole disk were: (S), zone diameters ≥ 17mm; SDD, 14 to 16mm; (R), zone diameters ≤ 13mm. Since no breakpoints have been published for amphotericin B, and caspofungin, breakpoints chosen were for amphotericin B disk were: (S), zone diameters ≥ 15mm; SDD, 14 to 10mm; (R), zone diameters ≤ 9mm and for caspofungin disk were: (S), zone diameters ≥ 16mm; SDD, 15 to 13mm; (R), zone diameters ≤ 12mm. Categorical agreement (CA) was assigned where the-tested methods classified the susceptibilities of the isolates within the same interpretive categories (S, SDD/I, or R) as the reference method.

In vitro susceptibility testing results of 46 *Candida* isolates against amphotericin B, fluconazole, voriconazole and caspofungin by DD method is shown in [Table/Fig-1]. The percentages of isolates in each category (S, SDD, and R) were 89%, 0%, and 11% and 100%, 0%, and 0% for fluconazole and voriconazole, respectively. For amphotericin B and caspofungin the percentages of isolates in each category (S, I, and R) were 57%, 39%, and 4% and 100%, 0%, and 0% respectively.

Invitro susceptibility testing results of 46 *Candida* isolates against amphotericin B, fluconazole, voriconazole and caspofungin by E-test method is shown in [Table/Fig-2]. The percentages of isolates in each category (S, SDD, and R) were 89%, 0%, and 11% and 100%, 0%, and 0% for fluconazole and voriconazole, respectively. For amphotericin B and caspofungin the percentages of isolates in each category (S, I, and R) were 24%, 74%, and 2% and 100%, 0%, and 0% respectively.

Invitro susceptibility testing results of 46 *Candida* isolates against amphotericin B, fluconazole, voriconazole and caspofungin by BMD method is shown in [Table/Fig-3]. The percentages of isolates in each category (S, SDD, and R) were 72%, 15%, and 13% and 100%, 0%, and 0% for fluconazole and voriconazole, respectively. For amphotericin B and caspofungin the percentages of isolates in each category (S, I, and R) were 28%, 72%, and 0% and 98%, 2%, and 0% respectively.

		AMB			FLC			VRC			CAS		
Isolate	No	S	I	R	S	SDD	R	S	SDD	R	S	I	R
C .tropicalis	25	14	11	0	25	0	0	25	0	0	25	0	0
C. albicans	9	7	2	0	9	0	0	9	0	0	9	0	0
C. glabrata	5	0	3	2	0	0	5	5	0	0	5	0	0
C. kefyr	3	3	0	0	3	0	0	3	0	0	3	0	0
C. lusitaniae	2	1	1	0	2	0	0	2	0	0	2	0	0
C. guilliermondii	2	1	1	0	2	0	0	2	0	0	2	0	0
Total (%)	46	26(57)	18(39)	2(4)	41(89)	0	5(11)	46(100)	0	0	46(100)	0	0

[Table/Fig-1]: Categorical results (%) of antifungal Susceptibility Testing of 46 *Candida* isolates by BMD Method
S; Susceptible, SDD; Susceptible Dose dependent I; intermediate, and R; resistant

		AMB			FLC			VRC			CAS		
Isolate	No	S	I	R	S	SDD	R	S	SDD	R	S	I	R
C .tropicalis	25	7	18	0	25	0	0	25	0	0	25	0	0
C. albicans	9	0	9	0	9	0	0	9	0	0	9	0	0
C. glabrata	5	0	4	1	0	0	5	5	0	0	5	0	0
C. kefyr	3	3	0	0	3	0	0	3	0	0	3	0	0
C. lusitaniae	2	1	1	0	2	0	0	2	0	0	2	0	0
C. guilliermondii	2	0	2	0	2	0	0	2	0	0	2	0	0
Total (%)	46	11(24)	34(74)	1(2)	41(89)	0	5(11)	46(100)	0	0	46(100)	0	0

[Table/Fig-2]: Categorical results (%) of antifungal Susceptibility Testing of 46 *Candida* isolates by E-test
S; Susceptible, SDD; Susceptible Dose dependent I; intermediate, and R; resistant

Isolate	No	AMB			FLC			VRC			CAS		
		S	I	R	S	SDD	R	S	SDD	R	S	I	R
C .tropicalis	25	9	16	0	20	4	1	25	0	0	25	0	0
C. albicans	9	3	6	0	9	0	0	9	0	0	9	0	0
C. glabrata	5	0	5	0	0	0	5	4	0	0	4	1	0
C. kefyr	3	0	3	0	1	2	0	3	0	0	3	0	0
C. lusitaniae	2	0	2	0	2	0	0	2	0	0	2	0	0
C. guilliermondii	2	1	1	0	1	1	0	2	0	0	2	0	0
Total (%)	46	13(28)	33(72)	0	33(72)	7(15)	6(13)	46(100)	0	0	45(98)	1(2)	0

[Table/Fig-3]: Categorical results (%) of antifungal Susceptibility Testing of 46 *Candida* isolates by DD Method
S; Susceptible, SDD; Susceptible Dose dependent I; intermediate, and R; resistant

Antifungal agents	Categorical results (n)			Categorical agreement (%)
	S	SSD/I	R	
AMB				
BMD	26	18	2	
E-test	11	34	1	65.2
DD	13	33	0	67.4
FLC				
BMD	41	0	5	
E-test	41	0	5	100
DD	33	7	6	82.6
VRC				
BMD	46	0	0	
E-test	46	0	0	100
DD	46	0	0	100
CAS				
BMD	46	0	0	
E-test	46	0	0	100
DD	45	1	0	97.8

[Table/Fig-4]: Qualitative categorical agreement of E-test, DD method with reference BMD assigned for 46 *Candida* isolates against 4 antifungal agents.
S; Susceptible, SDD; Susceptible Dose dependent I; intermediate, and R; resistant

[Table/Fig-4] Describes qualitative (S, SDD/I, R) categorical agreement (CA) of E-test, DD method with the established reference BMD method. A good agreement was found between the CLSI reference BMD method and agar based methods for voriconazole, and caspofungin susceptibility testing. A 100% CA result for voriconazole was seen for both E-test and DD when compared with the established BMD method. The CA result for caspofungin was estimated to be 100% and 97.8% respectively for E-test and DD method. A low level of agreement between the E-test and the BMD method for amphotericin B were 65.2% seen. A similar agreement level (67.4%) was also found between DD method and BMD method. The agreement between the results of BMD method and those of DD method was 82.6% only for fluconazole, although the level of agreement between E-test and the BMD method was 100%.

DISCUSSION

The comparison between E-test DD method and CLSI methodology has been studied by several authors. In this study comparison between methods for assessing the susceptibilities of *Candida* spp. against amphotericin B, fluconazole, voriconazole and caspofungin showed that categorical agreement was highest for voriconazole (100% by both E-test and DD) and caspofungin (100% by E-test and 97.8% by DD) as shown in [Table/Fig-4]. These results are in accordance to those obtained by Milici et al,. and Matar et al., [18,19]. Agreement was lowest for amphotericin B (65.2% by E-test and 67.4% by DD) according to [Table/Fig-4]. This result however differed from previous study result where the agreement percentages varied from 90% to 96% by E-test [20,21]. Around 33% of the isolates that tested susceptible by the reference BMD method were categorized wrongly as susceptible-dose dependent to amphotericin B by agar-based methods. The discrepancy might be due to lack of interpretive breakpoints for amphotericin B or interlaboratory differences due to the use of different media for E-test and DD methodology.

The categorical agreement for fluconazole by E-test was found to be 100% but remained 82.6% by DD method shown in [Table/Fig-4]. The present study supports the findings of previous comparisons between the E-test and broth micro dilution techniques for fluconazole [20,22,23].

CONCLUSION

Based on these results we can conclude that the agar-based E-test and DD methods are reliable alternatives to the CLSI M27-A3 reference BMD method for susceptibility testing of fluconazole, voriconazole and caspofungin. However, it cannot be considered, a substitute while testing of amphotericin for BMD method B, since a complete agreement between both methodologies has not been reached, as demonstrated by the present study. Thus susceptibility testing results must be interpreted with caution in case of amphotericin B.

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