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RESEARCH ARTICLE

Antifungal Susceptibility Pattern among *Candida species*: An Evaluation of Disc Diffusion and Micro Broth Dilution Method

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ABSTRACT

Objective: *Candida species* are emerging as a significant pathogen certain species of Candida like *Candida krusei* are inherently resistant to azoles. In vitro susceptibility testing is essential for guiding therapy. The present study aims to study the antifungal susceptibility pattern of Candida isolates by disc diffusion and micro broth dilution method and to evaluate the degree of agreement between both the techniques.

Methods: Candida isolated from specimens like Oropharyngeal swabs, blood, pus and wound swabs were included in the study. Speciation was done as per standard microbiological methods. Antifungal resistance was determined by disc diffusion method for fluconazole, Itraconazole, amphotericin B, nystatin and clotrimazole. Microbroth dilution method was performed for fluconazole, Itraconazole and amphotericin B. The degree of agreement between both the methods for the resistant isolates was analysed by deriving the kappa value.

Results: Out of the 156 Candida isolates obtained, *Candida albicans* was the most common species isolated. By disc diffusion method fluconazole and Itraconazole showed an overall resistance of 34 (21.7%) and 27 (17.3%), respectively. Using micro broth dilution method, Fluconazole and Itraconazole had a resistance percentage of 35 (22.4%) and 29 (18.5%), respectively. There was a good agreement between both the methods in detecting the percentage of resistant isolates for fluconazole and Itraconazole (κ=0.9).

Conclusion: It is essential to perform susceptibility testing for all the Candida isolates for providing crucial information about the resistance pattern and help in choosing the appropriate antifungal drug for therapy. Disc diffusion method which is easy to perform can be utilized for day to day practice. *J Microbiol Infect Dis 2018; 8(3):97-101*

Keywords: Broth micro dilution, Candida, Disc diffusion

INTRODUCTION

Candida is present as a normal commensal of the gastrointestinal tract. *Candida species* have become a significant nosocomial pathogen with raise in drug resistant isolates. Species level identification is essential as certain species like *Candida krusei* are inherently resistant to azole drugs [1].

Antifungal drug therapies are selected empirically, but in vitro susceptibility testing are essential for guiding or altering the therapy. Fluconazole is the common azole antifungal drug that is being prescribed to immune compromised individuals either as prophylaxis or for therapy [2].

Owing to the rise of drug resistant isolates the study of Antifungal drug resistance remains an area of significant importance [3]. The drug susceptibility testing methods must be precise, cost effective and easily reproducible. In the present study the degree of agreement between two antifungal susceptibility methods, disc diffusion method and Microbroth dilution method for the resistant isolates was analyzed.

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METHODS

This cross sectional study was done in the Department of Microbiology, Government Kilpauk medical college, Chennai, India, between June 2012 and December 2012. The study was approved by the institutional ethics committee. A total of 156 Candida spp isolated from various clinical specimens such as oropharyngeal swabs (HIV seropositive patients, diabetics, cancer patients), burns wound infection, diabetic foot ulcer, pus, blood and urine specimens were included in the study (Table 1). All other specimens where isolates other than Candida spp were obtained were excluded from the study. All the 156 isolates were subcultured onto Sabouraud dextrose agar and incubated at 37 ^{0}C for 24 to 48 hours and the growth of creamy white colonies were subjected to gram staining and further speciation was done as per standard Microbiological techniques (germ tube test, colour of the colonies in Chrom agar, morphology in corn meal agar, sugar assimilation test and sugar fermentation tests) [4]. Further antifungal susceptibility testing was performed by disc diffusion method and broth micro dilution method and the degree of agreement between both the methods for the resistant isolates were analyzed.

Antifungal susceptibility testing by disc diffusion method

Inoculum preparation: The colonies were mixed in 0.85% sterile normal saline (5 ml volume) and adjusted to a turbidity of 0.5 McFarland standards. Meuller Hinton agar with 2% glucose and 0.5 µg/ml of methylene blue was used.

Procedure: A sterile swab was used to inoculate the plate by making a lawn culture by rotating the plate 180 degree in three directions. The antifungal discs fluconazole 25 µg, Itraconazole 10 µg, amphotericin B 100 U, clotrimazole 10 µg and Nystatin10 µg were placed on the plate and incubated at 37 ^{0}C for 18-24 hours. The zone sizes were interpreted as per Clinical Laboratory Standards Institute (CLSI) guidelines [5].

Broth Micro dilution method

Medium used: RPMI (Rosewell park memorial institute medium) 1640 medium with glutamine, without bicarbonate in MOPS (3N-Morpholino propane sulphonic acid).

Inoculum preparation

The stock suspension was prepared by suspending individual colonies in 5 ml of sterile normal saline (0.5 McFarland Standard).The stock suspension was further diluted to 1:1000 in RPMI medium (contains 1×103 to 5×103 cells/ml).

Antifungal stock solution

Sterile distilled water was used to dissolve fluconazole powder and DMSO (dimethyl sulfoxide) was used for dissolving amphotericin B and Itraconazole. The antifungal stock suspension was prepared in the range as follows: fluconazole 64 µg/ml-0.125 µg /ml and amphotericin B and Itraconazole 16 µg-0.031 µg/ml.

Procedures

Disposable 96 well microtitre plates were used. Row 1 had the highest drug concentration and lowest concentration in row 10. Sterility control and growth controls were also included 100 µl of 2x concentration of inoculum was added to each well followed by 100 µl of 2x concentration of the drug were added to the corresponding wells (row 1 highest concentration to row 10 lowest concentration). The microtitre plates are incubated at 37 ^{0}C for 24 to 48 hours .The MIC (minimum inhibitory concentration) end point was observed and interpreted as per CLSI guidelines [6].

Candida albicans ATCC 90028 strains was used for quality control.

Statistical Analysis

The degree of agreement between both the methods for the resistant isolates was analyzed by deriving the kappa value.

RESULTS

A total of 156 Candida isolates were obtained from various specimens such as oral swabs (HIV positive patients and cancer patients), wound swabs from diabetic patients and burns patients, blood, us ,urine and perianal candidiasis (Table 1). *Candida albicans* 93 (59.6%) was the most common species isolated. Among non-albicans Candida, *Candida tropicalis* 30 (19.2%) was the commonly isolated species (Table 2).

By disc diffusion method fluconazole and Itraconazole showed an overall resistance of 34

(21.7%) and 27 (17.3%) respectively. Clotrimazole andnystatin showed a resistance of 12 (7.69%) and 9 (5.76%) respectively. Amphotericin B showed a lowest resistance of 2 (1.28%). Antifungal susceptibility of the individual species is depicted in (Table 3).

Table 1. Distribution of various clinical specimens.

Specimens	Number
Oropharyngeal swabs	
HIV seropositive patients	33
Diabetes mellitus patients	25
Others (carcinoma patients with oral lesions, oral ulcers in denture wearers)	18
Burns wound swab	28
Diabetic foot ulcer	18
Pus samples	17
Urine	8
Blood	6
Perianal samples	3
Total	156

By broth micro dilution method, fluconazole and Itraconazole showed a resistance percentage of 35 (22.4%) and 29(18.5%) respectively (Table 4). Non-albicans Candidas showed a higher resistance compared to *Candida albicans* by both disc diffusion and broth micro dilution method (Table 3 and 4).

Table 2. Species wise distribution of the *Candida* isolates.

Isolate	No. of isolates (%)
Candida albicans	93 (59.6)
Candida tropicalis	30 (19.2)
Candida krusei	11 (7.05)
Candida glabrata	10 (6.41)
Candida parapsilosis	9 (5.76)
Candida dubliniensis	3 (1.92)
Total	156 (100.0)

The strength of agreement between the disc diffusion method and broth microdilution methods was analyzed and the kappa value was derived. There was a good agreement between both the methods in detecting the percentage of resistant isolates (Table 5).

Table 3. Antifungal drug resistance determined by the disc diffusion method.

Antifungal drug	Fluconazole, n (%)	Itraconazole, n (%)	Nystatin, n (%)	Clotrimazole, n (%)	Amphotericin B, n (%)
Candida albicans, n=93	11 (11.8%)	10 (10.75%)	5 (5.37%)	7 (7.52%)	1 (1.07%)
Candida tropicalis, n=30	5 (16.66%)	7 (23.33%)	1 (3.33%)	4 (13.33%)	1 (3.33%)
Candida krusei, n=11	11 (100%)	3 (27.2%)	3 (27.2%)	1 (9.09%)	0
Candida glabrata, n=10	7 (70%)	7 (70%)	0	0	0
Candida parapsilosis, n=9	0	0	0	0	0
Candida dubliniensis, n=3	0	0	0	0	0
Non Candida albicans	23 (36.5%)	17 (26.9%)	9 (14.2%)	12 (19%)	1 (1.5%)
Total 156	34 (21.7%)	27 (17.3%)	9 (5.76%)	12 (7.69%)	2 (1.28%)

Table 4. Antifungal drug resistance pattern observed by Microbroth dilution method.

Antifungal drug	Fluconazole		Itraconazole		Amphotericin B
	n (%)	SDD	n (%)	SDD	
Candida albicans, n=93	10 (12.9)	2 (2.1)	11 (11.8)	4 (4.3)	1 (1.07)
Candida tropicalis, n=30	8 (26.6)	1 (3.33)	7 (23.3%)	1 (3.33)	0
Candida krusei, n=11	11 (100)	0	4 (36.3)	0	0
Candida glabrata, n=10	6 (60)	0	7 (70)	0	0
Candida parapsilosis, n=9	0	0	0	0	0
Candida dubliniensis, n=3	0	0	0	0	0
Non *Candida albicans*	24 (39.6)	0	18 (28.5)	0	0
Total 156	35 (22.4)	3 (1.9)	29 (18.5)	5 (3.2)	1 (1.07)

Table 5. Agreement between disc diffusion method and Microbroth dilution method in the identification of resistant isolates.

Strains No	Antifungal drug	Kappa value	Strength of agreement
1	Fluconazole	0.981	Very good
2	Itraconazole	0.936	Very good
3	Amphotericin B	0.316	Good

DISCUSSION

Because of the increasing incidence of Candida infection along with the emergence of drug resistant phenotypes, it is essential to provide the clinicians with the antifungal susceptibility pattern for better treatment outcomes. Various guidelines such as CLSI, European Committee on Antimicrobial Susceptibility Testing have proposed reference methods for performing and interpreting antifungal susceptibility tests.

Candida albicans was the predominant species isolated followed by *Candida tropicalis* similar to other studies in literature [7-9]. By disc diffusion method, fluconazole and Itraconazole showed a resistance of 21.7% and 17.3% respectively. Among the Candida isolates resistance percentage of 12 to 34% for fluconazole have been documented [3,10-12]. Clotrimazole showed a resistance percentage of 7.6%. *Candida albicans* showed a resistance of 11.8% for fluconazole similar to Jayalakshmi et al. [12]. *Candida krusei* and Candida glabrata showed a resistance of 100% and 70 % respectively. By broth micro dilution method, the overall fluconazole resistance was 22.4% similar to Adhikary et al. (25%) [13]. Itraconazole showed a resistance percentage of 18.5%. Rex et al has reported Itraconazole resistance as 25% [13]. Amphotericin B showed a resistance percentage of 1.2%. Khotari et al has reported a resistance of 8% for amphotericin B [14]. The advent of antifungal drug resistance and isolation of Non-albicans Candida with inherent drug resistance can be attributed to the indiscriminate usage of antifungal drugs, immunocompromised states like HIV and cancer chemotherapy requiring prophylactic and empiric antifungal therapy [15]. Shift in the spectrum of infection from *Candida albicans* to non-albicans Candidas can impose difficulties in controlling the infections.

In the present study there was a good agreement between the disc diffusion method and broth micro dilution method in identifying the drug resistant isolates for fluconazole and Itraconazole (kappa value 0.9) similar some previous studies [11,16,17].

Disc diffusion method is easy to perform and can be interpreted by 24 hours and can be used for diagnostic purposes on daily basis whereas broth micro dilution method is cumbrous as it requires more practical skills. Disc diffusion

method which is easy to perform can be utilized for day to day practice. It is essential to perform susceptibility testing for all the Candida isolates for providing crucial information about the resistance pattern and help in choosing the appropriate antifungal drug for therapy. Periodic antifungal resistance surveillance protocols must be formulated for studying the trend of antifungal resistance in a particular area. This also will guide in choosing the empiric/prophylactic drug before the antifungal resistance pattern is available.

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