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Original Article

COMPARISON OF TWO IN-VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING METHODS IN CANDIDA ISOLATED FROM CLINICAL SPECIMENS

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ABSTRACT

OBJECTIVE: This study aims to determine the antifungal susceptibility of clinically relevant Candida species isolated from clinical specimens against Amphotericin B, Ketoconazole, Fluconazole, and Clotrimazole by broth microdilution (BMD) as per CLSI guide line and also by MTT [3-(4, 5-dimethyle-2-thiazyl)-2,5-diphenyl-2H- tetrazolium bromide] method. It also aims to compare MTT with standard broth microdilution method. **METHODS:** This laboratory based prospective study was done in the department of Microbiology for 2 years. *Candida* strains were isolated from clinically relevant specimens and species identification was done by the standard techniques. All the isolates of *Candida* included in the study were subjected to MIC (Minimum Inhibitory Concentration) determination against Amphotericin-B, Clotrimazole, Fluconazole and Ketoconazole by BMD (M27-A2) according to the guidelines of the Clinical and laboratory Standard Institute (CLSI) and also by MTT method. **RESULTS:** Out of 150 various clinical samples processed 74 Candida species were isolated. By MTT method, 82.4% Candida isolates were susceptible to Amphotericin B, 83.7% to Ketoconazole and 75.6% to Clotrimazole and 86.4 % to Fluconazole, whereas by BMD method 82.4% were sensitive to Amphotericin B, 86.4% sensitive to Ketoconazole and 79.7% sensitive to Clotrimazole and 86.4% to Fluconazole. This showed that MICs by MTT method (read at 24 hours) generated comparable results with MICs by BMD method. **CONCLUSION:** The MTT method is superior to the broth microdilution method that it requires 24 hours to demonstrate good agreement with the results of the standard BMD method at 48 hours. It can be used as an alternative method to the CLSI method since it shows high level of agreement with the CLSI method.

Keywords: *Candida, Broth microdilution (BMD), MTT, Amphotericin B, Ketoconazole, Fluconazole, Clotrimazole*

INTRODUCTION:

Candida species are ubiquitous yeasts that cause disease ranging from superficial to disseminated mycosis and often fatal infection.[1,2] Non-albicans *Candida* (NCA) are of special concern, since some are highly virulent and are associated with treatment failure due to reduced susceptibility to antifungal agents.[3]

The combined effects of increasing numbers of fungal infections, growing number of patients at risk, the increased rate of fungal resistance, and the expanded antifungal armamentarium have led to an increased recognition of the need for standardized laboratory testing for antifungal drug susceptibility. [4]

The choice of appropriate antifungal treatment is important but limited to a few licensed agent, and testing for susceptibility to these agents has only recently been standardized for yeast.[5] Many techniques have been used to

determine antifungal susceptibility, including measurement of germ tubes, uptake of metabolites, flow cytometry, agar based methods and broth dilution. Most are considered impractical for large-scale use. [4]

The methods of antifungal susceptibility testing currently recommended by the NCCLS have major short comings. The standard method a broth macrodilution technique, is time consuming and labour intensive and requires 48 hours for determination of MICs for *Candida;* furthermore the method is subjected to variable interpretation of fungal growth. The microdilution technique proposed as an alternative method is easier to perform, but it does not have shortened turn-around time to eliminate trailing end points.[6]

One assay is by using the dye 3-(4, 5-dimethyle-2-thiazyl)-2,5-diphenyl-2H- tetrazolium bromide (MTT). This yellow tetrazolium salt is cleaved by dehydrogenase inside mitochondria or in other cellular locations possessing dehydrogenase activity to form its purple formazen derivative which can be measured spectrophotometrically 550nm. MTT is cleaved by all living ,metabolically active fungi independent of proliferation and irrespective of unicellular or multicellular growth and thus is a measure of metabolic activity.[7] This MTT assay might meet the need for a rapid, reliable, and easy to perform method of antifungal susceptibility testing that is independent of individual reader bias.[6]
With this background in view, it is planned to undertake the study to compare MTT with standard reference method.

METHODS:
This laboratory based prospective study was done in the department of Microbiology for 2 years. The study proposal received clearance from Institutional Research Ethics Committee. All the clinically relevant samples submitted to the laboratory for fungal culture & sensitivity was included in the study. The samples were inoculated onto Sabouraud's Dextrose agar (SDA), SDA with chloramphenicol, Sabouraud's dextrose broth and incubated at 37^0 C and 25^0 C. *Candida* strains were isolated from clinically relevant specimens and species identification was done by the standard techniques.[8] All the isolates of *Candida* included in the study were subjected to MIC (Minimum Inhibitory Concentration) determination against Amphotericin-B, Clotrimazole, Fluconazole and Ketoconazole by broth microdilution method (M27-A2) according to the guidelines of the Clinical and laboratory Standard Institute (CLSI) using synthetic medium RPMI 1640 with MOPS in sterile disposal 96-well (U-shaped) microdilution plates. The MIC is the lowest concentration of an antifungal drug that substantially inhibits the growth of the organism as detected visually. For conventional microdilution procedure, the amount of growth in each tube was compared with that of growth control (drug free medium). The MIC for Amphotericin B was defined as the lowest concentration in which a score of 0 (optically clear) was observed. For the azoles, the MIC was defined as the lowest concentration in which a score of 2 (prominent decrease in turbidity) was observed. The MIC values for fluconazole were interpreted to the CLSI interpretive guidelines on antifungal susceptibility testing.[9] The MICs of > 64 ug/ml , 16-32 ug/ml and < 8 ug/ml were considered as resistant, Susceptible Dose Dependent(S-DD) and susceptible respectively. The results of Ketoconazole and Amphotericin B were interpreted as per Chakraborty et al.[10,11] For Ketoconazole, MICs of <0.125 ug/ml, 0.25 - 0.5µg/ml > 1 were considered as susceptible, susceptible dose dependent and resistant respectively. For Amphotericin B, the MICs of <1µg/ml was considered as susceptible, 1-4µg/ml as intermediate and >4µg/ml as resistant. At present there are no established CLSI interpretive breakpoints criteria by which to designate a *Candida* isolate as either susceptible or resistant to Clotrimazole. As per Vazquez JA et al, MIC break points for Clotrimazole were ≤ 0.125 µg/ml (Susceptible), 0.25-0.5µg/ml (Intermediate) and ≥1.0µg/ml (Resistant). [12]

After 24 hrs of incubation, the set of plates which were used for visually determining the MICs, the same plates were used for MTT testing. 25 ul of RPMI 1640 medium containing 5 mg of MTT per ml was added to each well containing an inoculum and incubation was continued at 35°C for 3 h. A stock solution of acid-isopropanol (5 ml of 1 N HCl in 95 ml of isopropanol) was simultaneously warmed for 3 hours at 35°C. After 3 hours, the plates were centrifuged at 1,000 x g for 10 min and the supernatants were aspirated. 100 ul of warm acid-isopropanol was added to each well containing an inoculum. After 30 mins of re-incubation of the microtiter plate at room temperature and gentle agitation, the optical density (OD) was measured. The optical density (OD) was determined with a microplate spectrophotometer (Multiskan MCC; Titertek,.) at 500 nm. [6] The OD of the blank, which consisted of the uninoculated well, was subtracted from the ODs of the inoculated plate. The percentage of MTT conversion to its formazen derivatives for each well was calculated by comparing the OD at 500 nm (OD500) of the wells with that of drug free control based on the following equation:
A_{500} of wells that contained the drug / A_{500} of the drug free well X 100% [6, 7]

MIC was defined as the highest concentration of drug that was associated with the first precipitous drop in OD (Optical density). The ODs were compared with that of the drug-free well respectively and accordingly —S , —S-DD , —R were noted.as per MICs criteria mentioned in broth microdilution method.[7]

RESULTS
The present study was conducted in the department of Microbiology. 150 clinical samples received in the laboratory during the present study period were processed with all Biosafety measures for fungus culture and anti-fungal susceptibility testing. A total of 74 Candida species were isolated. The *Candida* isolates were identified by routine methods and the antifungal susceptibility was performed by BMD and MTT. Out of 74 Candida isolates, maximum

number of 36 (25.67%) were isolated from urine followed by 25 (22.97%) isolates from blood, 15 (10.01%) from cervical swab, 16 (9.45%) from sputum, 15 (5.4%) from high vaginal swab, 7(2.7%) from CSF and 8 (2.7%) from pus (Table 1). Among the various Candida species isolated, 47 (63.51%) were *Candida albicans, 20* (27.02%) C. parapsilosis, 5 (6.75%) *C. tropicalis,* 1 (1.35%) *C. krusei* and 1 (1.35%) *C. guilliermondii* (1%) (Table 2, Table 3). It was noted that, among the 74 patients from whom Candida species were isolated, the most common predisposing factor was antibiotic intake in 64 (86.4%) patients followed by intravenous channels in 61 (82.35%) patients (Table 4). The maximum of 24 (32.43%) *Candida* were isolated from the age group >12 to 30 years followed by 21 (28.37%) in 0 to 1 years age group (Diagram 1). It was seen that 41(55.40%) of *Candida* was isolated from females and 33 (44.59%) from males (Table 5). Antifungal susceptibility testing of Candida isolates by BMD against Fluconazole, Ketoconazole, Amphotericin B and Clotrimazole, showed that maximum number of 4 (5.4%) isolates were resistant against Clotrimazole, followed by 2 isolates (2.7%) resistant against each of Fluconazole and Amphotericin B and 1 (1.5%) Ketoconazole (1.35%) (Table 6) . It was seen that out of 47 C. albicans isolated, 2 (4.2%) were resistant against Clotrimazole and 1 (2.1%) each against Amphotericin B, Ketoconazole and Fluconazole. Amongst 20 C. parapsilosis isolated, 2 (10%) were resistant to Clotrimazole and 1 (5%) to Amphotericin B. C. tropicalis and C. guilliermondii showed no resistance to all four drugs. The only C. krusei isolated was resistant to Fluconazole (100%) (Table 7). Antifungal susceptibility testing of Candida isolates by MTT showed that out of 74 Candida species isolated, 6 (8.1%) were resistant against Clotrimazole, 5 (6.7%) against Fluconazole and 3 (4.05%) each against Amphotericin B and Ketoconazole. By MTT, out of 47 C.albicans 3 (6.3%) were resistant to Clotrimazole and 2 (4.2%) each to Amphotericin, Ketoconazole and Fluconazole (Table 8). Amongst 20 C. parapsilosis isolated, 3 (15%) were resistant against Clotrimazole. It was noted that, out of 5 C. tropicalis isolated, 1 (20%) was resistant to Fluconazole. The only C. krusei isolated was resistant to Fluconazole (100%). C. guilliermondii showed no resistance to all four drugs (Table 9).

It was seen that 61 (82.4%) Candida isolates were sensitive to Amphotericin B and Clotrimazole by both the BMD & MTT methods. 64 (86.4%) isolates were susceptible to Ketoconazole by BMD whereas 62 (83.78%) isolates were susceptible to Ketoconazole by MTT method. 64 (86.4%) Candida isolates were susceptible to Candida isolates by both BMD and MTT method. S-DD by MTT method against Amphotericin B, Ketoconazole, Clotrimazole and Fluconazole were shown by 10 (13.5%), 9 (12.1%), 12 (16.2%) and 5 (6.7%) of Candida isolates respectively. S-DD by BMD method against Amphotericin B, Keteconazole, Clotrimazole and Fluconazole were noted against 11 (14.8%), 9 (12.1%), 11(14.8%) and 8 (10.8%) Candida isolates respectively. Resistant to Amphotericin B, Ketoconazole, Clotrimazole and Fluconazole by BMD were shown by 2(2.7%),1 (1.35%), 4(5.4%) and 2 (2.7%) Candida isolates respectively. By MTT method, resistant to Amphotericin B, Ketoconazole, Clotrimazole and Fluconazole were seen against 3 (4.05%), 3(4.05%) , 6(8.1%) and 5(6.7%) Candida isolated respectively (Table 10).

Table 1-*Candida* isolated from various clinical specimen

Specimens	Number of specimen received	*Candida* isolated	Percentage (%)
Aural swab	5	1	1.35%
Ascitic fluid	6	1	1.35%
Blood	25	17	22.97%
BAL	4	1	1.35%
CSF	7	2	2.70%
Cervical swab	15	8	10.01%
Gastric aspirate	7	4	5.40%
Oral swab	4	1	1.35%
Pus	8	2	2.70%
Sputum	16	7	9.45%
Tracheal swab	11	4	5.40%
Throat swab	6	1	1.35%
Urine	36	19	25.67%
High vaginal swab	15	4	5.40%
Wound swab	8	2	2.70%
Total	150	74	

Table-2- Various *Candida* species isolated in study

***Candida* species**	**Number (%)**
Candida albicans	47(63.51%)
Candida parapsilosis	20(27.02%)
Candida tropicalis	5 (6.75%)
Candida krusei	1(1.35%)
Candida guilliermondii	1(1.35%)
Total	**74(100%)**

Table 3- Distribution of various *Candida* species in clinical specimens

S.No	**Samples**	***C. albicans***	***C. parapsiosis***	***C. tropicalis***	***C. krusei***	***C. guilliermondii***	**Total**
1	Aural swab	1	0	0	0	0	01(1.35%)
2	Ascitic fluid	1	0	0	0	0	01(1.35%)
3	Blood	11	4	1	0	1	17(22.97%)
4	BAL	1	0	0	0	0	1(1.35%)
5	CSF	2	0	0	0	0	2(2.70%)
6	Cervical swab	2	5	1	0	0	8(10.01%)
7	Gastric aspirate	4	0	0	0	0	4(5.40%)
8	Oral swab	1	0	0	0	0	1(1.35%)
9	Pus	2	0	0	0	0	2(2.70%)
10	Sputum	5	1	0	1	0	7(9.45%)
11	Tracheal swab	4	0	0	0	0	4(5.40%)
12	Throat swab	1	0	0	0	0	1(1.35%)
13	Urine	10	8	1	0	0	19(25.67%)
14	High vaginal swab	2	1	1	0	0	4(5.40%)
15	Wound swab	0	1	1	0	0	2(2.70%)
	Total	**47(63.51%)**	**20(27.02%)**	**5(6.75%)**	**1(1.35%)**	**1(1.35%)**	**74**

Table 4-Various predisposing factors in study population

S.No	Predisposing factors	Number of patients	% of patients
1	Known HIV infection	6	8.1%
2	Antibiotics	64	86.4%
3	Antifungal	2	2.7%
4	Antimalignancy drugs	5	6.75%
5	Steroid therapy	12	16.2%
6	Radiotherapy	5	6.75%
7	Pregnancy	19	25.65%
8	Diabetes mellitus	15	20.25%
9	Malignancy	5	6.75%
10	Preterm baby	16	21.6%
11	Low birth weight	21	28.35%
12	Pulmonary Tuberculosis	10	13.5%
13	Ventilator	16	21.6%
14	Intravenous channels	61	82.35%
15	Catheters	19	25.65%

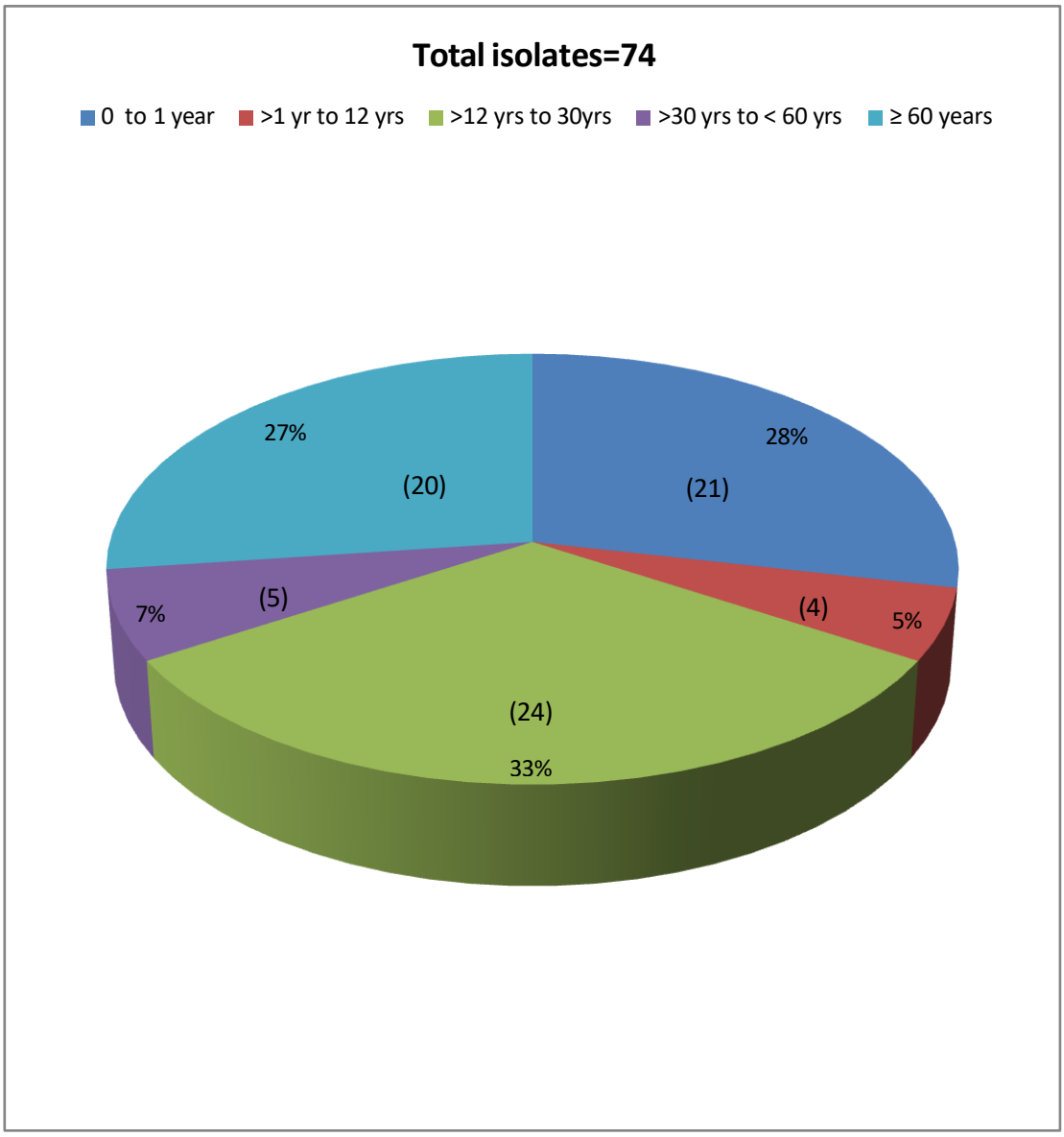

Diagram 1- Pie chart shows the number and percentages of the different Candida species isolated from the clinical specimens in the different age groups. Number in brackets shows the number of Candida isolates in each age group.

Table -5-Sex wise distribution of *Candida* isolates.

***Candida* isolates**	**Male**	**Female**
74	33(44.59%)	41(55.40%)

Table 6- Results of Antifungal susceptibility testing of *Candida* isolates by Broth microdilution method (n=74)

Drugs	**Results**		
	S	**S-DD**	**R**
Amphotericin B	61(82.4%)	11(14.8%)	2(2.7%)
Ketoconazole	64(86.4%)	9(12.16%)	1(1.35%)
Clotrimazole	59(79.7%)	11(14.8%)	4(5.4%)
Fluconazole	64(86.4%)	8(10.8%)	2(2.7%)

Table 7- Results of antifungal susceptibility of various *Candida* species by BMD method

Candida Species	Total No	Antifungals drugs	Results		
			S	S-DD	R
Candida albicans		Amphotericin B	40(85.1%)	6(12.7%)	1(2.1%)
	47	Ketoconazole	44(93.6%)	2(4.2%)	1(2.1%)
		Clotimazole	39(82.9%)	6(12.7%)	2(4.2%)
		Fluconazole	43(91.4%)	3(6.3%)	1(2.1%)
Candida parapsilosis		Amphotericin B	16(80%)	3(15%)	1(5%)
		Ketoconazole	14(70%)	6(30%)	0(0%)
	20	Clotrimazole	15(75%)	3(15%)	2(10%)
		Fluconazole	16(80%)	4(20%)	0(0%)
Candida tropicalis		Amphotericin B	3(60%)	2(40%)	0(0%)
		Ketoconazole	4(80%)	1(20%)	0(0%)
	5	Clotrimazole	4(80%)	1(20%)	0(0%)
		Fluconazole	4(80%)	1(20%)	0(0%)
Candida krusei		Amphotericin B	1(100%)	0(0%)	0(0%)
		Ketoconazole	1(100%)	0(0%)	0(0%)
	1	Clotrimazole	0(0%)	1(100%)	0(0%)
		Fluconazole	0(0%)	0(0%)	1(100%)
Candida guilliermondii		Amphotericin B	1(100%)	0(0%)	0(0%)
		Ketoconazole	1(100%)	0(0%)	0(0%)
	1	Clotrimazole	1(100%)	0(0%)	0(0%)
		Fluconazole	1(100%)	0(0%)	0(0%)

Table 8- Results of Antifungal susceptibility testing of *Candida* isolates by MTT method (n=74)

Drugs	Results		
	S	S-DD	R
Amphotericin B	61(82.4%)	10(13.5%)	3(4.05%)
Ketoconazole	62(83.78%)	9(12.1%)	3(4.05%)
Clotrimazole	56(75.6%)	12(16.2%)	6(8.1%)
Fluconazole	64(86.4%)	5(6.7%)	5(6.7%)

Table 9- Results of antifungal susceptibility of various candida species by MTT method

Candida species	Total No.	Antifungals drugs	Results		
			S	S-DD	R
Candida albicans		Amphotericin B	40(85.1%)	5(10.6%)	2(4.2%)
	47	Ketoconazole	43(91.4%)	2(4.2%)	2(4.2%)
		Clotimazole	38(80.8%)	6(12.7%)	3(6.3%)
		Fluconazole	43(91.4%)	2(4.2%)	2(4.2%)
Candida parapsilosis		Amphotericin B	15(75%)	4(20%)	1(5%)
		Ketoconazole	14(70%)	5(25%)	1(5%)
	20	Clotrimazole	14(70%)	3(15%0	3(15%)
		Fluconazole	16(80%)	3(15%)	1(5%%)
Candida tropicalis		Amphotericin B	4(80%)	1(20%)	0(0%)
		Ketoconazole	3(60%)	2(40%)	0(0%)
	5	Clotrimazole	3(60%)	2(40%)	0(0%)
		Fluconazole	4(80%)	0(0%)	1(20%)
Candida krusei		Amphotericin B	1(100%)	0(0%)	0(0%)
	1	Ketoconazole	1(100%)	0(0%)	0(0%)
		Clotrimazole	0(0%)	1(100%)	0(0%)
		Fluconazole	0(0%)	0(0%)	1(100%)
Candida guilliermondii		Amphotericin B	1(100%)	0(0%)	0(0%)
		Ketoconazole	1(100%)	0(0%)	0(0%)
	1	Clotrimazole	1(100%)	0(0%)	0(0%)
		Fluconazole	1(100%)	0(0%)	0(0%)

Table 10- Comparison of MTT with BMD

Method	Amphotericin B			Ketoconazole			Clotrimazole			Fluconazole		
	S	S-DD	R	S	S-DD	R	S	S-DD	R	S	S-DD	R
MTT	61 (82.4%)	10 (13.5%)	3 (4.05%)	62 (83.78%)	9 (12.1%)	3 (4.05%)	56 (75.6%)	12 (16.2%)	6 (8.1%)	64 (86.4%)	5 (6.7%)	5 (6.7%)
BMD	61 (82.4%)	11 (14.8%)	2 (2.7%)	64 (86.4%)	9 (12.1%)	1 (1.35%)	59 (79.7%)	11 (14.8%)	4 (5.4%)	64 (86.4%)	8 (10.8%)	2 (2.7%)

DISCUSSION

Candida is an asexual, diploid, dimorphic fungus that is present on humans and in their environment**.** These organisms are capable of causing a variety of superficial and deep- seated mycoses such as cutaneous, mucocutaneous, subcutaneous, or systemic candidiasis. The incidence of life threatening fungal infections is increasing with the number of patients suffering from severe neutropenia and general immunosuppression or immunodeficiency or because of the increasing use of cytotoxic immunosuppressive drugs and newer antibacterial agents.[13,14]

Predicting antifungal drug resistant of an organism to available drugs is the goal of antifungal susceptibility testing, which in turn, might aid in timely and successful intervention in these life threatening infections.[15] The antifungal susceptibility testing is indicated where therapy given to a particular patient is not responding in fungal disease.[16] A simple, rapid susceptibility test is needed to find out possible resistance of yeast to commonly used antifungal agents.[16] The demand for a simple, cost effective and reliable method for susceptibility testing of fungi is also growing.[17]

In present study, a total 74 *Candida* isolates were isolated during the study period from 150 various clinical samples like urine, high vaginal swab (HVS), wound swab ,throat swab, sputum, pus, oral swabs, gastrointestinal aspirates, blood, cerebro-spinal fluid (CSF), cervical swab, ascitic fluid, bronchoalveolar lavage (BAL), aural swabs. The commonest source of isolation of *Candida* species was urine (25.67%), followed by blood (22.97%) and cervical swab (10.01%). In a study conducted by Saldanha Dominic R.M.et al, high vaginal swab showed the highest number of isolates (38%) followed by blood (16%) and urine (12%).[18] Rizvi MW, Malik A et a in the year 2011 got the maximum number of *Candia albicans* from urine (30.3%),and cervical swabs 25% which is comparable with our study where we got maximum (25.67%) *Candida* isolates from urine. Comparative study of different species isolated in their studies by different workers showed that *Candida albicans* isolation was the highest in each of them except in Chakraborti et al which showed *C. tropicalis* was the highest (42%) and *Candida albicans* was 25% in his study. [19-24] In present study, the most frequent isolated species was *C. albicans* accounting for 63.51%. Speciation of *Candida* is important to provide a database for given area of study. The choice of antifungal is also dependent on the species of *Candida.* The azoles being effective against *C. albicans* and *C. tropicalis* are found to be ineffective against *C. krusei* and *C. glabrata.* Though Candidiasis can occur at all ages, studies by Dalal PJ and Kelkar SS et al at Mumbai showed the highest incidence of Candidiasis to be in the age group of 21 to 40 years. [25] This finding were comparable with those of our study, where maximum *Candida* isolates were found in age group of >12 years up to 30 years. In our study the maximum *Candida* species were obtained from females 41 (55.45%) than males. In a similar study conducted by K. C. Khandhan et al, the incidence among females was 61.2% and males it was only 38.8%. This could be due to higher number of samples which were conducted from female patient. [26]

We studied the distribution of *Candida* isolates with respect to predisposing factors and found that the highest number of *Candida* isolates were associated with multiple drugs intake mainly antibiotics and steroids. According to Rippon, there is some effect of the antibiotics on the host tissue, which predisposes it to invasion by organism.[27] The most important effect of antibiotics is the elimination and alternation of bacterial flora that holds the population of *Candida* in check. The other significant risk factors that were found in our study were presence of urinary and indwelling catheters (25.67%), pulmonary tuberculosis (13.5%), diabetes 20.25%, malignancy 6.75% and HIV (8.1%). Study by Annie S. Kao of USA in their study in June 1999 showed that the predisposing factors for Candidemia were malignancy (26%), abdominal surgery (14%), diabetes mellitus (13%) and HIV 10%.which they found in a total number of 837 incidence cases of Candidemia which is comparable with our study except that the percentage of diabetes in our study is higher than their study and percentage of malignancy is less in our study in comparison to them which may be due to their large sample size and geographical location.[28]

Many techniques have been described for determining antifungal susceptibility testing. NCCLS/CLSI has also recommended standard macrobroth dilution procedure to determine MICs of yeast.[29] However broth dilution procedure is quite cumbersome to use in routine clinical microbiology laboratory.[30] In our study by reading of MICs by using MTT dye and measuring the Optical density (OD) by spectrophotometer was compared with MICs by BMD method with the same isolates and QC strains and it was found that MTT method is comparable with BMD method.[6] In our study we found that by MTT method 82.4% *Candida* was susceptible to Amphotericin B, 83.7% to Ketoconazole and 75.6% to Clotrimazole and 86.4 % to Fluconazole, whereas by BMD method 82.4% was sensitive to Amphotericin B, 86.4% sensitive to Ketoconazole and 79.7% sensitive to Clotrimazole and 86.4% to Fluconazole which shows that MICs by MTT method (read at 24 hours) generated comparable results with MICs by BMD method. By MTT method 3 (4.05%) isolates showed resistance to Amphotericin B. By BMD 2.7% showed resistance to Amphotericin B. In case of Ketoconazole 1 (1.35%) showed resistance by BMD method but 3 (4.05%) showed resistant by MTT method. In case of Clotrimazole 4 (5.4%) showed resistance to Clotrimazole by BMD and 6 (8.1%) showed resistance by MTT method. For Fluconazole, 2 (2.27%) showed resistance by BMD method and 5 (6.7%) by MTT method. These variations may be due to subjective variation while reading BMD MICs visually by reading mirror.

Study by Cornelius J Clancy et al showed that for *Candida* both MTT method of getting MICs results and BMD method demonstrated excellent results and the MTT method generates MICs at 24 hours were comparable to those generated by the standard BMD method.[6] Their study also showed that *Candida tropicalis* isolated which was susceptible to Fluconazole by MTT method was not susceptible by BMD method and demonstrated that MTT method was superior to BMD method because it requires 24 hr to demonstrate results which were in good agreement with BMD method. They also demonstrated the level of comparison was superior in case of MTT method than BMD method. In another study by Bernhard Jahn et al found agreeable and comparable results with BMD method. [13] They did MICs determination by using MTT dye with Amphotericin B and Itraconazole.

The spectrophotometric determination of MICs is much more objective than the visual assessment required for BMD method. This is a particular advantage of the MTT assay given the well described phenomenon of Trailing agents. The advantage of the MTT assays method of determining MICs include easily interpretable end point determination, and the potential adaptability to use an automated system. The results of our study suggest that MTT assay method should be included in future investigations of alternatives to current BMD method. The MTT method relies on metabolic activity of the fungus. Any factor which influences the metabolic rate might have an effect on reduction of MTT even if the biomass remains the same, and also that several problems like trailing effect caused by partial inhibition of fungal growth and subjectivity of visual reading, time of incubation (48 hrs in BMD) may be the various factor of difference in our study with the BMD.[7] We have only studied the MICs of *Candida* by MTT method in a simplified method and not going into details study of other influencing criteria as demonstrated by other workers as found in various studies.

CONCLUSION:

The MTT method is superior to the broth microdilution method that it requires 24 hours to demonstrate good agreement with the results of the standard BMD method at 48 hours. MTT which is a photometric method of susceptibility testing not only provides good agreement with the standard BMD, but does so in 24 hours less time. The advantage of this MTT method includes its simplicity of procedure, the rapid turnaround time, the easily interpretable end point determination, and its potential adaptability to use as an automated system. MTT method should be included in future investigation of alternative to current BMD. This MTT method should be extendable to other medically important yeast and antifungal agents. It can be used as an alternative method to the CLSI method since it shows high level of agreement with the CLSI method. The spectrophotometric reading provides more detailed information on the antifungal activity in each well. Furthermore it may overcome many drawbacks.

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