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Susceptibility of *Candida* Species Isolated From HIV Infected and Newborn Candidaemia Patients to Amphotericin B

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Abstract: Problem statement: HIV infected and neonatal candidaemia is a serious condition where proper selection of the antifungal agent is the key factor in the prognosis of the disease. Thus in this study susceptibility of *Candida* species to Amphotericin B was explored in such patients. **Approach:** Forty different *Candida* isolates obtained from blood culture of such candidaemia cases were tested for Amphotericin B susceptibility by disc diffusion method and by microdilution method. **Results:** There was a good correlation between low MIC values and large inhibition zones among most isolates as expected, particularly with 24 h reading. Several outlier data were also observed during the study. After analysis it was found that 24 h microdilution method results were better for interpretation of susceptibility results. **Conclusion:** Microdilution method is the reliable method for susceptibility testing of *Candida* isolates from HIV infected and neonatal candidaemia patients to Amphotericin B.

Key words: Candidaemia, disc diffusion, microdilution

INTRODUCTION

Candidiasis is a common opportunistic infection in immunocompromised hosts and candidaemia causes significant morbidity and mortality in this group of patients (Richards *et al*., 1999). In recent past, life-threatening mycoses has been increasing in individuals with compromised immune system and there is a dramatic change in the incidence of different *Candida* spp. in candidiasis. During last five years (Nguyen *et al*., 1996). In general *Candida albicans* is the most abundantly isolated *Candida* spp. implicated in fungal infections. However, in South East Asian countries incidence of *Candida albicans* infection had gradually been decreased, while incidence of *Candida tropicalis* infection was almost equivalently increased during the same period, most of which were fluconazole resistant (Chakrabarty *et al*., 1999). Antifungal agents currently available for clinical use belong to three major classes: Polyenes, azoles and 5-flucytosine (Martin *et al*., 1992). Optimally, antifungals should exhibit selective toxicity; they should be able to inhibit the growth of the fungus without adversely affecting the host. It has been noticed that emergence of resistant strains of *Candida* spp. is due to extensive use of fluconazole as prophylactic treatment in immunocompromised patients (Rattan, 1999).

Even today, Amphotericin B is the gold standard medicine for treating invasive mycoses. It is concentrated primarily in liver and spleen; lesser amounts are accumulated in kidneys and lungs. The primary tissue reservoirs elute the drug back into the blood as plasma levels of the drug fall (Baginski and Czub, 2009). Amphotericin B is selectively taken up into the reticuloendothelial system and concentrated in the reservoir organs. Lipid-rich particles with Amphotericin B are also ingested by phagocytic monocytes; this process helps in targeting the drug to sites of infection or inflammation (Jill and Richard, 2002; Czumb and Baginksi, 2009). *In vitro*, Amphotericin B demonstrated potent and broad-spectrum fungicidal activity against clinically relevant fungi including *Candida* spp.

The M27-A2, NCCLS, USA (Second Edition) is a "reference" standard protocol for susceptibility testing of *Candida* spp. It is being developed through a consensus process to facilitate the agreement among laboratories in measuring the susceptibility of yeasts to antifungal agents. The NCCLS method for *in vitro* susceptibility testing is essential for standardization and to improve inter-laboratory reproducibility, it is difficult to use for all organisms or for routine use in clinical laboratories (National Committee for Clinical Laboratory Standards, 2002). Alternative methods such

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as the agar-based Disc Diffusion (DD) method are preferable because they are simple and cost effective.

The disease candidaemia is diagnosed when there is isolation of *Candida* spp. from one or more blood specimens (Edmond *et al*., 1999). Although oropharyngeal candidiasis being the most common fungal infection affecting patients with HIV and AIDS, candidaemia is most frequent in HIV infected adults particularly with borderline-stage AIDS (CD4+ counts <50 cells mm^{-3}) and after extensive prior therapy with Amphotericin B. The frequent use of azole in HIV-infected patients compels Amphotericin B to be used as the first line antifungal agent when candidaemia sets in (Abgrall *et al*., 2001).

Thus in this study, the applicability of the DD method for testing the susceptibility of *Candida* species to Amphotericin B was compared with the results of the broth microdilution method of the NCCLS (M-27-A2, NCCLS, USA) for *Candida* spp. strains isolated from blood of HIV-infected patients and some neonates, the two most susceptible groups for candida infection (Cuenca-Estrella *et al*., 2002).

MATERIALS AND METHODS

***Candida* spp. Isolates from blood culture:** All *Candida* spp. were isolated from candidaemia patients with HIV infection (65%) and from neonates (35%). Blood cultures of patients were done in automated blood culture system (Becton Dickinson, BD, BACTEC 9050) in MYCO/F-Lytic bottles. A total of 40 clinical Candida isolates and 3 international control strains comprising of *Candida albicans* (8), *Candida tropicalis* (18), *Candida glabrata* (10) and *Candida krusei* (4) were taken to study their antifungal susceptibility.

All the isolates were stored in cryogenic vials at -70°C as recommended (Espinel-Ingroff *et al*., 2004). Species identification were done by germ-tube test, colonial study on Corn Meal Agar (CMA) with 1% Tween 80/trypan blue, on candida chromogenic media and by carbohydrate assimilation, fermentation tests.

Two types of susceptibility testing-DD method: This test was done using Amphotericin B discs purchased from Himedia, India manufactured according to the M 44-A, NCCLS, USA guidelines (National Committee for Clinical Laboratory Standards, 2004). "Method for Antifungal Disc Diffusion Susceptibility Testing of Yeasts: Propose Guideline M44-A.NCCLS, Wayne, PA., USA)". The drug concentration of the discs was 20 mcg $disc^{-1}$. According to the guidelines, the medium required for the disc diffusion test was Mueller-Hinton Agar with 2% Glucose and 0.5 mcg mL^{-1} Methylene Blue Dye (GMB) Medium. Zone diameters (in mm) for the zone of complete inhibition were determined after 24 and 48 h of incubation at 35°C and compared with MICs determined by the M27-A2, NCCLS, USA (Second Edition) MD method. All the tested isolates showed good growth rates under these conditions which is a mandatory requirement under this protocol (Lozano-Chiu *et al*., 1999).

Microdilution method: The antifungal stock solutions were made of standard antifungal powder of Amphotericin B (Obtained from Himedia, India). Its generic name was Amphotericin (Molecular formula-$C_{47}H_{73}NO_{17}$ and Molecular weight-924.1) with potency of 750 mcg mg^{-1}. The inoculum suspensions were prepared as instructed in M27-A2, NCCLS, USA (Approved Standard Second Edition) (National Committee for Clinical Laboratory Standards, 2002). "Method for Antifungal Disc Diffusion Susceptibility Testing of Yeasts: Propose Guideline M44-A.NCCLS, Wayne, PA., USA)". Final concentrations of Amphotericin B were 0.0313-16 mcg mL^{-1} (The concentrations were 0.0313, 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 mcg mL^{-1}). To dilute the stock solution DMSO was used. These microwells were incubated at 35°C and MIC endpoints were determined after 24-48 h. The MICs of Amphotericin B were read by Thermo multiskan reader (Vantaa, Finland) as the lowest concentration that caused a significant diminution of growth compared with the control growth well. The medium used in this test was RPMI 1640, the endpoint was considered as a prominent decrease in turbidity corresponding to ~50% inhibition in growth as determined spectrophotometrically (Bannatyne and Cheung, 1977; Rodriguez-Tudela *et al*., 1996). It is demonstrated that the most reliable testing criteria for *Candida* are the use of RPMI broth and a prominent inhibition MIC endpoint. The two types of MICs were determined in this study, these were MIC_{50} (MIC that inhibits 50% of the study strain) and MIC_{90} (MIC that inhibits 90% of the study strain).

For both methods an arbitrary break point had to be established. Isolates with Diameter inhibition Zone (ZD) of <16 mm and MICs ≥16 mcg mL^{-1} were considered to have high MIC values and those isolates with MICs of <16 mcg mL^{-1} and Diameter inhibition Zone (ZD) ≥16 mm were considered to have low MIC values.

For quality control three international strains were used-*Candida albicans* (ATCC 10231), *Candida tropicalis* (ATCC 750), *Candida krusei* (ATCC 6258) in every batch tested for antifungal susceptibility (National Committee for Clinical

Laboratory Standards, 2002; 2004). "Method for Antifungal Disc Diffusion Susceptibility Testing of Yeasts: ProposeGuidelineM44-A.NCCLS, Wayne, PA., USA)".

Statistical analysis of this study: Arithmetic Mean (AMs) and MIC ranges were calculated in each genus-species combination to evaluate the comparative study between the disc diffusion and broth microdilution methods. The Mean, Standard deviation, Standard error of Mean were calculated in the data sets of different types of variables. As in this method paired data were obtained, so "paired t tests" of every possible combination were analyzed. The linear regression analysis was done in between the DD method and the MD method of antifungal susceptibility testing. Statistical analysis was calculated using Graph Pad and Vassar software.

RESULTS

There was no significant difference of susceptibility zones by DD method measured at 24 and 48 h (Table 1). Out of four species, *Candida albicans* and *Candida glabrata* showed high susceptibility zones (AM ≥18 mm) but *Candida tropicalis* and *Candida krusei* showed smaller zones (AM <13 mm) in the DD method (Table 2 and 3).

Similarly, AM and ranges of various *Candida* species studied by micro dilution method was expressed as MIC_{50}. (MIC that inhibits 50% of study strains), MIC_{90} (MIC that inhibits 90% of study strains) and their ranges are given in Table 2 and 3. In this study Amphotericin B exhibited high antifungal activity against all tested *Candida* species ($IC_{50} \leq 2$ mcg mL^{-1} in most cases) by the micro dilution method and in this method all species have showed high sensitivity ranges compared to DD method.

Paired 't' test value in between MIC_{50} and MIC_{90} in 24 h: The two tailed P value was less than 0.0001. By conventional criteria, this difference was considered to be extremely statistically significant. The standard error of difference was 0.168.

Table 1: Susceptibility testing results determined by disc diffusion (AM and range) of Amphotericin B

Species (No. of isolates)	Incubation period (h)	Inhibition zone (mm)	AM (range)
Candida albicans (8)	24	18	16-21
	48	18	17-22
Candida tropicalis (18)	24	11	10-13
	48	11	11-14
Candida glabrata (10)	24	21	18-22
	48	21	19-23
Candida krusei (4)	24	11	8-14
	48	11	9-15
Total (40)	24	15	8-21
	48	15	9-23

AM: Arithmetic Mean

Paired 't' test value in between MIC_{50} and MIC_{90} in 48 h: The two tailed P value was less than 0.0001. By conventional criteria, this difference was considered to be extremely statistically significant. The standard error of difference was 0.210. A good correlation was observed between low MIC values and large inhibition zones for most of the organism tested as expected. The correlation was better in between results of MD 24/DD 24 h than results of MD 48/DD 24 h (r = -0.334 and r = -0.270, respectively) (Fig. 1a and b). There were some aberrant results with few isolates having low MIC ranges after 48 h of incubation shown small inhibition zones after 24 h and having high MIC ranges after 24 h of incubation but showing high inhibition zones after 48 h.

Thus it was found that MD is a reliable method for susceptibility testing of *Candida* isolates to Amphotericin B in comparison to DD susceptibility testing. Most isolates with MIC sec ≤2 mcg mL^{-1} had >16 mm inhibition zones. However, there were few isolates of *C. tropicalis*, *C. glabrata* and *C. krusei* where this was not applicable. These isolates had inhibition zone of <13 mm but they had the MIC values of ≤0.25 mcg mL^{-1}. On the other hand, few isolates of *C. glabrata* and *C. albicans* have >20 mm zones but have ≥2 mcg mL^{-1} MIC values.

When susceptibility of 24 h DD was compared with 48 h MD (Fig. 1b) there were few isolates which had the MIC values of ≤0.25 mcg mL^{-1} but low ZD (<13 mm). The best correlation (95.5%) was observed between susceptibility results of MD 24 and DD 48 h. The percentage of false resistance/susceptibility was low in case of results with 24 MD 48 h^{-1} DD.

Table 2: The suceptibility of *Candida* spp. against Amphotericin B determined by micro dilution method (MIC_{50}, MIC_{90} and range) after 24 and 48 h of incubation

Species (No. of isolates)	Incubation period (h)	MIC_{50} (mcg mL^{-1})	MIC_{90} (mcg mL^{-1})	Range (mcg mL^{-1})
Candida albicans	24	1.22	2.80	1.0-4.00
(8)	48	1.22	3.20	1.0-4.00
Candida tropicalis)	24	0.86	3.26	0.25-4.0
(18)	48	0.84	3.90	0.25-4.0
Candida glabrata	24	0.82	2.30	0.5-4.00
(10)	48	0.86	2.80	0.5-4.00
Candida krusei (4)	24	1.20	3.20	0.5-4.00
(4)	48	1.20	3.80	0.5-4.00
Total (40)	24	0.97	2.90	0.5-4.00
	48	0.96	3.50	0.5-4.00

MIC_{50}: 50% inhibition of growth; MIC_{90}: 90% inhibition of growth; AM: Arithmetic Mean

Table 3: Susceptibility testing results after 24 and 48 h (Mean ± SD ± SEM)

MIC and DD types	N (Total no.)	Mean	SD	SEM
MIC_{50} in 24 h	43	0.9712	0.8777	0.1338
MIC_{90} in 24 h	43	2.9070	1.3419	0.2046
MIC_{50} in 48 h	43	0.9653	0.8817	0.1345
MIC_{90} in 48 h	43	3.5349	1.7368	0.2649
DD in 24 h	43	14.650	4.1100	0.6300
DD in 48 h	43	15.600	4.0900	0.6200

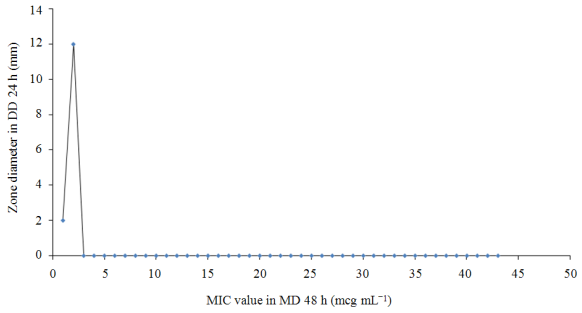

Fig. 1a: Scatterogram showing relationship of 24 h DD zones of inhibition with 24 h MIC 90 values. The regression equation was y = 17.63-1.02x, r = -0.334, r^2 = 0.111

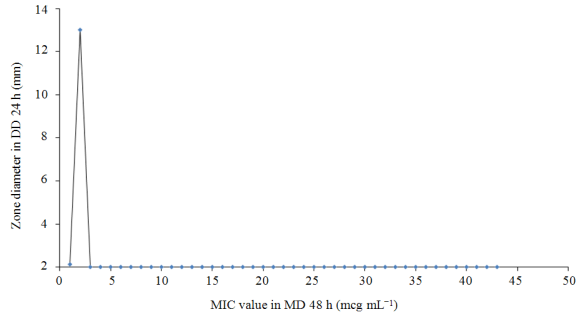

Fig. 1b: Scatterogram showing relationship of 24 h DD zones of inhibition with 48 h MIC90 values. The regression equation was y = 16.91-0.64x, r = -0.270, r^2 = 0.073

DISCUSSION

The ability of the MD method to express the true susceptibility of an individual isolate within 24 h is its great advantage but it has got a slight disadvantage in relation to its complex preparatory steps. On the contrary, the DD method is less time-consuming and could be a method of choice in a routine clinical laboratory but chance of false positive results are much higher than MD method (Lozano-Chiu *et al*., 1999). Thus, MD method appears to be superior to DD method.

Our aim to find out a comparative data in relation to an agar-based method and broth MD technique in yeast was thus ended with a conclusive result.

Our conclusion is that the MD method is a very useful and perfect method than the DD method was also supported by the regression analysis (Fig. 1a and b) between MIC values in 24 h broth MD method with 24 h susceptibility zone sizes. In the same analysis, the correlation between MIC values in 48 h broth MD method with 24 h susceptibility zone sizes was less significant than the previous combination (Ramirez *et al*., 2006).

CONCLUSION

The above results suggested that the MD method should be used for testing the susceptibility of *Candida* spp. isolated from blood to Amphotericin B and as it is possible to get the result in spectrophotometric reading within 24 h, it may help in prevention of many deaths of patients suffering from candidaemia (Menichetti *et al*., 1994). As most of the candidaemia patients are associated with immunosuppression, it is necessary for the physicians to choose the perfect antifungal drug as soon as possible where MD method appears to be very helpful (Petri *et al*., 1997). However, there is a need for further studies on *Candida* strains with high MIC values against Amphotericin B. In developing countries like India it is also important to set up quality control parameters in different clinical laboratories in relation to *in vitro* susceptibility test by microdilution method which is reliable and better than Disc diffusion method.

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