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Original Article

Emergence of Non-*albicans Candida* Species and Antifungal Resistance in a Tertiary Care Hospital

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SUMMARY: The spectrum of candidiasis has changed with the emergence of non-*albicans Candida* spp. and acquired antifungal resistance, especially in immunocompromised hosts. This changing scenario has necessitated routine antifungal susceptibility testing. In the present work, 102 *Candida* spp. isolates gathered during 2003-2004 were characterized by standard procedures, and antifungal susceptibility testing to amphotericin B, fluconazole and itraconazole was performed by broth macrodilution (BMD)-minimum inhibitory concentration (MIC) and disk diffusion (DD) methods. Among all isolates, 77.4% were from an ICU and 10.8% were obtained from a nursery. The majority of the isolates were *C. tropicalis* (48%), followed by *C. parapsilosis* (27.4%) and *C. albicans* (22.5%). Overall 6.9, 4.9 and 3.9% of all isolates were resistant to amphotericin B, fluconazole and itraconazole, respectively. Out of the 5 (4.9%) isolates resistant to fluconazole, 4 (3.9%) were from patients with AIDS on fluconazole prophylaxis. A discrepancy was observed between the results of susceptibility testing by DD and those by BMD-MIC: 15 (14.7%) isolates were reported to be resistant by DD despite having low MICs. Based on these results, it was concluded that initial antifungal screening of clinical isolates by the DD method followed by confirmation of resistant strains by the broth dilution method is desirable to optimize patient management.

INTRODUCTION

The incidence of the various fungal pathogens has increased dramatically over the past few decades. *Candida* spp. are the most common of these pathogens (1). These infections are often severe, rapidly progressive, difficult to diagnose and refractory to therapy. The combination of suppressed host defense and exposure to multiple risk factors is responsible for their emergence. *C. albicans* was previously responsible for nearly 80% of candidemia in many hospitals. But recent reports suggest that a shift has occurred in the distribution of infections, with non-*albicans Candida* spp. being increasingly detected (2-5).

The clinical manifestations of candidiasis are varied (6). A rise in invasive fungal infections (IFI) by less common *Candida* spp. associated with significant morbidity and mortality has been reported. Early and appropriate therapy may alter the course of these infections (7-9). The therapeutic options, though numerous, are characterized by high toxicity and a wide range of drug interactions, and thus remain limited to a few licensed antifungal agents for which these problems are slightly less prominent – e.g., amphotericin B, fluconazole, itraconazole and ketoconazole. Newer antifungals (lipid formulations of amphotericin B, voriconazole, caspofungins, micafungins), though less toxic still, are extremely expensive (10). The emergence of drug-resistant *Candida* spp., which is largely attributed to use of prolonged and inappropriate empirical therapy, has further complicated the patient management (11).

Available data suggest that routine susceptibility testing of fungi is desirable for clinical decision-making (12). Unlike with antibacterial susceptibility testing, the E-test is not recommended for antifungals owing to poorly defined endpoints, especially in testing azoles (13). The approved broth dilution test, which is relatively expensive and laborious, is used in few laboratories (14). Thus, the disk diffusion (DD) test on glucose methylene blue (GMB) Mueller-Hinton agar (MHA), which is an easy and practical technique to screen antifungal agents, is generally used as a routine procedure in clinically resistant isolates (15).

The present study was undertaken to determine the spectrum of candidiasis and antifungal susceptibility of *Candida* isolates. The broth macrodilution (BMD) and DD methods were compared to optimize routine antifungal testing of commonly used antifungals in the clinical laboratory.

MATERIALS AND METHODS

The present study was conducted for a period of 1 year and 2 months from September 2003 to November 2004 at the Department of Microbiology at Vardhman Mahaveer Medical College (VMMC) and the Safdarjung Hospital (SJH), a 1,700-bed referral hospital.

A total of 362 samples, including urine (183), blood (152), sterile fluids (18), oral scrapings (6) and stool (3) samples, were processed to determine their fungal etiology. Demographic and clinical data such as age, sex, site of infection, predisposing factors, history of exposure to antifungals and clinical outcome of the patients were noted. Samples were processed for microscopy and culture using standard mycological procedures (6). Repeat samples (3 or more) were processed wherever indicated. *Candida* isolates were characterized by staining and culture characteristics, growth in Sabouraud's dextrose broth, growth on CHROM agar

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Candida medium, and carbohydrate fermentation and assimilation patterns (13).

The isolates were screened for antifungal susceptibility by the DD method using amphotericin B (10 U), fluconazole (25 μg) and itraconazole (15 μg) on Muellar Hinton agar (MHA) supplemented with 2% glucose and methylene blue (GMB) 5 μg/ml. One half each of MHA- GMB (150 cm plate with 4 mm thickness) were inoculated with the test and control strain and incubated for 24 h at 37°C. Zone diameter endpoints were read at 80% growth inhibition and were interpreted as per the approved NCCLS (M44-A) guidelines (15).

The BMD-minimum inhibitory concentration (MIC) of the isolates was performed to the above mentioned drugs using RPMI medium and MOPS buffer. The test results were read visually and by spectrophotometer after 48 h of incubation. The optical density (OD) of the medium control tube was subtracted from the ODs of all other tubes, and the inhibitory concentration was computed mathematically. The MIC experiments were repeated three times and the mean was taken.

Briefly, the BMD-MIC of amphotericin B was determined as the lowest drug concentration with an OD corresponding to greater than or equal to 90% decrease in turbidity compared to that of growth control, and the MICs of the other drugs, corresponding to a 50% decrease in turbidity as in NCCLS (M27-A2) guidelines (16).

The culture media and antifungal disks (except fluconazole [25 μg/ in house]) were procured from Himedia Laboratory (Mumbai, India), and antifungal powders were obtained from Ranbaxy Research Foundation (Gurgaon, India). The quality control was performed by testing *C. parapsilosis* (ATCC 22019), *C. krusei* (ATCC 6258) and *C. albicans* (ATCC 90028) with each batch of clinical isolates.

RESULTS

Patient population and clinical data: The median age of the patients in the study was 33 years and the majority (72.5%) were male. One hundred and two *Candida* spp. isolates were deribed from the 362 samples. These were from patients admitted to the intensive care unit (ICU) (77.4%), nursery (10.8%), pediatric ward (5.9%), and medicine ward (5.9%). The clinical data for the 102 patients with candidiasis is shown in Table 1. Underlying disease conditions attributable to candidiasis were gastrointestinal perforation in 42 cases, followed by trauma in 22 and renal insufficiency in 14 cases. The mortality rate was highest (53.3%) for the patients with candidemia. There were six isolates from AIDS cases; these were adult male patients on antifungal prophylaxis, and their isolates were obtained from oral scrapings (3), urine (2) and stool (1).

Spectrum: The majority of the isolates were *C. tropicalis* (48%), followed by *C. parapsilosis* (27.4%), *C. albicans* (22.5%) and one each of *C. glabrata* and *C. krusei*. In blood

Table 1. Clinical data for 102 patients with candidiasis

Clinical data	*n*	*C. tropicalis* (49)	*C. parapsilosis* (28)	*C. albicans* (23)	*C. glabrata* (1)	*C. krusei* (1)
Site						
Urine	64	26	19	17	1	1
Blood	30	18	7	5	–	–
Sterile fluid	4	2	2	–	–	–
Oral scraping	3	3	–	–	–	–
Feces	1	–	–	1	–	–
Underlying clinical conditions						
GI perforation	42	19	4	17	1	1
Trauma	22	8	8	6	–	–
Renal insufficiency	14	4	2	8	–	–
Diabetes	12	2	1	9	–	–
Burns	4	1	2	1	–	–
AIDS	6	3	–	3	–	
Malignancy	8	4	3	1	–	–
Prematurity	4	4	–	–	–	–
Infective endocarditis	3	3	–	–	–	–
Predisposing factors						
Broad spectrum antimicrobials	102	49	28	23	1	1
CV Catheter	72	20	28	22	1	1
ICU, Nur stay	89	44	23	20	1	1
Steroid therapy	8	4	2	2	–	–
Major G1 surgery	42	20	9	12	–	1
Neutropenia	8	5	1	1	–	1
H/O antifungal Prophylaxis	6	3	–	3	–	–
TPN	62	22	26	12	1	1

GI, gastrointestinal; CV, central venous; ICU, intensive care unit; Nur, nursery; H/O: history of; TPN, total parenteral nutrition.

Table 2. In vitro susceptibilities of *Candida* spp. to three antifungal agents

Species (no. of isolates)[5]	Antifungal agent	BMD-MIC (μg/ml at 48 h)			Resistant[1] *n* (%)
		Range[2]	MIC 50[3]	MIC 90[4]	
C. tropicalis (49)	amphotericin B	0.25 - 4	1	2	3 (6.1)
	fluconazole	0.25 - 256	1	128	2 (3.9)
	itraconazole	0.008 - 4	0.25	8	2 (3.9)
C. parapsilosis (28)	amphotericin B	0.03 - 4	0.5	4	2 (7.1)
	fluconazole	0.25 - 256	1	1	0
	itraconazole	0.008 - 4	0.25	0.25	0
C. albicans (23)	amphotericin B	0.25 - 4	0.5	1	1 (4.3)
	fluconazole	0.25 - 256	2	32	2 (8.7)
	itraconazole	0.008 - 8	0.032	0.5	1 (4.3)

[1]: Resistant strains defined as follows: amphotericin B ≥4 μg/ml; fluconazole ≥64 μg/ml; and itraconazole ≥1 μg/ml.
[2]: The complete "range" of MIC tested for a given antifungal agent.
[3]: MIC 50: MIC at which 50% of isolates were inhibited.
[4]: MIC 90: MIC at which 90% of isolates were inhibited.
[5]: Single strain of *C. krusei* resistant at MIC: amphotericin B = 8 μg/ml; fluconazole = 128 μg/ml; itraconazole = 2 μg/ml. Single strain of *C. glabrata* isolate sensitive with MIC: amphotericin B = 1 μg/ml; fluconazole = 4 μg/ml; itraconazole = 0.5 μg/ml.

Table 3. Comparison of antifungal susceptibility testing by disk diffusion (DD) and broth macro-dilution (BMD)-minimum inhibitory concentration (MIC)

Species (no. of isolates)	Test method	Percentage of susceptibility to each antifungal							
		Fluconazole			Itraconazole			Amphotericin B	
		S	SDD	R	S	SDD	R	S	R
C. tropicalis (49)	BMD-MIC	91.8	4	4	93.4	2	4	93.9	6.1
	DD	85.5	0	14.5	92.0	–	8	92.0	8
C. parapsilosis (28)	BMD-MIC	100	0	0	100	–	0	92.9	7.1
	DD	100	0	0	100	–	0	85.7	4.8
C. albicans (23)	BMD-MIC	86.9	4.3	8.7	86.9	8.7	4.3	95.6	4.3
	DD	78.2	8.7	13.1	78.2	–	21.7	95.6	4.3
C. krusei (1)	BMD-MIC	–	–	100	0	0	100	0	100
	DD	–	–	100	0	0	100	0	100
C. glabrata (1)	BMD-MIC	100	–	0	100	0	0	100	0
	DD	100	–	0	100	–	0	100	0

S, susceptible; SDD, susceptible dose dependent; R, resistant.

and sterile fluids, *C. tropicalis* (58.8%) was the predominant isolate, followed by *C. parapsilosis* (29.4%).

Susceptibility: The in vitro susceptibilities of *Candida* spp. to the three antifungal agents are depicted in Table 2. By the BMD-MIC method, 7 isolates (6.9%) showed resistance to amphotericin B, 5 (4.9%) showed resistance to fluconazole, and 4 (3.9%) showed resistance to itraconazole. By the DD method, 7 isolates (6.9%) showed resistance to amphotericin B, 11 (10.8%) showed resistance to fluconazole, and 14 (13.7%) showed resistance to itraconazole. Out of the 5 (4.9%) isolates resistant to fluconazole by the BMD-MIC method, 4 (3.9%) were from patients with AIDS on fluconazole prophylaxis.

Comparison: The results of antifungal susceptibility testing by DD and BMD-MIC were compared (Table 3). The DD testing performed in accordance with NCCLS M44-A guidelines was comparable in 85.3% of strains. A discrepancy between the BMD-MIC and DD test results for susceptibility to amphotericin B, fluconazole and itraconazole was observed in 3 (2.9%), 6 (5.9%) and 6 (5.9%) of strains, respectively.

DISCUSSION

The spectrum of candidiasis has changed with the emergence of non-*albicans Candida* spp. and acquired antifungal resistance assisted by an increase in the high-risk population. In the present study, use of broad-spectrum antibiotics in a constituting combination therapy, use of a central venous catheter, and a stay in the ICU or nursery were important predisposing factors. In fact, in many situations multiple predisposing and underlying factors were perhaps responsible. These findings are in concordance with the reports of previous workers (17,18). In addition, six cases of AIDS were admitted to the medicine ward due to low CD4 count and were on antifungal prophylaxis. In 65.6% of the ICU patients from whom we derived urine isolates, patients had intestinal obstruction with perforation peritonitis, showing a suspected endogenous mode of infection as reported by other workers (19,20).

The predominance of non-*albicans Candida* spp. over *C. albicans* was a notable feature, with *C. tropicalis* being the most common isolate (48%), followed by *C. parapsilosis* (27.4%), *C. albicans* (22.5%), *C. krusei* (0.88%) and *C. glabrata* (0.88%). This finding is in agreement with several published reports from India and abroad (3,5,7). Some authors have attributed the emergence of *C. krusei* and *C. glabrata* to the use of fluconazole in transplant units and ICUs (21,22), whereas other reports have failed to find a clear

epidemiological association (4). This hospital does not have a transplant unit, and antifungal prophylaxis is not administered in high-risk areas in routine practice. However, we recognize that this finding may simply be a result of the small sample size, and that further studies with larger sample size are needed to verify it. It has been feared that the increased use of fluconazole for treatment of candidiasis will lead to resistance or a shift towards intrinsically resistant non-*albicans Candida* spp. (23). In the present study, eight isolates of *Candida* spp. were observed to be in resistant or susceptible dose dependent (SDD) range for fluconazole by BMD-MIC after 48 h of incubation. These were reduced to five isolates when the readings of SDD were merged with those of susceptible isolates. Previous studies have suggested that the distinction between susceptible and SDD is not necessary for patients with invasive candidiasis, as these patients are treated with high doses (>400 mg/day) of fluconazole (4).

The incidence of amphotericin B-resistant *Candida* spp. in our study was 6.9% (7 of 102 isolates), which is comparable to the incidences of 2.5 to 16.3% reported in recent studies (18,24-26). This is attributable to a reduction in membrane ergosterol in resistant mutants of *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis*. These mutants have been reported from immunocompromised patients who had received extensive amphotericin B, azoles and broad-spectrum antibiotics (12,17,27,28). Similar findings were observed in our study. Coresistance of amphoterecin B and fluconazole has been reported recently (24).

Four isolates that showed resistance (high MIC) to the above antifungals were from AIDS cases on irregular antifungal prophylaxis. One isolate of *C. krusei* that showed high-level resistance was from a non-AIDS patient. Reports on rising MIC to antifungals are few and are attributed to inappropriate antifungal usage, differences in antifungal prescriptions and infection control practices (1-3,16,24-26). Empiric antifungal therapy is not practiced in ICU patients in our hospital, which perhaps explains the low incidence of antifungal resistance in our non-AIDS patients.

It was observed during the present study that the reference NCCLS M27-A method for antifungal susceptibility testing is cumbersome and costly. We have compared DD and BMD-MIC techniques with three common antifungal agents. Overall, 5 (21.7%) and 1 (4.3%) of the *C. albicans* isolates showed resistance to itraconazole by the DD and BMD method, respectively. The DD testing performed in accordance with NCCLS M 44-A guidelines was comparable in 85.3% of strains. Among all isolates, 14.7% were reported to be resistant to the aforementioned antifungals by DD, although the MICs of these strains were later found to be in the sensitive range. Keeping this in mind, DD alone does not seem to suffice even as a routine procedure and needs to be confirmed by BMD-MIC to exclude false resistance. Similar findings have been reported by other workers (11,23,29-31).

Unlike antibacterial susceptibility testing, antifungal susceptibility testing is not widely used. Moreover, the emergence of non-*albicans Candida* spp. and their resistance to antifungal agents, especially in immunocompromised hosts, is a matter of concern. Based on the present results, we conclude that routine screening of *Candida* isolates by the GMB-MHA DD method followed by confirmation of resistant strains by the broth dilution method in a clinical microbiology laboratory is desirable for surveillance of emerging antifungal resistance to optimize patient management.

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