Original Article:

Common fungal isolates from routine clinical specimens: a two years' study from a tertiary care hospital in South India

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ABSTRACT

Background: Fungal infections are gaining prominence in recent years and are becoming a cause of significant morbidity as well as mortality. Reliable data from India about the spectrum of pathogens causing fungal infection in various body systems, and particularly about the antifungal susceptibility pattern of *Candida* spp., which is the most common isolate worldwide is not available.

Methods: We prospectively studied 48,155 clinical samples submitted for fungal work-up to the microbiology laboratory at our tertiary care teaching hospital. Standard procedures were followed for fungal identification. *Candida* isolates were differentiated into *Candida albicans* and non-albicans candida (NAC) by germ tube test. Antifungal susceptibility of *Candida* isolates was determined by disc diffusion technique using amphotericin B (10 µg), fluconazole (25 µg), and voriconazole (1 µg) discs.

Results: A total of 555 fungal isolates were obtained of which 541 were *Candida* spp, while the others were filamentous fungi. Male gender and age over 50 years were found to be independent risk factors. Proportion of NAC isolates (n = 384, 69.2%) were greater compared to *C. albicans* (n = 157, 28.3%). *Aspergillus* spp. was the second most frequent isolate. Azole resistance was significantly more in NAC group as compared to *C. albicans*. For fluconazole, 57.5% of the NAC showed resistance compared to 24.8% seen in strains of *C. albicans* while the corresponding figure for voriconazole was 56.8% Vs 22.9%. Overall resistance for amphotericin B was low (8.9%).

Conclusions: Our observations bring to light the spectrum of common fungal isolates and their susceptibility patterns. This information will be useful for health planners and policy makers, as early institution of appropriate antifungal treatment can be life saving.

Key words: *Mycoses, Antifungal susceptibility testing, Candida*

INTRODUCTION

Fungal infections are emerging as an important cause of morbidity and mortality especially in critically ill patients. Indiscriminate and inappropriate use of broad spectrum antimicrobial agents as well as use of immunosuppressive drugs in various diseases has contributed to the increased propensity for fungal infections caused by both yeasts and moulds.[1] *Candida* and *Aspergillus* species are the most common causes of fungal infection but other yeasts and filamentous fungi are emerging as pathogens. Among the filamentous fungi, apart from *Aspergillus* spp, others like *Fusarium* spp., *Scedosporium* spp., *Penicillium* spp. and Zygomycetes are becoming increasingly common.[2,3] Although *C. albicans* is the most prevalent species involved in causing fungal infections, the incidence of infections due to non-*albicans* species is increasing[4] particularly in patients treated in the

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intensive care unit (ICU) and is associated with significant morbidity and mortality. Epidemiologic studies have identified intravenous catheters, broad spectrum antibiotics, mucosal colonization, neutropenia, previous surgical procedures, total parenteral nutrition, extremes of age, and concomitant bacteraemia as significant risk factors for fungal infections.[5-7] Early diagnosis is challenging due to delays in, and low sensitivity of confirmatory blood cultures, or difficulty in discriminating colonization/ contamination from invasion in samples like sputum. Once diagnosed, early initiation of appropriate antifungal therapy is essential for reducing morbidity and mortality.

About one-half of all candidaemias occurs in ICU setting and in these patients death rates are almost double than those of patients in general wards. Attributable mortality due to candidemia has been shown to be significant, ranging from 14% - 49%[8,9] depending on the patient population studied. Apart from neutropenic patients with haematologic malignancies, the incidence of invasive aspergillosis is also increasing in non-neutropenic patients, such as those with chronic lung diseases or systemic disease treated with long-term immunosuppressive drugs and solid organ transplant recipients.[10] The mortality rate in 1990s was greater than 95%, but by the end of the decade, it had decreased to 55% - 80%.[11] Despite the recent advances in antifungal therapy, such as, the availability of extended spectrum triazoles and the echinocandins class, response rates remain suboptimal. This study was undertaken to find out the spectrum of fungal pathogens at our tertiary care teaching hospitals, and the distribution of these pathogens among the various systems affected.

MATERIAL AND METHODS

This prospective study included the fungal pathogens isolated in the microbiology laboratory of Sri Venkateswara Institute of Medical Sciences, a tertiary care teaching institute hospital, in Tiurpati, Andhra Pradesh in South India. The isolates were from the samples of the patients suspected to have fungal infections during period from January 2013 to December 2014. These fungal isolates were from various clinical specimens, which included urine, sputum, endotracheal aspirate, broncho-alveolar lavage, catheter tip, central venous catheter tip, pus, vaginal swab, and blood.

All samples were analyzed by direct microscopy and culture as per standard microbiological procedures. In the event of visualization of suspected *Aspergillus* spp. on microscopy and subsequent growth within 48 hours, a repeat sample was collected wherever possible to rule out contamination.

For direct microscopy, 10% potassium hydroxide (KOH) was used to visualize presence of any fungal element. For yeasts, Gram's staining was done to look for Gram-positive cells. For fungal culture, all samples were inoculated on two isolation media: one in Sabouraud's dextrose agar (SDA) and the other in SDA with chloramphenicol, in duplicate. All culture media and antibiotics were obtained from Hi-media Laboratories, Mumbai, India. The culture tubes were incubated at 25 ℃ and 37 ℃ and examined daily for six weeks.

The identification of fungi was done by macroscopic and microscopic evaluation of the fungal morphology by visual inspection of the cultures tubes, lacto-phenol cotton blue mount, and by slide culture technique. The characteristics considered for fungus identification were macroscopic aspects of texture, colour, growth rate and microscopic aspects such as mycelium and conidium types, relationship between hyphae and fructification organs by lactophenol cotton blue mount. Micro culture on slides technique was used for observation of filamentous fungi. The yeast isolates were identified by standard tests like

germ tube (Figures 1 and 2), different spore production on corn meal agar (CMA), and urease production.[12] Antifungal susceptibility testing was done by disc diffusion method against amphotericin B (10 µg) and azole group of antifungal; fluconazole (25µg), and voriconazole (1µg) (Hi-media, Mumbai, India). The inoculum was prepared by inoculating 3-4 colonies of the *Candida* isolate in saline. The turbidity of the suspension was matched to 0.5 McFarland standard. The yeast suspension was inoculated on Mueller-Hinton agar supplemented with 2% glucose and methylene blue (0.5 µg/mL) by the lawn culture method using a tipped cotton swab. The inoculum was allowed to dry and the strip was placed on the surface of agar with the help of forceps. The plates were incubated at 35 ^{0}C 24-48 hours. *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019 were used for quality control. The results of antifungal susceptibility test were interpreted as sensitive (S) and resistant (R). Interpretative criteria for azoles were those recommended by the Clinical and Laboratory Standard Institute (CLSI).[13] Due to the lack of defined breakpoints for amphotericin B, values based on other previous studies were used.[14,15]

RESULTS

The frequency distribution of various clinical samples from clinically suspected fungal infection cases is shown in Figure 3.
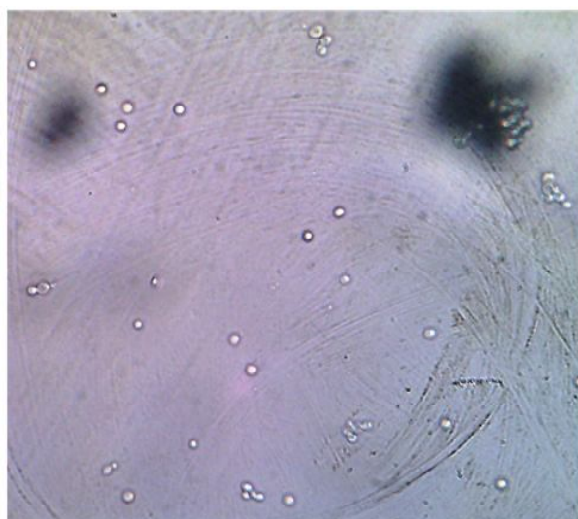

Figure 1: Photomicrograph of budding yeast suggestive of negative germ tube test ($\times$ 200)
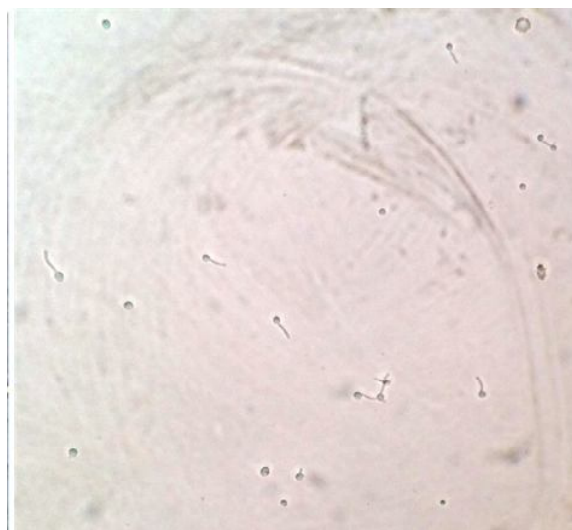
Figure 2: Photomicrograph of yeast cells showing germ tubes ($\times$ 100)

In the two year period of 2013 and 2014, we processed a total of 48,155 clinical samples for bacterial and fungal pathogens. The most frequent sample received in the laboratory was urine (n = 21,815), which also yielded the highest number of fungal isolates (n = 358, 1.6%). The frequencies of fungal isolates from other samples processed were as follows: blood (19/14,385, 0.1%); sputum (47/4598, 1.1%); pus and tissue (34/3,777, 0.9%); catheter tips (76/1905, 4%); various body fluids (18/1198, 1.5%); and stool (3/477, 0.6%).
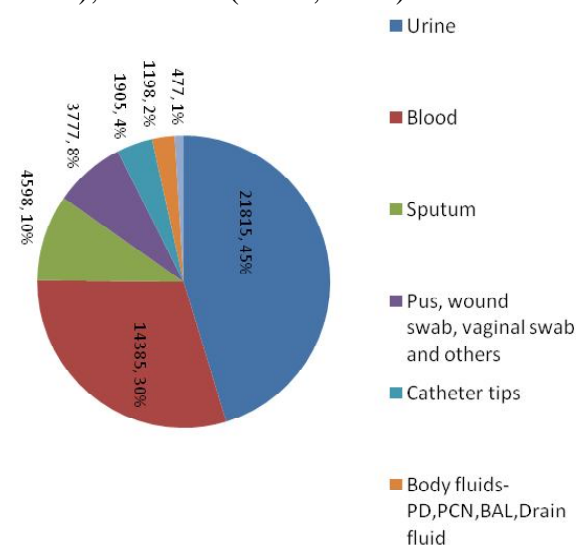
Figure 3: Distribution of various clinical samples obtained from patients suspected to have fungal infections
PD = peritoneal dialysis; PCN = percutaneous nephrostomy; BAL = bronchoalveolar lavage

Table 1 shows the number of specimens processed with the corresponding number of fungal isolates in the two years. A marginal increase of both the parameters is evident. The age and gender distribution of cases is shown in Table 2. Majority of the patients were adults, aged over 50 years. There was a slight male predominance [male:female=1.3]. The isolation rate was higher in patient population aged over 50 years among both genders.

The relative proportion of fungal isolates obtained from various clinical samples is shown in Table 3. Urine yielded the maximum isolates

Table 1: Annual frequency of fungal infections

Year	No. of samples	No. (%) positive
2013	23,975	265 (1.1)
2014	24,180	290 (1.2)
Total	48,155	555 (1.2)

Table 2: Age and gender-wise distribution of suspected cases

Age group (years)	Male No. (%)	Female No. (%)	Total No.
0-25	15 (5)	46 (17.9)	61
26-50	126 (42.3)	87 (33.9)	213
>50	157 (52.7)	124 (48.3)	281
Total	298 (53.7)	257 (46.3)	555

(n = 358), all belonging to the genus *Candida*. In urine as well as the other samples, *non-albicans candida* (NAC) were more frequently found than *C. albicans*. Among the various fungal isolates obtained during this study, NAC was found to be most frequent (384/555, 69.2%) followed by *Candida albicans* (n = 157, 28.3%), *Aspergillus niger* (n = 6, 1.1%), *Aspergillus flavus* (n = 5, 0.9%), *Curvularia species* (n = 2, 0.4%), dematiceous fungus (n = 1, 0.2%) and fungus from order Pleosporales (n = 1, 0.2%). Table 4 shows the antifungal susceptibility patterns of the Candida isolates against three drugs. Out of 541 candida isolates, 384 were NAC and the rest were *C. albicans*. Amphotericin B resistance was more common in NAC as compared to *C.albicans*. Resistance to the azole group of antifungal agents was also common in the NAC group. Out of 384 NAC isolates, 221 (57.6%) showed resistance to fluconazole and 218 (56.8%) were resistant to voriconazole (Figures 4 and 5).

DISCUSSION

Fungi are widely distributed in nature and incidence of life threatening fungal infections caused by true pathogenic or opportunistic

Figure 4: Non-albicans Candida antifungal susceptibility test showing resistantce to fluconazole and voriconazole and susceptible to amphotericin B,

Figure 5: *Candida albicans* antifungal susceptibility test showing susceptibility to fluconazole, voriconazole and amphotericin B

Table 3: Relative proportion of fungal isolates obtained from various clinical samples

Fungal isolates	Urine (n=358)	Sputum (n=47)	Blood (n=19)	Catheter tip (n=76)	Body fluids (n=18)*	Others (n=32)†	Tissue (n=2)	Stool (n=3)
Candida albicans	102	17	8	23	3	4		
Candida non-albicans	256	24	11	52	13	25		3
Aspergillus flavus		2			1	1		
Aspergillus niger		4		1			1	
Curvularia					1		1	
Dematiceous fungus						1		
Pleosporales						1		

* pleural = 5; PD fluid = 7, PCN fluid = 1; BAL fluid = 4; drain fluid = 1
† pus = 22; wound swab=3; vaginal swab=7
PD = peritoneal dialysis; PCN = percutaneous nephrostomy; BAL = bronchoalveolar lavage

fungi has been increasing since the past two decades. Fungal infections are often insidious and their diagnosis is often delayed due to co-existing illnesses. The emergence of these infections has created a challenge in their diagnosis and management.[16] Up to 7% patients dying in teaching hospitals have invasive aspergillosis.[17,18] *Candida spp.* accounts for 8%-15% of nosocomial blood stream infections and as per the study, was the fourth most common isolate among patients in the intensive care unit.[19] NAC species cause 35%-65% of all candidemias in the general patient population.[20] Specific patient groups have very high frequencies of fungal infections: 15% of allogenic haemopoietic stem-cell transplant recipients have a fungal infection;[21] about 20%

of lung transplant recipients are colonized and infected;[22] about 60% and 20% of acquired immunodeficiency syndrome (AIDS) patients have *Pneumocystis jiroveci* pneumonia or esophageal candidiasis, respectively;[23] cryptococcal meningitis is present in 30% of people with AIDS in Africa and southeast Asia;[24] and *Penicillium marneffei* infections are present in about 30% of people with AIDS in south-east Asia.[25]

In our study, a total of 48,155 samples yielded 555 fungal isolates. The largest numbers were from urine (n = 358) and catheter tip samples (n = 76). NAC increasingly being implicated, which was reflected in the present study. Among the various fungal isolates obtained during this study, NAC was found to be most

Table 4: Antifungal susceptibility pattern of the candida isolates

Candida Species	Drug susceptibility pattern					
	Amphotericin B (10 µg)		Fluconazole (25 µg)		Voriconazole (1 µg)	
	Sensitive No. (%)	Resistant No. (%)	Sensitive No. (%)	Resistant No. (%)	Sensitive No. (%)	Resistant No. (%)
Candida albicans (n=157)	147 (93.6)	10 (6.4)	118 (75.2)	39 (24.8)	121 (77.1)	36 (22.9)
Candida non-albicans (n=384)	346 (90.1)	38 (9.9)	163 (42.4)	221(57.6)	166 (43.2)	218 (56.8)
Total (541)	493 (91.1)	48 (8.9)	281 (51.9)	260 (48.1)	287 (53)	254 (46.9)

frequent amounting to 384 (69.2%) outnumbering *C. albicans* (n = 157, 28.3%).

In an earlier study,[19] *Candida* species was reported to be the seventh most common nosocomial pathogen and it accounted for 25% of all the urinary tract infections. The present study had a male preponderance, and the same was observed in another study[26] where male sex was found to be a risk factor for developing candidemia. Though candidiasis can occur at all ages, studies by one study has shown that the highest incidence of candidiasis was in the age group over 50 years.[27] This feature has been corroborated in our study where 281 (50.6%) of all fungal isolates, including *Candida* spp were from patients of age group over 50 years. Studies over the years have shown that there is a considerable increase in the NAC isolates. In the present study we observed that NAC (69.2%) predominated over *C. albicans* (28.3%), which is in agreement with another Indian study[26] where the NAC incidence (70.7%) was higher than that of *C. albicans* (29.2%). Similar findings were seen in two other studies where NAC incidences were 70.7% and 54.1% respectively.[28,29] These findings seem to suggest that NAC have emerged as important pathogens in the recent years.

The *in vitro* susceptibility testing of antifungal agents is becoming increasingly important because of the introduction of new antifungal agents and the recovery of clinical isolates that exhibit intrinsic resistance or acquire resistance to amphotericin B or the azole group of drugs during chemotherapy. Antifungal susceptibility testing was done for 541 Candida isolates by disc diffusion method. We could not perform antifungal susceptibility testing for moulds as definite guidelines are not available.

Antifungal resistance, once rarely documented, has increased in the recent years. The problem is compounded by aggressive immunosuppression (acquired or induced), an ageing population, and the emergence of virulent and intrinsically resistant organisms. In this study, amphotericin B resistance was less as compared to the azole group of antifungal agents. Azole resistance was significantly more in NAC group as compared to *C.albicans*. For fluconazole, 57.5% of the NAC showed resistance compared 24.8% strains of *C. albicans* while the corresponding figure for voriconazole was 56.8% versus 22.9%. In a large study[30] comprising of 1106 cases of candidemia over a nine year period, all the *C.albicans* strains were found to be susceptible to amphotericin B, fluconazole and voriconazole, while among the NAC, 88.6% were sensitive to voriconazole, and 68.8% to fluconazole. Similar higher frequencies of resistance among NAC compared to *C.albicans,* has been observed in recent years from Europe[31] and China.[32] Resistance to azole group of antifungal agents can be due to quantitative or qualitative modifications of target enzymes, low access of the drug to the target, or a combination of these mechanisms.[33] Resistance to the azole group of antifungal agents is of concern because azoles like fluconazole are among the most commonly used antifungal agents for the treatment of candidiasis.[34] These drugs are safe and effective for the treatment of all clinical types of candidiasis. The broad use of triazoles, especially fluconazole, has given rise to concerns regarding the emergence of resistance.[35] We found the overall resistance to amphotericin B to be 8.9%, which is similar to the findings of the German-Austrian multi-centre study[31] which showed nearly 11.2% Candida isolates to be amphotericin B-resistant. In such a scenario of high frequency of resistance, need of the hour is to ensure that identification, isolation of Candida species and evalution of treatment options should become an integral part of clinical microbiology services.[36]

The role of diagnostic mycology laboratory is important in the management of fungal

infections. An understanding of fungal infections in each set-up will help greatly in improving diagnostic and therapeutic approaches. *NAC* infections are also increasing globally making speciation necessary. Azole resistance in Candida spp is alarming. So culture and identification of fungal infections to the genus and species level is essential for commencement of suitable antifungal therapy. The clinician-microbiologist collaboration will help in improving patient care.

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