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Original Research Article

ANTIMICROBIAL RESISTANCE IN CANDIDA SPECIES ISOLATED FROM CLINICAL SPECIMEN IN A TERTIARY CARE HOSPITAL

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Abstract

Background: Incidence of candidiasis is increasing worldwide. Species identification of Candida is important as non-albicans Candida is increasing in number and is more resistant to antifungal drugs. The aim of the study was to find out the frequency of Candida from different clinical specimens and determine the susceptibility of antifungal drugs to isolated Candida species. **Materials and Methods:** Total 100 Candida strains isolated from different clinical specimens were included in the study. Isolation, culture and speciation of Candida were done by standard procedure as per CLSI guidelines. Antifungal susceptibility test was done according to the National Committee for Clinical Laboratory Standards 2011, method for antifungal disc diffusion susceptibility for yeasts with approved guideline M44-A2. **Result:** Five species of Candida were identified. Species distribution was C. albicans 44, C. tropicalis 30, C. glabrata 15, C. krusei 7 and C. gulliermondii 4. Most of the isolates were from female 54% as compared to male 46%. Candida isolates showed susceptibility to Fluconazole 74%, Itraconazole 55%, Amphotericin-B 86%, Clotrimazole 70%, Ketoconazole 59% and Voriconazole 82%. Amphotericin-B was the most effective antifungal drug followed by Voriconazole and Fluconazole. **Conclusion:** Non albicans Candida was more prevalent than Candida albicans. There is an increase in the resistance of the Candida isolates to commonly used antifungal agents. Therefore, this study highlights the need for speciation of Candida isolates up to species level and determines the antifungal susceptibility pattern to decrease the morbidity and mortality of the patients.

INTRODUCTION

The incidence of infection caused by Candida spp. has increased steadily over the last two decades, and Candida albicans remains the most common fungal pathogen isolated from various clinical samples.[1,2] Numerous records have documented the increased incidence of non-albicans species among hospitalized and immunosuppressed patients.[3] Candidiasis represents 80% of human fungal infections and most of the cutaneous, oropharyngeal & particularly vulvovaginal infections. The occurrence of chronic disease such as cancer, diabetes & cardiopathies, together with the lifestyle changes of the general population in major urban centres, constitute other reasons for increase in Candidiasis. An increase in other species C. tropicalis, C. glabrata, C. krusei & C. parapsilosis was recorded and C. glabrata has become a prominent pathogen in some institutions.[4] Non-albicans Candida species are emerging opportunistic pathogens and they exhibit varying degree of resistance, either intrinsic or acquired or both, to commonly used antifungal drugs.[5] Candida albicans and non-albicans Candida species are closely related but differ from each other with respect to epidemiology, virulence characteristics and antifungal susceptibility. All Candida species have been shown to cause a similar spectrum of disease ranging from oral thrush to invasive disease, yet differences in disease severity and susceptibility to different antifungal agents have been reported.[2] Indiscriminate and widespread use of fluconazole for the prophylaxis and treatment of candidiasis has led to a reduction of infections due to Candida albicans but that has led to the emergence of

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Candida infections caused by fluconazole resistant NAC.[6] Hence, speciation and antifungal susceptibility of clinical isolates of Candida has gained significance in the management of Candida infections.

MATERIALS AND METHODS

The present study was conducted in the Department of Microbiology, MGM Medical College and Hospital, Navi Mumbai, India, from January 2014 to December 2016. The research study was cleared and approved by Institutional Ethical committee for research on 27th October, 2014 vide letter no. MGM/HIS/RS/2014-15. Written consent was obtained from all study participants before collection of specimens. In case of minors, informed consent was taken from the patients guardians. Privacy and confidentiality was maintained in all cases.

Inclusion Criteria
Patients of all age group and both sex with clinically suspected candidiasis, attending Outpatients Departments (OPD) and Inpatients Departments (IPD) were included in the study.

Exclusion Criteria
Patients who were on antifungal treatment and those who refused to take part were excluded from the study.

Sample Size Calculation: Considering 95% confidence interval, 4% margin of error, Z score of 1.96 and prevalence rate of 4.03, the sample size was taken 100 for the study using the following formula:[7]

Sample size (n) = $(z^2pq)/d^2$

Where, z = Z-score, p = Prevalence rate, q = (1-p), d = absolute allowable error.

Identification and speciation of Candida isolates.[8-10]

Sample collection: Various clinical sample such as urine, sputum, throat swab, vaginal swab, stool, endotracheal tube, nail clipping, skin scrapping, wounds and pus samples were collected under aseptic precautions and processed following standard techniques.

Direct Microscopy: All samples were subjected to Potassium Hydroxide (KOH) wet mount for yeast cells with pseudohyphae and Gram stain to look for gram positive, budding yeast cells with or without pseudohyphae, pus cells, epithelial cells or bacteria.

Culture: All samples were cultured on Sabouraud's Dextrose Agar (SDA) and incubated at 25ºC and 37ºC for seven days. Culture was examined daily for the microbial growth. On SDA, Candida produced creamy, smooth, pasty and convex colonies within 24-72 hours. Some species required more than three days to appear on culture medium.[8-10]

Gram Staining: Isolated colonies obtained on SDA were further subjected to gram staining to identify the budding yeast cell and pseudohyphae.

Urease test: The test was done to rule out Cryptococcus neoformans which is urease positive. Criteria used to indicate Candida infection in various samples

- Urine: Quantitative culture with colony count of >10^5 Colony-Forming Unit (CFU)/ml of urine is associated with infection in patients without indwelling catheters and >10^3 CFU/ml for catheterised patients. Pyuria usually supports diagnosis of Candida infection. Low colony counts in presence of pyuria were considered significant. Repeat isolation in same patient was also considered significant.[9]
- Sputum: Considered acceptable on gram stain when 25 or more polymorphonuclear leukocytes were seen per oil immersion (100x) field with few (<10) squamous epithelial cells.[11]
- Nail clipping and skin scrapping: Direct demonstration of pseudohyphae along with yeast cells using KOH wet mount.[10]
- Wounds and pus: Direct demonstration of pseudohyphae along with yeast cells using KOH wet mount or gram stain.[12]
- Stool, throat or oral and vaginal swabs: Direct demonstration of pseudohyphae along with yeast cells using KOH wet mount or gram stain.[12]

Speciation of Candida
Conventional methods: Germ tube test, demonstration of chlamydospore formation on Cornmeal agar with Tween 80, sugar fermentation test and sugar assimilation test and growth on Hicrome Candida agar were employed for speciation.[8,10]

Temperature test (Growth at 45°C): This test was used to differentiate Candida albicans (growth) from Candida dubliniensis (no growth). The temperature test was performed using Yeast-Peptone-Dextrose (YPD) broth and SDA and incubated at 45ºC for 10 days.[13]

Hicrome Candida differential agar: The Candida isolates were subculture on Hicrome Candida differential agar for species identification according to the manufacturer's instructions [Figure 1].[14]

1) Candida albicans: Light green coloured smooth colonies.
2) Candida dubliniensis: Dark green coloured smooth colonies.
3) Candida tropicalis: Blue to metallic blue coloured raised colonies.
4) Candida glabrata: Cream to white smooth colonies.
5) Candida krusei: Purple fuzzy colonies.
6) Candida guilliermondii: Light pink to pink colonies.
7) Candida parapsilosis: Light pink colonies.

Antifungal susceptibility testing: It was done by disk diffusion method according to the CLSI (formerly National Committee for Clinical Laboratory Standards) 2011, method for antifungal disc diffusion (using Mueller Hinton Agar with 2% glucose & 0.5µg methylene blue) susceptibility for yeasts with approved guideline M44-A2 using

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commercially available 6 mm antifungal discs (Himedia, India).[15] The following antifungal discs were tested – Fluconazole (10µg), Itraconazole (10µg), Ketoconazole (10µg), Clotrimazole (10µg), Voriconazole (1µg), Amphotericin B (20µg) and the zone of inhibition size was measured (Figure 2). Due to the lack of defined breakpoints for itraconazole, ketoconazole and amphotericin B arbitrary values based on other studies and manufacturer instruction manual (HIMEDIA) guidelines were employed [Table 1].[12,16]

Interpretive Categories

Susceptible (S): The susceptible category implied that an infection due to the strain might be appropriately treated with the dose of antimicrobial agent recommended for that type of infection and infecting species, unless otherwise contraindicated.

Susceptible-Dose Dependent (S-DD): The susceptible-dose dependent category included isolates with antifungal agent Minimum Inhibitory Concentration (MIC) that approached usually attainable blood and tissue levels and for which response rates might be lower than for susceptible isolates.

Resistant (R): This category included those resistant strains which were not inhibited by the usually achievable concentrations of the agent with normal dosage schedules or when zone diameters had been in a range where clinical efficacy had not been reliable in treatment studies.[12,15,16]

Quality control: Every batch of media prepared was checked for sterility by incubating at 37°C for 24 hours. Candida albicans American Type Culture Collection (ATCC) 10231 was used as quality control strain for the antifungal susceptibility testing.

All the reagents & media used in this study were supplied by Hi-Media Laboratory, Mumbai. Culture media were prepared in laboratory from dehydrated powder according to manufacturer instruction. ATCC control strain of Candida species was obtained from Microbiologics Inc, USA i.e. Candida albicans ATCC 10231, Candida glabrata ATCC 15126, Candida krusei ATCC 6258 and Candida tropicalis ATCC 750.

Statistical analysis: Data collected was entered in Microsoft excel sheet. Data was analysed using descriptive statistics. Analytical statistics such as Chi-square (χ2) was done to test for association and p-value <0.05 was taken as significant.

RESULTS

Out of total 1152 clinical specimens 100 Candida strains were isolated, which shows the incidence of Candida was 8.7% in clinical specimens. Five species of Candida were identified. The species distribution was C. albicans (44), C. tropicalis (30), C. glabrata (15), C. krusei (7) and C. gulliermondii

(4). Non-albicans Candida (NAC) predominated (56%) over Candida albicans (44%). Most of the isolates were from female (54%) as compared to male (46%) patients. Females were affected more than male. Maximum number of Candida isolates were from the age group >61 years. Maximum number of sample received was urine (260), followed by sputum, stool, HVS, Throat swabs and Nail clippings. Incidence of Candida were higher in High vaginal swab 11/52 (21.1%) followed by Urine 30/260 (11.5%), Sputum 25/250 (10.0%), Skin scrapings 9/53 (9.5%), Nail clippings 3/34 (8.8%), Throat swabs 6/70 (8.6%), Stool 10/157 (6.5%), Eye swab 3/32 (6.4%), Ear swab 2/50 (6%), Endotracheal tube 2/35 (5.7%) and least was seen in Pus 3/160 (1.9%). Candida albicans was the major isolate from sputum. Candida tropicalis, Candida glabrata & Candida krusei was the major isolates from urine sample [Table 2]. Comparison of antifungal susceptibility of Candida albicans and Non-albicans Candida shows that, C. albicans were more sensitive to fluconazole, Itraconazole, Clotrimazole, Ketoconazole & Voriconazole where as Non-albicans Candida showed more sensitive to Amphotericin-B. Amphotericin-B was the most effective antifungal followed by Voriconazole, Fluconazole, Clotrimazole, Ketoconazole and Itraconazole [Table 3].
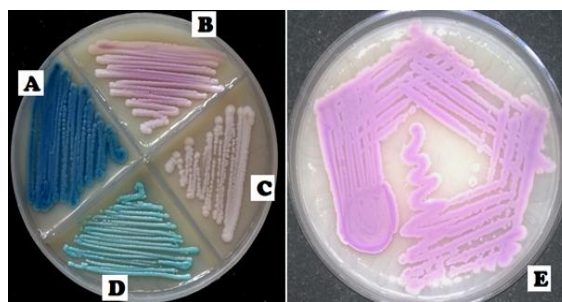
Figure 1: Growth of Candida species on Hicrome agar, (A) C. tropicalis, (B) C. gulliermondii, (C) C. glabrata, (D) C. albicans and (E) C. krusei.
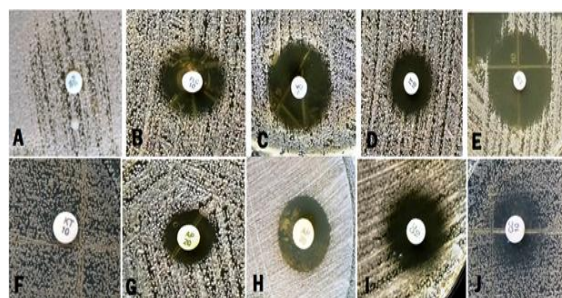
Figure 2: Antifungal susceptibility testing showing (A) Fluconazole resistant, (B) Fluconazole sensitive, (C) Voriconazole sensitive, (D) Itraconazole S-SD, (E) Itraconazole sensitive, (F) Ketoconazole resistant, (G) Amphotericin B S-SD, (H) Amphotericin B sensitive, (I) Clotrimazole sensitive and (J) Clotrimazole resistant.

Table 1: Antifungal susceptibility breakpoint zone diameter for Candida.[12,15,16]

S.N.	Antifungal agents	Disk Concentration	Zone diameter in mm		
			Resistance (R)	Susceptible Dose Dependent (S-DD)	Sensitive (S)
1	Fluconazole	10 µg	≤ 14	15 – 18	≥ 19
2	Itraconazole	10 µg	≤ 9	10 – 15	≥ 16
3	Amphotericin–B	20 µg	≤ 9	10 – 14	≥ 15
4	Clotrimazole	10 µg	≤ 11	12 – 19	≥ 20
5	Ketoconazole	10 µg	≤ 22	23 – 29	≥ 30
6	Voriconazole	1 µg	≤ 13	14 – 16	≥ 17

Table 2: Distribution of Candida species among different clinical specimens.

Candida species	HVS (%)	Urine (%)	Sputum (%)	Nail & Skin (%)	Stool (%)	Throat swab (%)	Pus (%)	ET tube (%)	Eye Swab (%)	Ear swab (%)
C. albicans (n=44)	5 (45.5)	12(39.4)	10 (40)	4 (50)	5 (50)	3 (50)	1 (33.3)	2 (100)	1(50)	1(33.3)
C. tropicalis (n=30)	2 (18.2)	9(30.3)	8 (32)	3 (33.3)	3 (30)	2 (33.3)	1 (33.3)	-	1(50)	1(33.3)
C. glabrata (n=15)	2 (18.2)	5 (18.2)	3 (12)	1 (16.7)	1 (10)	1 (16.7)	1 (33.3)	-	-	1(33.3)
C. krusei (n=7)	1 (9.1)	3 (9.1)	2 (8)	-	1 (10)	-	-	-	-	-
C. guillermondii (n=4)	1 (9.1)	1 (3)	2 (8)	-	-	-	-	-	-	-
Total (N=100)	11	30	25	8	10	6	3	2	2	3

Table 3: Antifungal sensitivity pattern of Candida isolates.

S.N.	Antifungal agents	Candida isolates (N=100)	C. albicans(n=44)	NAC(n=56)
1	Fluconazole 10µg Sensitive	74 (74%)	36 (81.8%)	38 (67.8%)
	Dose dependent	6 (6%)	2 (4.5%)	5 (8.9%)
	Resistance	20 (20%)	6 (13.7%)	13 (23.2%)
2	Itraconazole 10µg Sensitive	55 (55%)	25 (56.8%)	30 (53.6%)
	Dose dependent	8 (8%)	3 (6.8%)	5 (8.9%)
	Resistance	37 (37%)	16 (36.4%)	21 (37.5%)
3	Amphotericin B 20µg Sensitive	86 (86%)	36 (81.8%)	50 (89.3%)
	Dose dependent	9 (9%)	3 (6.8%)	5 (8.9%)
	Resistance	5 (5%)	5 (11.4%)	1 (1.8%)
4	Clotrimazole 10µg Sensitive	70 (70%)	35 (79.5%)	35 (62.5%)
	Dose dependent	4 (4%)	1 (2.3%)	3 (5.3%)
	Resistance	26 (26%)	8 (18.2%)	18 (32.2%)
5	Ketoconazole 10µg Sensitive	59 (59%)	30 (68.1%)	29 (51.7%)
	Dose dependent	5 (5%)	4 (9.1%)	1 (1.8%)
	Resistance	36 (36%)	10 (22.8%)	26 (46.5%)
6	Voriconazole 1µg Sensitive	82 (82%)	38 (86.3%)	44 (78.6%)
	Dose dependent	6 (6%)	2 (4.6%)	4 (7.1%)
	Resistance	12 (12%)	4 (9.1%)	8 (14.3%)

Table 4: Comparison of sensitivity pattern of Candida species to antifungal among various studies.
F: Fluconazole, V: Voriconazole, It: Itraconazole, Kt: Ketoconazole, AmpB: Amphotericin B

Authors, place and year of publication / Candida species with antifungal		Rajeevan S et al, Tamil Nadu, 2016.[34]	Gade N et al, Chhattisgarh, 2019.[35]	Talukdar A et al, Guwahati, 2020.[32]	Shwetha DC et al, Mandya, 2021.[28]	Chakraborty M et al, Kolkata, 2021.[33]	Verma S et al, Mumbai, 2021.[29]	Urvashi C et al, Manipur 2022.[21]	Our study, Navi Mumbai, India (%)
C. albicans	F	70	89.8	85	85.1	85	77	79.6	81.8
	V	-	100	100	85.1	100	78	84.1	86.3
	It	24	-	-	-	-	94	79.6	56.8
	Kt	27	-	-	-	-	-	63.6	68.1
	AmpB	100	97.9	100	100	56	97	93.2	81.8
C. tropicalis	F	89	89.8	28.5	93.8	43	79	31.3	73.3
	V	-	100	85.7	96.9	100	79	81.3	93.3
	It	82	-	-	-	-	93.5	56.3	53.3
	Kt	92	-	-	100	-	-	50	53.3
	AmpB	100	97.9	71.4		100	94.5	65.6	86.7
C. krusei	F	0	89.9	28	0	100	-	0	0
	V	-	100	28	66.7	100	100	100	71.4
	It	25	-	-	-	-	-	40	57.1

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	Kt	16	-	-	-	-	-	40	57.1
	AmpB	100	100	64	100	93	66	60	85.7
C. parapsilosis	F	29	-	-	-	93	60	50	-
	V	-	-	-	-	100	80	100	-
	It	21	-	-	-	-	-	25	-
	Kt	29	-	-	-	-	-	0	-
	AmpB	100	-	-	-	93	95	50	-

DISCUSSION

Candida is one of the most common emerging pathogen in immunocompromised patients and in patients with underlying risk factors. Therefore early detection of Candida species and their antifungal susceptibility testing are essential for the clinicians to choose the best therapeutic approach for the patients to reduce morbidity and mortality. A total 100 Candida species were isolated from various clinical specimens. These 100 Candida isolates were used for further study & analysis. Out of 100 Candida isolates, C. albicans were (44%) and Non-albicans Candida (NAC) spp. (56%).

Majority of isolates in the study were obtained from urine followed by sputum, high vaginal swabs, stool, throat swabs and skin. Moreover, Candida species are reported as seventh most common nosocomial pathogen in hospital settings causing 25% of all urinary tract infections (UTIs) in some of the previous studies.[17] Shaik N et al.,[18] and Joseph K et al.,[19] observed that maximum numbers of isolates were from urine 60% and 46.9% respectively followed by respiratory samples 17.3% and 20.4% respectively. However, Gopi A and Murthy NS,[20] observed maximum number of isolates from sputum 41.6% and urine sample 20.4%. Chongtham U et al,[21] observed majority of isolates were from sputum 43% followed by urine 34%. This might be due to the fact that the presence of fungi (yeasts and moulds) in sputum has been of increasing interest since the advent of antibiotics and steroids as common therapeutic agents.

In our study majority of Candida isolates were obtained from age group of >61 years (34%) and least was seen in <10 years (6%). This shows Prevalence increased with the age. Chongtham U et al,[21] showed maximum number of isolates were from age group >70 years (27%) and least seen in <10 years (5%) of age. Similar findings were found by Joseph K et al,[19] Goel R et al,[22] Predominance of Candida in elderly age group in the study might be due to the presence of significant co-morbid conditions like diabetes, hypertension, chronic obstructive pulmonary disease and prolonged antibiotics therapy. This study showed most of the Candida isolates were from female (54%) as compared to male (46%). This suggest that females are affected more than male which might be attributed to more number of cases in females with UTI, pregnancy, vaginitis, prolonged contact with water in housewives as in case of onchomycosis. Similar findings were obtained by Amar CS et al,[23] who reported 60.2% in females and 39.8% in male,

Chongtham U et al,[21] reported 53% in female and 47% in male. However, male preponderance has also been reported by Patel LR et al,[24]. In this study it was observed that prolonged antibiotics therapy was the most common predisposing risk factors followed by diabetes and HIV. Chakrabarthi A and Shivaprakash MR observed high rate of candidiasis in the patients with diabetes, HIV and patients with antibiotics administration of more than 7 days and receiving 3 or more antibiotics.[25] The occurrence of candidiasis in diabetic patients might be due to hyperglycaemic condition which favours the immune dysfunction and favours the growth of microorganisms there by increasing the susceptibility to various infections.

Our study showed NAC (56%) predominance over C. albicans (44%). C. tropicalis were the most common NAC. Similar studies were done by various authors from different place and year. Our study correlates with the findings of Pahwa N et al,[26] Pandit I et al,[27] Shwetha DC et al,[28] Verma S et al,[29] and Chongtham U et al,[21] However, various author Jayacharan AL et al,[30] Khadka S et al,[31] and Talukdar A et al,[32] reported significant predominance of C. albicans over NAC species. The distribution of Candida species is different in various regions and studies.

In our study, disc diffusion method for antifungal susceptibility testing of Candida was used. Among the azoles, voriconazole showed the maximum sensitivity 82% to Candida isolates and it was the most sensitive antifungal followed by fluconazole 71%, clotrimazole 70%, ketoconazole 59% and least in itraconazole 55%. However, amphotericin B showed sensitivity of 86% and resistance of 5% only. C. albicans showed 86.3% sensitive to voriconazole whereas, voriconazole showed resistance of 8% to Candida isolates.It has also been observed that all isolates of C. krusei were resistant to fluconazole as they are intrinsically resistant to fluconazole. Chongtham U et al,[21] reported maximum sensitivity to voriconazole 86%, amphotericin B 81% and least in ketoconazole 56% which is quite similar to our study. They also reported all isolates of C. krusei were resistant to fluconazole. Similarly, several antifungal sensitivity patterns were reported by other studies [Table 4]. While comparing the antifungal susceptibility of Candida albicans and Non-albicans Candida, C. albicans were more sensitive to amphotericin B, voriconazole, fluconazole and clotrimazole where as Non-albicans Candida showed more sensitive to Amphotericin-B, voriconazole and less sensitive fluconazole, itraconazole and ketoconazole. Use of Fluconazole should not be continued in patients with

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recurrent Candida infections. Rapid species identification from clinical specimens, standard drug susceptibility testing, constant monitoring and better interaction between the clinicians and the microbiologists would be an effective approach to decreased prevalence and drug resistance against candidiasis.

CONCLUSION

An increase in the prevalence of non-albicans Candida species has been noted during the last few decades. Increased incidence of systemic candidiasis along with antifungal resistance has become an important healthcare issue worldwide. Non-albicans Candida like C. tropicalis and C. glabrata exhibit a great degree of variation not only in their pathogenicity but also in their antifungal susceptibility profile. The change in epidemiology and pattern of antifungal susceptibility of Candida infection has made identification of aetiological agent compulsory along with its antifungal susceptibility. Resistance to antifungal agents is an alarming sign for the emerging common nosocomial fungal infections.

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