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ISOLATION, MOLECULAR CHARACTERIZATION AND ANTIFUNGAL SUSCEPTIBILITY OF CANDIDA SPECIES IN IMMUNOCOMPROMISED PATIENTS

Medical Science

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ABSTRACT

The emergence of non-albicans Candida species as significant pathogens has been well recognized and numerous records have documented the increased incidence of non-albicans species among hospitalized and immunosuppressed patients. Dissimilarity between species and strain identification through molecular characterization helps understanding the epidemiology of Candida species especially concerning about the method of transmission so that effective actions can be taken to prevent and control transmission of resistant pathogens. The present study was taken up with a view to get a better insight into the regional profile of Candida infections in immunocompromised patients from the native and catheter related sites, from hospitalized and non-hospitalized patients with suspected fungal infection and their correlation with patient parameters, which may help in determining effective treatment approaches. *C. albicans* is still the predominant isolate in immunocompromised patients but frequency of infection with non-albicans group is increasing especially *C. parapsilosis.* Fluconazole resistance was not seen in *C. albicans* group, but is increasing in non-albicans group. None of the *Candida* isolates showed resistance to Amphotericin B. Evidence suggests that some species of Candida have a great propensity to cause systemic, nosocomial, and superficial infections than other species.

KEYWORDS

Candida; Epidemiology; Genotyping; Identification; RAPD

INTRODUCTION

The genus *Candida* includes several species implicated in human pathology. *Candida albicans* is by far the most common species causing infections in humans. In the last decades Candida species have surfaced as a vital nosocomial pathogen in immunocompromised persons with severe underlying ailment and comorbidities, like in intensive care units (ICU) patients.[1,2] The emergence of non-*albicans Candida* species as significant pathogens has however been well recognized during the past decade. Numerous records have documented the increased incidence of non-*albicans* species among hospitalized and immunosuppressed patients. The common non-albicans species are *Candida glabrata, Candida tropicalis* and *Candida krusei*[3] whereas the newer species include *Candida Africana, Candida dubliniensis, Candida bracarensis* and *Candida nivariensis.*[4] Although this increased reporting may be caused by improved laboratory recognition, yet the emergence of these opportunistic pathogens is favored by the change in host susceptibility due to the growing number of immunocompromised individuals in the population as a result of the HIV pandemic and the use of long-term immunosuppressive therapy in cancer and organ transplant patients.

Candida albicans and *non-albicans* species are closely related but differ from each other with respect to epidemiology, virulence characteristics and antifungal susceptibility. All *Candida* spp. 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.. *Candidal* oesophagitis frequently occurs in AIDS and HIV infected patients, disseminated infection however, is common in other immunosuppressed patients.

Evidence suggests that some species of *Candida* have a great propensity to cause systemic, nosocomial, and superficial infections than do other species. *Candida* spp. identification is therefore important for successful management. Distinction between species and strain identification through molecular characterization facilitates the understanding of the epidemiology of *Candida* spp. particularly regarding the reservoir and mode of transmission which is a requirement for the development of effective measures to prevent and control transmission of resistant pathogens. Recognizing the local epidemiology of Candida is of great significance for the clinical management and treatment, particularly in hospitalized and non-hospitalized patients and their correlation with patient parameters, which may help in deciding effective treatment strategies. Therefore, it is important to confirm the prevalence and species of Candida, as well as the phenotypic and genotypic characteristics of these organisms.[5] From a variety of methods, the molecular techniques are the most suitable tools for epidemiological investigation of *Candida* species on both large-scale and small levels. Moreover they are accurate, easy to conduct, rapid and economical method of testing. The aim of this study was to assess the genetic relatedness among the Candida species isolated from hospitalized patients, by randomly amplified polymorphic DNA assay (RAPD).

MATERIALS AND METHODS

Study design

It was a prospective study in patients attending medicine OPD, ART centre, Radiotherapy OPD and patients admitted in Critical Care Medical Ward, ICU and Nephrology ward. The patients were screened and included according to the following inclusion criteria

Inclusion Criteria

a) HIV positive patients.
b) Cancer patients receiving Chemotherapy and/ or Radiotherapy.
c) Persistent Neutropenic patients
d) Patients with documented Bone Marrow Depression
f) Patients with Aplastic Anemia
e) Patients with Chronic Kidney Disease (CKD) on Dialysis with patent vascular accesses

Exclusion criteria:

a) Patients on chronic steroid therapy.
b) Diagnosed case of Hepatitis A, B and C
c) Patients of Bronchogenic Carcinoma
d) Patients of Fungal Lung disease/Aspergilloma.
e) Patients with Diabetes Mellitus.

One hundred and ten patients fulfilled the selection criteria and were enrolled in the study. The study was approved by the Institutional Ethics Committee and written informed consent was obtained from patients or their relatives.

A detailed history of tuberculosis, diabetes mellitus, steroid intake, antibiotic usage, immunosuppressive drugs, radiotherapy, chemotherapy, organ transplant and malignancy were taken, general physical examination and local examination of nail, genitalia and oral

cavity was carried out for every patient enrolled in the study. The patients were thoroughly investigated to ascertain their immunocompromised state and presence of *Candida* infection and were subjected to tests including complete haemogram, blood urea, serum creatinine, random blood sugar, urine- routine and microscopic, chest X-Ray PA view and upper G.I. endoscopy. CD4 count, isolation and culture characteristics including antifungal sensitivity and molecular characterization of isolated *Candida* species were also done. Oral swab culture and blood samples for blood culture were taken.

Media used: Sabouraud dextrose agar (SDA) [Emmon's modification], SDA with antibiotics, BHI Agar/ broth Biphasic medium for blood culture and CHROMagar.

Morphological and physiological studies

All the isolates were inoculated onto Sabouraud chloramphenicol agar (Bio-Rad, Marnes-La-Coquette, France) at pH 5 to 6 and were incubated at 30°C, 37°C, and 45°C for up to 7 days. The strains were sub-cultured on CHROMagar *Candida* medium (Becton Dickinson, Heidelberg, Germany), incubated at 37°C and examined after 24 to 72 h for colony color and morphology. Germ tube tests were performed by inoculating 2.0 ml of fresh, pooled, normal human serum with a fresh colony and incubation at 37°C for 2 h. To induce chlamydospores and pseudohyphal production, yeasts were incubated on potato carrot bile medium (Bio-Rad) and diluted milk medium for 24 to 48 h at 30°C.[6]

Characterization of Fungal Isolates

Identification & specification of the moulds was done based on the colony characteristics and morphology on lactophenol cotton blue preparation and gram's staining. Yeast isolates were identified on the basis of colony characteristics and further by germ tube production, morphology on corn meal agar (Hi Media), HiCrome *Candida* agar (Hi Media), urease test, carbohydrate fermentation tests [7] and assimilation tests using yeast nitrogen base agar (Hi Media). Triphenyl Tetrazolium Chloride salt reduction test was performed and different colonies of *Candida* were identified by the color they produce.[8] Subculture of all GTT positive and GTT negative isolates was done on CHROMagar and the colony morphology (colour) was noted to differentiate between *C. albicans* and *C. dubliniensis.* The urea hydrolysis test was also done in microtitre plates and was used to differentiate *Candida* species from cyptococcus.

Antifungal Susceptibility Testing

Susceptibility of all the isolates to antifungal fluconazole and Itraconazole were tested using the broth microdilution method. Minimum inhibitory concentrations (MIC) was recorded as the lowest concentration of the drug that produced a visible decrease in turbidity compared to that of the drug-free growth control which were determined according to Clinical and Laboratory Standards Institute (CLSI M27- A2 criteria).[9] All *C. albicans* isolates resistant to fluconazole were tested for itraconazole, ketoconazole and Amphotericin B by the broth microdilution method as per the CLSI guidelines. The quality control strains (*C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019) are tested in the same manner and included each time in the experiments. The MIC were recorded by visually using a plate-reading mirror and by the cell density.

Molecular Characterization

DNA Fingerprinting of the *C. Albicans* Isolates by Southern hybridization

Genomic DNA was isolated from each sample using conventional technique after lysis of the cells by Zymolyase (Seikagaku, Japan) treatment.[10] Around 2 µg chromosomal DNA from each isolates was digested with restriction enzyme EcoRI to completion and separated on agarose gel (0.8%). The gel was stained with Ethidium bromide (0.5(g/ml) and then denatured and neutralized.[11] The denatured DNA fragments were then transferred to nylon membrane (Sigma) then UV cross linked (Stratagene, USA) to the membrane and pre-hybridized in 300 mM phosphate buffer containing 7% SDS and 1 mM EDTA at 65ºC for 2–4 hours. In the next step immobilized fragmented DNA were hybridized with [^{32}P] dATP (Amersham Pharmacia Biotech, U. K.), labeled *C. albicans* specific probe CARE-2. Hybridization was carried out under high−stringency conditions following the manufacturer's recommendations.

Labeling of CARE-2 Probe

CARE-2 (*C. albicans* repetitive element -2) is a middle repetitive element isolated pathogenic yeast *C. albicans* and is interspersed and shows a high degree of RFLP. It is dispersed on all chromosomes, and southern blots hybridization demonstrates different copy numbers on different chromosome [12] CARE-2 distinguishes unrelated isolates and identifies the same strain in independent isolates.[13] For labeling of CARE-2 Probe, 5-10g of plasmid DNA (pRFL37) containing the CARE-2 sequence was digested with restriction enzyme *Kp*nI and *Pst*I [12] and the generated DNA fragments were separated on agarose gel (1%) in 1x TBE buffer and purified out of agarose. The purified DNA fragment was hybridized to random primers (Takara, Japan) and extended in presence of 200 M each of dGTP, dCTP, dTTP& 10 Ci [-^{32}P] dATP (6000 Ci / mmol.) by Klenow fragment at 30 °C for 30 minutes, labeled DNA fragment was then ethanol precipitated and washed several times with 70% ethanol to get rid of unincorporated nucleotides. Finally the precipitated DNA was dissolved in hybridisation buffer and used in subsequent step.

Dendrogram and cluster analysis

Dendrogram was generated by comparing the relatedness of DNA band pattern to compare the relatedness of the DNA banding patterns of different isolates in the autoradiogram, data was analyzed using the "PHYLIP" package (Version 3.65). The program "restdist" computed the distances using the character data and the output of this program was given as input to "neighbor" to cluster nearest neighbors. The output of this program was then fed to "drawgram" to generate the rooted tree.

RESULTS

Age and Sex Distribution among Patients and their clinical condition

The immunocompromised patients under study were of the age range 16 to 78 yrs with a mean age of 44.55±12.55 yrs. All the patients were divided into 7 age groups. Maximum numbers of patients were in the 41-50 years age group comprising of 42.7% of total no. of patients, followed by 31-40 years of age group (20.0%). Minimum numbers of patients were in the age group 71-80 years (1.8%). The study included 68 males (61.81%) and 42 females (38.19%).

The study enrolled patients of six clinical conditions in immunocompromised state. The maximum no. of patients was HIV positive (30%), followed by patients suffering from malignancies receiving chemotherapy or radiotherapy (25.45%), chronic kidney disease (19.09%) and neutropenia (18.18%) Patients of aplastic anemia (4.54%) and bone marrow depression (2.72%) constituted minimum number of patient included in the study

Frequency of Isolation of Different *Candida* Species from immunocompromised patients

Candida species were isolated from various sites from forty patients out of 110 patients included in the study. Thus the isolation rate was 36.36%. In the study population, *C.albians* predominated as the major isolate constituting of 52.5% of total isolation, whereas non-*albicans* group constituted 47.5% of the total isolates. Among non-*albicans* group, *C. parapsilosis* constituted the major species isolated (32.5%), followed by *C. tropicalis* (10%). *Candida glabrata* and *Candida krusei* were the rare species isolated in the study population (2.5% each)

Frequency of Isolation of Various *Candida* Species in Different Clinical Conditions Studied

The isolation rate was highest (69.69%) in HIV positive patients, lowest i.e 17.85% in malignancy patients and no *Candida* isolation observed in patients of bone marrow depression. [Table 1]

TABLE 1 frequency of isolation of various *candida* species in different clinical conditions

Clinical condition	Candida species isolated (%)	No isolation (%)	Total
HIV	23 (69.69%)	10 (30.31%)	33
Malignancy	05 (17.85%)	23 (82.15%)	28
Persistant Neutropenia	05 (25%)	15 (75%)	20
Aplastic Anemia	02 (40%)	03 (60%)	05
Bone Marrow Depression	00 (0%)	03 (100%)	03
Chronic Kidney Disease	05 (23.80%)	16 (76.20%)	21
Total	40 (36.36%)	70 (63.64%)	110

Distribution and frequency of Different Species Isolated, According to Body Site in Various Immunocompromised Patient Category

In the study population, out of the total no. of patients yielding positive isolates, 52.5% of total isolates (n=40) were from the single body site and 47.5% from multiple body sites. In only 2 patients, isolates were obtained from 4 body sites including blood.[Table 2, Fig 1]

TABLE 2: frequency of different species isolated, according to body site in various immunocompromised patient category

Patient Profile	Site	C. albicans	C.parapsilosis	C.tropicalis	C.glabrata	C.krusei
HIV (N=23)	Total	14	07	02	00	00
	Oropharynx	13	03	02	00	00
	Oesophagus	03	02	01	00	00
	Urine	03	06	01	00	00
	Blood	01	02	00	00	00
Malignancy on chemo. / Radiotherapy (N=5)	Total	02	02	00	00	01
	Oropharynx	02	02	00	00	00
	Oesophagus	01	01	00	00	00
	Urine	02	01	00	00	01
	Blood	00	01	00	00	01
Persistant Neutropenia (N=5)	Total	02	02	00	01	00
	Oropharynx	01	01	00	01	00
	Oesophagus	00	00	00	01	00
	Urine	02	01	00	00	00
	Blood	00	02	00	01	00
Aplastic Anemia(N=2)	Total	01	01	00	00	00
	Oropharynx	01	01	00	00	00
	Oesophagus	01	01	00	00	00
	Urine	01	01	00	00	00
	Blood	00	00	00	00	00
Chronic kidney Disesae(N=2)	Total	02	02	02	00	00
	Oropharynx	01	01	01	00	00
	Oesophagus	00	00	00	00	00
	Urine	02	02	01	00	00
	Blood	00	00	00	00	00
Bone marrow depression(N=0)	Total	00	00	00	00	00

– No of patients from which Candida species were isolated.

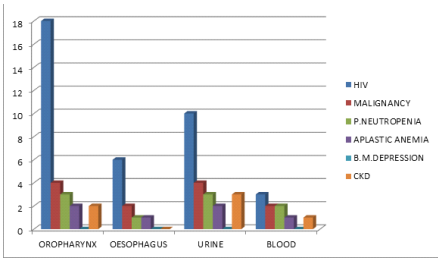

FIG 1 frequency of isolates according to body sites in different immunocompromised patients

Comparison of patient parameters according to positive and negative isolates and number of sites colonized

Positive isolate and negative isolate group were further studied for CD4 count, TLC and absolute neutrophil count. CD4 count and TLC were significantly low in the patient from which Candida species were isolated in comparison to the group which did not yield any positive isolate though ANC was high in the patient from which Candida species were isolated in comparison to the group which did not yield any positive isolate. [Table 3].

TABLE 3: Comparison of patients according to positive and negative isolates with CD4 count, TLC and ANC in immunocompromised patients

Parameters	Immunocompromised Patients			
	With Positive isolates(N=40) (mean±SD)	With Negative isolates(N=70) (mean±SD)	t value	p value
CD4 Count (per µL)	413.85±227.44	690.60±265.32	-5.11	0.000
TLC (per µL)	3107.75±1614.60	7831.78±30306.68	-1.30	0.198
ANC (per µL)	1587.10±1004.45	1089.21±658.21	2.81	0.007

TLC-Total leucocyte count, ANC-Absolute Neutrophil count.

Similarly CD4 count, TLC and ANC were low in the patient from which Candida species were isolated from multiple sites in comparison to the group in which positive isolates were obtained from single site. [Table 4]

TABLE 4: Comparison of various parameters of the patients with respect to single or multiple site yield of candida isolate

Parameters	Isolate Yield in Immunocompromised Patients			
	From single site(N=21) (mean±SD)	From multiple site(N=19) (mean±SD)	t value	p value
CD4 Count (perµL)	458.81±318.27	364.16±221.94	1.099	0.28
TLC (per µL)	3525.71±1460.31	2645.79±1688.13	1.75	0.088
ANC (per µL)	1847.19±805.33	1299.63±1139.84	1.74	0.092

TLC- Total leucocyte count, ANC- Absolute Neutrophil count.

Antifungal Susceptibility

All the 40 isolates of various *Candida* species obtained were tested for their antifungal susceptibility. The MIC was performed for amphotericin B, ketoconazole, fluconazole, and itraconazole. Amphotericin B (AMB) was the most active drug *in vitro*, with MIC_{50} and MIC_{90} values of 0.25 and 0.5 µg/ml respectively and all isolates were susceptible to AMB (MIC≤0.5 µg/ml). All isolates were susceptible to AMB and ketoconazole (MIC≤0.12 µg/ml), and all but one *C.albicans* isolate was also susceptible to fluconazole and itraconazole. Except for one, *C.tropicalis* isolate that was resistant to fluconazole (MIC>128 µg/ml) and itraconazole (MIC>64 µg/ml), all *C.albicans* and *C.parapsilosis* isolates were active against Ketoconazole (MIC_{90} 0.03 µg/ml) and Amphotericin B (MIC_{90} 0.12 µg/ml) respectively. MIC_{90} values for the other less frequently isolated spp. (including one isolates of *C.krusei*) was 8 µg/ml. Among all the azoles, amphotericin B was the most effective antifungal agent *in vitro* (MIC_{90} 0.25 µg/ml), followed by Ketoconazole (MIC_{90} 0.12 µg/ml). In general, the incidence of resistance to fluconazole was as low (1.3%) as the dose dependent susceptibility (2.5%). All *C.albicans* were susceptible for fluconazole (MIC_{90} 2 µg/ml) and all but one (susceptible dose dependent) *C.parapsilosis* isolates were also susceptible to fluconazole (MIC_{90} 1 µg/ml). Resistance among *C.tropicalis* for fluconazole was 1 out of 4 isolate tested (25%). All two of the less common *Candida* species isolated were resistant to Fluconazole. [Table 5]

Table 5: In-vitro antifungal activities of various Candida species.

Drug/Candida spp	No of isolate tested for for MIC/MFC	MIC50	MIC90	Range	MFC50	MFC90	Range
Amphotericin B							
C. albicans	21/21	0.125	0.5	0.03-0.5	0.25	0.5	0.25-1
C. parapsilosis	13/13	0.125	0.25	0.06-1	0.125	0.1	0.125-1
C. tropicalis	4/4	0.25	0.5	0.125-1	0.5	1	0.125-1
Other species	2/2	0.125	0.25	0.125-0.5	0.25	0.5	0.25-1
Ketoconazole							
C. albicans	21/21	0.03	0.03	0.03-1	0.06	0.06	≤0.12->12
C. parapsilosis	13/13	0.03	0.125	0.03-0.25	0.06	0.12	0.06-1
C. tropicalis	4/4	0.03	0.12	0.03-0.25	0.06	0.12	0.03-1
Other species	2/2	0.03	0.12	0.03-0.12	0.06	0.12	0.03-0.12
Fluconazole							
C. albicans	21/21	0.5	2	0.25-32	32	>64	2->64
C. parapsilosis	13/13	0.5	1	0.25-32	1	>128	1->128
C. tropicalis	4/4	0.5	64	0.125->64	>128	>128	>128
Other species	2/2	0.5	2	0.125-4	>32	>32	>128
Itraconazole							
C. albicans	21/21	0.06	0.25	0.03-0.5	>32	>32	1->32
C. parapsilosis	13/13	0.06	0.25	0.03-0.5	0.5	1	0.125-1
C. tropicalis	4/4	0.25	0.5	0.03-4	-	-	>64
Other species	2/2	0.25	0.25	0.03-0.25	-	-	>64
All organism (40)							
Ketoconazole (2.5%)		0.03	0.12	0.03-0.12	0.06	0.12	0.06-0.12
Fluconazole (2.5%)		0.5	64	0.125->64	1	>64	0.25->128
Itraconazole (10%)		0.06	0.5	0.03-4	>32	>32	0.125->64
Amphotericin B (0%)		0.25	0.5	0.03-1	1	1	0.125-1

Genotyping of the *Candida* Species Isolated from Immunocompromised Patients and their Cluster Analysis

The genetic relationships among the clinical isolates have been studied by DNA fingerprinting by employing southern blot hybridization. In this study the widely used middle repetitive DNA probe CARE-2 which is specific for *C. albicans* was used. When *Eco*RI digested genomic DNA of all the *Candida* isolates, were hybridized with [^{32}p] dATP-labeled CARE-2, the resultant hybridization profile were found to be relatively complex and each isolates was different and genetically unrelated irrespective of their origin. When these DNA fingerprinting patterns were further analyzed quantitatively using Dendron software as described by Pujol C *et al*[14] it was further confirmed that all the *C. albicans* isolates were genetically unrelated which indicates different source of infection. [FIG.2].

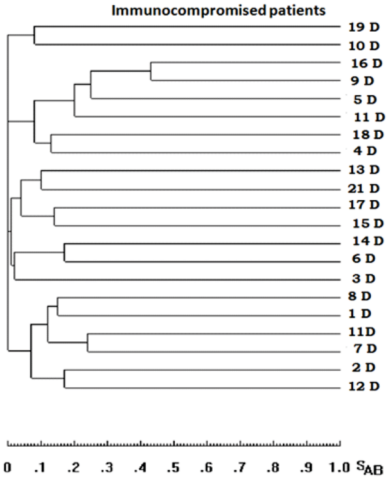

FIG.2 Dendrogram of the *C. albicans* isolates (total number 21) obtained from immunocompromised patients. The number on the right side of the figure indicates the isolates number.

RAPD Analysis of *non-albicans* Isolates

Since *C. parapsilosis* was the second most abundant species isolated from immunocompromised patients in our study, so we also typed some of the *C. parapsilosis* isolates to understand their relatedness. The method used to type the isolates was RAPD (Random amplification of polymorphic DNA), using the primer: NG2-5' CTGGCTTCTTCCAGCTTCA 3', as described by Vrioni and Matsiota-Bernard, 2001.[15] For RAPD analysis of *C. parapsilosis*, isolates were selected from different as well as the same patient. Similar to *C. albicans*, *C. parapsilosis* isolates also showed unrelatedness in isolates from different patients and high degree of relatedness in isolates from same patients as shown in [FIG. 3].

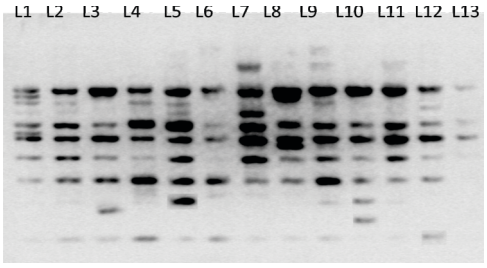

FIG 3: RAPD analysis of C. parapsilosis isolates from immunocompromised patients.

DISCUSSION

Over the past two decades, the incidence of the fungal infection has increased dramatically. Given the degree of immunosuppression that may be generated iatrogenically or secondary to HIV infection, any fungus present in the environment may cause localized or invasive infection when introduced into the appropriate host. As the population of immunosuppressed individuals increases, so do the numbers and types of fungal infections prominent in these patients. Although candidiasis remains the most common fungal infection in immunosuppressed patients, aspergillosis, zygomycosis, and other invasive filamentous fungal infections are major problems for certain groups of patients.

Over the past two decades many non-*Candida* a*lbicans Candida* (NCAC) species that are fundamentally different from each other and from *C. albicans* at the biological level have emerged as significant pathogens of clinical importance.[16] This diversity in the range of *Candida* species now associated with human infection has provided new challenges in the diagnosis and treatment of candidiasis and in the study of their virulence and biology.

The present study was conducted in a heterogeneous study group comprising of various diseased condition, clubbed together because of their immunocompromised state and thus rendering them susceptible for *Candida* infections.

With the mean age of 44.55 years, 62.7% of patients of this study were in the age group of 31 to 50 years. This section of the population formed the majority although extremes of age are considered immunocompromised, because of two reasons. Firstly neonates and infants were not included in the study, and only adult patients were

taken up for the study. Second, HIV positive patients forming the major immunocompromised state group in this study shifted the demographics towards youth and middle aged. These findings are consistent with the demographic data given by the National AIDS Control Organization (NACO) which reported mean age of 34 years, and also with studies elsewhere in India [17, 18] The Male: Female ratio in this study was 1.6:1, due to higher prevalence of HIV and lung malignancy in male sex, though no sex preponderance is seen with regards to *Candida* infection although vulvo-vaginal colonization provides an additional source in female patients.

The study evaluated on six clinical immunocompromised conditions. The maximum number of patients were HIV positive (30%), followed by patients suffering from malignancies receiving chemotherapy or radiotherapy (25.45%). Bone marrow depression and aplastic anemia constituted minimum number of patient included in the study. The reason for maximum number of HIV positive patients taken up for this study was their immunocompromised state and suspicion of *Candida* infection in them because mucocutaneus candidiasis is probably one of the commonest manifestations of HIV positive status worldwide with oropharyngeal candidiasis (OPC) being most widely reported. In India, other studies have reported its incidence from 50 -100 % [19,20]

In the present study *Candida* species were isolated from various sites from 40 patients out of 110 patients included in the study. Thus the isolation rate was 36.36%. Another study from the same geographical area showed an isolation rate of 10.6% but in that study high risk patients were taken up irrespective of their immune status. [21] *C. albicans* was found to be dominant as the major isolate constituting of 52.5% of total isolation, whereas non-albicans group constituted 47.5% of the total isolates. Among non-albicans group, *C. parapsilosis* constituted the major species isolated (32.5%), followed by *C. tropicalis* (10%). *Candida glabrata* and *Candid akrusei were* the rare species isolated in the study population (2.5% each). Grace et al in 2005 had also reported similar isolation rate of various *Candida* species, with *Candida albicans* in 43.15%, *Candida parapsilosis* in 21.05% and *C. tropicalis* in 9.47% of the isolates.[22]

HIV positive patients formed the major study population (30%) and the isolation rate was also maximum in this group (69.69%). Further maximum numbers of isolates were obtained from oropharynx (78.26%), which is lower than the western figure of about 90% [23,24] One can speculate that probably *Candida* carriage rate in Indian population is less.

The frequency of isolation of various *Candida* species was*C. albicans* (60.9%), *C. parapsilosis* (30.4%) and *C. tropicalis*(8.7%).Non-albicans *Candida* as an agent of oral candidiasis in HIV/AIDS patients is also documented in other studies. [18,25] In a study by Ismail H Sahand *et al.* on distribution of *Candida* isolates from oral swabs of HIV-infected patients similar results with *Candida* albicans isolated from 52% of patients and non-albicans *Candida* from the rest. [26] However another Indian study has reported 40% of all *Candida* isolates to be non-albicans.[18] Most interesting is that no *C. tropicalis* or *C. dubliniensis* was isolated. This is a surprising observation compared to the reports from developed world, where non-albicans Candidiasis is a major problem in HIV/AIDS.[27] Antifungal drug prophylaxis is rarely practiced in India and this may be the reason for this observed fact. Non-*C. albicans Candida* is not a major problem at this point of time in HIV/AIDS patients in this particular hospital where the study was conducted.

Although significant geographic variation is observed among cases of Candidaemia in different parts of the world, there appears to follow a specific pattern. In our study, non-albicans *Candida* bloodstream infections were more common than *C. albicans* bloodstream infections. This finding is consistent with other studies where non-albicans *Candida* species predominant in Asia, South Europe, South America and also in the subcontinent. [28] In our study among the non-albicans *Candida*, *C. parapsilosis* was isolated more frequently. Eiman Mokaddas *et al* have also reported similar incidence of *C. parapsilosis* candedemia. [29] Arora *et al* in a recent Indian study has also shown *C. parapsilosis*as an emergent cause of candemia which was 13% [30]

The incidence of invasive candidiasis has increased, largely propelled by advances in medical and surgical therapies that provide an optimum milieu for opportunistic infections in patients who are already immunocompromised. During the azole era, tremendous benefits were seen in curtailing *C. albicans* candedemia; however, more resistant *Candida* species emerged as a cause of disease. This change in epidemiology has been observed in individual institutions and in population-based surveillance programs also.

The observations made from our study showed that mean Total Leucocyte Count was low in the patient category yielding positive *Candida* isolate, in comparison to the patient group not yielding any isolate and lower the leucocyte count, more were the body sites inflicted. But these findings were not very significant. Though the mean absolute neutrophil count was low in the patient group from which *Candida* was isolated from multiple sites in comparison to those from which single site isolate was obtained, the comparison was not significant because of heterogeneous study group and different rate of isolation of *Candida* species from different clinically immunocompromised disease states. Further, infection with *Candida* species, esp. invasive has multiple and diverse risk factors and include haematological malignancy, central venous catheters, use of broad spectrum antibacterials, prolonged intensive care unit (ICU) stay, total parenteral nutrition, mucosal *Candida* spp. colonization and renal failure.

Our study revealed 100% and 97.4% susceptibility against AMB, and azoles respectively as also reported earlier. One of four (25%) isolates of C. tropicalis was resistant to flucocanozole and itraconazole individually. Some of the authors have shown reduced susceptibility of non albicans isolates against fluconazole. [31] In another prospective study, 349 *Candida* isolates obtained from colonization and systemic infections were analysed. [32] Resistance to fluconazole was observed in 3.4% of *C. albicans* isolates and in 30.7% of *C. glabrata* isolates. Only 2 strains (*C. glabrata* and *C. krusei*) were found resistant to amphotericin B (MIC =1 µg/mL). We observed very low rate of azoles resistance in our study which may be due to the reason that no topical or systemic antifungal agents are used prophylactically and their empiric use is restricted to only few cases in our centre.

The genetic relatedness of isolates of the same species has grown rapidly in recent years especially for epidemiology. The molecular typing of all the *C. albicans* isolates reported here revealed them to be unrelated to each other. The total unrelatedness of the isolates indicates the different source of each infection and the commensal status might become pathogen as immunocompromised state advances. Using the computer-based pattern analysis programs, like DENDRONE used in this study, it was possible to build up databases of DNA fingerprinting patterns and perform identity searches of new patterns in these databases.

The degree of unrelatedness of these isolates, as highlighted by cluster analysis further reaffirms relationship between commensalism and pathogenesis. Since no data and knowledge is available about the commensal status of the patients surveyed, it is not possible to speculate about the primary source of infection. The possibilities of a commensal organism becoming a pathogen, however, cannot be totally ruled out. Since, *C. parapsilosis*was the second most abundant species isolated from immunocompromised patients in our study, so we also typed some of the *C. parapsilosis* isolates to understand their relatedness. For RAPD analysis, *C. parapsilosis* isolates were selected from different as well as the same patient. Similar to *C. albicans*, *C. parapsilosis* isolates also showed unrelatedness in isolates from different patients and high degree of relatedness in isolates from same patients.

CONCLUSION

This is a non-interventional cross sectional observational study of various disease states in immunocompromised patients. *C. albicans*is still prevailing as the major isolate in immunocompromised patients but incidence of infection with non-albicans group is increasing. Among non-albicans group, *C. parapsilosis* constituted the major species isolated. Thus a changed pattern is observed in prevalence of different *Candida* species. Fluconazole resistance is not a major problem in *C. albicans* group, but there is increasing resistance in non-albicans group. No resistance to Amphotericin B was observed in any of the *Candida* isolate. This study is relevant in view of the changing pattern in the incidence of *Candida* species infections in different immunocompromised state, with changing drug resistance to antifungal agents especially in non-albicans group.

Conflict of Interest: There is no conflict of interest.

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