Molecular Speciation and Antifungal Susceptibility Profile of *Candida* Species in a Tertiary Care Centre in Central Kerala

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ABSTRACT

BACKGROUND
Candidiasis is a common life-threatening condition with various clinical manifestations. It can cause significant morbidity and mortality, especially in critically ill patients. Though mainly caused by *C. albicans*, it has shown a change in the epidemiological pattern with an increase in the prevalence of non albicans *Candida* (NAC) in the recent years. This change has also reflected in the profile of antifungal susceptibility since many of these species show high level of antifungal resistance associated with treatment failures. Thus, it is important to know the regional distribution of *Candida* species and also find their antifungal susceptibility profile to the commonly used antifungal agents.

METHODS
This is a descriptive study conducted for a period of two years on the clinical isolates of *Candida* species which satisfied the inclusion and exclusion criteria. Speciation was done by phenotypic methods which include germ tube test, chromogenic medium, corn meal agar and VITEK-2 system and by molecular methods using multiplex PCR. Antifungal susceptibility testing was done using automated method by VITEK-2 compact system.

RESULTS
Among the 80 isolates studied, on speciation *C .albicans* 25(31.25%) was the most common, followed by *C. tropicalis* 23 (28.75%), *C. parapsilosis* 13 (16.25%), *C. krusei* 12 (15%), *C. pelliculosa* 4 (5%), *C. auris* 2 (2.5%) and *C. glabrata* 1 (1.25%). Most isolates were susceptible to almost all the antifungal agents tested, but *C. krusei* showed high level of resistance.

CONCLUSIONS
C. albicans was the commonest individual species, but there was an overall predominance of non-albicans *Candida* (NAC). Speciation of *Candida* isolates is important as there is wide variation in their antifungal resistance pattern. Knowledge about the prevalent species and their antifungal susceptibility will help in early initiation of appropriate treatment, thus reducing the morbidity and mortality associated with *Candida* infections.

KEY WORDS
Candidiasis, Non Albicans *Candida* (NAC), Antifungal resistance, *Candida* Speciation

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BACKGROUND

Candidiasis is a common infection caused by yeast-like fungus, affecting both sexes, all age groups and is worldwide in distribution. Candidiasis includes diverse group of infections caused by *Candida albicans* or by other members of genus *Candida.* It produces infections that range from nonlife threatening mucocutaneous illness to invasive conditions which may involve virtually any organ. They are opportunistic pathogens in immunocompromised patients.[1]

There has been a significant increase in the number of reports of systemic and mucosal yeast infection over the last few years. These infections have also had a direct impact on the choice of empiric antifungal therapy and clinical outcome. Hence the clinical importance of species level identification and outcome has been recognized as the *Candida* species vary in the expression of virulence factors and antifungal susceptibility. *Candida* species are considered to be as the normal flora of human skin and mucosa but have been frequently reported as pathogens and the risk factors are due to excessive consumption of broad-spectrum antibiotics, underlying malignant diseases, HIV infections, organ transplantation, prolonged hospital stay and also exposure to invasive procedures.[2]

Species level identification of *Candida* species have become of utmost importance as many cases of Candida infections are being reported. For the past few years, an epidemiological shift has been noted from *Candida albicans* to non albicans *Candida* (NAC) species. As the conventional methods of identification of Candida species are often time consuming and may lead to inconclusive results, molecular diagnosis of Candidiasis has gained importance in the recent years because of its higher sensitivity and turn-around time.[3] The recent studies have suggested that with the introduction of fluconazole and itraconazole, there is increased prevalence of non albicans *Candida* (NAC) species. Infections with *Candida tropicalis*, *Candida glabrata*, *Candida krusei* and other *Candida* species are emerging as important opportunistic pathogens. This transition has a significant clinical impact due to the decreased susceptibility of many of these non albicans yeasts to antifungal agents. Thus, it is important to have an awareness about the different *Candida* species prevalent in a locality and their antifungal susceptibility pattern to start prompt and proper antifungal therapy thereby reducing the morbidity and mortality of such infections.

Mortality rates due to candidemia and disseminated candidiasis have not improved markedly over the last few years and have remained in the range of 30-40%, corresponding to 2,800-11,200 deaths annually. One of the studies published in India in 2015 has shown that India has one of the highest rates of *Candida* bloodstream infections in the world. Mortality varied from 35-75% that is about 40,000 deaths among 14.3 million ICU patients in India each year.[4]

Very few published data are available from Kerala on identification of *Candida* species. As the antifungal resistance is on a rise, we need to speciate the *Candida* species for proper treatment. In the present study, we aim to do identification of the *Candida* species both by conventional and molecular methods and also assessing the performance of the phenotypic methods for speciation of *Candida* species using molecular method as gold standard. In addition to that, we also aim to do antifungal susceptibility testing of the isolated *Candida* species. Therefore, early isolation, speciation and antifungal susceptibility will aid the clinicians to institute proper antifungal therapy thus decreasing morbidity and mortality.

METHODS

This descriptive study was performed in the microbiology department of a tertiary care hospital in Thrissur, Kerala during December 2017 to August 2019. Institutional ethics committee approval was obtained prior to the study. As per the suggestions from the statistician based on the prevalence observed in an earlier publication [5], a minimum of 76 *Candida* isolates had to be included in the present study. We included all clinically significant (as per the clinical history and microscopic picture of the samples) nonrepetitive isolates of *Candida* species, obtained from patient samples received in the routine microbiology laboratory, in the study. Isolates which appeared to be colonizers or commensals were omitted. The patient samples were sent by the treating doctors in appropriate containers and were cultured on routine media as per the laboratory SOP (standard operative procedures).

The isolates were subjected to speciation by conventional phenotypic methods which included Germ tube test and characteristics on Corn meal agar (Hi Media laboratories), colony colour on chromogenic medium (HiCrome agar by Hi Media laboratories), as well as by automated method by VITEK 2 compact system (Biomerieux Pvt Ltd) using ID-YST cards. All the instructions by the manufacturers were strictly followed during all the procedures. All isolates were also subjected to speciation by molecular method- Multiplex PCR (polymerase chain reaction) using the primers (Table. 1) and conditions given below. [6,7]

DNA Isolation

Fresh culture of the test isolates on Sabouraud's dextrose agar were used for colony PCR. A part of a single colony was picked with a sterile toothpick and suspended in 500 µl sterile nuclease free water in PCR tubes, then subjected to boiling and freezing at -80^{0}C, repeating this freeze thawing 3 to 4 times. PCR amplification: Multiplex PCR was carried out using the yeast specific primers and species-specific primers described in earlier studies.[4] The amplification was performed in a 25 µl volume consisting of 2x PCR buffer, 0.6875 µl of each primer, 10 µl of DNA template and remaining volume consisting of sterilized water. PCR was carried out in a thermocycler under the following cycling conditions: 40 cycles of 15 s at 94^{0}C denaturation, 30 s at 55^{0}C annealing, and 45 s at 65^{0}C extension, after a 10-min initial period of DNA denaturation and enzyme activation at 94^{0}C. The PCR products were analysed by electrophoresis with 2% agarose gels in TBE (tris-borate- EDTA) buffer. The gels were stained with ethidium bromide (75 µl in 500 ml distilled water) and PCR products were visualized with UV light using gel documentation system. Antifungal susceptibility testing: We also tested the susceptibility of these isolates to antifungal agents by automated VITEK 2 compact system using AST YS08 cards.

Statistical Analysis

On evaluation of the phenotypic methods with PCR as gold standard, the sensitivity and specificity of germ tube test (GTT), HiCrome *Candida* differential agar medium, corn meal

agar (CMA) and VITEK-2 system was calculated to be 88% and 100%; 91.25% and 100%; 83.75% and 100%; both 100% respectively.

RESULTS

A total of 80 clinically significant *Candida* isolates were enrolled in the study which satisfied the inclusion and exclusion criteria. Among the 80 isolates, 50 (62.5%) isolates were from males and 30 (37.5%) from females. Maximum number of samples belonged to the age group above 60 years (34) followed by neonates (23), 31-60 (17) and less than 30 years (6). The median age of study subjects was calculated to be 54 years. Thirty nine of these 80 *Candida* isolates were from blood samples, 19 from urine samples, 10 from pus and exudates and 12 from body fluids (table 2).

Speciation of Isolates

The phenotypic followed by molecular speciation (figure 1) of the isolates identified 25 isolates as *C. albicans* (31.25%). The rest 55 isolates of nonalbicans *Candida* species included 23 *C. tropicalis* (28.75%), 13 *C. parapsilosis* (16.25%), 12 *C. krusei* (15%), 4 *C. pelliculosa* (5%), 2 *C. auris* (2.5%) and one *C. glabrata* (1.25%). Isolates of *C. pelliculosa* and *C. auris* were identified in the mycology section of All India Institute of Medical Sciences, New Delhi since primers for these species were not included in our study and hence could not be identified.

Quality Control

Quality control tests was carried out with standard ATCC strains of *C. albicans* (ATCC 10231) and *C. tropicalis* (ATCC 750) along with 8 bacterial strains and 2 fungal strains which were: *E.coli, Klebsiella, Salmonella, Shigella, Pseudomonas, Staphylococcus, Streptococcus, Cryptococcus* and *Geotrichum* species. None of the negative controls have shown any bands in PCR. The clinical specimens from which different *Candida* species were obtained are shown in the Table 3. Among the 25 isolates of *C. albicans*, 7 were from pus samples, 5 from blood, 4 from urine and 9 from other body fluids. Non albicans *Candida* species were recovered from 34 blood samples, 15 urine samples, 3 pus and 3 other body fluids.

Candida Species	Primer Name	Sequence (5'-3')	Amplicon Size (bp)
Universal primers	UNI1 UNI2	GTCAAACTTGGTCATTTA TTCTTTTCCTCCGCTTATTG	
C. albicans	Calb	AGCTGCCGCCAGAGGTCTAA	583/446
C. glabrata	Cgla	TTGTCTGAGCTCGGAGAGAG	929/839
C. krusei	Ckru	CTGGCCGAGCGAACTAGACT	590/169
C. tropicalis	Ctro	GATTTGCTTAATTGCCCCAC	583/507
C. parapsilosis	Cpar	GTCAACCGATTATTTAATAG	570/370
C. guilliermondii	Cgui	TTGGCCTAGAGATAGGTTGG	668/512
C. lusitaniae	Clus	TTCGGAGCAACGCCTAACCG	433/329
C. dubliniensis	Cdub	CTCAAACCCCTAGGGTTTGG	591/217

Table 1. Primers Used for Identification of Candida Species

Antifungal susceptibility profile (table 4): Antifungal susceptibility testing was done for all isolates except the two *C. auris* for which guidelines are not available. All the *C. albicans* strains were susceptible to the antifungal agents tested except one which was resistant to amphotericin B. *C. tropicalis* and *C. pelliculosa* showed 100% susceptibility to all the agents tested. *C krusei* strains were 100% resistant to fluconazole, amphotericin B and *caspofungin.* All isolates were sensitive to micafungin. *C. parapsilosis* strains were 100% susceptible to fluconazole, amphotericin B, micafungin, caspofungin and flucytosine.

Samples	Number (%)
Blood	39(48.75%)
Urine	19(23.75%)
Pus	10(12.5%)
Other body fluids	12(15%)
Total	**80**

Table 2. Distribution of Samples

Candida Species		Total	Blood	Urine	Pus	Other Body Fluids
C. albicans (N=25)		25	5	4	7	9
Non albicans *Candida* (NAC) (N=55)	*C. tropicalis*	23	7	13	2	1
	C. parapsilosis	13	10	1	1	1
	C. krusei	12	11	0	0	1
	C. pelliculosa	4	4	0	0	0
	C. auris	2	2	0	0	0
	C. glabrata	1	0	1	0	0
	Total	**80**	**39**	**19**	**10**	**12**

Table 3. Distribution of Candida Species in Clinical Specimens

Candida Species	FLC	VRC	AMP B	MICA.	5FC	CASPO.
C. albicans (N=25)	25 (100%)	25 (100%)	24 (96%)	25 (100%)	25 (100%)	25 (100%)
C. tropicalis (N=23)	23 (100%)	23 (100%)	23 (100%)	23 (100%)	23 (100%)	23 (100%)
C. krusei (N=12)	0	12 (100%)	0	12 (100%)	1 (8.3%)	0
C. parapsilosis (N= 13)	13(100%)	12(92.3%)	13(100%)	13 (100%)	13 (100%)	13 (100%)
C. pelliculosa (N= 4)	4 (100%)	4 (100%)	4 (100%)	4 (100%)	4 (100%)	4 (100%)
C. glabrata (N=1)	0	1(100%)	1 (100%)	1(100%)	1 (100%)	0
Total (N=78)	65(83.3%)	77(98.7%)	65(83.3%)	78(100%)	67(85.9%)	65(83.3%)

Table 4. Antifungal Susceptibility of the Candida Species

*FLC- Fluconazole, VRC- Voriconazole, Amp B- Amphotericin B, Mica- Micafungin, 5FC- Flucytosine, Caspo- Caspofungin

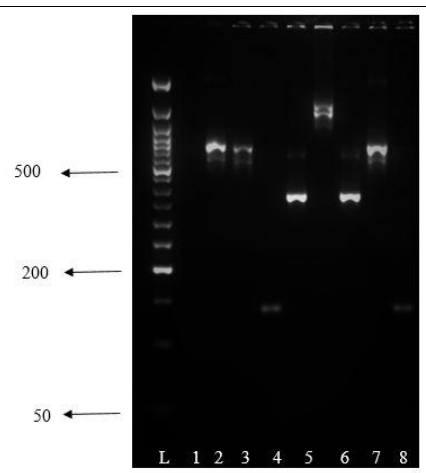

Figure 1. Agarose Gel Showing the Results Obtained for Multiplex PCR of Isolated Yeast Genomic DNA. Lanes: (L) 50-bp DNA Ladder, (1) Negative Control, (2) C. albicans, (3),(8) C. tropicalis, (4) C. krusei, (5),(7) C. parapsilosis, (6) C. glabrata

DISCUSSION

Candida infections have been increasingly reported as an important cause of mortality and morbidity in the recent years. Although *C albicans* had been the predominant yeast causing such infections, an emergence of other species of *Candida* with increasing resistance to the commonly used antifungal agents have been increasingly recognized in the

recent years globally. It is hence of utmost importance to have an awareness about the prevalent *Candida* species and their antifungal susceptibility pattern for prompt initiation of therapy and better clinical outcome.

The study was conducted to find out the distribution of various *Candida* species among the clinical isolates in our centre which is an 1800 bedded teaching hospital and their antifungal susceptibility pattern. A total of 80 *Candida* species isolated from clinical specimens that were received in the microbiology laboratory were enrolled in the study. The median age of the patients under study was 54 years. In the present study, males constituted 62.8% and females 37.1% of the patients. Among the 80 *Candida* isolates studied there were 25 (31.25%) *C. albicans* and 55 (68.75%) nonalbicans *Candida* species. The major non albicans species was *C. tropicalis* 23 (28.75%) followed by *C. parapsilosis* 13 (16.25%), *C. krusei* 12 (15%), *C. pelliculosa* 4 (5%), *C. auris* 2 (2.5%) and *C. glabrata* 1 (1.25%). Thus, in the present study though *Candida albicans* is still the commonest single isolate, overall, nonalbicans *Candida* species predominated.

Candida albicans had been the most commonly isolated species previously which accounts for 70-80% of the Candidiasis. During the last 10 years, this trend has been changing as NAC isolates are accounting for a major part of *Candida* infections.[7] This change has been reported in many studies from India[8] as well as abroad.[9,10] Bhattacharjee P had reported *Candida* albicans as the commonest species (48.57%) followed by *Candida tropicalis* (24.28%).[8] In a study from North American medical centres, a predominance of non-albicans species was noted; although *C. albicans* was the most frequently isolated species, it was followed by *C. glabrata* and other non- albicans *Candida species*.[11] Similar changes in the epidemiology have also been reported in studies from Latin American countries. In Chile, the prevalence of *C. albicans* has changed, with a progressive increase in non-albicans infections where *C. parapsilosis* was the most frequent species, followed by *C. tropicalis* and *C. glabrata*.[12,13] The reasons for this change in epidemiology could be severe immunosuppression, comorbidities, prematurity, prolonged antibiotic therapy, elderly patients, indwelling devices etc.

In our study, the commonest NAC species recovered was *Candida tropicalis* (28.75%). This finding is consistent with many similar Indian studies also.[12] A study from Mumbai in 2019 has shown highest isolation rates for *C. tropicalis* (10/29) followed by *C. parapsilosis* (8/29), *C. albicans* (3/29) and *C. krusei* (3/29)[14]. In a 3 years study conducted in trauma patients at All India Institute of Medical Sciences, *C. tropicalis*, 82 (38.7%) was the most common isolated followed by *C. parapsilosis*, 43 (20.3%) and *C. albicans,* 29 (13.7%).[15] A surveillance study conducted in around 39 countries had shown increase in the prevalence of *C. tropicalis* (4.6% in 1997 to 7.5% in 2003) and *C. parapsilosis* (4.2% in 1997 to 7.3% in 2003) with a rise in isolation rates of rare species like *C. guilliermondii, C. kefyr and C. rugosa*.[16]

Candida krusei (15%), Candida parapsilosis (16.25%) and *Candida tropicalis* (28.75%) are the predominant species from blood stream infections in our study. *Candida parapsilosis* has emerged as an important agent in nosocomial infections in several studies as reported in a review by Sardi et al.[17] In some other Indian studies like that of Singh et al.[18], Chakrabarti A et al.[19], Xess et al. [15], *C. tropicalis* is identified as the predominant species causing candidemia in North India[15] and Maharashtra[14]. In contrast to these findings *C. albicans* still constitute the most prevalent fungal species in candidemia (46.3%), followed by *C. parapsilosis* (19.5%), *C. glabrata* (15.9%), *C. tropicalis* (14.6%) in a study from China by Xiao et al.[20]

In the present study we got 4 isolates of *C. pelliculosa* also which were isolated from an outbreak of nosocomial fungaemia in neonatal intensive care unit (NNICU). A study by Chakrabarti A. et al in 2001 highlights the importance of *C. pelliculosa* as an emerging pathogen in neonatal ICUs where he reports 379 cases (4.2 % of admissions) over a period of 23 months.[21] During the study period we also got 2 isolates of *Candida auris*. *Candida auris* is an emerging multidrug resistant yeast which causes superficial and invasive infections with high mortality that has been reported globally and from India. The fungus *C. auris* was first noticed in India during a multicentric study of Candidemia across 27 ICUs in 2011.[22-24] It had caused infections in 5.3% of these episodes, thus becoming fifth in position of the agents causing candidemia.[23,24]

In our study, among the 19 *Candida* isolates from urine sample, *C. tropicalis* was the commonest (13/19) followed by *C. albicans* (4/19) and *C. parapsilosis* (1/19). A study conducted in Rajasthan in 2014 has shown the isolation rates of *Candida* in urine samples as 10.2%. Here, the non albicans *Candida* group showed a predominance (88.4%) over *C. albicans* (11.6%). Among the non albicans isolates, most common was *C. tropicalis*, 61 (54.5%) followed by *C. glabrata* 28 (25%).[25] In the present study all the isolates (except 2 isolates of *C. auris*) were tested for their antifungal susceptibility using automated method (VITEK 2 compact system) to Amphotericin B, Fluconazole, Voriconazole, Micafungin, Caspofungin and Flucytosine. All the 25 isolates of *Candida albicans* showed 100% susceptibility to all the above-mentioned agents tested except one which was resistant to Amphotericin B. Highest level of resistance was shown by *C. krusei. C. tropicalis, C. parapsilosis, C. pelliculosa* and *C. glabrata* showed 100% invitro sensitivity to amphotericin B whereas *C. krusei* was 100% and *C. albicans* was 4% resistant to amphotericin B. Although amphotericin B has good activity against most species its use has been restricted due to the nephrotoxicity associated with it.

Fluconazole is the most common antifungal agent used for treatment of disseminated candidiasis and also for prophylaxis, especially in low birth weight infants. Excellent oral bioavailability also makes it the most preferred agent for treating fungal infections. In the present study fluconazole showed excellent in vitro activity against all *C. albicans strains* but resistant to all isolates of *C. krusei* and one isolate of *C. glabrata*. However, *C. krusei* are intrinsically resistant to fluconazole.[8] Though some Indian studies have reported high rates of fluconazole resistance,[26] our findings show that it still can be continued as the first line agent in infections with *C albicans* in our geographical area. In contrast, for infections with non albicans *Candida* it can be used only after testing the susceptibility. In a similar study, overall fluconazole resistance was reported as 34.8% in various species.[8] Overuse of fluconazole might be one among the reasons for the NAC predominance in the recent years. Voriconazole was sensitive in all strains except one *C. parapsilosis* strain. Voriconazole has high susceptibility rates in many of the earlier studies.[14]

In our study, micafungin shows 100% sensitivity to all the 78 *Candida* isolates tested. Resistance to flucytosine and caspofungin was seen only for *C. krusei*. Caspofungin is reported to have good activity against many non albicans *Candida* and is now increasingly used for treatment of invasive infections especially health care associated infections with NAC. So, in our study, even though *C. albicans* is still the commonest individual species, there is overall predominance of NAC over *C. albicans*. Also, antifungal resistance was low as compared to other studies. It is important to know the prevalence of the *Candida* species in a geographical area which will help in proper management of the Candidiasis and also in the modification of antibiotic policies.

CONCLUSIONS

Candidiasis is recognized as an important cause of serious infections leading to high morbidity and mortality worldwide. The current changing epidemiological pattern and increasing incidence of non albicans *Candida* (NAC) have led to a hike in antifungal resistance pattern leading to failures in empirical therapy with the common antifungal agents. Knowledge about the prevalent *Candida* species in a geographical area will help the clinicians to institute proper antifungal therapy in appropriate dose thereby avoiding any treatment failures and mortality.

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