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Original Research Article

Isolation, Identification and Antifungal Susceptibility Testing of Various Candida Species in Tertiary Care Hospital

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Abstract

Background: Testing for antifungal susceptibility and identification of Candida to the species level has critical importance in the treatment of fungal infections. This study set out to do two things: (1) isolate and identify the species of Candida from various samples of individuals clinically suspected of having candidiasis; and (2) assess the susceptibility pattern of the Candida species isolates against the routinely used anti-fungal medications.

Material & Methods: From September 2021 through September 2022, a cross-sectional analysis of patients with clinically suspected candidiasis was done in the Department of Microbiology at a tertiary care center in Silvassa. Colony morphology on Saboraud's Dextrose agar and HiCrome Candida differential agar was used to identify Candida species isolated from various sources positively. Testing for antifungal susceptibility was conducted using the disk diffusion method as recommended by CLSI M44-A2.

Results: A total of 205 eligible samples of were included in this study. The mean age of the study participants was 37.05 ± 23.03 years. Majority were between 18 to 60 years of age group. Proportion of males and females were almost equal (49.3% and 50.7% respectively). The most of the samples was urine (62.9%) followed by blood (13.7%). Candida Albicans was the most prevalent Candida species in the analysed sample (65.9%), followed by Candida Krusei (13.7%), Candida Tropicalis (10.7%) and Candida Glabrata (9.8%). The amphotericin-resistance patterns of Candida albicans, Candida glabrata, Candida krusei, and Candida tropicalis were 24.44%, 25%, 25%, and 9.09%, respectively. In 20.74% of the samples, Candida albicans was resistant to fluconazole, 51.84% to clotrimazole, 11.11% to ketoconazole, and 12.59% to nystatin. Clotrimazole was the medication with the highest rate of resistance among the four Candida species.

Conclusion: In hospital settings, Candida spp. have emerged as the major pathogens responsible for opportunistic infections. To decrease morbidity and mortality, early isolation, species

identification, and antifungal susceptibility testing are critical for doctors to pick the optimal treatment strategy for patients.

Keywords: Isolation, Identification, Antifungal Susceptibility Testing, Candida Species, Tertiary Care Hospital

Introduction

Candida is a unicellular fungus that is crucial in various biological processes. It has an oval shape and belongs to a genus that encompasses several species capable of causing human infections, with Candida albicans being the most common occurrence [1,2].

Infections caused by Candida can appear in various parts of the body, such as the mouth, throat, digestive tract, and genitalia. The symptoms of these infections can range from mild to severe and include itching, burning, discomfort, and discharge. Several factors, including antibiotic usage, a weakened immune system, hormonal abnormalities, and poor hygiene, can influence the development and proliferation of Candida. In healthy individuals, Candida is typically controlled by the immune system and beneficial microorganisms in the gut. However, when these defenses are compromised, Candida can overgrow, resulting in an infection. The diagnosis of Candida infections usually involves clinical symptoms and laboratory testing to confirm the presence of the fungus. Treatment options may vary, depending on the severity of the infection, from topical antifungal creams to oral antifungal drugs [3-5].

One critical aspect of managing Candida infections is testing for antifungal susceptibility, which helps determine the most suitable antifungal medication for a particular patient. This test involves cultivating the Candida species obtained from a patient and examining its susceptibility to different antifungal medications. Antifungal susceptibility testing techniques include the broth microdilution method, the disk diffusion method, and the E-test. These methods determine the minimum inhibitory concentration (MIC) of an antifungal agent, which is the lowest concentration capable of inhibiting the growth of Candida species [6-8].

The sensitivity of Candida species to antifungal medications varies. The most common cause of Candida infections, Candida albicans, is typically susceptible to fluconazole and other azole antifungals. Nonetheless, certain Candida species, such as Candida glabrata, Candida krusei, and Candida parapsilosis, may exhibit reduced responsiveness to azoles and may require alternative antifungal therapies [9,10].

Antifungal resistance in Candida species is rapidly becoming an issue, especially with the emergence of azole-resistant strains. Several species of candida vary in their virulence and anti-fungal resistance; thus, it is now vital to identify the infective fungus up to the species level because of the shift in infection prevalence caused by these fungi following the widespread and extended use of anti-fungal medicines. Antifungal susceptibility testing should be performed often to ensure that the most effective treatment is being given to each patient [11,12].

Aims & Objectives

The objectives of this study were “to isolate and identify the species of Candida from different samples of clinically suspected candidiasis patients and to determine the susceptibility pattern of the Candida species

isolates against the commonly used anti-fungal agents from the clinical samples".

Materials and Methods

Study setting, study type and duration: A cross sectional study was conducted in the Department of Microbiology in a tertiary care centre, from September 2021 to September 2022.

Study participants: Patients of all age group and both sex with clinically suspected candidiasis, attending outpatient and inpatient Departments were included. Patients who were on antifungal treatment and who are identified with bacterial growth were excluded from the study.

Sample size and sampling technique: In the analysis, 205 samples of eligible participants who came to our setting in study duration were included.

Identification and Speciation of Candida Isolates

Established protocols were utilized to process various clinical specimens such as urine, blood, sputum, central line tip, oral swab, vaginal swab, nail clippings, and skin scrapings. To detect yeast cells with pseudohyphae and Gram-positive budding yeast cells, a potassium hydroxide (KOH) wet mount and Gram stain were employed respectively. Furthermore, all clinical specimens were cultured on Sabouraud's Dextrose Agar (SDA) and were placed in incubators at 25°C and 37°C. For blood culture, different inoculum sizes were used for adults, children, infants, and neonates, which were added to varying amounts of Brain Heart Infusion (BHI) broth and then incubated at 37°C for 7 days. The cultures were closely monitored daily for microbial growth and subcultured on SDA, which allowed Candida to produce creamy, smooth, pasty, and convex colonies within 24-72 hours. Notably, some species may require more than three days to appear on the culture medium. Gram staining was performed on isolated colonies on SDA to identify budding yeast cells and pseudohyphae.

Criteria used to Indicate Candida Infection in Various Samples

In patients who do not have catheters, a colony count of more than 105 Colony-Forming Units (CFU)/ml of urine in a quantitative culture indicates an infection. However, in catheterized patients, a count of over 103 CFU/ml is considered to be indicative of an infection. When pyuria is present, low colony counts are considered significant. Regarding sputum, a gram stain that reveals at least 25 polymorphonuclear leukocytes per oil immersion (100x) field and fewer than 10 squamous epithelial cells is regarded as proof of an infection. Candidemia is the presence of at least one positive blood culture, which exhibits pure growth of Candida species and clinically supportive characteristics. If a roll plate culture yields more than 15 CFU, the Catheter-Related Bloodstream Infection (CRBSI) diagnosis is confirmed. For oral and vaginal swabs, a KOH wet mount or Gram stain can be used to observe the presence of pseudohyphae and yeast cells directly.

Various methods were employed to identify the Candida species, including the germ tube test, demonstration of chlamydospore formation on Cornmeal agar with Tween 80, sugar fermentation test, and sugar assimilation test. Additionally, a temperature test was conducted to differentiate Candida albicans (which shows growth) from Candida dubliniensis (which does not). This test involved using Yeast-Peptone-Dextrose (YPD) broth, BHI, and SDA, and incubating them at 45°C for 10 days.

To achieve accurate species identification, Candida isolates were sub-cultured on HiCrome Candida differential agar (Figure 1), following the manufacturer's instructions. The colonies of Candida albicans were

smooth and light green. Meanwhile, Candida dubliniensis colonies were smooth and dark green. Candida tropicalis colonies appeared raised and blue to metallic blue, while Candida glabrata colonies were smooth and cream to white. Candida krusei colonies appeared fuzzy and purple, while Candida guilliermondii were light pink to pink, and those of Candida parapsilosis were light pink.

Figure 1: Various species of candida on HICHROME agar

Antifungal susceptibility testing:

The disk diffusion method was used to perform antifungal susceptibility testing following the CLSI (previously NCCLS) M44-A2 guidelines from 2009. For this purpose, commercially available 6 mm antifungal disks (Himedia, Mumbai, India) were used, including fluconazole 25 µg, voriconazole 1 µg, amphotericin B 20 µg, itraconazole 10 µg, and ketoconazole 30 µg. Since no defined breakpoints were available for itraconazole, ketoconazole, and amphotericin B, values were arbitrarily determined based on previous research and the manufacturer's (Himedia, Mumbai) instructions.

Interpretation of antibiotic susceptibility testing

The term "susceptible" (S) signifies that the specific strain of the infection can be effectively treated with the recommended dose of antimicrobial agent for that particular type of infection and infecting species, provided there are no contraindications. The "susceptible-dose dependent" (S-DD) category encompasses isolates with antifungal agent Minimum Inhibitory Concentrations (MICs) that approach the normal attainable levels in blood and tissue, and for which the response rates may be lower than those for susceptible isolates. The "resistant" (R) category includes strains not inhibited by the usual concentrations of the agent using normal dosage schedules, or when the zone diameters are within a range where clinical efficacy has not been established in treatment studies.

Antifungal agent	Disk content	Sensitive (S)	Susceptible-Dose Dependent (S-DD)	Resistant (R)
Fluconazole	25 µg	≥19 mm	15-18 mm	≤14 mm
Voriconazole	1 µg	≥17 mm	14-16 mm	≤13 mm
Amphotericin B	20 µg	≥15 mm	13-14 mm	≤12 mm
Itraconazole	10 µg	≥17 mm	14-16 mm	≤13 mm
Ketoconazole	10 µg	≥28 mm	27-21 mm	≤20 mm
Nystatin	50 µg	≥15 mm	14-10 mm	<10 mm

The sterility of each media batch was evaluated by incubating it at 37°C for 24 hours. To ensure the accuracy of antifungal susceptibility testing, the quality control strain used was Candida albicans American Type Culture Collection (ATCC) 90028.

Data analysis

The data were entered and analysed with Epi Info CDC version 7. Descriptive variables were expressed as frequency and percentages while continuous variables were expressed as mean and standard deviation.

Results

The average age of the subjects enrolled in the study was 37.05 ± 23.03 years, with the majority falling within the age group of 18 to 60 years. Almost an equal proportion of males and females participated in the study (49.3% and 50.7%, respectively). Among the samples collected for the study, the majority were urine (62.9%), followed by blood (13.7%) and pus (12.2%).

Table 1: Characteristics of study participants (n=205)

Variables	Number	Percentage
Age		
<1 year	27	13.2
1-18 years	20	9.8
18-60 years	114	55.6
>60 years	44	21.5
Gender		
Male	101	49.3
female	104	50.7
Samples		
Urine	129	62.9
Blood	28	13.7
Pus	25	12.2
Sputum	13	6.3
Vaginal swab	07	3.4
Et swab	3	1.5

Table 2: Species of candida found in samples (n=205)

Candida species	Numbers	Percentage
Candida Albicans	135	65.9
Candida Glabrata	20	9.8
Candida Krusei	28	13.7
Candida Tropicalis	22	10.7

Candida Albicans was the most prevalent Candida species in the analysed sample, accounting for nearly two-thirds of the isolates 135 (65.9%). Candida Krusei was the second most common species, with 28 isolates (13.7%), followed by Candida Tropicalis with 22 isolates (10.7%) and Candida Glabrata with 20 isolates (9.8%).

Table 3: Resistant pattern of Candida species

Candida species	Resistant	Sensitive	Total
Amphotericin	**(n=47)**	**(n=158)**	
Candida Albicans	33 (24.44%)	102 (75.56%)	135
Candida Glabrata	5 (25%)	15 (75%)	20
Candida Krusei	7 (25%)	21 (75%)	28
Candida Tropicalis	2 (9.09%)	20 (90.91%)	22
Fluconazole	**(n=43)**	**(n=152)**	
Candida Albicans	28 (20.74%)	107 (79.26%)	135
Candida Glabrata	5 (25%)	15 (75.00%)	20
Candida Krusei	8 (28.57%)	20 (71.43%)	28
Candida Tropicalis	2 (9.09%)	20 (90.91%)	22
Clotrimazole	**(n=108)**	**(n=97)**	
Candida Albicans	70 (51.85%)	65 (48.15%)	135
Candida Glabrata	12 (60%)	8 (40%)	20
Candida Krusei	16 (57.14%)	12 (42.86%)	28
Candida Tropicalis	10 (45.45%)	12 (54.55%)	22
Ketoconazole	**(n=19)**	**(n=186)**	
Candida Albicans	15 (11.11%)	120(88.89%)	135
Candida Glabrata	0 (0.0%)	20 (100%)	20
Candida Krusei	3 (10.71%)	25 (89.89%)	28
Candida Tropicalis	1 (4.55%)	21 (95.45%)	22
Nystatin	**(n=20)**	**(n=185)**	
Candida Albicans	17 (12.59%)	118 (87.41%)	135
Candida Glabrata	2 (10%)	18 (90%)	20
Candida Krusei	0 (0.0%)	28 (100%)	28
Candida Tropicalis	1 (4.55%)	21 (95.45%)	22

Of the 135 tested Candida albicans isolates, 33 (24.44%) were resistant to amphotericin, while the remaining 102 (75.56%) showed sensitivity to the drug. Candida glabrata, Candida krusei, and Candida tropicalis demonstrated an amphotericin-resistant pattern of 25%, 25%, and 9.09%, respectively. In the samples, Candida albicans exhibited resistance to fluconazole in 20.74% of cases, clotrimazole in 51.85%, to ketoconazole in 11.11%, and to nystatin in 12.59%. The highest percentage of resistance among all four Candida species was observed for clotrimazole.

Discussion

Candida albicans is the leading cause of candidiasis, accounting for 60-80% of all infections. In the last few decades, an increase in the number of non-albicans species that may serve as pathogens has been seen. Candida spp. are capable of causing numerous illnesses, including systemic infections such as blood stream infections (BSIs) and disseminated candidiasis [13,14].

The genus Candida has over 17 distinct Candida spp. which are responsible for a

variety of human diseases. Candida albicans, Candida glabrata, Candida tropicalis, and Candida krusei are responsible for over 90% of invasive infections. Recovery of yeasts from ordinarily sterile body fluids (blood, cerebrospinal fluid, etc.), recovery from patients with impaired immune systems due to chronic disorders, and repeated recovery from several specimens all indicate infection with yeasts [15,16].

Antifungal agents for treating systemic and invasive candidiasis are restricted to polyenes, allylamines, azoles, and the recently developed echinocandin class of molecules. Characterization to species level helps to identify those strains which might be intrinsically resistant to some antifungal agents. Incidence of antifungal resistance among Candida spp. is increasing over the past decade. Species level identification of Candida spp. is clinically important since they differ in virulence and antifungal susceptibility. Rapid identification of Candida spp. can also help with early management of antifungal therapy [17,18].

Most individuals who participated in our study fell within the age range of 18 to 60 years old. This age group also comprised a significant portion of the isolates in studies conducted by Azad M *et al* [3]. Urvashi Chongtham *et al* [19], and Bhattacharjee *et al* [20]. The proportion of males and females in our study was almost equal, with males accounting for 49.3% and females 50.7%. A similar gender distribution was observed in the study by Khadka S *et al* [21], while Azad M *et al* [3] reported a male predominance with a male to female ratio of 1.5:1, and Chongtham U *et al* [19], reported a higher number of females than males.

Most samples collected in the present study were urine (62.9%), with blood samples accounting for 13.7%. In a study conducted by Khara R *et al* [1]. Candida species were found to be most frequently isolated from blood (35%), catheterized urine (20%), sputum (19%), non-catheterized urine (14%), and ET tips/secretions (7%), with smaller percentages isolated from other specimens. Similarly, most Candida isolates were obtained from blood and urine samples in studies conducted by Azad M *et al* [3], Jayalakshmi L *et al* [5], and Sharma M *et al* [22] However, in contrast, most isolates in this study were obtained from sputum and urine, as reported in studies conducted by Khadka S *et al* [21] and Chongtham U *et al* [19].

The most frequently isolated Candida species in our study was Candida Albicans, which accounted for approximately 65.9% of the isolates. Candida Krusei was the second most common species (13.7%), followed by Candida Tropicalis (10.7%) and Candida Glabrata (9.8%). Our findings are consistent with those of other studies such as Khara R *et al* [1], Azad M *et al* [3], Chongtham U *et al* [19], Manjunath et a [l4], and Jayalakshmi L *et al* [5], which also found Candida Albicans to be the predominant species. However, non-albicans Candida (NAC) isolates are on the rise, as reported in studies by Kaviarasan *et al* [23], Adhikary R *et al* [24], Wang T Y *et al* [25], and Kanna VB *et al* [26]. In contrast, Chakraborti A *et al* [27] and Mokaddas EM *et al* [28] have reported that NAC species have a higher isolation rate than C. albicans, indicating the growing significance of non-albicans Candida species as important pathogens.

Using CHROM agar for Candida species identification based on color differentiation proved to be a quick, convenient, and reliable method compared to previous time-consuming techniques. In low-resource settings, CHROM agar can serve as a simple substitute for molecular-based assays for phenotypic testing. CHROMagar demonstrated a high level of sensitivity and specificity for Candida species identification. We selected this medium for Candida spp.

isolation based on the prevalence of these four species in our area, leading us to conclude that it was the optimal medium for Candida spp. isolation. This medium provides results within 24-48 hours and aids in identifying and detecting Candida species from mixed cultures [21-23].

The present study found that Candida albicans, Candida glabrata, Candida krusei, and Candida tropicalis species exhibited resistance to amphotericin, with resistance rates of 24.44%, 25%, 25%, and 9.09%, respectively. Candida albicans also showed resistance to fluconazole (20.74%), clotrimazole (51.85%), ketoconazole (11.11%), and nystatin (12.59%) in the samples. Similarly, in Khara R *et al*'s study [1], a higher resistance to most antifungal agents was observed, with clotrimazole (80%), ketoconazole (77%), fluconazole (63%), and itraconazole (62%) being the most resistant.

However, Khadka S *et al* [21] reported that clotrimazole had the highest level of susceptibility, whereas ketoconazole had the highest level of resistance. In our study, clotrimazole exhibited the highest percentage of resistance among all four species of Candida, which is consistent with the findings of Khara R *et al* [1] and Azad M *et al*. [3]

In general, the variation in the occurrence of Candida species, their specific types, and their susceptibility and resistance to antifungal agents may be attributed to factors such as geographical location, clinical environment (e.g. ICU vs hospital), patient demographics (e.g. age, underlying medical conditions), sample size and type, and different isolation and identification techniques (e.g. automated vs conventional/semi-automated methods).

Additionally, various studies have tested different Candida species and antifungal agents, which may contribute to discrepancies in results. It should be noted that many studies have only focused on Candida species that cause candidemia, whereas our study investigated all Candida species present in sepsis patients in order to determine the source of infection.

Conclusion

It is concluded from the study that Candida species have become increasingly common as the primary cause of opportunistic infections in healthcare facilities, with Candida Albicans being the most prevalent species and Clotrimazole showing the highest percentage of resistance among the four species tested. Early identification of Candida isolates is crucial as it can limit the unnecessary use of antifungal agents and aid clinicians in selecting appropriate treatment options for patients, as some Candida species are inherently resistant to specific antifungal agents.

Therefore, isolating and identifying Candida species and performing antifungal susceptibility testing at an early stage is crucial. This will assist clinicians in choosing the most effective therapeutic approach to reduce morbidity and mortality. A comprehensive multicenter study with a large sample size is necessary to determine the dominant Candida species and assist in developing guidelines for the empirical treatment of invasive fungal infections.

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