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CANDIDA TROPICALIS – A STUDY OF VIRULENCE FACTORS AND ANTIFUNGAL SUSCEPTIBILITY PROFILE OF THE CLINICAL ISOLATES OF AN EMERGING NON-ALBICANS CANDIDA SPP.

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ABSTRACT

Introduction: Non-albicans *Candida* spp. (NAC) accounts for more than half of the cases of non-superficial *Candida* infections. Over the past two decades, there is a significant shift, showing increased isolation of non albicans *Candida* and *Candida tropicalis* is one of the commonest among NAC. In few studies, *Candida tropicalis* has superseded *Candida albicans* as the commonest cause of invasive Candidial infections. In this study we determined the virulence factors and antifungal susceptibility profile of the *Candida tropicalis* isolates obtained from clinical specimens. **Methods and results:** All the *Candida tropicalis* isolates were identified based on Corn-meal agar morphology, sugar assimilation test and CHROMagar. Biofilm formation (Tube method/48hr/2% safranin staining) was seen in 33.34% of the isolates. All the isolates (100%) elaborated esterase enzyme (Tween 80 hydrolysis test), hemolysin (Blood agar plate assay) and showed pseudohyphae formation. Hemolytic activity (Hz) and Esterase activity (Ez) were measured. The antifungal susceptibility of these isolates were determined using VITEK 2 system. The activity of fluconazole, voriconazole, caspofungin, micafungin, amphotericin B and flucytosine against the study isolates has been noted. Though all the studied isolates were susceptible to the above mentioned agents, the minimum inhibitory concentration of amphotericin B has been found to be increased (0.5µg/ml) in 44.44% of the isolates. **Conclusion:** The identification of virulence attributes and antifungal susceptibility profile of these isolates will aid in the understanding of the pathogenesis and better management of the infections caused by these organisms.

KEYWORDS: *Candida tropicalis*, virulence factors, anti-fungal susceptibility, esterase, hemolytic activity.

INTRODUCTION

Incidence of fungal infections has increased significantly over the last decades and a number of factors have been implicated in this increased occurrence of fungal disease. It is generally accepted that the increased and widespread use of certain medical practices, such as immunosuppressive therapies, invasive surgical procedures and use of broad-spectrum antibiotics are significant attributable factors.[1]

Of the fungi regarded as human pathogens, the members of the genus *Candida* are most frequently recovered from human fungal infection.[1,2] Though, *Candida albicans* is generally considered as the major pathogen among the *Candida* species, there is a significant shift over the past two decades showing increased isolation of non-albicans *Candida* species (NAC) from invasive candidial infections.[3] The reasons for this alteration in the pattern of *Candida* species distribution has not yet been completely understood, but could be attributed to the resistance of the NAC species to antifungal agents, which are used for relatively long periods during hospitalization.[4]

Non-albicans *Candida* spp. (NAC) accounts for more than half of the cases of non-superficial *Candida* infections globally.[5] In Indian subcontinent, more than two-third of nosocomial candidemia cases were due to NAC species of which *Candida tropicalis* was the most common species.[6]

Candida tropicalis is the second most prevalent *Candida* species after *C. albicans* in a wide spectrum of infections such as superficial, cutaneous, subcutaneous and systemic candidiasis.[6] *Candida tropicalis* has been shown to be involved significantly in various human pathologies such as fungemia, urinary tract infections, post-surgical wound infections, osteomyelitis, endocarditis, etc.[3]

In few studies, *Candida tropicalis* has superseded *Candida albicans* as the commonest cause of invasive Candidial infections.[5] This species appears to display higher potential for dissemination in the neutropenic host than *C. albicans* and other NAC species. This propensity for dissemination in some way may explain the reported relatively high mortality associated with *C. tropicalis*.[4]

The following virulence factors like adhesins, phenotypic switching, biofilm formation, hemolysin and production of enzymes like esterase, phospholipase, proteinase, etc. contribute to the pathogenicity of candidiasis.[3] Most of these have been found to be expressed by the strains of *Candida tropicalis*.

The susceptibility testing of fungi to antimycotic drugs, including that of yeasts, was until recently performed only rarely because of the following factors: the cases necessitating systemic antifungal treatment were relatively rare; the number of antifungal drugs was limited; the incidence of resistance to the drugs was rare; most importantly, correlation between in vitro and in vivo results was questionable. However, recently there is a marked increase in the incidence of mycotic diseases, especially disseminated infections, the number of available drugs has increased, and continues to increase, and an innate or acquired resistance of certain yeast species to several drugs has been reported. Thus, susceptibility testing of yeasts has been standardized and published by Clinical Laboratory Standards Institute.(M27-A3).[7]

The resistance to azole group of antifungal drugs were found to be increasing among *Candida tropicalis* and this is of concern because the species is one of the most commonly isolated NAC spp. and azoles are the most common antifungal agents used for the treatment of various types of candidiasis. [8,9]

There are several factors probably responsible for the development of drug resistance in various clinical conditions and few mechanisms have been described by Sanglard and Odds.[9]

Though, *C. tropicalis* remains a significant cause of candidial infections, relative to the wealth of information available on *C. albicans*, very much less is known about *C. tropicalis* and there are very few studies examining the virulence factors of *C. tropicalis*. Therefore, this study was undertaken with an objective to determine the virulence factors expressed by the *Candida tropicalis* isolates obtained from clinical specimens in our population and profiling of their antifungal susceptibility pattern.

MATERIALS AND METHODS

This was a hospital based prospective study conducted in the Department of Microbiology of our institute. A total of 18 *C. tropicalis* isolates from various clinical samples were included in the study.

The isolates were identified based on the colony morphology on Sabouraud's Dextrose agar (SDA), gram stain, germ tube test (GTT), corn-meal agar (CMA) morphology and sugar assimilation test and CHROMagar.

The virulence factors studied were exoenzymatic activity (coagulase and esterase), biofilm formation, pseudohyphae formation and hemolysin production. The antifungal susceptibility of these isolates were determined using VITEK 2 system.

A. *Coagulase activity*

Coagulase production by *C. tropicalis* was detected by the method of Yigit et al.[10] Approximately 0.1mL of an overnight culture of *C. tropicalis* was aseptically inoculated into a tube containing 500 μL of sheep plasma. The tubes were incubated at 37°C and observed for clot formation after 2, 4, 6, and 24h. The presence of a clot that could not be resuspended by gentle shaking indicated positive coagulase test. *Staphylococcus aureus* ATCC 25923 and *S. epidermidis* ATCC 14990 were used as positive and negative controls, respectively.

B. *Esterase activity*

The esterase activity was studied using an agar containing 1% peptone, 0.5% NaCl, 0.01% $CaCl_2$, and 1.5%/L at 6.8 pH. When heated at 45–50°C, 5 mL Tween‑80 was added. The round‑shaped strains with 10‑mm in diameter were incubated at 30°C for 10 days. The presence of a transparent halo around the inoculum site indicated positivity. The zone sizes were measured and the ratio of zone diameter to colony diameter was used to compare the enzyme activity(Ez).[11]

C. *Hemolysin Production*

Hemolytic activity of *C. tropicalis* was screened on sheep blood Sabouraud dextrose agar plate by the method described by Manns et al.[12] Approximately 10 μL of standard inoculum (10^8 *Candida* cells/mL) was aseptically inoculated onto the medium. The culture plates were incubated at 37°C for 48h. *Streptococcus pyogenes* (Lancefield group A) was used as positive controls for beta hemolysis. The presence of a zone of hemolysis around the colony indicated hemolysin production. Hemolytic activity (Hz) was calculated in terms of the ratio of diameter of the colony to that of the translucent zone of hemolysis.

D. *Pseudohyphae formation*

Pseudohyphae formation was defined as a cell bearing a rounded outgrowth with a length greater than or equal to the diameter of the parent cell, with a constriction at the base. The percentage of cells in pseudohyphae form, against blastospores, was determined by microscopy counting after 2 h of cell growth in serum and the pseudohyphae formation was also appreciated in the corn-meal agar test.

E. *Biofilm formation*

The ability of *C. tropicalis* isolates to form biofilms was assessed by the tube method described by Yigit et al.[13] Colonies of *C. tropicalis* from Sabouraud dextrose agar were inoculated in saline and incubated overnight at 37°C. 0.5mL of this saline suspension was added into screw capped conical polystyrene tubes containing 5mL of Sabouraud dextrose broth supplemented with glucose (final concentration of 8%). The tubes were incubated at 37°C for 48 h without agitation. After incubation the broth from the tubes was aspirated gently using Pasteur pipette. The tubes were washed twice with distilled water and stained with 2% safranin. The stain was decanted after 10 min. The tubes were rinsed with distilled water to remove excess stain. Presence of visible adherent film on the wall and at the bottom of the tube indicated biofilm formation. Ring formation at the liquid interface was not considered as an indication of biofilm production.

F. *Antifungal susceptibility testing*

The antifungal susceptibility of these isolates were determined using VITEK 2 system. AST-YS07 cards were used and manufacturer's instructions were followed for the study of antifungal susceptibility profile. The activity of fluconazole, voriconazole, caspofungin, micafungin, amphotericin B and flucytosine against our isolates has been studied.

RESULTS

A total of 18 patient-specific non duplicate isolates were obtained. The mean age of patients was 46.17(±19.28) and male-female ratio was 1:2. Majority of the isolates were obtained from urine samples (33.34%) and tracheal secretions (33.34%). Rest were obtained from sputum (22.22%) and high vaginal swabs (11.11%).

Expression of virulence factors under study by *C. tropicalis* are shown in Table 1. All the eighteen isolates (100%) showed hemolysis with a mean Hz activity of 1.548(±0.19). Esterase production tested by Tween 80 agar test showed 100% expression among all the isolates with a mean enzymatic (Ez) activity of 1.923(±0.23). Biofilm formation was observed in 4 (22.22%) isolates and none of the isolates showed coagulase production. Pseudohyphae formation was seen in all the eighteen isolates studied. The positive tests for hemolytic activity, esterase activity and biofilm formation were shown in Fig 1.

Antifungal susceptibility profile of *Candida tropicalis* isolates under study are shown in Table 2.

TABLES

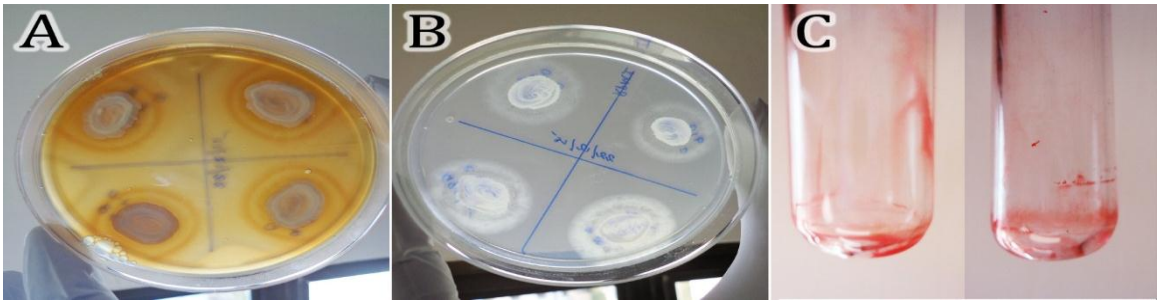
Table 1: Production of various virulence factors by *C. tropicalis* and its correlation with the sample of origin

	Urine (n=6)	ET (n=6)	Sputum (n=4)	HVS (n=2)	Total (n=18)
Hemolysin production	6 (100%)	6 (100%)	4 (100%)	2 (100%)	18 (100%)
Esterase production	6 (100%)	6 (100%)	4 (100%)	2 (100%)	18 (100%)
Biofilm formation	2 (33.34%)	2 (33.34%)	0 (0%)	0 (0%)	4 (22.22%)
Pseudohyphae formation	6 (100%)	6 (100%)	4 (100%)	2 (100%)	18 (100%)
Coagulase production	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Table 2: Antifungal susceptibility profile of *Candida tropicalis*

	Sensitivity (%)	MIC
Amphotericin B	100%	≤0.25 µg/ml (55.56%) and 0.5 µg/ml (44.44%)
Caspofungin	100%	≤0.25 µg/ml (100%)
Fluconazole	100%	≤1 µg/ml (100%)
Flucytosine	100%	≤1 µg/ml (100%)
Micafungin	100%	≤0.06 µg/ml (100%)
Voriconazole	100%	≤0.12 µg/ml (100%)

Figure



(a) Hemolytic activity, (b) Esterase activity and (c) biofilm production

DISCUSSION

Traditionally *Candida albicans* has been considered as the most virulent and clinically most important *Candida* causing human infections. However, recent epidemiological shifts have shown that non-albicans *Candida* (NAC) species have surpassed *C. albicans* in terms of significant clinical infections in many centers. *Candida tropicalis* is the second most common *Candida* (& most common NAC) isolated in many centers and in few centers it is the most common *Candida* species isolated from clinical infections.[5]

C. tropicalis represents 3-66% of all *Candida* bloodstream infections worldwide.[5] *C. tropicalis* appears to be as virulent as *C. albicans* and has been found to cause increased overall crude mortality when compared with other *Candida* spp.[21, 22]

Candida spp. have many virulence attributes which assist in invasion of host tissues. These include adherence to host tissues, production of extracellular enzymes, particularly proteases, the formation of hyphae to aid in evasion of host immune defenses and biofilm production.[5] In rare cases, *C. tropicalis* can form true hyphae, which is a character uniquely shared with *C. albicans*.[16]

Biofilm formation is a potential virulence factor for the development of candidiasis.[17] *C. tropicalis* isolates were shown to produce biofilms in higher proportion when compared with other *Candida* spp. Many studies have reported production of biofilms in range of 40%-100% of *C. tropicalis* isolated.[2,8,18,19] In our study 22.22% of the *C. tropicalis* isolates produced biofilm; 11.11% were from urine samples of catheterized patients and 11.11% were from tracheal secretions of intubated patients.

The ability of pathogenic microorganisms to acquire elemental iron has been shown to be of pivotal importance in their survival and ability to establish infection within the mammalian host.[20] It has been established that *Candida albicans* produces hemolytic factor significantly when grown on glucose-enriched blood agar. Similar approach has been tried with other NAC and few studies have reported more stronger expression of hemolytic activity by *C. tropicalis*.[21] In our study, all the isolates (100%) produced hemolysis on glucose enriched blood agar with a mean hemolytic activity of 1.548(±0.19). Studies done by Mane et al, Deorukhkar et al and Negri et al have shown that *C. tropicalis* isolates are prolific producers of hemolytic factor similar to the findings in our study.[2,8,22]

Extracellular hydrolytic enzymes play an important role in the pathogenesis of candidiasis and these enzymes have been found to facilitate adaptation to distinct types of infection and enhance survival of the pathogen.[13, 30] Coagulase production and esterase production and esterase production has been least studied among the extracellular enzymes produced by *Candida* spp. In different studies the coagulase activity has been found to be different. Few studies have reported low coagulase activity among *C. tropicalis* isolates and few have reported high activity.[8,10,24,25] In our study, none of the isolates exhibited coagulase activity and this can be attributed to the sheep plasma we used, as it has been shown in the many studies referred above that rabbit plasma yielded better results when compared with the sheep plasma in the detection of coagulase activity of *Candida* spp.

Esterase activity among *Candida* spp. has been found to be highest among *C. albicans* and *C. tropicalis*.[18, 33, 34] In our study, all the isolates (100%) exhibited esterase activity in Tween 80 agar test. Similarly, pseudohyphae formation has been found to expressed by all the isolates (100%) similar to the findings in other studies.[2,5]

Antifungal susceptibility testing of *Candida* spp. has been standardized by CLSI and broth microdilution method is recommended. However, the method is very complex and laborious to use as a routine method. Alternatively, disc diffusion method for selected antibiotics has been studied and recommended by CLSI. In our study we evaluated the antifungal susceptibility profile of the *C. tropicalis* isolates using Vitek 2 compact system. *C. tropicalis* was for a long time regarded as a species largely susceptible to fluconazole and amphotericin B (>95–98%), but resistance to fluconazole has been increasingly reported among *C. tropicalis* in various studies and it's role has become questionable in few centers.[5, 6, 13, 16] In our study, all the test isolates were sensitive to fluconazole with MIC ≤1 µg/ml. Another azole agent tested was voriconazole and it is a highly active drug against C. tropicalis.[5] All the isolates were sensitive to voriconazole.

Amphotericin B had been the mainstay of therapy against systemic fungal infections and *C. tropicalis* had shown good susceptibility to it. Resistance(MIC >2 µg/ml) is rare and only few strains have been reported with high level resistance to this agent.[29] In our study 10 isolates (55.56%) isolates showed MIC ≤0.25 µg/ml and 8 isolates (44.44%) showed MIC 0.5 µg/ml.

All the study isolates were susceptible to Flucytosine (MIC ≤1 µg/ml). However, resistance had been reported to develop during the course of treatment in upto 58% of strains in some centers and combination therapy with other antifungals is recommended.[30] Echinocandins have good activity against *Candida tropicalis* isolates and all the isolates in our study showed susceptibility to caspofungin and micafungin with MIC of ≤0.25 µg/ml and ≤0.06 µg/ml respectively. Though, few cases of Caspofungin resistant *Candida tropicalis* have been reported, echinocandins demonstrate good clinical response, better than azoles.[31]

CONCLUSION

C. tropicalis accounts for a significant proportion of isolates encountered in invasive candidiasis. Owing to the expression of wide range of virulence factors and increasing resistance to commonly used antifungal agents by *Candida tropicalis*, morbidity and mortality associated with this species is increasing considerably. It is important to further enhance our knowledge on the virulence and resistance mechanisms associated with this emerging pathogen for prevention and identification of novel therapeutic targets.

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