RESEARCH ARTICLE

NON ALBICANS CANDIDA SPECIES: ITS ISOLATION PATTERN, SPECIES DISTRIBUTION, VIRULENCE FACTORS AND ANTIFUNGAL SUSCEPTIBILITY PROFILE

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ABSTRACT

Background: The change in epidemiology and antifungal susceptibility has generated interest of Clinical Microbiologists in identification of Candida up to species level along with antifungal susceptibility pattern. Non-albicans Candida (NAC) has emerged as an important opportunist pathogen. Extracellular hydrolytic enzymes are one of the important virulence attributes of Candida species.

Aims & Objective: The present study aimed to determine the species distribution, virulence factors and antifungal susceptibility profile of NAC spp. isolated from various clinical specimens.

Material and Methods: Speciation of Candida was done by assessing the germ tube formation, assimilation and fermentation of sugars and colony color on HICHROM Candida agar. In-vitro extracellular hydrolytic enzymes production in NAC spp. was assessed. Antifungal susceptibility testing of the isolates was performed by Hicomb minimum inhibitory concentration (MIC) test.

Results: Majority of the isolates were obtained from urine sample (35.6%). C. tropicalis (29.4%) was the major isolate. Maximum extracellular hydrolytic enzymes activity was seen in C. tropicalis. A total of 79 (27.3%) isolates were resistant to fluconazole. Amphotericin B resistance was noted in 17 (5.8%) isolates.

Conclusion: NAC spp. cannot be overlooked as mere containment or non-pathogenic commensals as most of them show reduced susceptibility to commonly used antifungal drugs. Extracellular hydrolytic enzymatic activity of NAC Spp. would be an important tool to prove the relation between the infective species of Candida and infection.

KEY-WORDS: Antifungal Susceptibility; Extracellular Hydrolytic Enzymes; Fluconazole; Non- Albicans Candida Species

Introduction

Infections caused by Candida have been increased in the past few decades.[1] The increase in frequency of therapeutic applications of immunosuppressive drugs, excessive use of broad-spectrum antibiotics and the emergence of HIV/ AIDS are important among the various the contributing factors.[2]

Although among the Candida species, Candida albicans remains the most frequent aetiological agent encountered in various clinical forms of candidiasis, recent studies have documented a shift towards non-albicans Candida (NAC) species.[3] The clinical manifestations caused by various pathogenic Candida spp. are not distinguishable but the problem becomes acute because different species of NAC exhibits varying degree of resistance either intrinsic or acquired or both to the commonly used antifungal drugs.[4] The increased isolation rates of NAC spp. and gradual shift in the antifungal susceptibility profile underlines the need of early and accurate diagnosis of infecting Candida spp. along antifungal susceptibility testing for selecting the most appropriate antifungal agent for therapy.

The virulence attributes of Candida includes hyphal switching, germination, surface recognition and production of extracellular hydrolytic enzyme.[1] Among these, extracellular hydrolytic enzymes play an important role in adherence and infection of Candida.[5]

The present study was planned at rural tertiary care hospital with an aim to determine the isolation pattern, species distribution, virulence factors and antifungal susceptibility profile of NAC spp. isolated from various clinical samples.

Materials and Methods

The present study is part of a PhD thesis and was approved by the Institutional Ethics Committee (Registration No.FN.32/2010). A total of 289 NAC spp. isolated from various clinical specimens processed in the Department of Microbiology were included in the study. Speciation of Candida was done by assessing the germ tube formation, assimilation and fermentation of sugars and colony colour on HICHROM Candida agar.

Virulence Factors

The virulence factors of NAC spp. studied included production of extracellular hydrolytic enzymes (phospholipase, proteinase and coagulase) and haemolysin.

1. Determination of Phospholipase Activity

The NAC spp. was screened for phospholipase production by method of Samaranayake et al.[6] The phospholipase activity was detected by measuring the size of precipitation zone after the growth on egg yolk agar. This medium contained Sabouraud's dextrose agar (SDA) (13.0 g), NaCl (11.7 g), $CaCl_2$ (0.11 g) and 10% sterile egg yolk and distilled water (184 ml). The components were mixed and sterilized using autoclave prior to addition of egg yolk. The egg yolk was centrifuged at 500 rpm for 10 min at room temperature and 20 ml of the supernatant was added to the sterilized medium.

Standard inoculum of the test and control Candida (C. albicans ATCC 10231) [5 µl, with 10^8 yeast cells (ml saline)$^{-1}$] were deposited onto the egg yolk agar medium and left to dry at room temperature. The culture plate was incubated at 37^0 C for 48 h. The assay was conducted on three separate occasions for each isolate.

The presence of visible precipitation zone around the colony indicated phospholipase production. The value of phospholipase activity (Pz) was measured by the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone.

A Pz value of 1 denotes no activity, and less than one (Pz < 1) indicated the phospholipase activity. The lower the Pz value, the higher the enzymatic activity.

2. Determination of Proteinase Activity

Proteinase production was measured in terms of bovine serum albumin (BSA) degradation according to the technique described by Staib et al.[7] The suspension of $1X10^6$ cells ml^{-1} was prepared from Candida isolate. 10 µl suspension was inoculated on 1% BSA medium plate. The BSA medium consisted of dextrose 2%, KH_2PO_4 0.1%, MgSO4 0.05%, agar 2% and 1% BSA solution.

The plate was incubated for 5 days at 37^0 C. After incubation, the plates were fixed with 20% trichloracetic acid and stained with 1.25% amidoblack. Decolourisation was performed with acetic acid. Opaqueness of the agar, corresponding to a zone of proteolysis around the colony that could not be stained with amidoblack indicated degradation of the protein. The assay was conducted on three separate occasions for each Candida isolate tested.

The proteinase activity (Pr_z) was analyzed in the terms of the ratio of the colony to the diameter of the proteolytic unstained zone. A Pr_z value of 1 denotes no activity, and less than one (Pr_z<1) indicated the proteinase activity.

The lower the Pr_z value, the higher the enzymatic activity. Reference strains of C. albicans ATCC 10231 served as positive controls.

3. Determination of Coagulase Production

Coagulase production of NAC spp. was assessed by using EDTA-rabbit plasma by a classical tube test. 0.1 ml of an overnight culture of each isolate in Sabouraud's dextrose broth was inoculated into a tube containing 500 µl of EDTA- rabbit plasma. The tubes were incubated for 4 h at 35^0 C. The presence of a clot that could not be resuspended by gentle shaking indicated positive coagulase test. If no clot formed, the tube was reincubated and reexamined after 24 h.

Staphylococcus aureus ATCC 25923 and S. epidermidis ATCC 14990 were used as positive and negative controls.

4. Determination of Haemolysin Activity

Haemolysin activity of NAC spp. was detected by blood agar plate assay as described by Manns et al.[8] The media was prepared by adding 7 ml aseptically collected fresh sheep blood to 100 ml SDA supplemented with glucose at a final concentration of 3% (w/v). 10 µl of standard inoculum [10^8 yeast cells (ml saline)$^{-1}$] prepared from both the test and the control Candida isolates was deposited onto the medium. The blood agar plate was then incubated at 37^{0}C in 5% CO_2 for 48 h.

Haemolysin activity (Hz) was determined by calculating the ratio of the diameter of the colony to that of the translucent zone of haemolysis (in mm). The assay was conducted on three separate occasions for each isolate

C. albicans ATCC 90028 was used as positive control. One strain each of Streptococcus pyogenes (Lancefield group A) and Streptococcus sanguis, which induce beta and alpha haemolysis, were used as positive controls.

Antifungal Susceptibility Test

Antifungal susceptibility testing of the isolates was performed by Hicomb MIC test (Himedia Laboratories Mumbai). The antifungal agents used were amphotericin B (range 0.002-32 mcg), fluconazole (range 0.016-256 mcg), itraconazole (range 0.002-32 mcg) and ketoconazole (range 0.002-32 mcg). The manufacturer's instructions were adhered to throughout the test.

The suspension of the isolate to be tested was prepared in 0.85% saline. The turbidity of each suspension was adjusted to 0.5 Mc Farland standard. The suspension was inoculated on agar plates containing RPMI 1640 supplemented with glucose using sterile cotton tipped swab. The antifungal strips were placed on the media and the plates were incubated for 48 h at 35^{0}C. The minimum inhibitory concentration (MIC) of each isolate against each antifungal tested was read after 24 and 48 h.

C. albicans ATCC 90028 and C. parapsilosis ATCC 22019 were used for the purpose of quality control. The antifungal susceptibility of the isolates was reported as sensitive (S), dose dependent-susceptible (DDS) and resistant (R). For fluconazole and itraconazole the results were evaluated as per the interpretive susceptibility criteria recommended by Clinical and Laboratory Standard Institute (CLSI) (formerly known as National Committee for Laboratory Standards (NCCLS)) M27-A2 standard guidelines.[9] Due to the lack of defined breakpoints for amphotericin B and ketoconazole, arbitrary values based on the studies of other researchers were used.[10]

Results

Figure 1 shows the sample wise distribution of NAC spp. Majority of the isolates were obtained from urine sample (35.6%) followed by vaginal swabs (23.8%). C. tropicalis (29.4%) was the major isolate followed by C. glabrata (20.7%). patients (Figure 2).

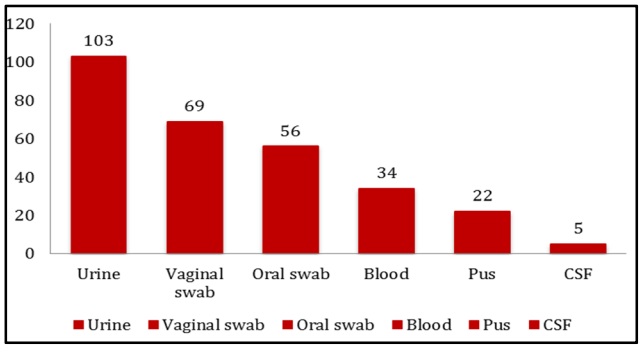

Figure-1: Sample wise Distribution of NAC Species

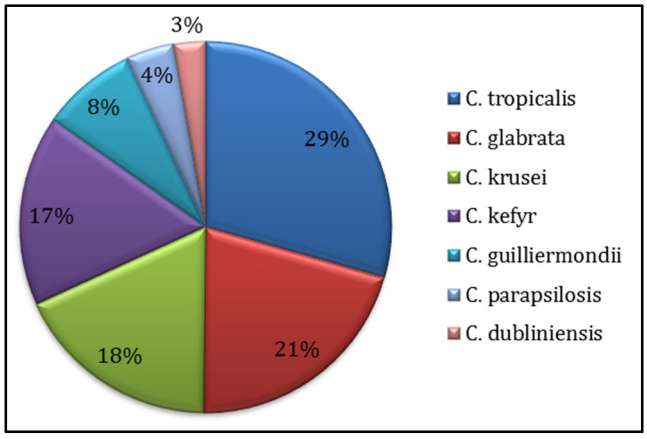

Figure-2: Number of Non Albicans Candida Isolates

As shown in Table 1, maximum phospholipase and proteinase activity was seen in C. tropicalis isolates followed by C. glabrata. C. kefyr showed minimum phospholipase and proteinase production. Coagulase was produced by 80% of C. tropicalis isolates. In our study coagulase enzyme was not produced by C. guilliermondii isolates. C.

tropicalis (76.4%) followed by C. glabrata (15%) showed maximum haemolytic activity. Haemolysin was not produced by C. kefyr, C. guilliermondii and C. parapsilosis isolates.

Table-1: Extracellular hydrolytic activities of NAC spp.

Species	No. of Isolates	Phospholipase Production (%)	Proteinase Production (%)	Coagulase Production (%)	Haemolysin Production (%)
C. tropicalis	85	74 (87.1)	74 (87.1)	68 (80)	65 (76.4)
C. glabrata	60	23 (38.3)	22 (36.6)	14 (23.3)	09 (15)
C. krusei	52	08 (15.3)	07 (13.4)	03 (5.7)	04 (7.6)
C. kefyr	48	03 (6.2)	02 (4.1)	-	-
C. guilliermondii	24	02 (8.3)	02 (8.3)	01 (4.1)	-
C. parapsilosis	12	01 (8.3)	01 (8.3)	-	-
C. dubliniensis	08	01 (12.5)	02 (25)	01 (12.5)	01 (12.5)
Total	289	112 (38.7)	110 (38.1)	87 (30.1)	79 (27.3)

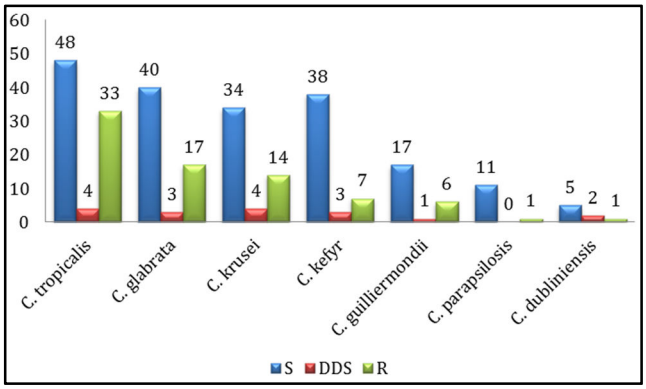

Figure-3: Antifungal Profile of NAC spp. (Fluconazole)

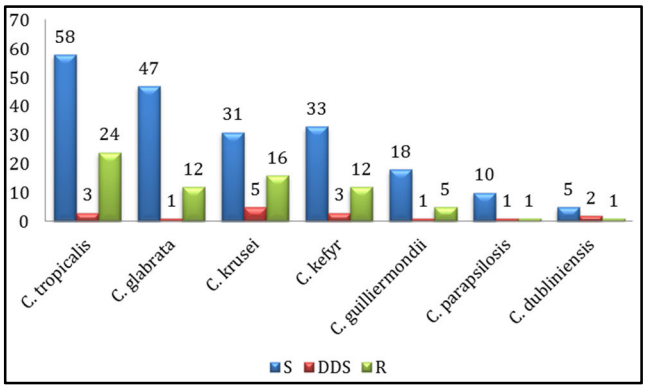

Figure-4: Antifungal Profile of NAC spp. (Ketoconazole)

A total of 79 (27.3%) isolates were resistant to fluconazole. Fluconazole resistance was more in C. tropicalis followed by C. glabrata (Figure 3). Maximum resistance to ketoconazole was shown by C. krusei followed by C. tropicalis (Figure 4). As shown in figure 5 itaconazole resistance was more in C. parapsilosis followed by C. tropicalis. Amphotericin B resistance was noted in 17 (5.8%) isolates. Maximum resistance was shown by C. krusei followed C. glabrata and C. kefyr. Resistance to amphotericin B was not noted in C. guilliermondii, C. parapsilosis and C. dubliniensis isolates.

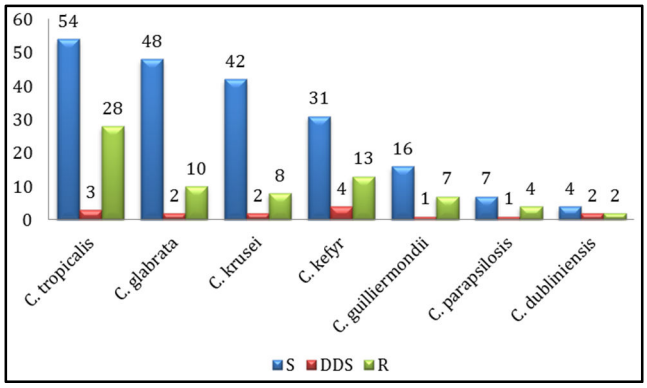

Figure-5: Antifungal Profile of NAC spp. (Itraconazole)

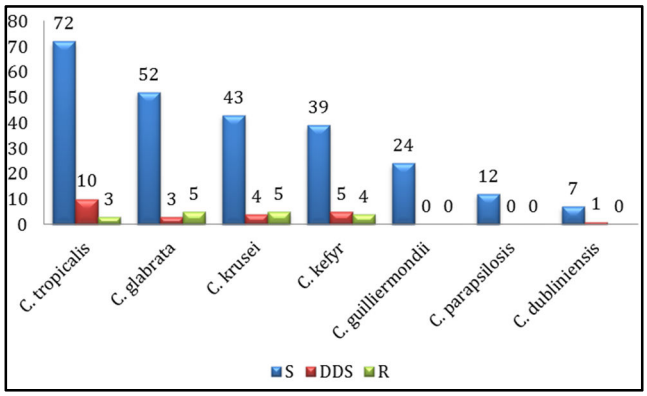

Figure-6: Antifungal Profile of NAC spp. (Amphotericin B)

Discussion

The changing patterns of the Candida isolation from various clinical samples has made identification of Candida spp. producing virulence factors compulsory for diagnostic microbiology service. In the present study C. tropicalis was the most common NAC spp. isolated from clinical specimen. Other researchers have also reported C. tropicalis as the most common emerging pathogen from the group of NAC spp.[11,12] Factors like increased use of antifungal drugs, use of broad spectrum antibiotics, long term use of catheters and increase in the number of immuno-compromised patients contributes to the emergence of C. tropicalis.[13] C. glabrata was the second most common NAC spp. isolated in the present study. Infections caused by C. glabrata are difficult to treat as it is resistant to many azole group of antifungal agents.[14] C. glabrata infections are common in immunocompromised hosts and diabetes mellitus patients. It is also associated with high-mortality rates in at risk

hospitalized and immunocompromised patients.[14]

Among the various putative factors important for invasion of host tissue and subsequent infection by Candida spp. the major role is played by extracellular hydrolytic enzymes. These enzymes derange the cell membrane constituents of the host leading to its dysfunctioning and facilitate the invasion of the host.[15] Most of the available studies on hydrolytic enzymes is focused on C. albicans.[16,17]

In our study phospholipase activity was noted in 38.7% of NAC spp. Maximum phospholipase production was seen in C. tropicalis (87.1%), which is similar to the observation of Thangam et al.[18] In contrast to our observation Samaranayake et al[6] reported, no production of phospholipase by C. tropicalis. The variation in different strains or the difference in the method of media preparation may be the reason for discrepancy observed by different workers in the phospholipase activity of the NAC spp.[19] Proteinase activity was seen 38.1% of NAC spp. Proteinase of Candida spp. evades host defense by degrading enzymes and complement proteins.[20] The correlation between production of proteinase and virulence shows that, the most virulent NAC spp. like C. tropicalis produces more proteinases in-vitro than less virulent spp.[15] This observation was also noted in our study.

The most of research on Candida hydrolytic enzyme activities is focused on proteinases, phospholipases and haemolysins. There are few studies available on coagulase production in Candida.[21] In our study 30.1% of NAC spp. produced coagulase after incubation at 24 h. Maximum coagulase production was noted in C. tropicalis (80%). No strains of C. kefyr and C. parapsilosis produced coagulase. This finding was consistent with study conducted by Rodrigues et al.[21] In Candida secretion of haemolysin followed by iron acquisition helps in penetration into deep tissues.[8] In the present study 27.3% of NAC spp. showed β-type of haemolytic colonies on blood agar. Our observation is in constrast to that of Luo et al[22] where on α-type of haemolysis was seen on glucose-free sheep blood agar.

Drug resistance although rare in fungi two decades ago, is becoming a major problem.[23] In case of Candida infection antifungal resistance was previously noticed in few isolates from patient receiving prolonged treatment for chronic mucocutaneous candidiasis.[24] Fluconazole resistance was observed in 27.3% of NAC spp. in our study. The maximum fluconazole resistance was observed in C. tropicalis was also noted by Myoken et al[25], whereas in a study conducted by Pfaller et al[26] moderate level of fluconazole resistance was seen in C. tropicalis isolates. The resistance to fluconazole is of concern not only because it is cost effective drug but it is also the most common azole used for the treatment of candidiasis. Though only 5.8% isolates of NAC spp. were resistant to amphotericin B, the high frequency of renal toxicity and several other adverse effects limits its use.[27]

Conclusion

The change in epidemiology and pattern of antifungal susceptibility of Candida infection has made identification of aetiological agent compulsory along with its antifungal susceptibility. NAC spp. cannot be overlooked as mere containment or non-pathogenic commensals. Research on extracellular hydrolytic enzymatic activity of NAC Spp. would be an important tool to prove the relation between the infective species of Candida and infection.

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