



Original Research Article

Prevalence of *non albicans Candida* in diabetic subjects and its extracellular enzymatic profilesSiva Prasad B V¹, Chandra Sekhar A², Vijaya Raghava Prasad D¹, Vijaya Lakshmi D^{1,*}¹Dept. of Microbiology, Yogi Vemana University, Kadapa, Andhra Pradesh, India²Dept. of Biotechnology, Yogi Vemana University, Kadapa, Andhra Pradesh, India

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ABSTRACT

Aims: Epidemiology of Candidiosis their prevalence, identification and evaluation of extra cellular enzymes patterns in *non albicans Candida* group from the oral washings of diabetic subjects.**Materials and Methods:** A total of 125 oral samples were collected and analyzed. The Candida species were identified by means of morphological and by molecular methods. The production of virulence factors assessed by using standard protocols.**Results:** Total of 70 different yeast like fungal strains were isolated and identified. The selected 6 virulence factors were investigated and compared critically among the NAC species. The overall observations were indicating that the virulence factors are fluctuated randomly with in the strains tested. Maximum extent of the virulence factors were reported as Phospholipase [*Candida parapsilosis*], protease [*Candida tropicalis-2*, *Candida parapsilosis*], Haemolysin [*Candida tropicalis-3*], Coagulase [*Candida tropicalis-3*], and Biofilm formation [*Candida parapsilosis*] respectively.**Conclusions:** In the present study we focused mainly on NAC species and their credible factors responsible for the progression of infections in diabetic subjects.© This is an open access article distributed under the terms of the Creative Commons Attribution License (<https://creativecommons.org/licenses/by/4.0/>) which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

1. Introduction

Oral candidiasis is an opportunistic fungal infection have increasingly recognized and represent as relevant cause of high mortality and morbidity in growing segment of immuno-compromised subjects for the last few decades.¹ Most of the fungal infections occur in patients with chronic obstacles like cancer, AIDS, neutropenia as well as diabetes. Both type –I and II diabetes comes under immuno-compromised state, moreover it is also an emerging threat to global health in the twenty-first century.² According to Indian diabetic federation (IDF) statistics, approximately 463 million adults age ranging from 20-79 years were suffering from diabetes throughout the world, perhaps it will rise up to 700 million tentatively by 2045 and 10% of global

health expenditure is spent on diabetes exclusively.³

Candida albicans are the most common fungal pathogens capable of triggering infections in immunocompromised individuals with high incidence rate. Similarly the asymptomatic state of other than *C.albicans* can also cause most severe to lethal infections ranging from muco-cutaneous over growth (bio-film) to life threatening disseminated candidemia. The predisposing conditions are increasingly associated with spectrum of patients with age, food habits, associated risk factors related to impaired immunity, dysbiosis etc. These conditions make the patients to vulnerable for infections due to *Non albicans Candida* (NAC) rather any other species. The relationship between diabetes and Candidiosis has been widely studied by many researchers^{4,5} and concluded that the frequency of infection is more adequate in insulin depended patients. Interestingly in recent past most of the researches concentrating on NAC

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species due to availability of scanty information at the same time which are natural resistant or attain resistance to available antifungal drugs.

The progression of *Candida* from non virulent to potent virulent strain is facilitated by variety of influencing factors related to both pathogen as well as host. These factors exclusively enhance the virulence of an organism, mainly depends on the secretion level of extracellular hydrolytic enzymes and bio-film formation. However among the recognized host conditions for *Candida* colonization and subsequent infection prevailed due to adhesion to epithelial cell surfaces, elevated salivary glucose levels,⁶ reduced salivary flow,⁷ micro vascular degeneration, and impaired anticandida activity of neutrophils. These conditions are particularly serious in the presence of high glucose levels,⁸ secretion of several degradative enzymes^{9,10} or even in a generalized immunosuppressant state of the patient.^{11,12} Although there has been extensive research done to detect the pathogenic features in *Candida albicans*, but relatively less information is available on *non Candida albicans*. Therefore the main objective of the present study was to isolate and identify the clinical significance of *non Candida albicans* from the oral washings of diabetic subjects and also to study their efficacy to produce important extra cellular hydrolytic enzymes such as phospholipase, Protease, Haemolysin and Coagulase and Bio-film formation.

2. Materials and Methods

2.1. Clinical samples

The present research study protocols was approved by the Institutional ethical committee 1841/Go/Reg/S/CPCSEA: DATED 18/11/2015, Clinical samples were collected from patients who were regularly visiting at Rajiv Gandhi Institute of Medical Sciences, Kadapa, Andhra Pradesh, India by providing a consent form. Total 125 oral washings were collected in sterile containers and transported to the laboratory immediately for analysis.

2.2. Preparation of *Candida* suspension

Isolated *Candida* species were inoculated on Sabouraud's Dextrose broth (SDB) with chloramphenicol, incubated at 37°C for 24 to 48h. Further culture was harvested into sterile 0.1M phosphate buffered saline (PBS). Allow the turbidity equal to optical density (OD) of 0.5 McFarland tube and measured for cell count and adjusted to 1×10^6 cells/ml.¹³

2.3. Phospholipase activity

Phospholipase production levels were detected by using egg yolk agar medium by precipitation zone method with slide modifications.¹⁴ The agar medium consist of Sabouraud dextrose agar (65g), NaCl (55.3g), CaCl₂ (5.5 g) and 10% sterile egg yolk. Ten micro liters of saline suspension (10^6

cells/ml) was inoculated as spots in triplicates and incubated at 37 °C for 7 days. Phospholipase index (Pz) was calculated as described by Price et al.

2.4. Protease activity

Extracellular protease (Prz) was estimated by fungal base medium supplemented with 0.2% Bovine Serum Albumin (BSA) according to Staib.¹⁵ The medium was adjusted to pH 5.0, sterilized by filtration & mixed with sterilized medium. Aliquots (10 µl) of 48 h old fungal cells were placed on the culture plate like spots and incubated at 37 °C for seven days. Clear zone around the colony ensure the proteolytic activity.

2.5. Haemolysin activity

Haemolysin production was demonstrated by plate assay method according to Manns et al.,¹⁶ In brief, 10 µl of the culture suspension was inoculated in triplicates on a sheep blood agar medium enriched with sugar (3%) and incubated in 5% CO₂ incubator at 37°C for five days. The transparent/semitransparent zone at site of inoculation, considered as positive for haemolysin production. The enzyme activity was measured by EAI (HI).

2.6. Coagulase activity

Coagulase activity was measured by tube method according to Isenberg.¹⁷ 0.1ml of culture suspension and 500µl of filter-sterilized human plasma was inoculated to the tubes and incubated at 37°C. The tubes were observed for clot formation with in 24 hrs along with controls. Non suspended clot after gentle shaking at appropriate incubation were treated as positive.

2.7. Bio-film formation

The test organisms were investigated for their bio-film formation, by growing on polystyrene tubes.¹⁸ 200µl of each test organism was inoculated in to the wells containing SD broth with 8% glucose and incubated at 37°C for 24hrs at static condition. Later the tubes were washed with PBS and stained with 1% Safranin.

2.8. Calculation of Extracellular enzyme activity index and Statistical analysis

Selected NAC strains were screened for the production of extracellular enzymes i.e. Phospholipase [Pz], Protease [Prz], Haemolysin [Hz] and Coagulase using the standard protocols. The activity index of Phospholipase was calculated using the ratio of colony diameter to colony diameter plus diameter of sediment (in mm). The enzymatic activity was scored into four categories: a Pz of 1.0 indicated no enzymatic activity; a Pz between 0.99 and 0.90 indicated weak enzymatic activity; Pz between 0.89 and

0.70 corresponded to moderate activity; and low Pz values ≤ 0.69 meant strong enzymatic activity. For assessment of protease and haemolysin activities, the diameter of translucent zone was used instead of sediment zone. For Coagulase activity non suspended clot after gentle shaking at 24hrs of incubation was treated as positive.

The experimental data analyzed statistically using one-way analysis of variance (ANOVA) as well as both Standard deviation and Standard error method used to compare the EAI levels between *Candida* species. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Prevalence of non albicans *Candida* species

In the present study we analyzed a total of 125 oral samples from both diabetic and non diabetic subjects (67 Male and 58 Female, Smokers (24) and Non smokers (101)) with age range from 21 to 75 years (Table 1). Among the tested subjects 22.4% cases showed *Candida* infection from which 20 male & 8 female cases. From the total samples we isolated 70 different yeast like fungal species including; 22 (31.5%) *Candida albicans* and 48 (68.5%) non-*Candida albicans*. The most prevalent and potential species of NAC were significantly noticed as *C. tropicalis* (23), *C. ontarioensis* (8), *C. parapsilosis* (10), and *Dipodascus capitatus* (7) (Table 2). The isolated *Candida* species were further conformed by 18S rRNA sequence analysis and also by using PCR based markers RAPD and ISSR's. Among the non albicans strains, we selected only predominant isolates based on their virulence as well as clinical significance: of *Candida tropicalis*-1, *Candida tropicalis* -2, *Candida tropicalis*-3, *Candida tropicalis*-4, *Candida parapsilosis* and *Candida ontarioensis* for further progression of the study (Figure 1).

3.2. Evaluation of extracellular hydrolytic enzymes

Fig. 2 Summarized the secretion levels of an extracellular enzymes in different species of non albicans group. *C. parapsilosis* and *C. ontarioensis* species secreted the maximum Phospholipase enzyme with enzyme activity index of Pz 0.01 and Pz 0.32 respectively. In *C. tropicalis* group except *C. tropicalis*-4 remaining strains are rated as medium production on activity index i.e. *C. tropicalis*-1 (Pz-0.40), *C. tropicalis*-2 (Pz-0.42), *C. tropicalis*-3 (Pz-0.56) respectively. Very low Phospholipase activity was recorded with only one member i.e., *C. tropicalis*-4 (Pz-0.62).

Similarly tested protease production activity of *C. parapsilosis* and *C. tropicalis*-2 species secreted maximum extent of protease enzyme with an index of Prz 0.32 and Prz 0.34 respectively. *C. tropicalis*-1, 3, 4 and *C. ontarioensi* are noticed with medium enzyme activity with enzymatic activity index (Prz 0.49, 0.52, 0.44 and 0.4 respectively). Simultaneously, haemolysin activity tested

and their fluctuations were noticed in the form of Hz values. Except *C. ontarioensis* all most all test organisms were showed the very strong haemolytic activity with high Hz values. *C. parapsilosis* (Hz=0.31) reported as scanty producer. Finally, the highest and lowest coagulase activity was observed with *C. tropicalis*-3 and *C. ontarioensis*.

All the NCA strains were correlated for their bio-film formation capability by polystyrene tube method and the results were reported in Figures 3 and 4. From the Figure 4 it was evident that the maximum bio-film formation was showed by *C. parapsilosis* (0.62). The mean calculation of the bio-film formation activity for group was 0.513 with $P < 0.05$ significant level.

4. Discussion

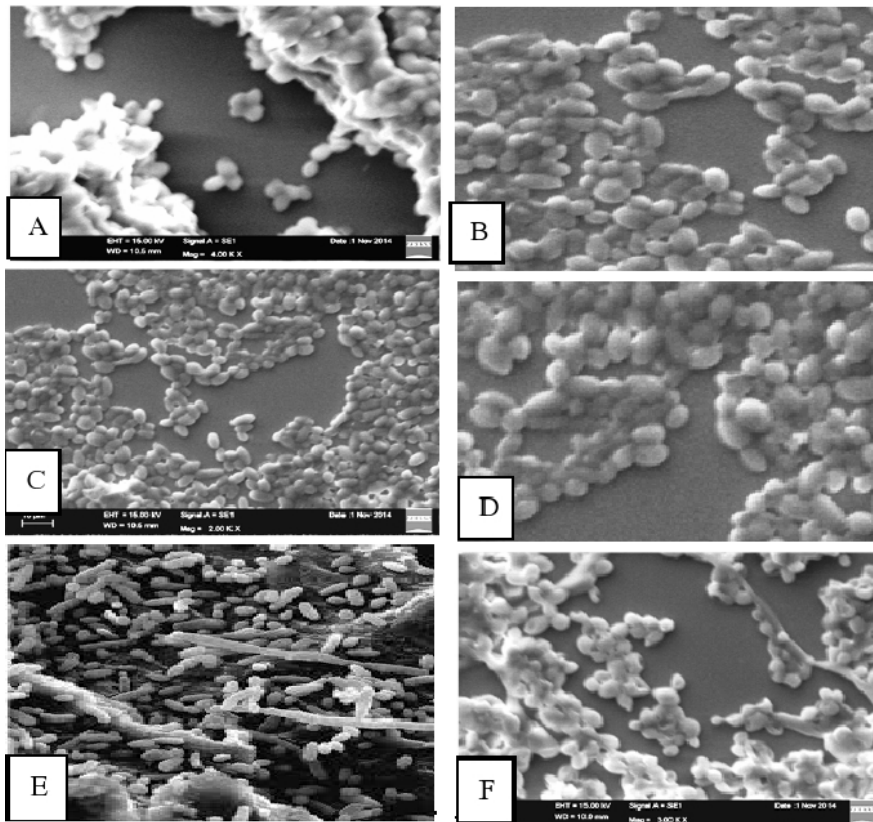
The epidemiology reports had undergone profound changes in the past few decades. In most of the cases candida species exists in the host as non pathogenic form because of ecological and physiological variations non pathogenic forms have been emerged as pathogens are of high morbidity and mortality worldwide. The most important predisposing condition for the conversion of non pathogen to severe pathogens is an ever-expanding population with mucosal or cutaneous barrier disruption, as well as quantitative or qualitative dysfunction of neutrophils, overall decreasing cell-mediated immunity and metabolic disorders¹⁹ in view of these facts, the present study was conducted to determine the prevalence of NAC in oral infections of diabetic subjects. We analyzed a total of 125 oral washings. Among 70 different strains were isolated and identified by using the conventional (CHROM agar) and molecular methods and conformed that 31.5%, strains having the homology to *Candida albicans* and remaining 68.5% strains were belongs to NAC. Among the NAC group *C. tropicalis* (47.9%), *C. ontariensis* (20.8%) *C. parapsilosis* (16.8%) and *Dipodascus* (14.5%). We also included healthy individuals as control group towards the demonstration of prevalence of *C. albicans* (10%) due their poor oral hygienic conditions. Similar to our study Chouhan et al.,²⁰ worked on diabetic and non diabetic people, they isolated the high frequency of non albicans species namely *C. glabrata* and *C. tropicalis*. According to Sachin²¹ conducted a study on isolation of candida species from various clinical samples for a period of three years stating that the reports as 36.7% were *C. albicans* and 63.3% were NAC spp. Among the NAC spp., *C. tropicalis* followed by *C. krusei* and *C. glabrata* are more prevalent. In another research the scientists emphasized clearly on the candidosis, working on the patients suffering with candiduria had hemoglobin A1c (HbA1c) levels above 7%. In this case they noticed that the rate of candiduria was relatively high in type 2 diabetic patients suffering from a improper blood glucose levels. During this condition the prevalence of non albicans species *C. albicans* (46.4%), *C. glabrata* (42.8%), *C. kefyer* (7.2%)

Table 1: Demographic data of samples analyzed according to gender and smoking

Variable	Male	Female	Smokers	Non smokers
Diabetic (n=100)	52	48	24	76
Healthy individuals (n=25)	15	10	0	25
Total number samples analyzed (n=125)	67 (53.6%)	58 (46.4%)	24(19.2%)	101 (80.8%)

Table 2: Total number of strains isolated in the present study

S.No	Name of the organism isolated	Number isolated
1.	<i>Candida albicans</i>	22
2.	<i>Candida tropicalis</i>	23
3.	<i>Candida parapsilosis</i>	10
4.	<i>Candida ontarioensis</i>	8
5.	<i>Dipodascus capitatus</i>	7
		70

**Fig. 1:** Scanning electron microscopic pictures of the different isolate **A:** *Candida tropicalis* 1, **B:** *Candida tropicalis* 2; **C:** *Candida tropicalis* 3; **D:** *Candida tropicalis* 4; **E:** *Candida parapsilosis*; **F:** *Candida ontarioensis*

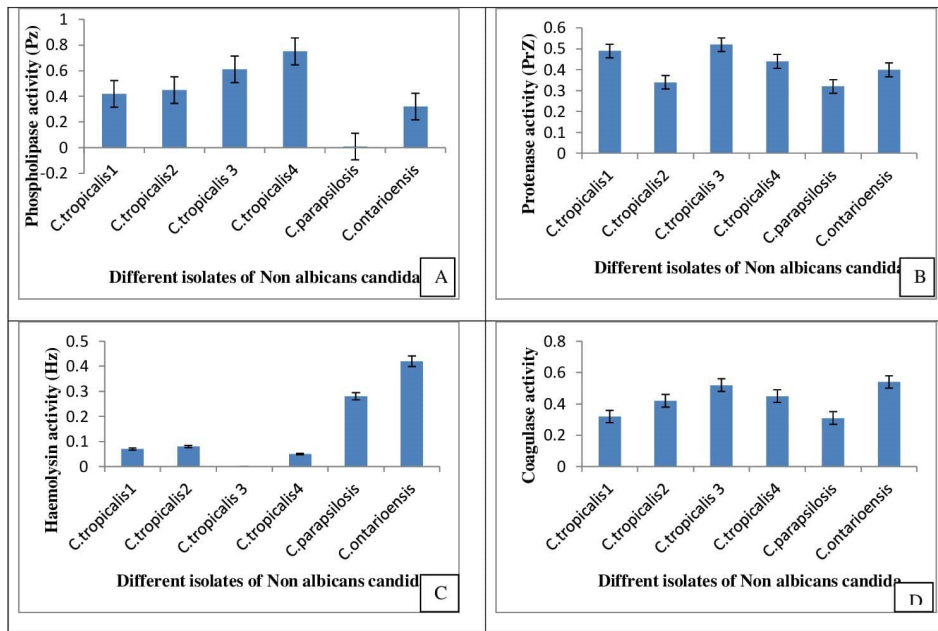


Fig. 2: Expression levels of different Exo enzymes in different isolates of *non albicans Candida*; **A:** Phospholipase activity; **B:** Protease activity; **C:** Haemolysin activity; **D:** Coagulase activity.

and *C. krusei* (3.6%). Seventy-three (88%) profoundly responsible for clinical manifestations with symptomatic conditions.²²

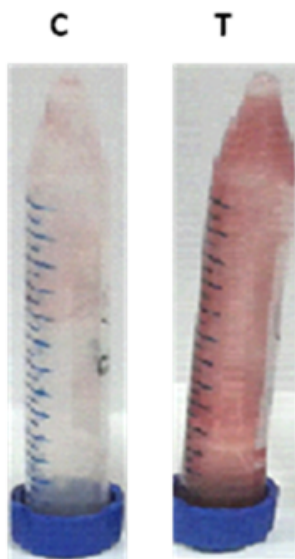


Fig. 3: Biofilm formation showed by a thin layer which is stained attached to the polystyrene tube. C= Control, T= Test organism

The production of extracellular hydrolytic enzymes such as Phospholipase, protease, haemolysin and Coagulase are the major virulence factors known to contribute to *Candida* pathogenesis had been characterized extensively

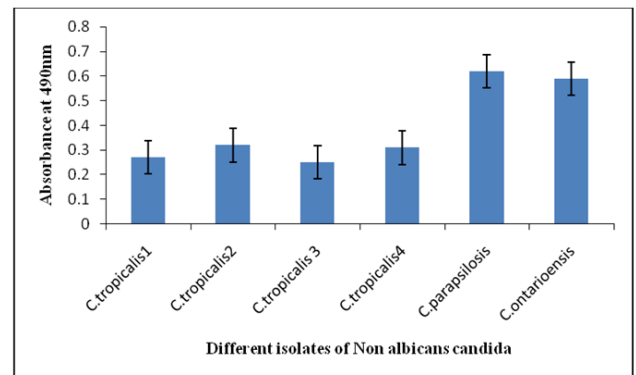


Fig. 4: Bio-film formation capacity of various *non albicans Candida* species

in *C. albicans* by a many of investigators. These enzymes play an important role in the degradation of cellular component of host tissues and further facilitate their survival, adhesion, invasion, and dissemination. Phospholipase, protease, lipase, esterase, haemolysin, etc., are the major hydrolytic enzymes secreted by most of the fungal pathogens. Phospholipase and protease enzymes prominent virulence factors which contribute to the host-*Candida* interaction. Usually the phospholipase enzyme degrades the phospholipid bi layers of the cell membrane, resulting to cell damage and lysis that enhances the frequency of dissemination. Similarly unlike other enzymes, protease specifically degrades the surface proteins and

disrupts the local immunity in order to invade the tissue.

The efficacy of extracellular hydrolases varies among the candida species and also depends on the source, location of infection.²³ Much and more these virulence factors mainly produced by all the Candida species in general and *C. albicans*, which is considered the most pathogenic member of the genus.²⁴ However, quite a few research articles refer to virulence factor produced by NAC spp. In the present study we found that the *C. parapsilosis* produced maximum extent of enzyme where as *C. tropicalis*-4 produced low amount of phospholipase enzyme when compared with other members present in that group. *C. ontarioensis* was considered as the optimal producer for the phospholipase enzyme.

Several researchers have reported contradictory findings regarding phospholipase activity of NAC group, particularly Thangam et al.²⁵ has been reported high phospholipase activity in *C. tropicalis* while Samaranayake et al.²⁶ noticed with zero activity. Substantially, we assumed that the above inconsistency and erratic observations may be due to biological differences among the strains tested. However this could be one of the possible reasons for minimal or no ability to cause invasive infections for some strains of NAC. Protease enzyme promotes the *Candida* invasion and colonization of host tissue by disruption of host membrane by degrading the defense proteins. In general a total of 52% of *C. tropicalis* producing protease enzyme. In the present study we concluded that all test organisms secreted medium to high levels of protease enzyme. These findings were in harmony with other researchers like Deorukhkar and Saini,²⁷ Mane et al.,²⁸ and Dosta et al.²⁹

Haemolysins are proteins that cause lysis of red blood cells by disrupting the cell membrane. It plays a central role in the lyses of RBC by complement fixation and opsonization²³ and enhances the hyphal invasion in systemic candidiasis.²⁴ Hence it is considered as an essential virulence factor which enables the pathogen survival and prolonged persistence in the host. In the present study, we recorded the maximum haemolysin production by *C. tropicalis* strains and medium in *C. parapsilosis* and *C. ontarioensis*. Sachin et al.,²¹ and Mane et al.²⁸ reported that 30.4% of *C. tropicalis* showed maximum hemolytic activity than other NAC isolated from HIV infected individuals. Generally Coagulase enzyme triggers a cascade of reactions that stimulate the plasma clotting. The present study reported that the coagulase production was very high in *C. tropicalis* 3 and *C. ontarioensis* when compared with other strains. Our findings are also comparable to that of Yigit et al.,³⁰ and Rodrigues et al.³¹

The bio-film formation ability of different clinical isolates of NCA species was evaluated and the results showed that all NAC species studied (*C. tropicalis*, *C. parapsilosis* and *C. ontarioensis*), formed bio-films on polystyrene surfaces under the favorable conditions. Similar

to our findings shin et al., 2002³² and Ramage et al., 2006³³ confers the bio-film formation by NAC on a biotic surfaces including polystyrene.^{32,33}

5. Conclusions

Present research work mainly showed the attributes of oral Candidiasis with relatively high prevalence in diabetic subjects. Most of them are suffering from the unstable or fluctuations in blood glucose levels. The prevalence of non albicans candida species especially *C. tropicalis* is quite high in all the respects including the efficacy of virulence factors. However rapid detection of Candida species from clinical samples, accurate in identification is key source to pave the way in order to prevent the invasion or dissemination into the tissue are the major perspectives.

6. Source of Funding

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7. Conflicts of Interest

The authors declare that there is no conflict of interest.

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