International Journal of Medicine
pISSN 1468-3814 | eISSN 2667-7008
https://theinternationalmedicine.com/

Original Research Article *DOI: 10.61336/im/26-06-120*

Unveiling the virulence determinants of Candida species from diverse clinical sources

[1] Dr Nagarajuu Vanaparti, [2] Dr.Venkateswarlu.Mudu, [3] Dr.Anil kumar Porandla

[1]*Associate Professor, Department of Microbiology, Nova Institute of Medical Sciences and Research Centre, Jaffereguda (V), Abdullapurmet (M), Telangana, India*

[2] *Assistant Professor, Department of Microbiology, Arundhati Medical College,Gandimisamma Dundigal village Medical-Malkajgiri district Telangana, India.*

[3] *Assistant Professor, Department of Biochemistry,Arundhati Medical College, Gandimissama Dundigal village Medchal Malkajgiri District, Telangana, India*

Correspondence:
Dr Nagarajuu Vanaparti

Received: **May 09, 2026**
Revised: **May 25, 2026**
Accepted: **June 08, 2026**
Published: **June 20, 2026**

Abstract

Background: Only few decades back, it was believed that Candida spp. have only passive or almost no role in the pathogenesis of disease and therefore immunocompromised status of the host was the only factor considered as crucial in pathophysiological process of candidiasis. Recently, this concept is changed and it is evident that Candida spp. can actively contribute to establishment and further progression of disease through aggression mechanism termed as virulence factors. This study aimed to study the virulence factors of Candida spp. isolated from various clinical specimens. ***Material and methods:*** Virulence factors like production of extracellular hydrolytic enzymes, haemolysin activity and biofilm formation were studied. Extracellular enzymes included phospholipase and proteinase. ***Results:*** Study revealed that haemolysin production (81.1%) was is the major virulence factor of Candida spp. followed by biofilm formation (79.7%). Proteinase activity was shown in 70.5% of isolates. Phospholipase activity was demonstrated by 74.4% of Candida isolates from various clinical specimens. ***Conclusion:*** The virulence attributes like exoenzymatic activity and biofilm formation once attributed only to C. albicans, is also noted in Non albicans Candida species. As there was a variation noted in type of virulence factor produced as per the species of Candida, infecting strain and the site of infection varies greatly with species, strain and site of lesions, identification and characterization of these virulence attributes is extremely essential to understand the pathogenesis and epidemiology of candidiasis.

Keywords: Candida, biofilm formation, phospholipase, haemolysin, proteinase, virulence factors.

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INTRODUCTION

For many decades, fungi were considered as innocuous environmental inhabitants saprophytes with no or minimal role in human disease. However this concept has revamped. It has become apparent that a number of fungal species previously considered as saprophytes are in fact capable of causing life threatening infections.1 Various factors are known to predispose to fungal infections but modern medical interventions and immunosuppressive diseases are particularly important.

Annually billions of people are infected by fungi worldwide. Fungal infections are more common in individuals with defective cellular immunity compared to those with deficient humoral immunity.2 Fungi though a substantial contributor to morbidity and mortality, have always received less attention as compared to other infective causes of human disease.3

Among various fungal infections, candidiasis is a major threat. Probably, Candida species is the only opportunistic fungal pathogen capable of causing a wide spectrum of clinical manifestations that ranges from muco-cutaneous overgrowth to life threatening disseminated infections like blood stream infection (BSI), meningitis etc.4

Only few decades back, it was believed that Candida spp. passively participates in the overall process of establishment and progression of the disease and hence organic weakness or compromised immune status of the host was the only mechanism considered for candidiasis. 4 However, recently the concept is changed and now it is confirmed that Candida participates actively in pathophysiology of the disease through aggressive mechanism termed as virulence factors. 4

These virulence factors are all traits required by the pathogen to establish and further exacerbate the infection process in a susceptible host.5 Direct interaction of

virulence factors with host cells leads to cellular damage.5

Adhesion or adherence to host tissues and medical devices, biofilm formation, the secretion of extracellular hydrolases, thigotrophism, polymorphism and phenotypic switching are important virulence factors attributing to pathogenicity of Candida spp.4 The present study was conducted with an aim to evaluate the virulence activity of Candida spp. isolated from various clinical specimens.

MATERIALS AND METHODS

The present descriptive cross sectional study was conducted in the Department of Microbiology for a period of 3 years. Candida spp. isolated from various clinical specimens were included in the study. Candida isolates were identified upto species level by standard mycological protocol.6

Virulence factors like production of extracellular hydrolytic enzymes, haemolysin activity and biofilm formation were studied. Extracellular enzymes included phospholipase and proteinase.

1. Extracellular hydrolytic enzymes

1.1. Phospholipase activity.

In clinical isolates of Candida spp., the phospholipase activity was screened on egg yolk agar by the method described by Samaranayake et al. (1984).7 A 5 μL of standard inoculum of Candida strain containing 108 yeast cells/mL was aseptically inoculated onto egg yolk agar. The inoculated plates allowed to dry at room temperature for 2 minutes and then incubated at 350C for 3 days.

The phospholipase activity (Pz) was indicated by presence of a precipitate zone around the colony and was expressed as the ratio of the colony to the diameter of the colony plus the precipitation zone.

A Pz value of 1 indicated no phospholipase activity, whereas Pz<1 indicated phospholipase expression by the isolate. C. albicans ATCC 10231 (positive) and C. kefyr ATCC 25412 (negative) were used quality control strains

1.2. Proteinase activity.

Proteinase activity (Prz) of Candida spp. was determined by Bovine serum albumin agar (BSA) by the method of Aoki et al (1990). 10 μL of standard inoculum containing 106 yeast cells/mL was aseptically inoculated onto 1% BSA agar plate.

Inoculated plates were incubated at 370C for 7 days. Post incubation, the further proteinase production of Candida spp. was inhibited by adding 20% trichloroacetic acid and the petri plate was stained with 1.25% amido-black.

RESULTS

The diameter of the colonies was measured before staining and the diameter of the clear zones was measured after staining.

Prz was measured in terms of the ratio of the colony to the diameter of unstained zone. A Prz value of 1 denoted no proteinase activity, whereas Prz<1 indicated proteinase expression by the isolate. C. albicans ATCC 10231 (positive) and C. kefyr ATCC 25412 (negative) were used for the purpose of quality control.

2. Haemolysin production.

The clinical isolates Candida spp., were screened for haemolysin production as per the method described by Luo et al (2001).9 Sheep blood Sabouraud dextrose agar (SB-SDA) plate was used and 10 μL of standard inoculum (108 Candida cells/mL) prepared from test strain was inoculated on it.

Inoculated SB-SDA plates were then incubated at 370 C in 5% CO2 for 2 days. The presence of a distinct translucent halo around the inoculum site indicated haemolysin production.

Haemolytic activity (Hz) was determined by calculating the ratio of the diameter of the colony to that of the translucent zone of haemolysis.

C. albicans ATCC 90028 and C. parapsilosis ATCC 22019 were used as positive and negative controls, respectively

3. Biofilm formation.

Tube method described by Yigit et al (2011) was used for detection of biofilm formation in Candida isolates.10 Colonies of test strain were inoculated in saline and incubated for 24 h at 350C. 1.5mL of this saline suspension was transferred to screw capped conical polystyrene tubes containing 5mL of Sabouraud dextrose broth supplemented with glucose (final concentration, 8%).

The tubes were incubated at 370C for 24h without agitation. After incubation, the content from the tube was gently aspirated using a sterile Pasteur pipette. The tube was washed thrice with phosphate buffer saline (PBS) (pH7.2) and stained with 1%safranin.The stain was decanted after 15 min and the tube was rinsed with PBS to remove excess stain.

Visible adherent film on the wall and the bottom of the tube indicated biofilm formation by the isolate. C. albicans ATCC 90028 and C. albicans ATCC 10231 were used as positive and negative control strains respectively.

During the study period, a total of 207 *Candida* spp. were isolated from various clinical samples. The isolation of non *albicans Candida* spp. was significantly high (Fisher exact test *P* value 0.05).

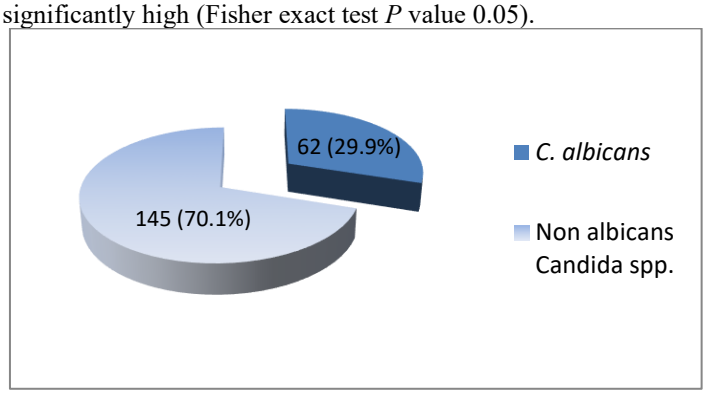

Figure 1: *Candida* isolates from various clinical specimens.

NAC spp. isolated in the present study is shown in figure 2. *C. tropicalis* was the predominant NAC spp.

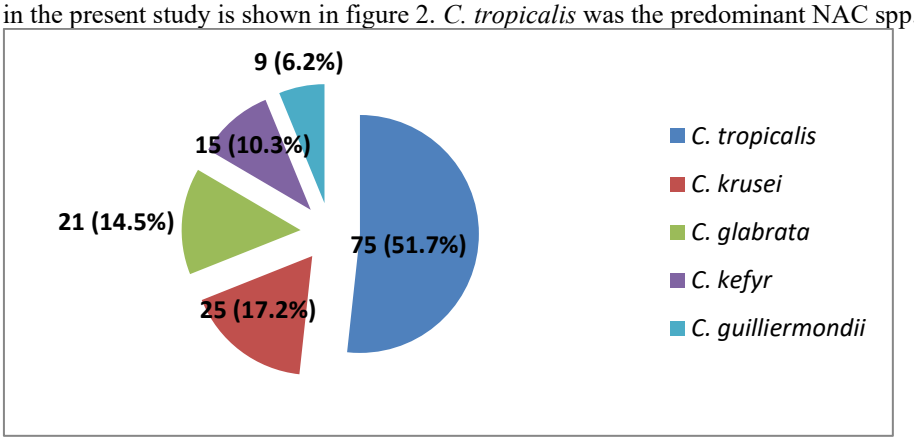

Figure 2: NAC spp. isolated from various clinical specimens.

Table 2: Virulence factor production in *Candida* isolates.

***Candida* spp. (N)**	**Extracellular hydrolytic enzymes (%)**		**Haemolysin production (%)**	**Biofilm production (%)**
	Phospholipase	Proteinase		
C. albicans (62)	57 (91.9)	59 (95.2)	62 (100)	60 (96.8)
C. tropicalis (75)	68 (90.7)	64 (85.3)	74 (98.7)	70 (93.3)
C. krusei (25)	14 (56)	09 (36)	11(44)	12 (48)
C. glabrata (21)	13 (61.9)	08 (38.1)	13(61.9)	16 (76.2)
C. kefyr (15)	06 (40)	05 (33.3)	06 (40)	05 (33.3)
C. gulliermondii (9)	02 (22.2)	01 (11.1)	02 (22.2)	02 (22.2)
Total (207)	**154 (74.4)**	**146 (70.5)**	**168 (81.1)**	**165 (79.7)**

As shown in table 2, in *Candida* spp. haemolysin production (81.1%) was the major virulence factor followed by biofilm production (79.7%). Proteinase production was seen in 70.5% of isolates whereas 74.4% of *Candida* isolates showed phospholipase activity.

The phospholipase activity was significantly high in *Candida* spp. isolated from systemic infection compared to superficial and mucocutaneous infections (Mann Whitney Test, *P* value <0.05).

Proteinase activity was significantly high in isolates from superficial and mucocutaneous infections (Mann Whitney Test, *P* value <0.05). There was no significant difference noted in haemolysin activity of isolates from different sites (Mann Whitney Test, *P* value >0.05). Majority of biofilm forming isolates showed significantly high phospholipase activity (Fisher's Exact test, *P* value<0.05).

Candida spp. isolated from disemminated infections showed significantly high ability of biofilm formation compared to those isolated mucocutaneous infections (Chi square test, *P* value <0.05)

DISCUSSION

In recent years, hitherto unknown microorganisms with minimal or no pathogenic role have emerged as an important cause of both community and hospital associated infections. The incidence of serious mycotic infections in general and candidiasis in particular has dramatically increased in recent yeras.

During the study period, a total of 207 Candida spp. were isolated from various clinical samples. The rate of isolation of C. albicans was 29.9% whereas, 70.1% of Candida isolates were NAC spp. NAC spp. were the predominant isolates from various clinical specimens. The apparent increased isolation of NAC spp. may be partly related to improvement in diagnostic techniques like use of chromogenic media, adaptation of commercial identification kits and molecular methods for identification of unusual Candida spp. 11 However, the increased involvement of NAC spp. in human infections could also be related to rampant use of antifungal drugs.

C. tropicalis was the predominant isolate in the present study. The predominance of C. tropicalis among various Candida isolates was also reported in the study of Pahwa et al (2014)12 and Deorukhkar et al (2014).13 In recent years, among NAC spp., C. tropicalis alone, or in association with other species, is more frequently implicated in human infections.14 It is often isolated from patients ICU patients, especially those requiring prolonged catheterization, treated with broad-spectrum antibiotics or with malignancies.15 C. tropicalis has higher potential for dissemination in neutropenic patients compared with C. albicans and other NAC spp.15

Several virulence factors contributing to colonization and invasion of host tissues has been described in Candida. Extracellular hydrolases like phospholipases, lipases and proteinases play an important role in adherence, tissue penetration, invasion, and the destruction of host tissue.

In the present study, phospholipase was seen in 74.4% isolates. Early studies conducted with aim to evaluate the phospholipase production in different Candida spp. reported activity in C. albicans only. In the present study, phospholipase production was seen in both C. albicans and NAC spp. These enzymes cleave the phospholipids, alter the membrane stability and lyse the host cell.16 Phospholipases are connected to germ tube formation, transition of yeasts into hyphal forms, and tissue destruction. 16

In the present study, phospholipase activity was significantly high among Candida spp. isolated from disseminated infections compared to mucocutaneous infections (Mann Whitney Test, P value <0.05). Similar findings were reported by ElFeky et al (2016).17

Proteinase, plays a major role in the establishment and exacerbation of candidiasis.4 This putative virulence factor deteriorates epithelial and mucosal barrier proteins such as collagen, keratin and mucin. Additionally also degrades complement, cytokines and immunoglobulins.18 These hydrolases also co-regulates other virulence factors like biofilm formation, adhesion and invasion of host tissue and filamentation.

Earlier studies have reported variable proteinase activity among different Candida spp. 18 In the present study proteinase activity was detected in both C. albicans and NAC spp. Similar observation was reported by ElFeky et al (2016).17

Haemolysin activity is one of the least studied virulence attributes of Candida spp. Production of haemolysin enables Candida spp. to survive and persist in host. 5 It degrades haemoglobin and facilitates recovery of the elemental iron from host erythrocytes87.

Although haemolysin activity in C. albicans is well studied, very few studies have evidenced the secretion of haemolysin in NAC spp. In the present study, haemolysin production was noted in both C. albicans and NAC spp. Our observation is similar to that of Luo et al (2001) 19 and Mane et al (2011) 20.

As the versatility of Candida spp. in adaptation to variety of different habitats and biofilm formation enhance its ability to adhere to various surfaces including medical devices and cause infection, it is one of the important virulence factors contributing to pathogenicity of Candida. 4 Candida spp can form biofilm on most, if not all, medical devices. Biofilm forming Candida isolates often demonstrate high level of resistance against most commonly prescribed antifungal drugs. 4
In the present study, 79.7% of Candida spp. isolated from various clinical specimens showed ability to form biofilms. C. albicans biofilm has been extensively studied as compared to NAC spp. In this study, biofilms were produced by both C. albicans and NAC spp. As Candida spp. isolated from disemminated infections showed significantly high ability of biofilm formation compared to those isolated mucocutaneous infections.

CONCLUSION

To conclude, non albicans Candida (NAC) species are emergent opportunists and are leading among various clinical types of Candida infections. . These ‘cryptic’ NAC species are noted to capable of producing virulence factors once only attributed to C. albicans. As the type of virulence factor produced varies greatly with species, strain and site of lesions, its identification is absolutely necessary to understand the pathogenesis and epidemiology of Candida infections.

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