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Exoenzymes Activity and Biofilm Production in *Candida* Species Isolated from Various Clinical Specimens in Benha University Hospital, Egypt

Enas Sh. Khater[1*] and Mohamed H. Al-Nory[2]

[1]Department of Microbiology and Immunology, Faculty of Medicine, Benha University, Egypt.
[2]Department of Intensive Care Unit, Faculty of Medicine, Benha University, Egypt.

Authors' contributions

This work was carried out in collaboration between both authors. Author ESK planned and designed the study, wrote the protocol, collected the samples, performed the practical laboratory activities, participated in the interpretation of the results and analysis, drafted and critically revised the manuscript. Author MHAN participated in planning and designing the study, sample collection, participated in the interpretation of the results. Both authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Background: The frequency of severe systemic fungal diseases has increased in the last few decades.
Aims: This study was done to speciate the candida isolates, to determine their antifungal susceptibility pattern and to detect biofilm formation and exoenzymes (phospholipase and proteinase) production.
Place and Duration of Study: This is a Six-months Cross sectional study conducted in ICU and Microbiology & Immunology departments, Benha University, Egypt
Methodology: The study was conducted on 75 Candida spp. isolated from various clinical samples of patients admitted in ICU. The Candida isolates were identified upto species level. Antifungal susceptibility testing was done by disc diffusion method. The biofilm formation was assessed by inoculating the isolates in conical polystyrene test tube containing Sabouraud's dextrose broth supplemented with glucose. Proteinase activity was detected by using plates containing bovine serum albumin (BSA) agar. Phospholipase activity was detected by using egg yolk agar.
Results: Seventy five Candida spp. were isolated from different clinical samples. *C.*

**Corresponding author: Email: in4ssss@yahoo.com;*

albicans was isolated from 39(52%) samples. Non-albicans Candida (NAC) spp. were isolated from 36 (48%) clinical specimens. Forty one (54.7%) out of 75 Candida species isolates obtained from the clinical isolates produced biofilm. Out of 39 C. albicans isolates 20 (51.3%) produced biofilm, while out of 36 NAC species isolates 21 (58.3%) produced biofilm. The number of total proteinase positive isolates were 50 (66.7%). C. albicans was higher than that of the NAC isolates (29 [66.7%] versus 21 [58.3%]). Phospholipase positive isolates of *C. albicans* was higher than that of the NAC isolates (32[82.1%] versus 37[49.3%]). All isolates were susceptible to amphotericin B and ketoconazole. Resistance to fluconazole was found in 8 isolates (22.2%) of NAC spp. and 2 isolates (5.1%) of C. albicans isolates.

Conclusion: The isolation of C. albicans were 39 (52%) in different clinical samples and isolation of NAC spp. were 36(48%). So NAC spp. is no longer overlooked as these organisms are emerging pathogens. The number of NAC producing proteinase, phospholipase and biofilm are more than the number of *C. albicans* producing these virulence factors. The *C. albicans* and NAC showed 100% susceptiblity to amphotericin B and ketokonazole while fluconazole showed resistance in 22.2% of NAC spp. and 5.1% of *C. albicans* isolates. All resistant Candida species to fluconazole were biofilm producers.

Keywords: Candida species; biofilm; phospholipase; proteinase.

1. INTRODUCTION

Candidaemia is associated with considerable morbidity in critically ill patients leading to an overall prolonged ICU stay, a longer duration of mechanical ventilation and haemodialysis [1]. The attributable mortality is 38%, although it can vary between 5% and 71% [2].

Candida is a part of normal flora of the human body colonizing various anatomical sites like oral cavity, digestive tract, vagina and skin [3]. The transition of *Candida* from a harmless commensal to disease causing pathogen depends on the immune system of the host and virulence factors of *Candida* [4].

The virulence factors expressed by *Candida* species, to cause infections may vary depending on the type of infection, the site and stage of infection, and the nature of the host response. The main virulence factors are biofilm formation, production of acid proteinase, phospholipase, etc [5].

Biofilms are produced when microorganism adhere to a surface and produce extracellular polymers [6]. *Candida* can form biofilm on most of medical devices like stents, shunts, implants, endotracheal tubes, pacemakers and catheters [7]. The strong adherent character of biofilm-producing ability of *Candida* on medical devices makes them a persistent source of infection [8].

Aspartyl proteinases are secreted by pathogenic species of *Candida In vivo* during infection [9] the enzymes are secreted *In vitro* when the organism is cultured in the presence of exogenous protein (usually bovine serum albumin) as the nitrogen source. Proteinase production is believed to enhance the ability of the organism to colonize and penetrate host tissues and to evade the host immune system [10].

Phospholipase enzymes are associated with membrane damage of the host cells, adherence and penetration. Invasion of host cells by microbes entails penetration and damage of the

outer cell envelope. Early data suggest that direct host cell damage and lysis are the main mechanisms contributing to fungal virulence. [5].

Phospholipase and proteinase production in *Candida* spp. can serve as an important parameter to distinguish invasive pathogenic strain from non-invasive colonizing strains [11]. Amphotericin B, a polyene fungicidal agent, has been standard treatment for candidal infections for decades, but the toxicity of the conventional form and the coast of its lipid forms limit its use. More recently, azole antifungal compounds, with lower toxicity and perfect efficacies, have emerged as the principal drugs used in treatment of candidal infection. Disk diffusion has served as simple, rapid and cost-effective method for screening the susceptibility patterns of the yeasts [12].

The aim of the study:

1. To speciate the *Candida* isolates and to determine antifungal susceptibility pattern among *Candida* spp. isolated from patients admitted in ICU of Benha University Hospital.
2. To detect biofilm formation and exoenzymes (phospholipase and proteinase) activities of the *Candida* spp. isolates.

2. MATERIALS AND METHODS

2.1 Samples Collection

A total of 75 clinical isolates of *Candida* spp. were collected from different patient sources; urine (25), blood (17), vaginal discharge (14), vascular (V.) catheter tip (7), sputum (6), pleural fluid (2) pus (2) and endotracheal tube (ETT) (2) from ICU patients of Benha University during the study period (February to July 2013). Samples were obtained from patients with clinically proven or suspected systemic *Candida* infection. The patients' symptoms and characteristics included persistent fever, unresponsiveness to broad spectrum antibiotic therapy, immunocompromization and undergoing invasive surgical procedures. Samples were collected from patients that were isolated in ICU units for a period not less than 48 h and have not taken any azole.

2.2 Reference Strains

For quality control, American Type Culture Collection (ATCC) strains were used: *Candida albicans* ATCC 14053 ^{TM}, *Candida glabrata* ATCC 2950 ^{TM}, *Candida krusei* ATCC 6258^{TM} and *Candida parapsilosis* ATCC 22019 ^{TM} (bioMérieux, France).

2.3 Cultivation [14]

The samples were inoculated on Blood, MacConkey, Chocolate and Sabouraud's dextrose agar (SDA) supplemented with 0.05g / L Chloramphenicol. After 48 hours incubation at 37ºC, growth on Sabouraud's dextrose agar were examined for pasty, creamy and smooth white colonies of yeasts which were further identified.

2.4 Yeast Identification

Yeast identification was done to each positive growth on SDA as follows:

2.4.1 Germ tube test [13]

The elongated daughter cells from the round mother cell without constriction at their origin were referred to as germ tubes, and constricted hyphae at the round mother cell were referred to as pseudohyphae. All germ tube positive isolates were considered as *C. albicans* or *C. dubliniensis* and these two isolates were further classified on the basis of CHROM agar.

2.4.2 Cultivation on the selective medium [14]

CHROMagar™ *Candida* is a selective differential medium for the rapid isolation and identification of clinically important *Candida* species. CHROMagar ™ *Candida* was purchased as powdered medium from the CHROMagar Company, Paris, France. In addition to peptone (10g/liter), glucose (20 g/liter), and agar (15 g/liter), the medium contained chloramphenicol (0.5 g/liter) and chromogenic mix (2 g/liter). The medium was prepared according to the manufacturer's instructions.

All positive cultures on SDA were plated on CHROMagar™ *Candida* Agar for identification of NAC species. Plates were incubated aerobically at 30ºC and inspected for the growth of *Candida* species at 24, 48 and 72 hours. Identification was based on colony color and morphology. Using this medium, strains were identified as following: *C. tropicalis* (wet, metallic blue colonies), *C. krusei* (dry, pink, fuzzy) and *C. albicans* (green colonies,). *C.glabrata* and *C. parapsilosis* appear as a variety of white to mauve colors.

2.4.3 Sugar assimilation test [15]

It was performed to differentiate between *C.glabrata* and *C. parapsilosis* as they give the same colors (white to mauve) on CHROMagar™ *Candida* Agar. The query colonies on CHROMagar™ *Candida* agar were incubated each in tube containing 2 ml saline at room temperature for about 24 hours to exhaust the carbohydrate reserves so that the sugar supplemented will be properly utilized and this rules out false negative results. A lawn culture of the preincubated saline was made on the yeast extract agar 01497(Sigma Aldrich) and the sugar disks (paper disks soaked in 1% (w/v) solutions of various carbohydrates, glucose, maltose, sucrose, lactose) were put and incubated for 24-72 hours at 25°C. Positive results showing enhanced growth around the disks were noted and tabulated. *C. glabrata* gives positive glucose and maltose assimilation test and lactose sugar assimilation and *C. parapsilosis* is positive for glucose, maltose and sucrose

2.5 Antifungal Susceptibility Testing [16]

Disk diffusion (DD) testing will be performed by Kirby Bauer disk diffusion method for fluconazole (25µg/disk), amphotericin (10µg/disk) and ketoconazole (15 µg /disk) (AB biodisc, Solna, Sweden) as described previously per CLSI guidelines 2009[16]. Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg/ml methylene blue dye was prepared according to the manufacturer recommendation. Inocula were prepared by picking five distinct colonies of approximately 1 mm in diameter from a 24-hour-old culture of *Candida*

species. Colonies were suspended in 5 ml of sterile saline (8.5 g/L NaCl). The resulting suspension was vortexed for 15 seconds and its turbidity was visually adjusted to that of a 0.5 McFarland standard producing a yeast stock suspension of 1 x 10^6 to 5 x 10^6 cells per ml. Inoculation of the dried agar plates using a sterile cotton swab dipped into suspension was performed by streaking the swab over the entire agar surface. Anti-fungal discs amphotericin B, ketoconazole and fluconazole were dispensed onto the surface of the inoculated agar plate. The plates were inverted and placed in an incubator at 35ºC and examined after 24 hours.

The interpretive criteria for the disk test were as follow: amphotericin B: disk zone dz≥15mm, susceptible; 14≥dz≥10mm, susceptible dose dependent and dz≤9mm, resistant. Fluconazole: dz≥19mm, susceptible; 15≤dz≤18mm, susceptible dose dependent and dz≤14mm, resistant. As for ketoconazole: dz≥20mm, susceptible; 10<dz<20mm, susceptible dose dependent and dz≤10mm, resistant.

2.6 Biofilm Formation

Biofilm formation was assessed by visual method described by Yigit et al. [17]. The isolate to be tested for production of biofilm was inoculated in conical polystyrene test tube containing Sabouraud's dextrose broth supplemented with glucose (final concentration 8%).The tubes were incubated at 35ºC for 48 hours. After incubation the broth from the tubes were gently aspirated using Pasteur pipette. The tubes were twice washed with distilled water to remove non-adherent cells. The tubes were stained with 2% safranin for 10 min. Excess of stain was removed by rinsing with distilled water and the tubes were examined for the presence of adherent layer. The isolate was considered positive for biofilm formation when a visible film was seen on the wall and bottom of the tube. The formation of ring at the liquid interface was not considered as an indication of biofilm production. *Staphylococcus epidermidis* ATCC 35984 (bioMérieux, France) served as positive control.

2.7 Detection of Exoenzymes Production

2.7.1 Preparation of inocula [18]

The inocula of yeast cells were prepared from stock cultures and incubated for 18 hours at 37ºC in Brain Heart Infusion (Oxoid) and turbidity was visually adjusted to that of a 0.5 McFarland standard producing a yeast stock suspension of 1 x 10^6 to 5 x 10^6 cells per ml.

2.7.2 Detection of protinase production was performed according to Júnior et al. [18]

2.7.2.1 Preparation of media

The test medium consisted of plates containing bovine serum albumin (BSA) agar. Sixty milliliters of a solution containing 0.04g MgSO4.7H2O, 0.5g K2HPO4, 1g NaCl, 0.2g yeast extract, 4g glucose and 2g bovine serum albumin (BSA) (Sigma - Aldrich). The pH was adjusted to 4 and the solution was sterilized by filtration and then mixed with 140 ml of sterile molten agar.

2.7.2.2 Seeding

10 µl of suspension of yeast culture at a density of 10^6 yeast /ml was inoculated onto a 1% BSA agar plate. The plates were incubated at 37ºC for 5 days. Before incubation, the BSA-agar was slightly opaque. The plates were observed daily for subsequent clearing due to hydrolysis by the acid proteinases of the fungi. Staining with 1.25% amidoblack (Sigma - Aldrich) for 15 min was performed on the fifth day followed by washing with 90% (v/v) methanol/water destaining solution. Clear zones around the disks, could not be stained with amidoblack indicated proteinase activity.

2.7.2.3 Interpretation

Proteinase activity was measured and calculated according to the method described by Price et al. [19]. Activity zone (ZA) was calculated as the ratio of the colony diameter to the colony plus the clear zone of proteolysis. ZA equal to or greater than 1.0 detects no proteinase activity, ZA lower than 1.0 detects proteinase activity.

2.7.3 Detection of phospholipase was performed according to Price et al. [19]

2.7.3.1 Preparation of media

The test medium is SDA supplemented with 1 M sodium chloride, 0.005 M calcium chloride and 2% egg yolk. All components except egg yolk were sterilized at 121ºC for 20min. Egg yolk was added to cooled medium at (45-50ºC), mixed and dispensed in plates.

2.7.3.2 Seeding

An aliquot (10µl) of the yeasts suspension was inoculated on the centre of test medium which was then incubated at 37ºC for four days to check and measure the formation of an opaque halo around the colony.

2.7.3.3 Interpretation

Activity zone (ZA) was calculated by dividing colony diameter by the sum of the colony diameter and size of precipitation zone. ZA equal to or greater than 1.0 detects no phospholipase activity, ZA lower than 1.0 detects phospholipase activity.

2.8 Statistical Analysis

The collected data were analyzed using SPSS version 16 software. Data were presented as numbers and percentages. _ Z ˝ test for 2 variables and ˝ χ2 _ (Chi square) test for more than two were used as tests of significance. P value of <0.05 was considered statistically significant.

3. RESULTS

Table 1 shows the sample wise distribution of *Candida* species. A total of 75 *Candida* spp. were isolated from different clinical specimens. *C. albicans* was isolated from 39(52%) samples. NAC spp. were isolated from 36 (48%) clinical specimens. Among NAC spp., *C. tropicalis* was the major isolate 50% (18/36) followed by *C. parapsilosis* 19.4% (7/36), *C.*

krusei 16.7% (6/36) and the least was *C. glabrata* 13.9% *(*5/36). Vaginal samples revealed *C. albicans* as a predominant isolates 71.4%(10/14), while the predominant species recovered from urine, blood and vascular catheter tip samples were non-albicans spp 52% (13/25), 58.8%(10/17) and 57.1%(4/7) respectively. Pus reaveled only *C.* albicans. Endotracheal tube, sputum and pleural fluid yielded *C. albicans* and non-albicans spp. equally.

Table 1. Distribution of *Candida* species in relation to various clinical specimens

***Candida* species**	**Urine**	**Blood**	**Vaginal**	**Sputum**	**Pleural fluid**	**Pus**	**V. catheter tip**	**ETT**	**Total**
C. albicans	12	7	10	3	1	2	3	1	39
C. trobicalis	8	5	2	-	-	-	2	1	18
C. parasiliosis	2	2	1	1	-	-	1	-	7
C. krusei	2	2	-	1	1	-	-	-	6
C. galbrata	1	1	1	1	-	-	1	-	5
Total NAC	13	10	4	3	1	-	4	1	36
Total	**25**	**17**	**14**	**6**	**2**	**2**	**7**	**2**	**75**

Table 2 shows that 41 (54.7%) out of 75 *Candida* species isolates obtained from the clinical isolates produced biofilm. Out of 39 *C. albicans* isolates 20 (51.3%) produced biofilm, while out of 36 NAC species isolates 21 (58.3%) produced biofilm. There was no significant difference in the number of biofilm producing isolates of *C. albicans* and NAC species (P=0.539). Biofilm production was high in *C. krusei* 4(80%) followed by *C. tropicalis* 12(66.7%), *C. parasiliosis* 3(42.9%) and the least producer was *C. galbrata* 2(40%).

Table 2. Biofilm production by *Candida* species

***Candida* species**	**Positive biofilm**		**Negative biofilm**	
	NO	**%**	**NO**	**%**
C. albicans=39	20	51.3**%**	19	48.7**%**
C. tropicalis=18	12	60**%**	6	40**%**
C. parasiliosis=7	3	42.9**%**	4	57.1**%**
C. krusei=6	4	80**%**	2	20**%**
C. galbrata=5	2	40**%**	3	60**%**
Total NAC=36	21	58.3**%**	15	41.7**%**
Total=75	**41**	**54.7**	**34**	**45.3**

Comparing the C. albicans and non-albicans spp. regarding the biofilm positivity Z= 0.613 and P =0.539.

Table 3 shows that biofilm producer strains isolated from blood 70.6% [12/17] , urine 56% [14/25], vascular catheter tip 85.7% [6/7] and endotracheal tube 100% more than isolated from vaginal 28.6% [4/14], sputum 33.3% [2/6], pleural fluid 0% and pus 50% [1/2].

Table 3. Biofilm formation in various clinical samples

***Candida* species**	**Urine**	**Blood**	**Vaginal**	**Sputum**	**Pleural fluid**	**pus**	**Vascular Cath. tip**	**Endotracheal tube**	**Total**
Biofilm positive	14 (56%)	12 (70.6%)	4 (28.6%)	2 (33.3%)	0 (0%)	1 (50%)	6 (85.7%)	2 (100%)	41
Biofilm negative	11 (44%)	5 (29.4%)	10 (71.4%)	4 (66.7%)	2 (100%)	1 (50%)	1 (14.3%)	0 (0%)	34
Total	**25**	**17**	**14**	**6**	**2**	**2**	**7**	**2**	**75**

Table 4 shows that the number of total proteinase positive isolates were 50 (66.7%). There was no significant difference in the number of proteinase producing isolates of *C. albicans* and NAC species (p=0.141). *C. albicans* was higher than that of NAC isolates (29 [66.7%] versus 21 [58.3.]). Highest proteinase producers among NAC isolates were *C. tropicalis* 12(66.7%), followed by *C. krusei* 3 (50%), *C. glabrata* 2(40%) and the least producer was *C. parasiliosis* 1(14.4%). The total number of phospholipase positive isolates were 37 (49.3%). There was significant difference in the number of phospholipase producing isolates of *C. albicans* and NAC species (P=0.003). the number of phospholipase positive isolates of *C. albicans* was higher than that of NAC isolates (32[82.1] versus 37[49.3%]). Highest phospholipase producers among NAC isolates were *C. tropicalis* 11(61.1%), followed by *C. krusei* 2(40%) and the least producer was *C. parasiliosis* 1(28.6%) while *C. glabrata* has no phospholipase activity.

Table 4. Exoenzymes production by *candida* species

***Candida* species**	**Proteinase Positive**		**Proteinase Negative**		**Phospholipase Positive**		**Phospholipase Negative**	
	NO	**%**	**NO**	**%**	**NO**	**%**	**NO**	**%**
C. albicans=39	29	66.7%	10	33.3%	32	82.1%	7	17.9%
C. tropicalis=18	12	66.7%	6	33.3%	11	61.1%	7	38.5%
C. parasiliosis=7	2	28.6%	5	71.4%	2	28.6%	5	71.4%
C. krusei=6	4	66.7%	2	33.3%	2	40%	4	60%
C. galbrata=5	3	60%	2	40%	-	-	5	100%
Total NAC=36	21	58.3**%**	15	41.7**%**	15	41.7%	21	58.3%
Total=75	**50**	**66.7**	**25**	**33.3%**	**37**	**49.3%**	**38**	**50.7%**

Comparing the C. albicans and non-albicans spp. regarding the Proteinase positivity (Z= 1.471 and P=0.141) and Phospholipase positivity (Z= 3.612 and P=0.003)

Table 5 shows that all isolates were susceptible to amphotericin B and ketoconazole. Resistance to fluconazole was found in 8 isolates (22.2%) of NAC spp. and 2 isolates (5.1%) of *C. albicans* isolates. All resistant *Candida* species to fluconazole were biofilm producers.

Fig. 1 shows phospholipase activity of *C. albicans* in Sabouraud's dextrose agar supplemented with egg yolk.

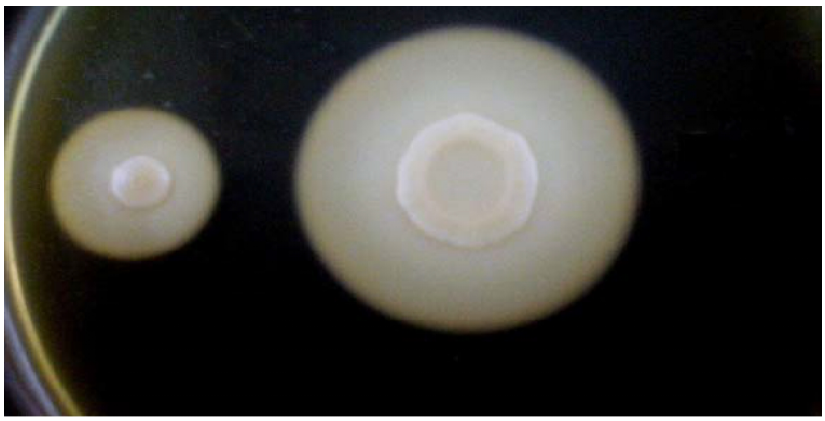

Fig. 1. Phospholipase activity of *C. albicans* in Sabouraud's dextrose agar supplemented with egg yolk

Table 5. Antifungal susceptibility pattern among *Candida* species

Candida species	Fluconazole			Ketoconazole		Amphotericin B	
	S	**S-DD**	**R**	**S**	**R**	**S**	**R**
C. albicans 39	36(92.3%)	1(2.6%)	2 (5.1%)	39(100%)	0	39(100%)	0
C. tropicalis 18	15(83.3%)	2(11.1%)	1(5.6%)	18(100%)	0	18(100%)	0
C. parasiliosis 7	4(62.4%)	0	3(42.9%)	7(100%)	0	7(100%)	0
C. krusei 6	4(66.7%)	0	2(33.3%)	6(100%)	0	6(100%)	0
C. galbrata 5	3(60%)	0	2(40%)	5(100%)	0	5(100%)	0
Total NAC 36	26(72.2%)	2(5.6%)	8(22.2%)	36(100%)	0	36	0
Total=75	**62**	**3**	**10**	**75**	**0**	**75**	**0**

S, susceptible; S-DD susceptible dose dependent; R; resistant

4. DISCUSSION

Candida species are ubiquitous colonizers of human mucosal surfaces; they are the part of the normal microbial flora of human. These, normal microbial floras adapt a pathogenic role under compromised conditions and manifested in number of distinct clinical forms collectively known as candidiasis or moniliasis. In Human, species of *Candida* producing broad range of infection ranging from most common superficial infection of the oral cavity or vagina, that involves the formation of whitish mucoid plaques known as biofilm on the mucous membranes; to acute or chronic invasive infection affecting single organ or disseminated resulted into candidaemia [20].

The present study showed that 75 *Candida* spp. were isolated from different clinical specimens. *C. albicans* was isolated from 39(52%) samples. NAC spp. were isolated from 36 (48%) clinical specimens. Similar to our results Saher and Ziab, 2013 [21] also reported the predominance of *C.albicans* 61% (63/103) than non-albicans spp. Nerurkar et al. [22] also reported the most commonly isolated species was *C. albicans* (61.36 %) . On the other hand in a study conducted by Vijaya et al. [23] non-albicans *Candida* predominated (54.1%) over *C. albicans* (45.9%). Also lower prevalence rates (39.5% and 25%) of *C. albicans* was reported by Mokaddas et al. [24] and Chakrabarti et al. [25], respectively. In our study Among NAC spp., *C. tropicalis* was the major isolate followed by *C. parapsilosis*, *C. krusei* and the least was *C. glabrata*. Sachin and Santosh, [26] reported also among NAC spp. *C. tropicalis* was the major isolate followed by *C. parapsilosis* and *C. glabrata*.

In this study vaginal samples revealed *C. albicans* as a predominant isolates 71.4% (10/14). Emam et al. [27] also reported *C. albicans* as a predominant species isolated from pregnant women and non-pregnant women with vulvovaginitis. While the predominant species recovered from urine, blood and vascular catheter tip samples were non-albicans spp 52% (13/25), 58.8% (10/17) and 57.1% (4/7) respectively. These findings are consistent with study of Jain et al. [28] who showed that non-albicans spp., especially *C. tropicalis* predominate in urine samples in many regions. Mohandas and Ballal, [5] also revealed that predominant species isolated from the blood samples were non-*Candida albicans*. These results are nearly similar to those reported by Hasana et al, 2009 [29]. The proportion of such infection due to non-albicans spp. is persistently rising [30].

Biofilms represent the most prevalent type of microbial growth in nature and are crucial to the development of clinical infections [31]. While many studies of biofilm development and species interaction have focused largely on bacterial species, relatively little is known about fungal biofilms. *C. albicans* biofilms share several properties with bacterial biofilms, including

their structural heterogeneity, the presence of expolymeric material, and their decreased susceptibility to antimicrobial agents and biocides [32].

In the present study a total of 41 (54.7%) out of 75 *Candida* species isolates obtained from the clinical isolates produced biofilm. Amer, [33] also found that 61(50.8%) of all *Candida* species were biofilm producers. Also in Luciana, [34] study (28) 56.7% of *Candida* isolates obtained were biofilm positive.

In our study out of 39 *C. albicans* isolates 20 (51.3%) produced biofilm, while out of 36 non albicans *candida* species isolates 21 (58.3%) produced slime. Biofilm production was high in *C. krusei* 4(80%) followed by *C. tropicalis* 12(66.7%), *C.parasiliosis* 3(42.9%) and the least producer was *C. galbrata* 2(40%).Similar results were obtained in a study conducted by Vijaya D. et al. [23] who reported that non-albicans *Candida* predominated (54.1%) over *Candida albicans* (45.9%). Mohandas and Ballal [5] also reported that strong biofilm production was seen in *C. krusei* and *C. tropicalis.*

This study shows that biofilm producer strains associated with blood 70.6% [12/17], urine 56% [14/25], vascular catheter tip 85.7% [6/7] and endotracheal tube 100% more than with vaginal 28.6% [4/14], sputum33.3% [2/6], pleural fluid 0% and pus 50% [1/2]. This may be due to the source of infection in the first group which is usually catheter associated that led to candidemia, UTI or LRTI. The ability to form extensive biofilms on the surface of catheters, and other prosthetic devices, also contributes to the high prevalence of the organism as etiologic agent of intravascular nosocomial infections. [35]

Aspartyl proteinases are secreted by pathogenic species of *candida in vivo* during infection. Secreted aspartic proteinases are responsible for the adhesion, tissue damage, and invasion of host immune responses. Proteinases fulfill a number of specialized functions during the infective process, they include digesting molecules for nutrient acquisition, digesting or distorting host cell membranes to facilitate adhesion and tissue invasion, and digesting cells and molecules of the host immune system to avoid or resist antimicrobial attack by the host [10].

In the present study the number of total proteinase positive isolates were 50 (66.7%). There was no significant difference in the number of proteinase producing isolates of *C. albicans* and NAC species (P=0.141). *C. albicans* was higher than that of the non-C. albicans isolates (29 [66.7%] versus 21 [58.3%]). Highest proteinase producers among NAC isolates were *C. tropicalis* 12(66.7%) and *C. krusei* 4(66.7%), followed by *C. glabrata* 3(60%) and the least producer was *C. parasiliosis* 2(28.6%). The enzymatic production of *C. albicans* and other species isolated from different clinical samples was studied and results of 62.5-100% for proteinase activity were determined by Ruchel et al. [36], Samaranayake et al. [37], Maffei et al. [38] and Pichova et al. [39]. Kantarcioğlu and Yücel, [40] reported also proteinase enzymes as important virulence factors in *C. tropicalis* and *C. parapsilosis* during mucosal and disseminated fungal infections.

The term “phospholipases” refers to a heterogeneous group of enzymes that share the ability to hydrolyze one or more ester linkage in glycerophospholipids. Since phospholipase targets membrane phospholipids and digests these components, leading to cell lysis; direct host cell damage and lysis has been proposed as a major mechanism contributing to microbial virulence [41].

In our study there was significant difference in the number of proteinase producing isolates of *C. albicans* and NAC species (P=0.003). The total number of phospholipase positive isolates were 37 (49.3%). There was significant difference in the number of phospholipase producing isolates of *C. albicans* and NAC species (P=0.003). The number of phospholipase positive isolates of *C. albicans* was higher than that of NAC isolates (32 [82.1] versus 37[49.3%]). Highest phospholipase producers among NAC isolates were *C. tropicalis* 11(61.1%), followed by *C. krusei* 2(40%), and the least producer was *C.parasiliosis* 1(28.6%) while *C. glabrata* has no phospholipase activity. The result in this study agrees with the reports of Ibrahim et al. [42] in proving that *C. albicans* isolated from the blood samples showed greater extracellular phospholipase activity. High phospholipase activity in *C. albicans* was also observed by Tsang et al. [43] and Thangam et al. [44]. Among NAC spp. maximum phospholipase activity was noted in *C. tropicalis* (65.5%). Thangam et al. [44] also reported the increased phospholipase activity in *C. tropicalis* isolates among NAC spp.

Evidence suggests that biofilms have dramatically reduced susceptibility to antifungal drugs [45]. Consequently, biofilm-related infections are inherently difficult to treat and to fully eradicate with normal treatment regimens. Antifungal susceptibility testing represents a means of predicting therapeutic concentrations of antifungal drugs used to treat a variety of *Candida* infections [46].

In this study shows that all isolates were susceptible to amphotericin B and ketoconazole. Resistance to fluconazole was found in 8 isolates (22.2%) of NAC spp. and 2 isolates (5.1%) of *C. albicans* isolates. All resistant *Candida* species to fluconazole were biofilm producers. In a study conducted by Punithavathy, [47] biofilm forming cells showed increased resistance to fluconazole. A study conducted by Ramage et al. [7] showed that biofilms from all *C. albicans* strains tested were intrinsically resistant to fluconazole. The resistance to amphotericin B was less pronounced and more variable between the isolates tested [48].

5. CONCLUSION

The present study showed predominance of *C. albicans* 39(52%) in different clinical samples, while isolation of NAC spp. were 36(48%) so NAC spp. is no longer overlooked as these organisms are emerging pathogens. The number of NAC producing proteinase, phospholipase and biofilm are more than the number of *C. albicans* producing these virulence factors. The *C. albicans* and NAC showed highly susceptiblity to amphotericin B, ketokonazole followed by fluconazole.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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