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Molecular Identification and Virulence Factors Determination in *Candida* Species Isolated from Egyptian Patients

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Abstract Candida-related infections are becoming a universal threat to the health of human who undergo immunosuppressive therapy or aggressive medical intervention. **Objectives**: The aim was to study the distribution of *Candida* species among winter and summer seasons and to determine the expression of their virulence factors. **Methods:** A total of 164 *Candida* isolates were collected from clinical specimens at Mansoura University Hospitals. *Candida* species were identified by polymerase chain reaction (PCR). Extracellular phospholipase, secretory aspartyl proteinase (SAP) and coagulase enzymes and biofilm formation were determined. *SAP 9* and *10* genes were detected by PCR. **Results**: Non-albicans (NAC) isolates were more dominant than *C. albicans* isolates (P value < 0.0001). *C. tropicalis* was the most prevalent (59.2%) followed by *C. albicans* (31.1%), then *C. glabrata*, *C. krusie*, unidentified NAC and *C. kefyr* in 3.7%, 2.4%, 2.4% and 1.2% respectively. Extracellular phospholipase activity was detected in 31.7% of *Candida* isolates. All C. *albicans* had phospholipase activity (100%) and one isolate of *C. tropicalis* was positive while other species were negative. SAPs activities were determined in 61.6% of *Candida* isolates and were detected in 70.1% and 62.7% among *C. tropicalis* and *C. albicans* isolates respectively. *SAP9* and *SAP 10* genes were detected in 27.7% and 12.9% of *Candida* isolates showed positive SAPs activity respectively and they were all *C. albicans* strains. Other species did not harbor either *SAP9* or *SAP10*. Coagulase activity was detected in 80.4% of *Candida* isolates with higher activity in *C. albicans* (88.2%), followed by *C. tropicalis* (81.4%), then other NAC isolates. Biofilm formation was determined in 69.5% of *Candida* isolates and was more prevalent in C. *tropicalis* (82.5%) followed by *C. albicans* (19.6%), *C. krusie* (100%), unidentified *NAC* (75%), *C. glabrata* (33.3%) and *C. kefyr* (50%). **Conclusion:** NAC with a preponderance of *C. tropicalis* was the most common isolated *Candida* species. Biofilm production, proteinase, phospholipases and coagulase enzymes were observed in both *C. albicans* and NAC. *SAP9* and *SAP* 10 genes were detected only in *C. albicans* strains.

Keywords: *candida, polymerase chain reaction, phospholipases, proteinase, coagulase, biofilm, SAP 9, 10 genes*

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1. Introduction

Candida species are member of the human normal microbiota, colonizing the oral cavity, the gastrointestinal and genitourinary tracts in most of healthy individuals [1]. However, these species can become pathogenic in case the host's normal flora is disrupted or the immunity is impaired [2]. *Candida* species are the most common cause of fungal infections in humans [3] with *Candida albicans (C. albicans)* being the most prevalent pathogen [4,5]. When host defenses become compromised due to hospitalization, treatment with antibiotics, surgery and the use of catheters and prosthetic devices, *C. albicans* can cause symptomatic infections, ranging from superficial skin or mucosal infections to life-threatening systemic ones [1,6,7].

The expanding spread of antifungal agents and the frequent use of broad-spectrum immune-inhibitors, changes the epidemiology of candidiasis with a shift in the prevalence of *Candida* species so that a reduced proportion of *C. albicans* and an increase in non-*albicans Candida* (NAC) species can be seen [2]. Emerging NAC species are causing increasingly high morbidity and mortality [5,7]. The rise in the incidence of *Candida* infections is complicated by the antimicrobial resistance and the limited number of available anti-fungal drugs [8].

The rapid and correct identification of *Candida* species can narrow therapy options by preventing treatment with potentially toxic antifungal agents, thus reducing costs of hospitalization and improving negative patient outcomes [2,9,10]. The precise identification of the strains at species and sub-species levels is highly demanded to perform epidemiological investigations and control the outbreaks [2]. Phenotypic and biochemical assays are frequently used for species identification. However, these assays are time-consuming, prone to error and delay appropriate antifungal treatment. Nowadays, polymerase chain reaction (PCR) is regarded as a standard platform in many clinical laboratories due to its affordability and reproducibility [9,11,12].

Candida species pathogenicity and its ability to cause infection is attributed to a set of virulence factors, including the ability to escape host defense, phenotyping switching, adhesion, biofilm formation and secretion of coagulase and hydrolytic enzymes [7,8,13,14,15]. Hydrolytic enzyme secretion during infection increases the ability of organisms for adhesion, invasion, as well as destruction of immune factors in the host, in addition to acquisition of nutrients. These enzymes include secreted aspartyl proteinases (Saps), phospholipases and hemolysins [6,16].

Secreted aspartic proteases (Saps) are encoded by a family of 10 *SAP* genes (*SAP1- SAP 10*) which have a vital role in virulence of *Candida* by degrading host tissue proteins as well as adhere to epithelial host tissue [5,8]. They degrade proteins related to immunological defense such as antibodies, complement and cytokines, allowing the opportunistic fungus to escape from the first line of host defenses [1]. Sap1 to Sap8 are fully secreted to the extracellular environment while Sap9 and Sap10 remain attached to the cell wall via a glycosylphosphatidylinositol anchor [17]. Sap9 and Sap10 enzymes maintain cell surface integrity of the *Candida* cell wall, and promote biofilm formation [8]. Sap9 is the most highly expressed Sap in strains isolated from patients with both oral and vaginal *Candida* infections [17]. All *Candida* species secrete proteinases, but non-*C. albicans* appear to do so at a lower level. These genes exhibit differential expression profiles at different stages and sites of infection [6].

Extracellular phospholipases are responsible for lipids digestion for nutrient acquisition, adhesion to host tissues, synergistic interactions with other enzymes, nonspecific hydrolysis and initiation of inflammatory processes by provoking cells of the immune system and self-defense [1]. The phospholipases catalyze the hydrolysis of phospholipids, the main part of cell membranes in humans, causing cell lysis that facilitate adhesion and penetration [6,18].

Compared with other enzymes, coagulase activity of *Candida* is the least studied with very few researches enumerated its activity. Coagulase is a protein enzyme which enables conversion of plasma fibrinogen to fibrin [19]. Candidal coagulase activity was first reported by Rodrigues *et al* [20] and previous studies have demonstrated that *Candida* species show varied coagulase activities against different animal plasma [21]. The exact mechanism of candidal coagulase enzyme in disease pathogenesis is not clearly elucidated [22].

Biofilms formed by *Candida* are a complicated three-dimensional structure, with yeast, hyphal, and pseudohyphal cells embedded in extracellular matrix [5,23]. Living within a biofilm has many advantages, which include protection against the environment, resistance to physical and chemical stresses, metabolic cooperation and joint regulation of gene expression for the associated microorganisms [1]. *Candida* presents in biofilm structure show a decrease in susceptibility to some anti-fungals and a reduction in killing by the host immune system [8]. Therefore, *Candida* infections associated with biofilms are often refractory and recurrent [23].

Expression of *Candida* virulence factors may vary depending on the infecting species, geographical origin, type of infection, the site and stage of infection, and host reaction [24]. Therefore, understanding the virulence factors will be a main tool to determination pathogenesis of candidiasis and also will help discovery new antifungal drug targets for improved therapeutic regimens [18].

Seasonality in pathogen dynamics influences the impact of disease on populations and can enhance pathogen spread [25]. Seasonal differences in host habitat can affect the transmission and persistence of pathogens by changing the contact with infective stages in the environment. Seasonal diversities in host immune function can also modify the growth of the pathogens within hosts [26]. Sociality fluctuates seasonally in many species and varies the transmission by increasing or decreasing contact rate [27]. Understanding the patterns and drivers of seasonality increases our understanding of disease effect on inhabitants and the rate of spread of invading pathogens [26].

The aim of this work was to study the prevalence of different *Candida* species isolates and their distribution among seasons using PCR for identification and to determine the expression of virulence factors by different species of *Candida*.

2. Methods

2.1. *Candida* Isolates

In this study, 164 isolates of different *Candida* species were isolated from Mansoura University Hospitals from different specimens including urine, sputum, bed sores, oral and throat swabs. Standard strain *C. albicans* (ATCC 10231) was used as control. The isolates were collected in two seasons; group I collected in May-August representing summer season isolates and group II collected from October-January representing winter isolates. All samples were inoculated on Sabouraud dextrose agar. *Candida* species were inoculated in yeast peptone dextrose broth with 20% glycerol and stored at -20°C [28].

2.2. Identification of *Candida* Species

Candida isolates were identified by their colony morphology, Gram staining, germ tube test and subculture on morphologic media (brilliance *Candida* agar) [29]. Molecular identification of *Candida* species were performed by the PCR using specific oligonucleotide primers (Table 1). For *Candida* DNA extraction, a colony was collected and inoculated into 20 µl of TE buffer. The mixture was heated for 10 minutes in water bath at 100ºC

and, then heated in microwaves at high power for 2 minute [28]. *Candida* species were detected by the PCR using specific oligonucleotide primers (Table 1).

DNA samples were amplified in a reaction mixture that contained (2.5 µl) genomic DNA samples, 5 µl 5X Green buffer, 0.75 µl (10mM) dNTP Mix, 1 µl forward primer (10µM), 1µl reverse primer (10µM) (Table 1), 0.125µl Taq Polymerase, 1.5 µl $MgCl_2$ solution (25Mm), and distilled water (13.125 µl). For *C. albicans, C. dubliniensis, C. glabrata, C. parapsilosis* and *C. tropicalis* DNA was amplified in a PCR thermal cycler (Eppendorf PCR Master cycler) by running one cycle at 95°C for 3 minutes, then 40 cycles as follows: 60 s of denaturation at 94°C, 30s of annealing at 55°C and 45s of primer extension at 72°C. Following the last cycle, additional 10 minutes incubation at 72°C was carried out to ensure the complete polymerization of any remaining PCR products [30]. While for *C. krusie* and *C. kefyr* the PCR cycle parameters were as follows; preheating at 96°C for 2 minutes; then 30 cycles of 96°C for 30s, 57°C for 3s and 74°C for 60s [31]. The amplified genes were visualized by 1.5% agarose gel electrophoresis and ethidium bromide staining under UV transilluminator. The standard strain *C. albicans* (ATCC 10231) was used as control.

2.3. Virulence Factors of *Candida* Species

2.3.1. Detection of Extracellular Phospholipase Activity

An aliquot (10µl) of the yeasts suspension was inoculated onto Sabouraud egg yolk agar and incubated at 37°C for four days. Colony diameter and precipitation zone plus colony diameter were measured and interpreted for each isolate according to Mahmoudabadi *et al* [32]; negative (Pz value = 1), weak (+) (Pz value <0.90- 0.99), poor (++) (Pz value =0.80-0.89), moderate (+++) (Pz value =0.70-0.79) and strong (++++) (Pz value <0.70). The standard strain *C. albicans* (ATCC 10231) was used as control.

2.3.2. Detection of Aspartic Proteinase Activity

Candida isolates were suspended in saline to produce turbidity equivalent to a 0.5 McFarland standard. A 6 mm sterile filter paper discs were impregnated with 10 µl of the suspension and placed on the surface of the bovine serum albumin agar. The plates were incubated at 30°C up to 7 days. Enzyme activities were scored according to the criteria by Patil *et al* [33]; (1+) when the zone of agar clarification around the margin of the colony was 1-2 mm in diameter and (2+) when the zone was >2 mm (3 to 5 mm). Standard strain *C. albicans* (ATCC 10231) was used as control.

2.3.3. Molecular Detection of *Sap9* and *Sap10* Genes by PCR

Sap9 and *Sap10* genes were detected by the PCR using specific oligonucleotide primers (Table 2).

DNA samples were amplified in a reaction mixture (12.5 µl) that contained (1.25 - 2.5 µl) genomic DNA, 2.5 µl 5X Green buffer, 0.375 µl dNTP Mix (10 mM), 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM) and (1.25- 2.5µl) template DNA and 0.0625 µl Taq Polymerase (500 µl), 0.75 µl MgCl2 solution (25 Mm), and distilled water (6.563 - 5.3125 µl). DNA was amplified in a PCR thermal cycler by running one cycle at 95°C for 5 minutes, and then 40 cycles as follows: 5s of denaturation at 95°C, 10s of annealing at 59°C and 30 s of primer extension at 72°C. Following the last cycle, additional 3 minutes incubation at 72°C was carried out to ensure the complete polymerization of any remaining PCR products.

The amplified genes were visualized by 1% agarose gel electrophoresis and ethidium bromide staining under UV transilluminator [34]. Standard strain *C. albicans* (ATCC 10231) was used as control.

Table 1. Primers for PCR amplification used for identification of *Candida* species and their oligonucleotide sequences

Species	Primer	Sequence	Sizes of PCR product (bp)	Reference
C. albicans	INT1	5'-AAGTATTTGGGAGAAGGGAAAGGG-3'	310	[30]
	INT2	5'-AAAATGGGCATTAAGGAAAAGAGC3'		
C. dubliniensis	CDf	5'-AGTATTGGGAGAGGGAAAGACC-3'	262	
	CDr	5'-ACAGGGAAGTCGATTCTTGC-3'		
C. glabrata	CGf	5'-ACATATGTTTGCTGAAAAGGC-3'	406	
	CGr	5'-ACTTTTTCTTAGTGTTCAGGACTTC-3'		
C. parapsilosis	CPf	5'-AGGGATTGCCAATATGCCCA-3'	113	
	CPr	5'-GTGACATTGTGTAGATCCTTGG-3'		
C. tropicalis	CTf	5'-TGATAGTTAGGAAAGATCAGGTG-3'	147	
	CTr	5'-AACATATCCCATGTGTGTGT-3'		
C. krusie	CKSF35	5'-GAGCCACGGTAAAGAATACACA-3'	227	[31]
	CKSR57	5'-TTTAAAGTGACCCGGATACC-3'		
C. kefyr	CKFF35	5'-CTTCCAAAGGTCAGAAGTATGTCC-3'	532	
	CKFR85	5'-CTTCAAACGGTCTGAAACCT-3'		

Table 2. Specific amplification and sequencing primer sets for *SAP9* and *SAP10 genes.*

Gene	Primers	Secuence (5´-3´)	Size of amplified product (bp)	Reference
SAP9	*SAP*9F	ATTTACTCCACAGTTTATATCACTGAAGGT	80	[34]
	*SAP*9R	CCACCAGAACCACCCTCAGTT		
SAP10	*SAP*10F	CCCGGTATCCAATAGAATCGAA		
	*SAP*10R	TCAGTGAA TGTGACGAA TTTGAAGA		

2.3.4. Detection of Coagulase Enzyme Activity

Rabbit plasma was filter-sterilized and aliquots of the filtrates were placed in screw-cap tubes and frozen at -20°C until used [21]. *Candida* strains were cultured overnight in Sabouraud dextrose broth. Approximately 0.1 ml of each culture was inoculated into tubes containing 500 µl of rabbit plasma. The tubes were incubated at 25, 37 or 45°C and observed for clot formation after 2, 4, 6 and 24 hours. The standard strain *C. albicans* (ATCC 10231) was used as control.

2.3.5. Quantification of Biofilm Formation by Crystal Violet

Quantitative analysis of biofilm production was based on method described by Jin *et al* [35]. Briefly, a 20 ml aliquot of *Candida* cell suspension containing $3x10^7$ CFU/ml was inoculated into wells of microtiter plate containing 180 ml Sabouraud glucose broth and incubated at 35°C for 24 hours without agitation. The biofilm coated wells were washed twice with 200 µl of sterile distilled water and stained with 110 µl of 0.4% aqueous crystal violet solution for 45 minutes. Afterwards, the wells were washed 4 times with 350 µl of sterile distilled water and destained with 200 µl of 95% ethanol. After 45 minutes of destaining, 100 µl of destaining solution was transferred to a new plate. The amount of the crystal violet stain in the destaining solution was measured with a microtiter plate reader at 595 nm. According to Stepanovic *et al* [36], the classification of the results obtained has four categories; non-adherent (0), weakly (+), moderately (++), or strongly (+++) adherent, based upon the optical density (ODs) of bacterial films. The standard strain *C. albicans* (ATCC 10231) was used as control.

3. Results

3.1. Identification of *Candida* Species by Polymerase Chain Reaction (PCR)

In this study, 164 of *Candida* isolates were collected in two seasons; group I isolates (80/164; 48.8%) in summer season and group II isolates (84/164; 51.2%) representing winter season. The most prevalent *Candida* species was *C. tropicalis* with isolation rate of 59.2% (97/164) followed by *C. albicans* in 31.1% (51/164), then *C. glabrata*, *C. krusie* and *C. kefyr* isolates in 3.7% (6/164), 2.4% (4/164) and 1.2% (2/164) respectively (Figure 1). Four (4/164; 2.4%) isolates could not be identified (unidentified NAC) by PCR. *C. tropicalis* was statistically significant prevalent than *C. albicans* (P value = 0.000004). Also, the total number of NAC isolates was significantly higher than that of *C. albicans* isolates (P value < 0.0001).

Distribution of isolated *Candida* species among the clinical specimens is illustrated in Table 3. *Candida* isolates were mostly recovered from urine specimens (77.4%; 127/164) followed by sputum (12.8%; 21/164) then bed sore, oral and throat swabs in 4.3% (7/164), 3.7% (6/164) and 1.8% (3/164) respectively. The distribution of *Candida* species among different season's groups is illustrated in Table 4. It was found that *C. albicans* and *C. kefyr* have a higher significance occurrence among summer group (P value = 0.01 and 0.04 respectively), while *C. glabrata* was higher among winter group (P value = 0.0005).

Among the 164 studied isolates, 86 (52.4%) isolates were collected from males and 78 (47.6%) isolates from females. For male's isolates, 62 (63.9%) were *C. tropicalis* followed by *C. albicans* (19; 37.3%), then *C. glabrata* (1; 16.6%), *C. kefyr* (1; 50%) and unidentified NAC (2; 50%). For female's isolates, *C. tropicalis* was detected in 35 (36.08%), followed by *C. albicans* (32; 62.7%), then *C. glabrata* (5; 83.3%), *C. krusie* (4; 5.1%), *C. kefyr* (1; 50%) and unidentified NAC (2; 2.7%). There was significant difference between female and male group concerning the number of *C. tropicalis* (P value = 0.0001) as it was more prevalent among male group. *C. glabrata*, *C. albicans* and *C. krusie* were more prevalent among females (P value = 0.02, 0.01 and 0.0046 respectively).

3.2. Virulence Factors Determination in *Candida* Species

3.2.1. Detection of Extracellular Phospholipase Activity

Extracellular phospholipase activity was demonstrated in 31.7% (52/164) of *Candida* isolates. All *C. albicans* had phospholipase activity (51/51; 100%) and only one (1/97; 1%) isolate of *C. tropicalis*. Other species showed negative phospholipase activity. The level of phospholipase activity among *Candida* species and according to different seasons is illustrated in Table 4. There was significant difference between total positive phospholipase activity between summer and winter groups as summer group showed higher prevalence (P value = 0.006), while no significant difference were detected between summer and winter seasons among *Candida* isolates expressing strong, moderate or week levels of phospholipase activity.

3.2.2. Detection of Secretory Aspartyl Proteinases Activity

Secretory aspartic proteinase activities was determined in 61.6% (101/164) of *Candida* isolates and was detected among 70.1% of *C. tropicalis* (68/97), 62.7% of *C. albicans* isolates (32/51) and 25% of unidentifiable NAC isolates (1/4). No activity was detected among other species. Table 4 showed the level of secretory aspartic proteinase activity among *Candida* in different seasons. There was no significant difference between summer and winter groups concerning secretory aspartyl protease activity and their levels (P value >0.05).

3.2.3. *Sap 9* and *10* genes Detection by Polymerase Chain Reaction

SAP9 and *SAP 10* genes were detected in 27.7% (28/101) and 12.9% (13/101) of *Candida* isolates showed positive aspartyl protinase activity respectively (Table 4, Figure 2). All of them were *C. albicans* isolates. *SAP9* or *SAP10* were determined simultaneously in 4.9% (5/101). Other species did not harbor either *SAP9* or *SAP10*. There was no significant difference between summer and winter groups concerning *SAP9* and *10* genes (P value > 0.05).

Table 3. Distribution of isolated *Candida* species among the clinical specimens.

Specimens		*C. tropicalis* No (%)	*C.albicans* No (%)	*C. glabrata* No (%)	*C. krusie* No (%)	*C. kefyr* No (%)	*Unidentified NAC* No (%)
Urine	(no.=127)	83 (65.4%)	32 (25.2%)	6 (4.7%)	1 (0.8%)	2 (1.6%)	3 (2.4%)
Sputum	(no.=21)	8 (38.1%)	10 (47.6%)	0 (0%)	2 (9.5%)	0 (0%)	1 (4.8%)
Bed sore	(no.=7)	3 (42.9%)	4 (57.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Oral swabs	(no.=6)	1 (16.7%)	4 (66.6%)	0 (0%)	1 (16.7%)	0 (0%)	0 (0%)
Throat swab	(no.=3)	2 (66.7%)	1 (33.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total	(no=164)	97 (59.1%)	51 (31.1%)	6 (3.7%)	4 (2.4%)	2 (1.2%)	4 (2.4%)

Table 4. The distribution of *Candida* species and virulence factors among different season's groups

	Winter group No (%)	Summer group No (%)	P Value
***Candida* species (no. of isolates)**			
C. tropicalis (97)	55 (65.4%)	42 (52.5%)	0.06
C. albicans (51)	19 (22.6%)	32 (40%)	0.01
C. glabrata (6)	6 (7.1%)	0 (0%)	0.0005
C. kefyr (2)	0 (0%)	2 (2.5%)	0.04
C. krusie (4)	1 (1.19%)	3 (3.75%)	0.1
Unidentified NAC (4)	3 (3.57%)	1 (1.25%)	0.1
Total (164)	84 (51.2 %)	80 (48.8%)	-
Extracellular phospholipase activity level (no of positive isolates)			
C. albicans (51)			
Poor (2)	0 (0%)	1 (3.03%)	0.157
Weak (5)	1 (5.26%)	4 (12.1%)	0.057
Moderate (10)	4 (21.05%)	6 (18.75%)	0.37
Strong (35)	14 (73.6%)	21 (63.3%)	0.09
C. tropicalis (1)			
Strong (1)	0 (0%)	1 (3.03%)	0.157
Total (52)	19 (36.5%)	33 (63.5%)	0.006
Aspartic proteinase activity level (no. of positive isolates)			
C. albicans (32)	13 (40.6%)	19 (59.3%)	0.1
1+ (25)	12 (23.07%)	13 (26.5%)	0.7
2+(7)	2 (3.8%)	5 (10.2%)	0.1
C. tropicalis (68)	41 (60.2)	27 (39.7%)	0.016
1+(62)	37 (67.2%)	25 (54.3%)	0.03
2+(6)	4 (7.2%)	2 (4.3%)	0.2
Unidentified NAC (1)	1 (1.8%)	0 (0%)	0.1
1+ (1)	1 (1.8%)	0 (0%)	0.1
Total (101)	55 (54.5%)	46 (45.5)	0.2
***Sap genes* detection by PCR (no. of positive isolates)**			
SAP9 (28)			
C. albicans (28)	17 (60.7%)	11 (39.3%)	0.1
SAP10 (8)			
C. albicans (8)	6 (75%)	2 (25%)	0.3
Total (36)	23 (63.9%)	13 (36.1%)	0.3
Coagulase enzyme activity (no. of positive isolates)			
C. albicans (45)	15 (33.3%)	30 (66.6%)	0.001
C. tropicalis (79)	43 (54.5%)	36 (45.5%)	0.2
C. kefyr (1)	0 (0%)	1 (100%)	0.1
C. krusei (3)	0 (0%)	3 (100%)	0.01
C. glabrata (3)	3 (100%)	0 (0%)	0.01
Unidentified NAC (1)	1 (1%)	0 (0%)	0.1
Total (132)	70 (53.03%)	62 (46.96%)	0.3
Biofilm formation (no. of positive isolates)			
C. albicans (10)	7 (70%)	3 (30%)	0.07
Weak adherence (10)	7 (70%)	3 (30%)	0.07
C. tropicalis (94)	54 (57.4%)	40 (42.6%)	0.04
Weak adherence (10)	6 (60%)	4 (40%)	0.3
Moderate adherence (13)	7 (53.8%)	6 (46.2%)	0.6
Strong adherence (71)	41 (57.7%)	30 (42.3%)	0.06
C. glabrata (2)	2 (100%)	0 (0%)	0.04
Weak adherence (1)	1 (100%)	0 (0%)	0.1
Moderate adherence (1)	1 (100%)	0 (0%)	0.1
C. krusie (4)	1 (25%)	3 (75%)	0.1
Weak adherence (2)	0 (0%)	2 (100%)	0.04
Moderate adherence (1)	1 (100%)	0 (0%)	0.1
Strong adherence (1)	0 (0%)	1 (100%)	0.1
C. kefyr (1)	0 (0%)	1 (100%)	0.1
Weak adherence (1)	0 (0%)	1 (100%)	0.1
Unidentified NAC (3)	2 (66.7%)	1 (33.3%)	0.4
Weak adherence (3)	2 (66.7%)	1 (33.3%)	0.4
Total (114)	66 (57.9%)	48 (42.1%)	0.017

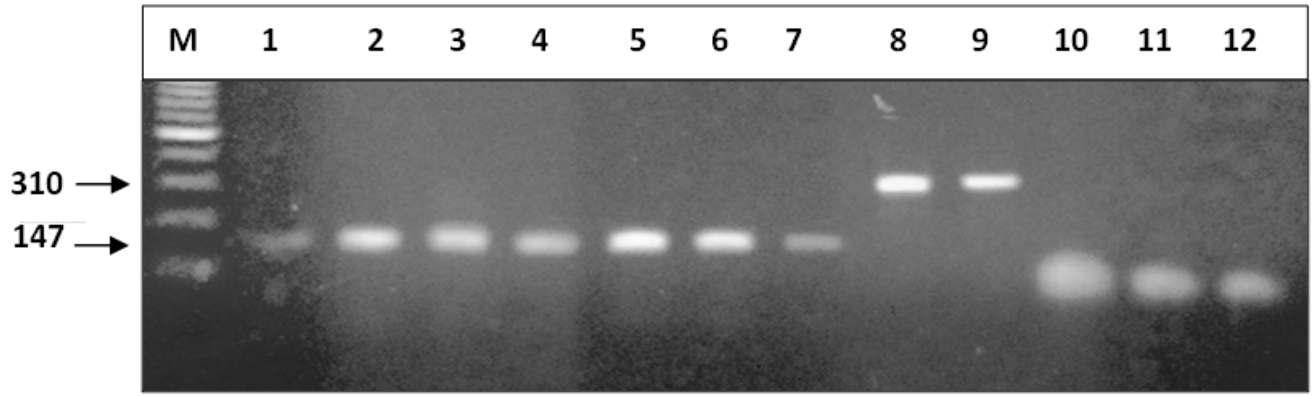

Figure 1. Agarose gel electrophoresis of polymerase chain reaction. M: DNA marker (100 bp), lanes (1-7) were identified as *C. tropicalis*, lanes (8-9) were identified as *C. albicans* and lanes (10-13) were negative for *C. parapsilosis* identification

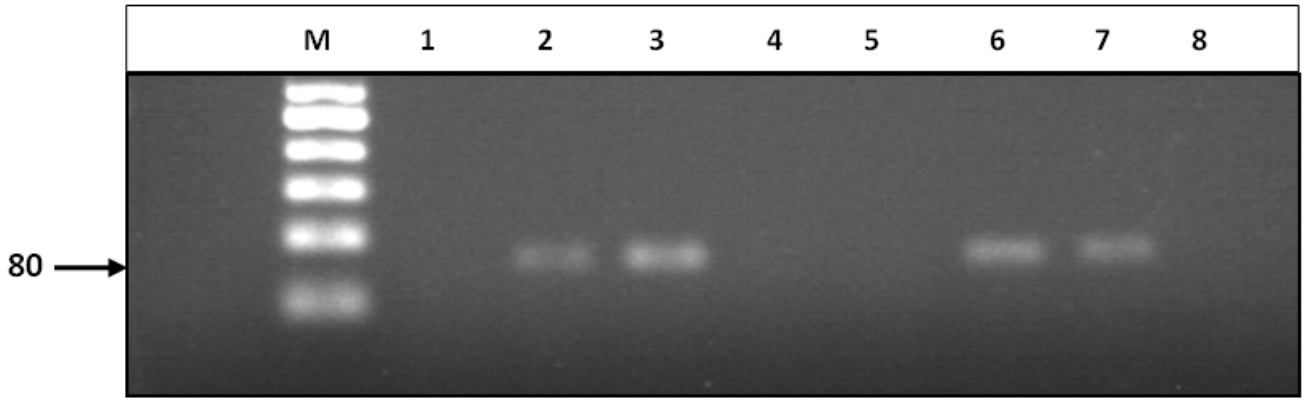

Figure 2. Agarose gel electrophoresis of polymerase chain reaction for *SAP9* gene detection. M: DNA marker (50 bp), Lanes 2, 3, 6 and 7 were positive for *SAP9* gene and lanes 1, 4, 5 and 8 were negative

3.2.4. Detection of Plasma Coagulase

Coagulase activity was detected in 80.4% (132/164) of *Candida* isolates. *C. albicans* isolates showed coagulase activity in 88.2% (45/51) followed by *C. tropicalis* (81.4%; 79/97), *C. krusie* (75%3/4), *C. glabrata* 50% (3/6), *C. kefyr* (50%; 1/2) and unidentified NAC in 33.3% (1/3). There was no significant difference in coagulase producing *Candida* species in winter group versus summer group among *C. tropicalis*, *C. kefyr* and unidentified NAC. There was significant difference in coagulase activity of *C. albicans* and *C. krusei* (P value= 0.001 and 0.01 respectively) with higher predominance in summer season while *C. glabrata* showed higher prevalence in winter (P value = 0.01) (Table 4).

3.2.5. Detection of Biofilm Formation

Biofilm formation was determined in 69.5% (114/164) of *Candida* isolates and were significantly more prevalent in NAC (91.2%; 104/114) species than in *C. albicans* isolates (8.8%; 10/114) (P value = 0.00001). Among the 114 biofilm producer *Candida* species, biofilm formation was more frequent in C. *tropicalis* of (82.5%; 94/97) followed by *C. albicans* (19.6%; 10/51), *C. krusie* (100%; 4/4), unidentified NAC (75%; 3/4), *C. glabrata* (33.3%; 2/6) and *C. kefyr* (50%; 1/2). Biofilm production among *Candida* species with their categories and statistically significant values in different seasons is illustrated in Table 4. Biofilm formation were more dominant among the total *Candida* species, *C. tropicalis* and *C. glabrata* in winter season than in the summer (P value = 0.017, 0.04, 0.04 respectively) whereas weakly adherence *C. krusie* isolates have more occurrence in summer season than the winter (P value = 0.04).

4. Discussion

Candida species are prevalent opportunistic fungi that become pathogenic in patients with reduced immune competence or in individuals with an imbalance of competing bacterial microflora [13]. *Candida*-related infections are becoming a universal threat to the health of human who usually undergo immunosuppressive therapy or aggressive medical intervention [9].

C. albicans is the most prevalent pathogen in mucosal and systemic fungal infections. However, NAC species, are now emerging as important contributors in series of disturbs ranging from mildly superficial mucosal discomforts to deadly disseminated bloodstream and deep-seated tissue infections [5].

Considering the variation of *Candida* species in susceptibility to antifungal agents, the rapid and accurate identification of the species may assist in finding an appropriate therapy for candidiasis **[2]**. Polymerase chain reaction (PCR) has been developed to help establish an early diagnosis of infection with the aim of allowing prompt initiation of antifungal therapy and improving patient outcomes [12].

The virulence of the *Candida* species is attributed to a wide variety of mechanisms including adherence, biofilm formation, extracellular enzymes secretion, and dimorphism

[18]. Hydrolytic enzyme include secreted aspartyl proteinases (SPAs) and phospholipases [6]; SPAs have a potential role in pathogenicity through facilitating the invasion and counteracting the host defense system [8] and extracellular phospholipase lyses host cells to facilitate adhesion and penetration [18]. Biofilms protect *Candida* from the harmful effects of the host and the natural environment and increase their chance of survival [15].

In the present study, the prevalence of different *Candida* species isolates and their distribution among seasons was detected using PCR for identification and the expression of virulence factors by different species of *Candida* were determined.

In this study, 164 isolates of Candida species were collected in two seasons as group I (80/164; 48.8%) summer season isolates and group II (84/164; 51.2%) representing winter isolates. The isolation rate of *Candida* species was higher in the winter which was consistent with the results of other studies [37,38]. Edi-Osagie and Emmerson [39] evaluated the season of birth as a risk factor for the development of invasive *Candida* in preterm low-birth weight infants and found that 73% of *Candida* infections occurred during the months from September to February and they recommended the consideration of seasonal associations when targeting selective antifungal chemoprophylaxis. Our results was different from other reports who stated that the isolation rate of *Candida* species was highest in April and lowest in February [40,41]. Fungal infections by *Candida* occur easily in a body with a weakened immune system. Based on this fact, it is thought that the immune system is significantly weakened at the change of seasons leading to a susceptible condition of infection.

In our study, *C. tropicalis* (59.2%) was the most prevalent *Candida* species followed by *C. albicans* (31.1%). Less frequently isolated *Candida* species were *C. glabrata*, *C. krusie* and *C. kefyr* (3.7%, 2.4% and 1.2%) respectively. This result is in accordance with many studies that identified *C. tropicalis* as the predominant widespread pathogenic yeast species of the non-albicans (NAC) group [16,42,43]. The incidence of infection attributed to *C. tropicalis* species were reported to be varying in a range of 20-45% depending on infection site and geography [44]. In India, epidemiological data showed that 67-90 % of nosocomial candidaemia were as a result of NAC species, of which *C. tropicalis* was the most predominant [45]. Formerly, *C. albicans* was thought to be the predominant species that causes candidiasis in immunocompetent and immunocompromised individuals. However, infections due to NAC including *C. tropicalis* have elevated dramatically on a universal scale. Therefore, *C. tropicalis* is proclaimed to be emerging pathogenic yeast [5,44]. On contrary, other studies reported *C. albicans* as the most frequently identified species [46,47].

In our study, the virulence activity of different *Candida* species was determined with the detection of seasonality effect on the virulence expression. Extracellular phospholipase activity was demonstrated in 31.7% of *Candida* isolates. All *C. albicans* had phospholipase activity (100%) and only one isolate of *C. tropicalis* was positive while other species were negative. Our results were in agreement with many studies who stated that 100% of *C. albicans* demonstrated phospholipase activity [24,32,48,49]. Pinto *et al* [50] reported that phospholipase activity was detected only in *C. albicans* strains in a rate of 99.4%. Vidotto *et al* [51], believed that the correlation between phospholipase activity and germ tube formation in *C. albicans* facilitate the mucosa penetration. Several studies have shown that clinical isolates of *C. albicans* have higher levels of expression of extracellular phospholipase activity that allows *C. albicans* to acquired nutrients in host nutrient-poor niches and contributes to invasion [3,16,18]. Moreover, few studies reported phospholipase positivity among NAC isolates with low enzymatic activity [3,48,52] while Gokce *et al* [53] found that all NAC strains were phospholipase negative.

In the present study, secretory aspartic proteinase activities (Saps) was determined in 61.6% of *Candida* isolates with detection rates of 70.1% and 62.7% among *C. tropicalis* and *C. albicans* isolates respectively. Our results were in agreement with Kaur *et al* [54] who reported that the SAPs activity was determined in 69.23% of *Candida* isolates where *C. tropicalis* and *C. albicans* exhibited SAPs activity in 77% and 61.1% respectively. Another study by Sachin et al. [55] declared that SAPs activity was detected in 52% of *C. tropicalis* isolates. Nawaz *et al* [56] reported that NAC strains have higher protease activity than *C. albicans* where *C. tropicalis* showed the highest proteolytic activity. Controversy, another study stated that the highest proteinase expression was seen in *C. albicans*, followed by the NAC species [18]. The absence of SAPs activity in our study among other identified NAC species was in accordance with the previous reports [3,57]. The production of SAPs is a critical virulence trait in *C. albicans*, which destruct the surface proteins (albumin, keratin) and degrade the locally protective IgA and C3 component. This enables tissue invasion and resistance to the antimicrobial attack by the host [3,18].

In this study, *SAP9* and *SAP 10* genes were detected in 27.7% and 12.9% of *Candida* isolates showed positive aspartyl protinase activity respectively and all of them were *C. albicans* strains. Other species did not harbor either *SAP9* or *SAP10*. These results are in agreement with Ikonomova *et al* [17] who stated that Sap 9 is the most highly expressed Sap in strains isolated from patients with both oral and vaginal *Candida* infections. Another study reported that *Sap5 and Sap9* are the major SAPs expressed in vivo in mucosal biofilms [58]. Other *Candida* species in our results did not harbor either *SAP9* or *SAP10* which agreed with other reports who documented that *C. glabrata* and *C. krusei* do not possess any *SAP* genes [8,57]. While Sap1 to Sap8 are fully secreted to the extracellular environment, Sap9 and Sap10 remain attached to the cell wall via a glycosylphosphatidylinositol (GPI) anchor [17,58]. Sap9 seems to be predominantly located in the cell membrane, and Sap10 is located in the cell wall and membrane [5]. In contrast to all other members of the *Saps* family, the *Saps* 9-10 proteases monitored under *in vitro* and *in vivo* conditions are independent of pH and morphotype [59]. *Sap* 9 and *Sap*10 influence distinct cell wall functions by proteolytic cleavage of covalently linked cell wall proteins, which mediate biofilm formation and promote adherence to host cells and invasion into epithelial cell layers [8,58]. The

effects of SAPs on *C. albicans* virulence can be supported by the activation of other genes such as *HWP1* that encodes the hyphal cell wall protein promoting *C. albicans* adhesion to different surfaces [5]. Kadry *et al* [8] declared that non-pathogenic *Candida* species usually has fewer genes encoding *SAP* than pathogenic species and this fact was confirmed by gene sequencing.

In this study, coagulase activity was detected in 80.4% of *Candida* isolates with higher activity in *C. albicans* (88.2%), followed by *C. tropicalis* (81.4%), then other NAC isolates. Our results is agreed with Rodrigues *et al* [20] who detected high coagulase activity in *C. albicans* (88.5%) and *C. tropicalis* (82.6%), but lower activities in other species using the coagulase tube test with rabbit plasma after incubation for 24 hours. Yigit *et al* [60] reported that coagulase activity of *C. albicans* (64.7%) isolates were higher than other species. In previous study, Padmajakshi *et al* [22] detected higher coagulase activity among *C albicans* isolates (68%) followed by *C tropicalis* (59%). In our study, the rabbit plasma were used in the coagulase tube test for the detection of coagulase activity as many studies [19,21,22] stated that the rabbit plasma appeared to give the best indication of coagulase activity, whereas sheep plasma was less sensitive and human plasma expressed no activity for any of the *Candida* species. These data indicate that rabbit plasma is the most appropriate medium for coagulase testing of *Candida* strains). Variations in coagulase production by *Candida* species may be related to their pathogenicity. Thus, laboratory detection of coagulase activity in clinical isolates of *Candida* species may help in the diagnosis of *Candida*-related infections [21].

In the current study, biofilm formation was determined in 69.5% of *Candida* isolates and was more prevalent in NAC species (91.2%) species than in *C. albicans* isolates (8.8%). These results were in accordance with Lahkar *et al* [18] who reported that of a total 33 (62.3%) biofilm positive isolates, significant production was observed in the NAC species. In a study by Mohandas and Ballal [61], a total of 73% of Candida species produced biofilm where only 51% of *C. albicans* isolates formed biofilm, which was significantly lower than the percentage of all NAC species. The later authors found that strong biofilm production was seen in *C. krusei* and *C. tropicalis* while weak biofilm production was detected in *C. albicans*. Several previous reports were in accordance with these results [62,63]. On the other hand, another study declared that *C. albicans* biofilms were bulkier than the ones formed by *C. glabrata* [3]. *Candida* biofilms formed both *in vitro* on abiotic surfaces and *in vivo* on biotic surfaces such as the oral and vaginal mucosa [8]. Because biofilms are associated with a protective extracellular matrix, the cells in biofilms are more resistant to conventional antifungal drugs and host immune factors [64]. Further, cross contamination through medical devices via biofilm is a major source of infection in hospitals [65]. It was found that SAP 9 and SAP 10 enzymes maintain cell surface integrity of the *Candida* cell wall, and promote biofilm formation [8].

5. Conclusion

The present study showed predominance of NAC in different clinical samples. Biofilm production, proteinase and phospholipases were observed in both *C. albicans* and NAC. The number of NAC isolates producing biofilm is more than the number of *C. albicans* producing this virulence factor. This result suggests that the biofilm production is more important for NAC strains and that *C. albicans* possess mechanisms other than biofilm production to establish infections. Our study showed that the percentage of NAC producing proteinase is higher than *C. albicans*, whereas *C. albicans* are higher producers of phospholipase than NAC. The knowledge on how the pathogen regulates the production of different virulence factors contributes to better understanding of the pathogenesis. Thus, innovative strategies to develop newer and safer antifungal agents are needed to suppress and eradicate the virulence factors involved in the pathogenicity of life threatening *Candida* species.

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