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ORIGINAL ARTICLE

Evaluation of Virulence Factors of *Candida* Species Isolated from Superficial Versus Systemic Candidiasis

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Key words:

Virulence factors, Candida species, Aerobic conditions, Anaerobic conditions, Superficial candidiasis, Systemic candidiasis

ABSTRACT

Background: *Various virulence factors are contributing to the colonization and pathogenicity of* Candida *during both superficial and systemic infections* ***Objectives:*** *The current study was conducted to investigate production of extracellular hydrolytic enzymes, hemolysis and biofilm formation among Candida species isolated from superficial versus systemic candidiasis and to compare the production of these virulence factors among albicans and non-albicans Candida species, in addition to comparing hemolysis and hydrolytic enzymes production under aerobic and anaerobic conditions.* ***Methodology:*** *A total of 51 Candida strains isolated from vulvovaginal candidiasis and 44 strains isolated from systemic candidiasis were included in the study. Germ tube test and Hi Crom Candida Differential Agar medium were used for species identification of the isolates. The Candida isolates were tested for expression of hemolytic activity, phospholipase, proteinase and esterase production under aerobic and anaerobic conditions, in addition to their ability of biofilm formation.* ***Results:*** *In both groups, 55 isolates were identified as Candida albicans (C. albicans), while 44 isolates were identified as non-albicans species. Hemolytic activity and biofilm formation were significantly higher among the systemic Candida isolates than superficial isolates, while expression of proteinase activity was significantly higher among superficial isolates. Expression of phospholipase and esterase was significantly higher among C. albicans isolates than non-albicans isolates. Expression of proteinase and esterase activities was significantly better under aerobic conditions than under anaerobic conditions.* ***Conclusions:*** *Hemolytic activity and bioflm formation were more evident among the systemic isolates while proteinase production appears to be more evident among the vaginal isolates. Phospholipase production is almost an exclusive characteristic feature of C. albicans, while C. albicans is the leading species in terms of esterase production. Aerobic conditions favor expression of proteinase and esterase activities.*

INTRODUCTION

Candida species are among the normal flora of skin and mucous membranes of healthy people. These opportunistic pathogens cause infections that range from superficial infections in immunecompotent individuals, such as thrush in babies and vaginal infections in women, to life-threatening systemic infections in immunocompromised and immunosuppressed people [1,2] Despite their frequency and associated morbidity, superficial *Candida* infections are non-lethal. In stark contrast, systemic candidiasis is associated with a high crude mortality rate, even with first line antifungal therapy [3]. Though *Candida albicans* (*C. albicans)* has been associated mostly with human infections, there has been an increase in the prevalence of infections due to non-albicans *Candida* [4].

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Various virulence factors are contributing to the colonization and pathogenicity of *Candida* during both superficial and systemic infections including the expression of adhesins and invasins on the cell surface, yeast-hyphal morphogenetic transformation, formation of biofilms, phenotypic switching and the secretion of hydrolytic enzymes[5]. Extracellular hydrolytic enzymes are considered to be one of the major virulence factors that play a major role in overgrowth of *Candida*, since these enzymes pave way for adherence, penetration and for tissue invasion[6]. Phospholipases facilitate the invasion of host mucosal epithelia by hydrolysing one/more ester linkages in glycerophospholipids [7]. Secreted aspartyl proteinases play an important role in pathogenesis of *Candida* species. They have proposed to facilitate active penetration into the cells through degrading vital immunological and structural proteins. In addition, they are thought to enhance the efficiency of extracellular nutrient acquisition [3,8]. Furthermore, hemolysin production is considered to be one of the important attribute contributing to survival and ability

of *Candida* species to establish infection within humans being related to the acquisition of iron [9,10]. The production of esterase has been documented as virulence character among clinical isolates of *Candida,* however, little is known about the pathogenesis. Recent investigations suggest the mechanism of virulence is due to cytotoxic effects of lipases and esterase in the host tissues [11]. Moreover, it has been reported that biofilm formation also plays an essential role in the pathogenecity of *Candida* species [9]. Biofilm producing *Candida* are known to be more resistant to immune response and antimicrobial agents, which lead to treatment failure [12].

Numerous virulence attributes of *Candida* have been postulated to facilitate the transition of a mucosal colonizer to a fatal disseminating pathogen. While adhesions help the yeast in adhering to the host cell surfaces, hydrolytic enzymes like proteinases, phospholipases, and lipases along with hyphal forms promote the penetration through cells. In addition to these virulence attributes of *Candida*, host immunity plays a major role in restricting these infections to either a localized form as seen in immunocompetent hosts or a deep-seated and disseminating form of infections common among immunocompromised hosts [4]. It has been reported that studies on virulence factors of pathogenic fungi are still needed [9]. Some studies [11] demonstrated that *Candida* isolated from immunocompromised patients has significantly higher production of hydrolytic enzymes than immunocompotent patients. Other studies [4,13,14] showed that production of virulence factors varies widely not only between *Candida* species but also within the same species according to their source of isolation.

In view of this, the current study was conducted to investigate production of extracellular hydrolytic enzymes, hemolysis and biofilm formation among *Candida* species isolated from superficial versus systemic candidiasis and to compare the production of these virulence factors among albicans and non-albicans *Candida* species, in addition to comparing hemolysis and hydrolytic enzymes production under aerobic and anaerobic conditions.

METHODOLOGY

Candida isolates:

A total of 99 *Candida* isolates from various clinical samples were included in the study; 51 strains were isolated from superficial candidiasis (vulvovaginal candidiasis), and 44 strains were isolated from systemic candidiasis (36 isolates from blood and sputum specimens and 12 isolates from infected diabetic foot ulcers). Germ tube test and culture on Hi Crom Candida Differential Agar (HiMedia, India) incubated at 37°C for 48 hours were used for species identification of the isolates. The *Candida* isolates were tested for expression of hemolytic activity, phospholipase, proteinase and esterase production under aerobic and anaerobic conditions, in addition to their ability of biofilm formation. Fresh cultures of isolates on Sabouraud dextrose agar (SDA) (Oxoid, UK) incubated at 37 °C for 24–48 h were used for species identification as well as for detection of virulence factors. Saline suspensions of the isolates from the fresh cultures were prepared and adjusted to 0.5 McFarland turbidity standard (1×10^7 cells/mL) to be used for further testing for virulence factors [2,9]. For testing for hemolytic, phospholipase, proteinase and esterase activities, 10µL of the prepared suspension of each isolate were inoculated onto 2 plates of the corresponding culture media and incubated at 37°C with one plate in aerobic and the other in anaerobic conditions.

1. *Assessment of hemolytic activity:*

The test was performed on SDA (Oxoid, UK) supplemented with 7% sheep blood and 3% glucose and adjusted to a pH of 5.6 ± 0.2 [9]. The inoculated plates were incubated for 48 hours. After incubation, a transparent/semitransparent zone around the inoculation site was considered as positive for hemolytic activity[9,15].

2. *Assessment of phospholipase activity:*

Modified egg yolk agar media described in earlier studies [16,17] was used for this test. This culture media contains 1 L of SDA (Oxoid, UK) supplemented with 1 M NaCl, 0.005 M $CaCl_2$ and 10% sterile egg yolk emulsion (Oxoid, UK). The inoculated plates were incubated for 5 days. The presence of a precipitation zone around the colonies was considered as evidence of phospholipase activity (Pz). Pz was calculated as the ratio of the colony diameter to the diameter of the colony plus precipitation zone and scored as negative (Pz = 1), positive (Pz = 0.64–0.99), and very strong (Pz ≤ 0.63) [16].

3. *Assessment of proteinase activity:*

Bovine-serum albumin agar described in earlier studies [18] was used for this test. The agar contained 0.1% KH2PO4, 0.05% MgSO4, 2% agar, and 1% bovine serum albumin (Sigma-Aldrich, USA) and adjusted to a pH of 4.5. The inoculated plates were incubated for 10 days. After incubation, the plates were fixed with 20% trichloracetic acid, stained with 1.25% amidoblack and then decolorized by 15% acetic acid [2,19]. The proteinase activity (Pz) was evident by the presence of unstained zone (areas of proteolysis) around the colonies. Pz was calculated as the aratio of the colony diameter to the diameter of the colony plus the proteolytic unstained zone [19] and scored as negative (Pz = 1), positive (Pz = 0.64–0.99), and very strong (Pz ≤ 0.63) [16].

4. Assessment of esterase activity:

Tween-80 agar described in earlier studies [20] was used for this test. It is consisted of 1% peptone, 0.5% NaCl, 0.01% $CaCl_2$, and 1.5% agar adjusted to a pH of 6.8 to which 0.5% of Tween-80 (Sigma, USA) was

added after cooling. After incubation of the inoculated plates for 10 days, esterase activity was considered as positive by the presence of a halo pervious to light around the inoculation site [20].

5. Assessment of biofilm formation:

The ability of *Candida* isolates to form biofilm was assessed by the microplate method using sterile 96-well microplatesas described in earlier studies [2,21]. Briefly, each isolate was inoculated into 2 mL of brain heart infusion broth (BHIB) medium supplemented with 0.25% glucose and incubated at 37 °C for 24 hours. After incubation, all tubes were diluted at a ratio of 1:20 using freshly prepared BHIB. For each isolate, 200 µL from the final dilution were placed into the corresponding well. Sterile plane BHIB was used as a negative control.Each isolate as well as the negative control were tested in duplicate. The plates were incubated at 37 °C for 24 hours. After incubation, the microplates were rinsed 3 times with phosphate buffered saline (PBS) and then inverted to blot. After that, 200 µL of 1% crystal violet was added to each well and incubated at 37 □ C for 15 min. After incubtion, the microplates were again rinsed 3 times with PBS. Then 200 µL of ethanol: acetone mixture (80:20 w/v) was added to each well. They were read using an enzyme-linked immunosorbent assay (ELISA) reader at 450 nm and the optical density (OD) was recorded for each well. The arithmetical mean of OD readings of the 2 wells for each isolate as well as the negative control was used for further analysis. The cutoff value was calculated as follows; mean OD of the negative control +2standard deviation. Samples with an OD higher than the cutoff value were considered positive, whereas those with lower value than cutoff were considered negative [9].

RESULTS

The current study included 51 *Candida* strains (51.5 %) isolated from patients with superficial candidiasis, and 48 *Candida* strains (48.5 %) isolated from patients with systemic candidiasis. Table 1 shows the distribution of different *Candida* species among both groups. In both groups, 55 isolates (55.6%) were identified as *C. albicans*, while 44 isolates (44.4%) were identified as non-albicans species.

Expression of virulence factors among isolates in the superficial and systemic groups (Table 2)**:** A total of 67 isolates express hemolytic activity either in aerobic or anaerobic conditions (figure 1). Higher proportion of the systemic isolates (91.7%) express hemolytic activity than the superficial isolates (45%) with a statistically significant difference between both groups (p-value= < 0.001). It was observed that the type of hemolysis reported among the systemic isolates was predominantly beta hemolysis (42 out of 44), while 13 and 10 isolates express beta and alpha hemolysis, respectively among the superficial group. A total of 33 isolates express phospholipase activity either in aerobic or anaerobic conditions (figure 2). Comparable proportion of the systemic and the superficial isolates express phospholipase activity (31.3% and 35.3%, respectively) with no statistical difference between both groups (p-value= 0.67). In addition, a total of 43 isolates express proteinase activity either in aerobic or anaerobic conditions (figure 3) with higher proportion of the superficial isolates (64.7%) express proteinase activity than the systemic isolates (20.8%) with a statistically difference between both groups (p-value= 0.001). For esterase enzyme, a total of 49 isolates express esterase activity (figue 4) either in aerobic or anaerobic conditions. Although higher proportion of the systemic isolates (58.3%) express esterase activity than the superficial isolates (41.2%), however, there was no statistically significant difference between both groups (p-value= 0.22). A total of 40 isolates were able to form biofilm. Higher proportion of the systemic isolates (56.3%) had the ability to form biofilm than the superficial isolates (25.5%) with a statistically significant difference between both groups (p-value= 0.001).

Expression of virulence factors by albicans and non-albicans *Candida* species (Tables 3 and 4)**:** Expression of all the tested virulence factors was higher among *C. albicans* isolates than non-albicans isolates, however this was much evident for expression of phospholipase and esterase (56.4% and 69.1% of the albicans group, respectively, versus 4.5% and 25% of the non-albicans group, respectively), with statistically significant differences between both groups as regards the expression of both enzymes (P-value= 0.001 for both). This was also reported among the superficial and systemic isolates for phospholipase (P-value= < 0.001), while for esterase, there was a statistical significant difference between albicans and non-albicans groups only among the superficial isolates (P-value= < 0.001) but not among the systemic isolates. In this study, phospholipase was almost exclusively expressed by *C. albicans* (31 out of 33 positive isolates), among them, 20 isolates express strong activity. In terms of esterase activity, *C. tropicalis* (47.1%) comes next to *C. albicans* (69.1%).

There were no statistically significant differences between abicans and non albicans species as regards the expression of hemolytic activity, proteinase and biofilm formation (P-value= 0.442, 0.39, 0.93, respectively). This was also reported among superficial and systemic groups. The highest hemolytic activity was reported among *C. krusei* isolates (88.9%), while *C. albicans* and *C. tropicalis* had almost the same proteinase activity (47%). It was found that among the isolates positive for proteinase production, only 2 isolates express strong activity, both of them were non-albicans species. The highest biofilm forming activity was reported among *C. glabrata* isolates (60%), while *C. albicans* was reported to have the least activity (40%).

There were statistical significant correlations between the production of esterase with both phospholipase and proteinase (p-value<0.001 and 0.002, respectively). In addition, there was a statistical significant correlation between proteinase production and biofilm formation (p-value=0.03).

Expression of virulence factors under aerobic and anaerobic conditions (Table 5)**:** Expression of proteinase and esterase activities was better under aerobic conditions than under anaerobic conditions with statistically significant differences (P-value= 0.001 and < 0.001, respectively). It was observed that aerobic conditions favor the expression of proteinase mainly among *C. albicans* since 25 isolates (45%) express proteinase in aerobic conditions versus 10 (18.2%) under anaerobic conditions; while for non-albicans species, 13 isolates express it under aerobic conditions (29.5%) versus 12 (27.3%) under anaerobic conditions. This was also true for esterase since 38 isolates (69.1 %) of *C. albicans* express esterase in aerobic conditions versus 9 (16.4%) under anaerobic conditions; while for non-albicans species, 11 isolates express it under aerobic conditions (25%) versus 7 (15.9%) under anaerobic conditions. Only 2 isolates express strong proteinase positivity, one of them express it in aerobic and the other in anaerobic conditions with no statistically significant difference.

On the other hand, expression of hemolytic and phospholipase activities was comparable under aerobic and anaerobic conditions with no statistical significant difference (P-value= 0.269 and 0.157, respectively). However, it was observed that expression of β hemolysis was better in anaerobic conditions (50 versus 40); while expression of α hemolysis was better in aerobic conditions (12 versus 5) regardless the species with a statistical significant difference (p-value= 0.005). In addition, it was noted that expression of strong phospholipase activity was better under anaerobic conditions (19 isolates) than in aerobic conditions (10 isolates) but with no statistical significant difference (p-value=0.06).

Table 1: Distribution of *Candida* species among *Candida* isolates from superficial and invasive candidiasis:

	Superficial Candida isolates ***N (%)***	***Systemic Candida isolates*** ***N (%)***	***Total*** ***N (%)***
C. albicans	26 (50.98%)	29 (60.4%)	55 (55.6%)
Non-albicans *Candida*	25 (49.01%)	19 (39.6%)	44 (44.4%)
C. tropicalis	4 (7.8%)	13 (27.1%)	17 (17.1%)
C. glabrata	8 (15.7%)	2 (4.2%)	10 (10.1%)
C. krusei	5 (9.8%)	4 (8.4%)	9 (9.1%)
Other species	8 (15.7%)	0 (0%)	8 (8.1%)
Total	51 (100%)	48 (100%)	99 (100%)

Table 2: Expression of virulence factors by *Candida* isolates from superficial and invasive candidiasis

	Superficial candidiasis (n=51) ***N (% among the group)***	***Invasive candidiasis(n=44)*** ***N (% among the group)***	***P-value***
Hemolytic activity	23(45%)	44 (91.7%)	<0.001*
Phospholipaseactivity	18 (35.3%)	15 (31.3%)	0.67
Proteinase activity	15 (64.7%)	10 (20.8%)	<0.001*
Esterase activity	21 (41.2%)	28 (58.3%)	0.221
Biofilm formation	13 (25.5%)	27 (56.3%)	0.004*

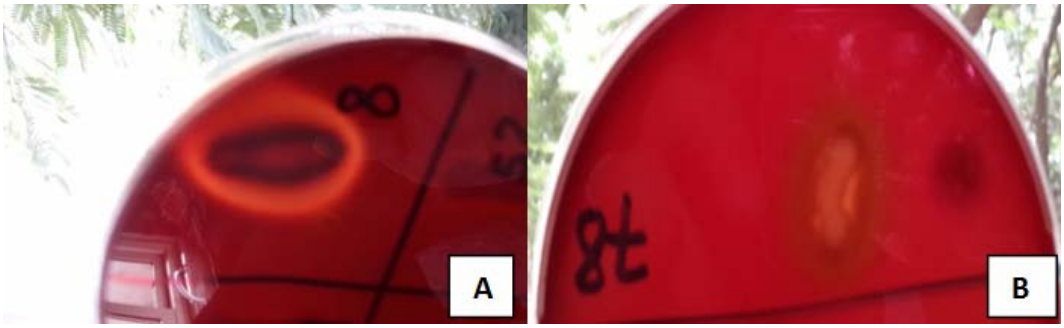

Fig. 1: A. *Candida* isolate expressing beta-haemolysis, B. *Candida* isolate expressing alpha-haemolysis on sheep blood agar

Table 3: Expression of virulence factors by *Candidaalbicans* and non-albicans species

	Candida albicans(n=55) ***N (% among the group)***	***Non-albicans* Candida*(n=44)*** ***N (% among the group)***	***P-value***
Hemolytic activity:			
• Total	39 (70.9%)	28 (64.6%)	0.44
• Superficial group	11 (42.3%)	12 (48%)	0.69
• Systemic group	28 (96.5%)	16 (84.2%)	0.13
Phospholipase activity			
• Total	31 (56.4%)	2 (4.5%)	0.001*
• Superficial group	16(61.5%)	2 (8%)	0.001*
• Systemic group	15 (51.7%)	0 (0%)	0.001*
Proteinase activity:			
• Total	26 (47.3%)	17 (35.4%)	0.39
• Superficial group	21 (80.8%)	12 (48%)	0.14
• Systemic group	5 (17.2%)	5 (26.3%)	0.45
Esterase activity:			
• Total	38 (69.1%)	11 (25%)	0.001*
• Superficial group	19 (73%)	2 (8%)	<0.001*
• Systemic group	19 (65.5%)	9 (31%)	0.52
Biofilm formation:			
• Total	22 (40%)	18 (40.9%)	0.75
• Superficial group	6 (23%)	7 (28%)	0.475
• Systemic group	16 (55.2%)	11 (57.9%)	0.68

Fig. 2: A. *Candida* isolate expressing phospholipase activity on egg yolk agar, B. *Candida* isolate showing no phospholipase activity on egg yolk agar.

Table 4: Expression of virulence factors by different *Candida* species isolated from superficial and systemic candidiasis

	Hemolytic activity			**Phospholipase activity**			**Proteinase activity**			**Esterase activity**			**Biofilm formation**		
	S	**I**	**T N (%)**	**S**	**I**	**T N(%)**	**S**	**I**	**T N (%)**	**S**	**I**	**T N (%)**	**S**	**I**	**T N (%)**
C. albicans (n=55)	11	28	39 (70.9%)	16	15	31 (56.4%	21	5	26 (47.3%)	19	19	38 (69.1%)	6	16	22 (40%)
Non-albicans *Candida* (n=44)	12	16	28	2	-	2	12	5	17	2	9	11	7	11	18
C. tropicalis (n=17)	1	11	12 (70.6%)	-	-	-	4	4	8 (47.1%)	1	7	8 (47.1%)	1	7	8 (47.1%)
C. glabrata (n=10)	2	1	3 (30%)	1	-	1 (10%)	1	-	1 (10%)	1	1	2 (20%)	4	2	6 (60%)
C. krusei (n=9)	4	4	8 (88.9%)	-	-	-	2	1	3 (33.3%)	-	1	1 (11.1%)	2	2	4 (44.4%)
Other species (n= 8)	5	-	5 (62.2%)	1`	-	1 (12.5%)	5	-	5 (62.5%)	-	-	-	-	-	-
Total (n=99)	23	44	67 (67.7%)	18	15	33 (33.3%)	33	10	43 (43.4%)	21	28	49 (49.5%)	13	17	40 (40.4%)

Table 5: Expression of different virulence factors in aerobic and anaerobic conditions

Virulence Factors	*Aerobic N (%)*	*Anaerobic N (%)*	*P-value*
Haemolytic Activity:	54 (54.5%)	55 (55.5%)	0.27
Phospholipase Activity:	32 (32.3%)	31 (31.3%)	0.16
Proteinase Activity:	38 (38.4%)	22 (22.2%)	0.001*
Esterase activity	49 (49.5%)	16 (16.2%)	<0.001*

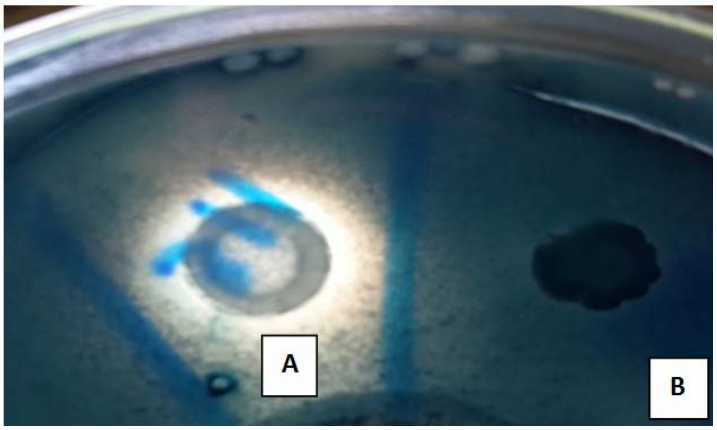

Fig. 3: A. *Candida* isolate expressing proteinase activity on bovine serum albumin agar, B. *Candida* isolate showing no proteinase activity on bovine serum albumin

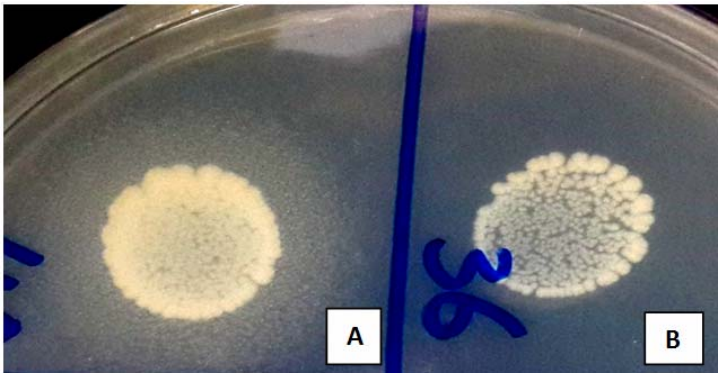

Fig. 4: A. *Candida* isolate expressing esterase activity on Tween 80 agar, B. *Candida* isolate showing no esterase a ctivity on Tween 80 agar.

DISCUSSION

The transition of Candida spp. from a commensal to a potent pathogen is contributed by several factors including host predisposing factors and virulence attributes of infecting species[22]. In view of limited number of suitable and effective antifungal drugs with increasing incidence of multidrug resistant *Candida* species, development of antifungal therapies against selective target virulence factors is very crucial [2,23]. This study was conducted to investigate production of extracellular hydrolytic enzymes, hemolysis and biofilm formation among *Candida* species isolated from superficial versus sytemic candidiasis and to compare the production of these virulence factors among albicans and non-albicans *Candida* species, in addition to comparing production of hemolysis and hydrolytic enzymes under aerobic and anaerobic conditions.

In the current study, higher proportion of the systemic isolates (91.7%) express hemolytic activity than the superficial isolates (45%) with a statistically significant difference between both groups. It was observed that the type of hemolysis reported among the systemic isolates was predominantly beta hemolysis, while almost equal proportion of the superficial isolates express beta and alpha hemolysis. Other studies also reported that 90% of the invasive candida isolates express hemolytic activity; all of them were beta hemolysis [9], while others [2] reported higher rates of

hemolytic activity (63%) among superficial *Candida* oral isolates. The type of hemolysis in both superficial and systemic *Candida* isolates may account for virulence to the host tissues, however due the unknown underlying mechanisms of these variants; it is not possible to predict their pathogenic mechanisms to the hosts [11].

Biofilm formation is one of the most extensively investigated virulence factors of *Candida* species [9]. In the current study, higher proportion of the systemic isolates was able to form biofilm (56.3%) than the superficial isolates (25.5%) with a statistically significant difference between both groups. Some studies [4] reported almost similar rate of biofilm production among blood stream infection isolates (52%) but higher rate (54%) among vulvovaginal candidiasis isolates with no statistically significant difference between both grouops, while others [2] reported higher rate (78%) for biofilm production among oral *Candida* isolates. The lower rate of biofilm formation among vaginal isolates in the current study might be explained by the different species of the isolates.

Secreted aspartyl proteinases (SAP) are considered to be one of the major virulence factors of *Candida* [6]. The ability to express proteinases varies not only among different species of *Candida* but also differs among the strains of same species isolated from different body sites [22]. The SAP family comprises ten members, Sap1–10. Sap1–8 are secreted and released to the surrounding medium, whereas Sap9 and Sap10 remain bound to the cell surface [3]. SAP1–SAP3 gene family appears to be essential for mucosal infection (SAP1–SAP3), while SAP4–SAP6 appear to be involved in both systemic and mucosal infections [13,23].

In the current study, higher proportion of the superficial isolates (64.7%) express proteinase activity than the systemic isolates (20.8%), with a statistically difference between both groups. In accordance with our results, some studies reported significantly high proteinase activity among *Candida* isolates from patients with *Candida* vaginal [23] and oral infections [2]. However, some studies reported higher rates of proteinase production among invasive *Candida* isolates (35%-90%) [4,9,12], while others reported that proteinase production was more common among invasive isolates in comparison with high-vaginal isolates [4].

It was reported that proteinases possess distinct differences in pH optima, with Sap1–Sap3 (yeast associated) having optimum activity at lower pH values, and Sap4–Sap6 (hyphal associated) having optimum activity at neutral pH values [13]. Under most proteinase-inducing conditions in the laboratory, the major proteinase gene expressed is SAP2 [23]. Studies showed that expression of SAP 2 is required for disease development in an animal vaginitis model [1]. In accordance with this, studies have reported that SAP1 and SAP2 were the most detected genes among vaginal isolates [13,24]. This could explain the high rate of proteinase production in this study among the vaginal isolates.

In the current study, although higher proportion of the systemic isolates (58.3%) express esterase activity than the superficial isolates (41.2%), however, there was no statistically significant difference between both groups. Similarly, other studies reported that esterase production was comparable among vaginal and blood cultures isolates with no statistically significant difference [4]. Others [2] reported comparable rate (50%) of esterase production among oral *Candida* isolates.

Phospholipase enzymes, is an important virulence factor associated with the function related to host cell damage, adherence and penetration [2]. The family of phospholipases consists of four different classes (A, B, C and D).71 Only the five members of class B (PLB1–5) are extracellular and may contribute to pathogenicity via disruption of host membranes [3]. In the current study, comparable proportion of the systemic and the superficial isolates express phospholipase activity (31.3% and 35.3%, respectively) with no statistical difference between both groups. Almost similar rates of phospholipase production by systemic *Candida* isolates were reported in other studies [9], however, other studies [4] reported that phospholipase production was more common among high-vaginal isolates (28%) than in blood isolates (4%). However, the high phospholipase activity in vaginal isolates in the latter study was possibly due to higher number of *C. albicans* in this group [4]. This finding was not observed in our study possibly because of almost comparable proportion of *C. albicans* among both systemic and vaginal isolates.

Non-albicans *Candida* species once overlooked as mere contaminants or non pathogenic commensals have emerged as potent pathogens [22]. In this study, expression of all the tested virulence factors was higher among *C. albicans* isolates than non-albicans isolates, however this was much evident for expression of phospholipase and esterase.

Earlier studies demonstrated that *C. albicans* produced extra-cellular phospholipase alone. However, further studies showed that non-albicans Candida produced also phospholipase but at a lesser extent [12]. In accordance with this finding, the current study reported that phospholipase was almost exclusively expressed by *C. albicans* (31 out of 33 positive isolates) with very little activity among the non-albicans isolates. It was found that among isolates positive for phospholipase production, 20 of them express strong activity, all of them were *C. albicans*. Similarly, some studies reported that the rate of phospholipase positivity was statistically higher in the *C. albicans* isolates than non-albicans *Candida* isolates with little or no phspholipase activity among non-albicans species isolated from different clinical samples [25,26,27], while others reported high phsopholipase production among non-albicans Candida species [22], however, the *Candida* isolates from the latter study were from oral infection in HIV patients

Studies have reported that *C. albicans* and *C. tropicalis* are the most common strains exerting esterase activity [12]. In agreement with these findings, in the current study, expression of esterase was hig her among than *C. albicans* (69.1%) than non-albicans isolates (25%) with a statistically significant differences both groups. *C. tropicalis* comes next to *C. albicans* in terms of esterase activity (47.1%), followed by *C. glabrata* (20%) and lastly *C. krusei* (11.1%). Similarly, other studies reported that the rate of esterase positivity was statistically higher in the *C. albicans* isolates than non-albicans *Candida* isolates [2,12].

In the current study, there was no statistically significant difference between albicans and non-albicans *Candida* groups as regards the expression of hemolytic activity. The highest hemolytic activity was reported among *C. krusei* isolates (88.9%) followed by C. tropicalis and C. albicans (70%). Other studies reported comparable rates of haemolysis production among *C. tropicalis* and *C. krusei* [2,22] but higher rate among *C. albicans* [9], however, the latter study investigate invasive isolates.

C. albicans is not the only *Candida* species known to produce extracellular proteinases. Many of the pathogenic *Candida* such as *C. dubliniensis, C. tropicalis* and *C. parapsilosis* species have been shown to have SAP genes. Less pathogenic or nonpathogenic *Candida* species do not appear to produce significant amounts of proteinase, even though they may possess aspartyl proteinase genes [23]. In the current study, no statistically significant difference between albicans and non-albicans *Candida* groups as regards proteinase production (47.3% versus 35.4%). Only 2 isolates express strong activity, both of them were non-albicans species. *C. albicans* and *C. tropicalis* had almost the same proteinase activity (47%). Other studies reported that proteinase positivity for *C. albicans* isolates is higher than non-albicans species [9,12,22,28] while others reported high proteinase activity among non-albicans [22]. These inconsistencies in observations may be due to biological differences among the isolates tested [22].

In the current study, there was no statistically significant difference between albicans and non-albicans *Candida* groups as regards biofilm formation. The highest biofilm forming activity was reported among *C. glabrata* isolates (60%), followed by *C. tropicalis* (47.1%), and *C. krusei* (44.4%), while *C. albicans* reported the least activity (40%). Similarly, biofilm formation was reported to be higher among non-albicans than *albicans Candia* species; however, highest activity was reported for *C. parapsilosis* [9,12]. In our study, no *C. parapsilosis* isolates were included among the systemic isolates, while those included in the superficial isolates had no biofilm production. This could be explained by the different source of the isolates. Other studies [2,4] reported that *C. albicans*, *C. tropicalis* and C. glabrata were the leading producers of biofilm

In this study, there were statistical significant correlations between the production of esterase with both phospholipase and proteinase among the studied *Candida* isolates. In addition, there was a statistical significant correlation between proteinase production and biofilm formation. In disagreement with our results, some studies have reported that production of more than one virulence factor among different *Candida* species did not show any statistical significance [4]. However, this study reported that coproduction of proteinase and esterase was observed in blood isolates, but not in vaginal isolates.

It is known that *Candida* spp. have the ability to grow in both aerobic and anaerobic conditions. Moreover, *Candida* spp. possess some adaptive mechanisms to survive in both situations [9]. In this study, it was reported that expression of proteinase and esterase activities was better under aerobic conditions (38.4% and 49.5%, respectively) than under anaerobic conditions (22.2% and 16.2%, respectively) with statistically significant differences. It was observed that aerobic conditions favor the expression of proteinase mainly among *C. albicans*. This was also true for esterase. Only 2 isolates express strong proteinase positivity, one of them express it in aerobic and the other in anaerobic conditions with no statistically significant difference. In accordance with results of the current work, studies have also reported that esterase activity substantially decreased in anaerobic conditions [29]. Other studies reported that there was a significant difference favoring aerobic conditions in terms of proteinase activity among all isolates while expressing strong proteinase activity were not affected by atmospheric conditions; however, in this study, no difference was found among *C. albicans* strains in terms of proteinase activity according to atmospheric conditions, while proteinase activity decreased in non-albicans *Candida* species in anaerobic conditions [9]. Unlike the findings in the current study, some investigators [30] reported that none of the studied *Candida* isolates expressed proteinase activity in anaerobic conditions. On the other hand, other studies [29] reported that proteinase activity increased in anaerobic conditions in *C. albicans* oral isolates.

In the current study, although expression of hemolytic activity was comparable under aerobic and anaerobic conditions for both albicans and non albicans species with no statistical significant difference, however, regardless the species, expression of β hemolysis was better in anaerobic conditions; while expression of α hemolysis was better in aerobic conditions with a statistical significant difference. In disagreement with our results, some studies reported that expression of beta hemolysis was better under

aerobic rather than anaerobic conditions with a significant difference favoring aerobic conditions [9,29].

Although expression of phospholipase activity was comparable under aerobic and anaerobic conditions with no statistical significant difference, however, expression of strong phospholipase activity was better under anaerobic conditions but with no statistical significant difference. Other studies also reported no significant difference between aerobic and anaerobic conditions in terms of phspholipase expression [9], while others [30] reported no phospholipase activity in anaerobic conditions.

The differences between production of virulence factors under aerobic and anaerobic conditions among studies may result from variations in the sources of isolates, incubation periods, and methods used to achieve anaerobic conditions [9].

In conclusion, hemolytic activity and bioflm formation were more evident among the systemic isolates while proteinase production appears to be more evident among the vaginal isolates. Phospholipase production is almost an exclusive characteristic feature of *C. albicans* rather than non-albicans species, while *C. albicans* is the leading species in terms of esterase production. Aerobic conditions favor expression of proteinase and esterase activities, while expression of strong phospholipase activity was better under anaerobic conditions. Anaerobic conditions appear to favor the expression of β hemolysis while aerobic conditions appear to favour expression of α hemolysis.

Limitation of the study include the lack of molecular characterization for the differential expression of virulence genes responsible for individual proteinase and phospholipase enzymes production which could otherwise emphasize the role of each of them in different types of *Candida* infection or among albicans or non-albicans species in addition to the small number of isolates of individual non-albicans species.

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