Virulence Attributes of Clinical *Candida glabrata* (Sensu Stricto) Isolates in the West Region of Cameroon

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Authors' contributions

This work was carried out in collaboration among all authors. Authors JPD and CBT designed the experiments. Authors CN, AND, CLK and AIE performed the experiments and wrote the manuscript. Authors JPD and CBT supervised the work. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Background: Studies on the assessment of the virulence factors of *C. glabrata* sensu stricto strains are on the rise. This is partly due to the increase in recurrent *C. glabrata* infections that have contributed to increased mortality rates. Published data on the virulence characteristics of *C. glabrata* strains in Cameroon are very rare.
Aims: This study aimed at assessing some virulence characteristics, including the capacity to form biofilms and hydrolytic enzymes (protease, esterase and phospholipase).
Methods: Fifty-four (54) molecularly (MALDI-TOF) identified non-duplicate *C. glabrata* sensu stricto clinical isolates initially collected in a previous study, were used in the present study. These isolates were obtained from stool (S), urine (U), oro-pharyngeal (OPS) and cervico-vaginal (CVS) swabs of pregnant women, diabetic patients (both types 1 and 2 diabetes mellitus), HIV/AIDS and

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other patients who had neither of these diseases. Phospholipase, protease, esterase and biofilm activities were assessed using previously described methods.

Results: Our results revealed that our isolates were more able to produce phospholipase (37.04%) than they were able to produce protease (1.85%) and esterase (0%). The high producers of phospholipase (Pz < 0.7) originated mostly from oro-pharyngeal swabs (41.17%) of some diabetic patients and pregnant women. Also, all our isolates were formers of biofilm, most (74.42%) of which had lower (< 100%) biofilm formation activity compared to our reference strain. To be able to give a significant conclusion about the virulence characteristics of *C. glabrata* strains in the west region, we recommend that more studies be carried on a larger number of strains.

1. INTRODUCTION

C. glabrata is the most important non - *C. albicans* pathogenic yeast [1] responsible for about 26.7% of *Candida* infections, and the second most frequent cause of mucosal and disseminated candidiasis [2-5]. Decades ago, it was assumed that yeasts passively contributed to the process of pathogenesis in the establishment of a fungal infection. Consequently, organic weakness or an immunocompromised host was considered the only mechanism responsible for the establishment of an opportunistic infection. Nowadays, this concept has been modified and it is known that these organisms dynamically participate in the pathophysiology of the disease process using mechanisms of aggression called virulence factors. These principally include; biofilm formation capacity (on host tissues and on medical devices), and the production of tissue damaging hydrolytic enzymes such as phospholipases, proteases, lipases, and hemolysins [6,7,8].

Research has shown that the virulence capacities of *C. glabrata* sensu stricto strains are strongly dependent not only on the strain type, but also on the environmental conditions or geographical location [6],[9]. Based on this, few studies have been carried out in some few African countries to evaluate the virulence capacities of the strains involved in *C. glabrata* sensu stricto infections, but little or no information is available concerning the strains in Cameroon, especially in the West Region of Cameroon. In this light, we aimed at assessing some of the virulence attributes of *C. glabrata* sensu stricto clinical isolates, including proteinase, esterase, phospholipase and biofilm formation.

2. MATERIALS AND METHODS

2.1 Yeast Strains

Fifty-four (54) molecularly (MALDI-TOF) identified non-duplicate *C. glabrata* sensu stricto clinical isolates initially collected in a previous study [10], were used in the present study. These isolates were obtained from stool (S), urine (U), oro-pharyngeal (OPS) and cervico-vaginal (CVS) swabs of pregnant women, diabetic patients (both types 1 and 2 diabetes mellitus), HIV/AIDS and other patients who came for consultation and had neither of these diseases.

2.2 Assessment of Virulence Attributes

The virulence attributes of *C. glabrata* sensu stricto put under assessment were; production of hydrolytic enzymes (phospholipase, esterase, protease) and biofilm formation capacity.

2.2.1 Production of hydrolytic enzymes

2.2.1.1 Phospholipase activity

The phospholipase activity was determined using an egg yolk agar medium following the protocol described by Price *et al*. [11]. The egg yolk agar medium was prepared by adding 65 g of sabouraud dextrose agar (SDA), 58.4 g NaCl, and 5.5 g CaCl$_2$ to 980 ml distilled water, and sterilising at 121°C for 15 minutes. The culture medium was then cooled to about 45-50°C followed by the addition of 2 ml supernatant of an initially centrifuged 8% egg yolk at 5000 g for 30 minutes. The prepared culture medium was then poured on petri dishes and allowed to solidify.

After solidification of the culture medium, about 10 µl of yeast suspension (about 10^7 cells) was spot inoculated on the egg yolk agar medium using a platinum wire loop, and incubated at 37°C for 4 days, after which the diameters of the

colonies formed together with the precipitation zones around the colonies were determined using a graduated transparent ruler. Phospholipase activity (Pz) was then calculated using the following equation:

Phospolipase activity (Pz) = colony diameter/ (colony diameter + zone of precipitation)

On the basis of Pz value, results of phospholipase activity were grouped under 4 categories as seen below.

Pz value = 1, test strain is negative for phospholipase,
$0.80 \leq Pz\ value \leq 0.99$ = weak phospholipase activity,
$0.70 \leq Pz\ value \leq 0.79$ = moderate phospholipase activity, and
Pz value <0.70 = high phospholipase activity [12,13]

2.2.1.2 Esterase activity

Esterase production was assayed using tween agar plates according to the protocol described by Aktas *et al*. [14]. In this light, our culture medium which constituted of 0.5% yeast extract, 1% peptone, 0.01% CaCl2, 1.5% agar, and 0.1% Tween 80, was sterilised at 121°C for 15 minutes. The medium was then poured in petri dishes and allowed to cool.

To determine enzymatic activities, suspensions (about 10^7 cells) of 48 hours old yeast cells were spotted on the surface of each agar medium and incubated at 37°C for up to 7 days. The formation of a precipitation zone around colonies was indicative of a positive esterase activity.

2.2.1.3 Proteinase activity

Proteinase activity was evaluated in bovine serum albumin agar, as previously described by Mohan and Ballal [15]. A mixture of 11.7 g of yeast carbon base, 0.1 g of yeast extract, and 2 g of bovine serum albumin was added in 200mL of distilled water. The solution was then sterilized, filtered, and added to a previously sterilized stock solution of 16 g of agar in 800 mL of distilled water. The mixture was then poured in petri dishes and allowed to solidify, after which inoculation was done and the plates were incubated at 37°C. The results were recorded after 6 days of incubation and the enzyme activity was represented by the diameter of the lytic area surrounding the colonies on the culture medium. The value of the zone of proteinase activity (Pz) was evaluated as the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone, and it was scored and categorized as follows:

Pz value = 1 (negative for phospholipase),
Pz value = 0.75 - 0.99 (low producers);
Pz value = 0.51-0.74 (moderate producers); and
Pz value = 0.35- 0.5 (high producers).

2.2.2 Evaluation of biofilm formation capacity

The evaluation of biofilm formation in our isolates was done following the method described by Weerasekera *et al*., [16]. RPMI 1640 (Mediatech Inc, USA) culture medium was initially prepared as directed by the manufacturer (dissolving 10.4 g in 1000 mL of sterile distilled water). Few colonies obtained on SCA (Sabouraud Chloramphenicol Agar) after culture, were inoculated into a polystyrene bottle containing 10 ml of SDB and incubated for 18 h at 37°C. The turbidity of the yeast cells was then adjusted to 10^6 cells /ml in RPMI 1640. This was followed by the distribution of 100 µl of the suspension in the wells of a 96-well flat-bottomed polystyrene microtitre plate and incubating for 48 h at 37°C. The wells were then washed twice with distilled water to remove the non-adherent yeast cells. The reference strain *Candida albicans* ATCC 9002 was used as a positive control and the RPMI-1640 medium as a negative control.

2.2.2.1 Quantification of the biofilm formed: Revelation with 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT)

After washing the plates, 200 µL of MTT solution (200 µg / ml) prepared in RPMI 1640 was introduced into each well, and incubated at 37°C for 4 hours. After incubation, the MTT solution was carefully aspirated, and then 100 µL of DMSO was added to each well to dissolve the formazan product. The absorbance was then measured at a wavelength of 630 nm using a microplate reader (VERSAmax). Each experiment was done four times and their means were calculated and used to determine the percentage of biofilm formed. This was done using the formula:

Percentage biofilm formation= O.D test - O.D blank/ O.D control-O.D blank*100

Where, O.D = Optical density (in nm)

Given that most of our knowledge on biofilm formation in *Candida* species results from the study of *C. albicans* as a model [17], we used the percentage biofilm formation result of the reference strain; *Candida albicans* ATCC 9022 to interpret results.

3. RESULTS

3.1 Production of Hydrolytic Enzymes

No esterase activity was detected among our isolates. On the other hand, proteinase and phospholipase activities were demonstrated as seen in Fig. 1.

Fig. 1. Positive hydrolytic enzyme activity

Fig. 1 reveals a positive test for hydrolytic enzyme activity (phospholipase or proteinase), translated by the formation of a precipitation zone around isolates. Our isolates were positive at different rates for the various hydrolytic enzymes as shown in Table 1.

Out of our 54 isolates, 20 (37.04%) isolates showed a positive test for phospholipase activity, while only 1 (1.85%) isolate showed a positive test for proteinase activity. The only isolate that had a positive proteinase activity had a Pz value = 0.4 showing a very high proteinase activity. As for phospholipase activity, the distribution with respect to the isolates from the various samples, was as seen in Table 2.

The table above shows that most (85%) of our isolates demonstrated high phospholipase activity. This was observed more in isolates from oro-pharyngeal swabs (41.17% of the high producers). The mean phospholipase activity was 0.53 ± 0.18. The distribution of phospholipase activity per participants' groups was as seen in Table 3.

Table 3 shows how the 20 *C. glabrata* sensu stricto isolates with positive phospholipase activity were distributed among the different participants' groups. The high producers of phospholipase originated mostly from oro-pharyngeal swabs of diabetic patients (17.65%) and pregnant women (17.65%).

3.2 Biofilm Formation Activity

Due to loss of the viability of 11 isolates with time, the remaining 43 isolates were tested for biofilm formation activity. The results on microtitre plates were observed as shown on Fig. 2.
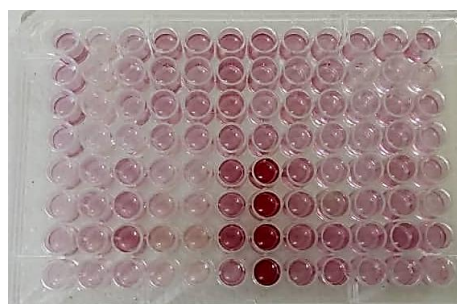

Fig. 2. Microtiter plate assay indicating biofilm production

Biofilm forming activity was expressed in percentages as earlier mentioned and results were normalized to the control (Ca ATCC 9022) as 100%. The percentages ranged from 7.3% to 402.3%. Our reference strain; Ca ATCC 9022, with 100% biofilm forming activity, was used to group the biofilm forming activities of *C. glabrata* sensu stricto isolates in Table 4.

Table 1. Hydrolytic enzyme activity

	Esterase	Proteinase	Phospholipase
Number of isolates	0	1	20
Percentage	0%	1.85%	37.04%

Table 2. Distribution of phospholipase activity per sample

	OPS	CVS	S	U	Total	Percentage
High producers *(Pz < 0.7)*	7	4	3	3	17	85.00%
Moderate *(0.70 ≤ Pz value ≤ 0.79)*	0	1	1	0	2	10.00%
Low *(0.80 ≤ Pz value ≤ 0.99)*	0	0	1	0	1	5.00%

The biofilm activity of the reference strain *Candida albicans* ATCC 9022 with 100% biofilm forming activity (B.A), was used to compare and interpret the biofilm forming activities of our *Candida glabrata* sensu stricto isolates. In this regard, 74.42% (32) of our isolates had lower (< 100%) biofilm forming activity, meanwhile 23.26% (10) of the isolates had higher (>100%) biofilm forming activity. Isolates with biofilm forming activity were most present in cervico-vaginal swabs (41.86%), followed by those from stool samples. On the other hand, urine samples had the lowest number of isolates with biofilm formation activity. Furthermore, isolates with biofilm formation activity greater than 100%, were most present in cervico-vaginal swabs and least present in oro-pharyngeal swabs.

4. DISCUSSION

The pathogenicity of *Candida glabrata* sensu stricto species is associated with a multitude of virulence factors, among which are; the ability to produce tissue-damaging hydrolytic enzymes such as proteases, esterases and phospholipases, and the ability to form biofilms. The ability to express these virulence factors has been associated with high pathogenicity. Previous studies have established that the virulence factors of *C. glabrata* sensu stricto species are strongly dependent not only on the strain type, but also on the geographical location or environmental conditions [6]. Along this line of thinking, we assessed the virulence factors of our isolates in order to get valuable information that could contribute in elaborating better disease prevention or control strategies.

Out of 54 *C. glabrata* sensu stricto isolates, 20 (37.04%) isolates showed a positive test for phospholipase activity (Mean Pz value = 0.53 ± 0.18), while only 1 (1.85%) isolate showed a positive test for proteinase activity [Pz value = 0.4 (high activity)]. On the other hand, no isolate showed a positive test for esterase activity. Most (85%) of our isolates demonstrated a high phospholipase activity. This was observed more in isolates from oro-pharyngeal swabs (41.17% of the high producers). Deorukhkar and Saini [18] revealed a positive phospholipase test in 30.2% of *C. glabrata* isolates obtained from diverse sources including urine, cervico-vaginal and oro-pharyngeal swabs. This result is very close to the results obtained in our study, as 37.04% of our isolates displayed a positive phospholipase activity. On the other hand, our results for phospholipase and protease activities differ from those obtained by Figueiredo-Carvalho *et al*. [19] in Brazil, who detected phospholipase and protease activities in 0% and 95.6% (87) of strains respectively, among 91 *C. glabrata* strains isolated from a great diversity of samples including stool, urine, cervico-vaginal and oro-pharyngeal swabs. It was interesting to notice that 41.17% of the high producers of phospholipase (Pz < 0.7) in our study originated from the oro-pharyngeal swabs of diabetic patients and pregnant women.

No (0%) esterase activity was detected among our isolates, contrary to the positive esterase activity detected in 56.0% of *C. glabrata* sensu stricto strains in a study conducted by Figueiredo-Carvalho *et al*. [19]. Results for esterase activity in our study concord with those of a study in Turkey which revealed that only one strain out of 14 *C. glabrata* sensu stricto strains isolated from bloodstream infection, had a positive esterase activity [19].

The variation or concordance of phospholipase, protease and esterase activities between isolates in our study and those of other studies, confirms that the virulence attributes of *C. glabrata* sensu stricto strains are highly heterogeneous depending on the source of the clinical material or the geographic region from which the strains were isolated.

Table 3. Distribution of phospholipase activity per subjects' group

	HIV-infected patients				Diabetic Patients				Pregnant women				Others				Total	%
	PCV	PB	S	U	PCV	PB	S	U	PCV	PB	S	U	PCV	PB	S	U		
High	2	0	2	1	0	3	0	1	1	3	1	1	1	1	0	0	17	85
Moderate	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	2	10
Low	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	5
TOTAL	2	0	2	1	0	3	0	1	2	3	2	1	1	1	1	0	20	100
(%)	10	0	10	5	0	15	0	5	10	15	10	5	5	5	5	0	100	

Table 4. Biofilm formation activity

	Number of isolates with biofilm forming activity (NBA)			
	NBA <100%	NBA = 100%	NBA >100%	%NBA
Stool *(n = 11)*	8	0	3	25.58%
Urine *(n = 6)*	4	0	2	13.95%
OPS *(n = 8)*	6	1	1	18.60%
CVS *(n = 18)*	14	0	4	41.86%
Total *(n=43)*	32	1	10	
Percentage	74.42%	2.33%	23.26%	

NBA: Number of isolates with biofilm forming activity; n: number of isolates

Biofilm formation is also one of the most important virulence factors that contribute to the pathogenicity of *C. glabrata* sensu stricto [8]. Given that *C. albicans* is the species that is most frequently associated with biofilm formation, and that most of our knowledge on biofilm formation in *Candida* species results from the study of *C. albicans* as a model [17], we used the reference strain; *Candida albicans* ATCC 9022 (100% biofilm formation activity) in our study to interpret biofilm results. In this regard, 74.42% (32) of our isolates had lower (< 100%) biofilm formation activity, compared to our reference strain. On the other hand, 23.26% (10) of the isolates had higher (>100%) biofilm formation activity. Isolates with biofilm formation activity were most present in cervico-vaginal swabs (41.86%), followed by those from stool samples. Furthermore, urine samples had the lowest number of isolates with biofilm formation activity, and isolates with biofilm formation activity greater than 100%, were most present in cervico-vaginal swabs and least present in oro-pharyngeal swabs. According to the literature, mucosal infections such as oropharyngeal candidiasis and vaginal candidiasis have been associated with biofilm formation as they involve the development on epithelia of 3D communities of *Candida* cells in association with commensal bacteria and host components [17]. The presence Of *C. glabrata* isolates during an infection (especially bloodstream infections) has been associated with higher morbidity and mortality rates compared to isolates that are unable to form biofilm.

C. glabrata sensu stricto displays the highest number of biofilm cultivable cells and its strains show similar biofilm forming ability [8]. Previous studies show that biofilm formation in *C. glabrata* sensu stricto contributes significantly to the colonization of tissues and indwelling medical devices [6,7]. Thus, the ability of our *C. glabrata* sensu stricto isolates to form biofilms has important clinical repercussions, as this is associated to their increased resistance to antifungal therapy, as well as the increased ability of the yeast cells within the biofilms to withstand host immune defences [6,8]. Their ability to form drug-resistant biofilms is an important factor in their contribution to human disease [7].

5. CONCLUSION

With the continuous increase of recurrent *C. glabrata* sensu stricto infections, many studies have been carried out to study the virulence characteristics of *C. glabrata sensu* stricto strains in different geographical locations. In Cameroon, published data on the virulence characteristics of *C. glabrata* strains are very rare. We therefore carried out this study to assess some virulence characteristics, including the capacity of formation of biofilm and hydrolytic enzymes (protease, esterase and phospholipase). Our results revealed that our isolates were more able to produce phospholipase (37.04%) than they were able to produce protease (1.85%) and esterase (0%). The high producers of phospholipase (Pz < 0.7) originated mostly from oro-pharyngeal swabs (41.17%) of some diabetic patients and pregnant women. Also, all our isolates were formers of biofilm, most (74.42%) of which had lower (< 100%) biofilm formation activity compared to our reference strain. To be able to give a significant conclusion about the virulence characteristics of *C. glabrata* sensu stricto strains in the west region, we recommend that more studies be carried on a larger number of strains.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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