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Identification of Virulence Factors Produced by *Candida* Isolates from HIV Seropositive and HIV Seronegative Pregnant Women

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Authors' contributions

This work was carried out in collaboration among all authors. Authors BIE, KAAN and OOK did the study design. Authors BIE and BRA did the laboratory work. Statistical analysis and literature searches was done by author BIE while author KAAN did the manuscript write up. Authors WAA and OOK did the proof reading of the manuscript. The final manuscript was read and approved by all authors.

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ABSTRACT

Background: *Candida* infection has been shown to be a source of morbidity in pregnancy and in immunocompromised individuals due to elaboration of virulence factors.
Study Design: The study was designed to identify various *Candida* species that were isolated from the vaginal and oropharyngeal cultures of HIV seropositive and HIV seronegative pregnant women in their third trimester of pregnancy as well as the virulence factors of the *Candida* isolates.
Methodology: A total of 240 pregnant women were enrolled in the study. They were screened for HIV seropositivity with HIV-1/2 strip and confirmed by Abbott enzyme-linked immunosorbent assay

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(ELISA). One hundred and fourteen (114) of the women were found to be HIV seropositive, while 126 were seronegative. Vaginal and oropharyngeal swabs from the two cohorts of pregnant women were cultured on mycological agar that was supplemented with streptomycin. *Candida* isolates were identified by sugar assimilation and fermentation procedures and further speciated using *Candida* Ident chromogenic agar. Assays for virulence factors were conducted using standard techniques.

Results: A total of 106 *Candida* isolates were recovered from the cultures, with 76.4% coming from vaginal cultures and the remaining 23.6% from oropharyngeal swabs. About 59.7% of the vaginal isolates and 92.3% of the 25 oropharyngeal isolates came from HIV seropositive pregnant women. Various *Candida* species were isolated from 28.9% of 114 seropositive women, compared to 18.3% of 126 seronegative women. *Candida albicans* and *C. glabrata* were the two predominant isolates from the two groups of pregnant women. The results show a higher degree of vaginal colonization among the HIV seropositive women. Interestingly, one of the *C. albicans* isolate from the vaginal cultures from the HIV seronegative women produced haemolysin. However, 55% to 91% of *C. albicans* and *C. glabrata* from the same cultures produced coagulase, phospholipase and biofilm. Bile hydrolysis was the most predominant virulent factor expressed by the *Candida* isolates recovered from all sites in our study.

Conclusion: *C. albicans* and *C. glabrata* were the two most predominant isolates that were recovered from both HIV seropositive and HIV seronegative women in our study. The degree of colonization of *Candida* isolates recovered from the two anatomical sites was higher in HIV seropositive women (59.7%), compared to 40.3% in HIV seronegative controls. The significant higher rate among HIV seropositive women underscores the potential burden of immunosuppression by HIV infection.

Keywords: HIV; pregnancy; high vaginal swabs; oropharynx; Candida isolates; virulence factors.

1. INTRODUCTION

Candida species are often described as commensals in healthy individuals but are becoming important causes of opportunistic infections especially in immunocompromised individuals [1]. They are the most predominant causes of cutaneous, oral, bloodstream and deep-tissue fungal infections. HIV/AIDS, extensive use of broad-spectrum antibiotics, increased administration of immunosuppressive drugs and implantation of catheters and artificial joints have significantly increased the incidence of candidiasis in the general population, but particularly in immunocompromised population [2]. Catheter-associated *Candida* biofilms are a major source of bloodstream infections, while the mortality rates of device-associated *Candida* infections has been estimated to be as high as 30% [2]. While *C. albicans* is the most documented as a major cause of human infections, other *Candida* species are just as capable of causing such human diseases as oral thrush, esophagitis, endocarditis, meningitis, vaginitis and urinary tract infections [3]. The ability of *Candida* species to cause diseases depends on their potential to produce virulent factors and the immune status of the host. These factors include evasion of host defenses, adherence to target tissues, biofilm formation and production of haemolysins and hydrolytic enzymes [4]. The elaboration of hemolysin, phospholipase and DNAse enables them to degrade red and white blood cells as well as host cellular DNA [5]. Although most yeast infections are attributed to *C. albicans*, the other non-*albicans Candida* species possess similar virulence factors [6,7]. Oral and vaginal candidiasis are common opportunistic infections that are prevalent in HIV seropositive patients [8]. It has been estimated that about 90% of *Candida* infections occur as oral thrush in immunocompromised individuals [9]. There is not much information on the virulence factors of various *Candida* species that are isolated from clinical patients in Nigeria. It is expected that identification of such factors would increase the understanding of *Candida* pathogenesis particularly in pregnant women and assist clinicians to better manage patients with candidiasis in this region. This study was therefore designed to identify, characterize and compare selected virulence factors of *Candida* isolates that were recovered from vaginal and oropharyngeal swabs from HIV seropositive and HIV seronegative pregnant women who attended antenatal clinics in Akure metropolis of Ondo state in southwestern Nigeria.

2. MATERIALS AND METHODS

2.1 Study Centres

The study, conducted at the antenatal clinics of four selected healthcare centres in Akure south and Ifedore local government areas in Ondo State, Nigeria between November 2014-December 2015, included 240 pregnant women at the third trimester of pregnancy. Approval for undertaking the study was obtained from the Ondo State hospital management and ethical board.

2.2 Inclusion Criteria and HIV Serostatus

Informed consent, pregnancy in the third trimester and participation in the highly active antiretroviral therapy (HAART) were criteria for inclusion in the study. Study participants were required to keep all physician appointments throughout the study. HIV-1/2 strip (Determine Test, Alere, London, England, United Kingdom (UK) was used for the preliminary determination of HIV serostatus. Confirmatory test for HIV infection was performed using the Abbott enzyme-linked immunosorbent assay (ELISA) procedure (Abbott Laboratories, Chicago, IL, USA). This led to the establishment of two cohorts of 114 HIV seropositive pregnant women and 126 HIV seronegative pregnant women.

2.3 Sample Collection and Analyses

Sterile cotton-tipped applicator was used to obtain oropharyngeal and high vaginal swabs (HVS) from the study participants. .The swabs were streaked onto mycological agar that was supplemented with streptomycin (Difco, Detroit, MI, USA). Cultures were incubated at 35℃ for 24 h for *Candida* growth and 72 to 120 h for the growth of molds. Sugar assimilation and fermentation were used to identify the yeast colonies that grew on the mycological agar. Each *Candida* isolate was speciated using *Candida* Ident Agar Modified Chromogenic Agar (Sigma-Aldrich St. Louis, MO, USA). Pure cultures of the isolates were maintained in freshly prepared slant bottles and used for determination of biofilm formation and production of hydrolytic enzymes by the isolates. All tests were done in triplicates.

2.4 Haemolysin Production

Haemolysin activity was assessed by the method of Manns et al. [10] on blood agar plate. Isolates were streaked onto freshly prepared blood agar plates supplemented with glucose at a final concentration of 3% (w/v). The plates were incubated at 35^{o}C for 24 - 48 h and observed for haemolysis. Beta (β)-haemolysis was indicated by a clear, colourless zone surrounding the colony of the organism and alpha (α)-haemolysis showed a greenish zone. Gamma (γ) haemolysis was indicated by an absence of any apparent haemolysis or discolouration. The ratio of the diameter of the colony to that of the translucent zone of haemolysis was used as the haemolytic index (Hz value) to represent the extent of haemolysin activity by different *Candida* isolates [11]. *Streptococcus pyogenes* (Lancefield group A) and *S. sanguis*, were used as positive and negative control organisms.

2.5 DNAse Activity

DNAse activity was measured by the modified methods of Jeffries et al. [12] and Barrow and Feltham [13], using DNAse medium supplemented with 1% glucose. Each colony from the 24 h growth was streaked unto DNAse agar plates and incubated at 35℃ for 48 h for growth. After incubation, DNAse medium was read for DNAse activity by flooding each plate with 1N HCl. The diameter of the clear zone around the line of streak was measured.

2.6 Bile Hydrolysis

A 24-hour culture of test organism was streaked onto freshly prepared bile agar (Oxoid) plates supplemented with 1% glucose and incubated at 35℃ for 48 h. The blackening of the medium by test organism indicated a positive reaction for bile hydrolysis.

2.7 Coagulase Activity

Coagulase production by *Candida* isolates was detected for each test isolate. A 0.5 ml of mycological broth in test tubes was inoculated with an overnight culture of *Candida* isolate and incubated at 35℃ for 24 h. A 0.5 ml of undiluted plasma was added to the test tube and incubated at 35℃. Tubes were examined after 2, 4, 6 and 12 h respectively for coagulation. Positive result was indicated by a definite clot when tubes were slanted at an angle and could not be resuspended. Negative tubes were left at room temperature overnight and then re-examined for coagulation. *Staphylococcus aureus* ATCC 25923 and *S. epidermidis* ATCC 14990 were

used as positive and negative controls respectively.

2.8 Phospholipase Activity

The phospholipase activity of *Candida* isolates was detected by the method of Samaranayake et al. [14] using the egg yolk medium. A 5 µL aliquot of standard inoculum of *Candida* isolate containing 10^8 *Candida* cells/mL was aseptically inoculated onto egg yolk agar. The plates were dried at room temperature and then incubated at 35℃ for 48 h. A visible precipitation zone around the colony on the plate was indicative of a positive phospholipase activity. *C. albicans* ATCC 10231 was used as a positive control. The phospholipase index (Pz) was defined as the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone. A Pz value of 1 denoted no phospholipase activity and a Pz < 1 indicated phospholipase production by the isolate. The lower the Pz value, the higher the phospholipase activity [15]. The assay was conducted in triplicates.

2.9 Biofilm Formation

Biofilm formation by *Candida* isolates was assessed by the tube method as described by Yigit et al. [16]. Colonies of *Candida* isolates from mycological agar plates were inoculated in saline and incubated overnight at 35℃. A 0.5 mL aliquot of the saline suspension was added into screw capped conical polystyrene tubes containing 5 mL of mycological broth supplemented with glucose at a final concentration of 8%. The tubes were incubated at 35℃ for 48 h without agitation. After incubation the broth from the tubes were aspirated gently with Pasteur pipette, washed twice with distilled water and stained with 2% safranin. The stain was decanted after 10 mins. The tubes were rinsed with distilled water to remove excess stain. Presence of a visible adherent film on the wall and at the bottom of the tube indicated biofilm formation. Ring formation at the liquid interface was not considered as an indication of biofilm production [17]. *Staphylococcus epidermidis* ATCC 35984 was used as a positive control.

3. RESULTS

A total of 114 HIV seropositive and 126 seronegative pregnant women were examined for vaginal and oropharyngeal colonization by *Candida* species. Table 1 shows that *Candida* isolates were recovered from 33 HIV seropositive women, giving a prevalence of 28.9%. On the other hand, vaginal and oropharyngeal *Candida* isolates were recovered from 23 HIV seronegative pregnant women, with a comparative prevalence of 18.3%. Vaginal *Candida* isolates came from 23 of the 33 HIV seropositive women as compared to 21 of the 23 HIV seronegative women. Only two of the 23 seronegative women were positive for oropharyngeal cultures as compared to ten (10) HIV seropositive women.

Table 2 shows that a total of 106 *Candida* isolates were recovered from all cultures, with 81 (76.4%) coming from vaginal cultures of HIV seropositive and HIV seronegative pregnant women (p=0.176) and the remaining 25 (23.6%) from oropharyngeal swabs. About 48 (59.3%) of the 81 vaginal isolates and 21 (84.0%) of the 25 oropharyngeal isolates came from HIV seropositive pregnant women. Only two of the oropharyngeal *Candida* isolates were recovered from HIV seronegative pregnant women. Among the HIV seropositive subjects, 20 of the 48 Candida isolates (41.6%) that were recovered from high vaginal swabs were speciated as *Candida albicans*. Among the 33 *Candida* isolates from HIV seronegative women, eleven (33.3%) were identified as *C. albicans. C. albicans* and *C. glabrata* represent the majority (65%) of *Candida* species that were recovered from the vaginal cultures of HIV seropositive women, compared to 57.5% of the vaginal cultures of HIV seronegative women. The oropharyngeal cultures yielded fewer *Candida* isolates, with *C. glabrata* and *C. albicans* also as the majority isolates. About 21 (91.3%) of the oropharyngeal isolates came from HIV seropositive women (p=0.05).

The results in Tables 2 and 3 also show 14 (70%) of the *C. albicans* and 10 (91%) of *C. glabrata* from the vaginal cultures of HIV seropositive women produced esculin for bile hydrolysis, while 55% of both *C. albicans* and *C. glabrata* produced coagulase, phospholipase and biofilm. However, 14 (70%) of the *C. albicans* and 10 (91%) of *C. glabrata* from the vaginal cultures of HIV seropositive women produced esculin for bile hydrolysis, while 55% of both *C. albicans* and *C. glabrata* produced coagulase, phospholipase and biofilm. On the other hand, only a smaller proportion of the vaginal cultures of *C. albicans* and *C. glabrata* from HIV seronegative women produced coagulase, phospholipase and biofilm.

Interestingly, none of the *C. albicans* and *C. glabrata* isolates from the oropharyngeal cultures of HIV seronegative women produced any of the six virulence factors.

C. krusei, *C. tropicalis* and *C. pseudotropicalis* were among the other isolates that were also recovered from various cultures. But none of the five (5) *C. tropicalis* isolates recovered from HIV seropositive subjects produced haemolysis. Three isolates each produced esculin and phospholipase while two other isolates were positive for biofilm formation. Among the six (6) *C. krusei* isolates recovered from vaginal swabs of HIV seronegative counterparts, none of the isolates showed haemolysis, but two each produced DNAse, coagulase, phospholipase and biofilm. Tables 2 and 3 also show that the exoenzymatic activities of *C. pseudotropicalis* are similar to those of *C. tropicalis.* None of the *C. pseudotropicalis* isolates recovered from high vaginal swab cultures produced DNAse or phospholipase enzymes.

4. DISCUSSION

The study examined vaginal and oropharyngeal colonization by *Candida* species among two cohorts of 114 HIV seropositive and 126 seronegative pregnant women in Akure metropolis of southwestern Nigeria. The study participants ranged in age from 19 to 43 years of age and were all in their third trimester of pregnancy. Various *Candida* species were isolated from 28.9% of seropositive women, compared to 18.3% of seronegative women (Table 1). *Candida albicans* and *C. glabrata* were the two predominant isolates from the two groups of pregnant women. The results show a higher degree of vaginal colonization among the HIV seropositive women.

The study also investigated production of virulence factors of *Candida* isolates that were recovered from high vaginal swabs and oropharynx of pregnant women. These factors include evasion of host defenses, adherence to target tissues, biofilm formation and production of haemolysins, coagulase, phospholipase and DNAse. However, 55 to 91% of *C. albicans* and *C. glabrata* from the same cultures produced coagulase, phospholipase and biofilm.

A number of studies have shown that extracellular hydrolytic enzymes play an important role in the pathogenesis of *Candida* infections [18] and that haemolysin production facilitates deeper tissue invasion [19]. Studies by Deorukhkar and Saini [15] have also documented phospholipase production by *C. albicans* isolates from bloodstream infections. Mane et al. [20] reported high haemolysin production by *C. tropicalis* isolates from HIV infected individuals. But our results show that only a relatively low percentage (2.8%) of our *Candida* isolates produced haemolysin and that the majority of haemolysin activity demonstrated by the isolates were from HIV seropositive subjects. Sachin et al. [21] reported that haemolysin activity was higher in *C. albicans* (94.8%) and *C. dubliniensis* (60%) isolates that were recovered in their study. The results of our study are at variance with their findings because majority of our haemolysin production occurred in *C. glabrata* that were cultured from both high vaginal swabs and oropharynx. The differences in haemolysin production may be due to epigenetic effects of HIV coinfection and to differences in the geographical strains of the *Candida* isolates. Furthermore, our investigation showed that bile hydrolysis was the most predominant virulent factor expressed by the *Candida* isolates recovered from all sites in our study. This was followed by phospholipase and coagulase production. Production of these virulence factors are known to enhance the pathogenicity of *Candida* and other microbial pathogens [16,22].

Table 1. Prevalence of vaginal and oropharyngeal Candida colonization among HIV seropositive and seronegative pregnant women in Akure, southwestern Nigeria

	HIV positive	HIV negative	Total no.
No. patients	114	126	240
Patients with HVS *Candida*	23 (20.2%)	21 (16.7%)	44 (18.3%)
Patients with ORP *Candida*	10 (8.8%)	2 (1.6%)	12 (5.0%)
No. *Candida* isolates	81 (76.4%)	25 (23.6%)	106 (100%)

HVS= High vaginal swab; ORP= Oropharyngeal

Table 2. Distribution of virulence factors among *Candida* isolates recovered from high vagina swabs of HIV seropositive and seronegative pregnant women

Candida isolates	Virulence factors													
	HIV seropositive							HIV seronegative						
	Total no.	Haemolysin	DNAse	Bile hydrolysis	Coagulase	Phospholipase	Biofilm formation	Total no.	Haemolysin	DNAse	Bile hydrolysis	Coagulase	Phospholipase	Biofilm formation
Candida albicans	20	0	2	14	11	11	11	11	1	1	7	2	4	4
Candida glabrata	11	1	1	10	6	5	5	8	0	0	7	5	2	2
Candida krusei	5	0	2	1	1	4	1	6	0	2	1	2	2	2
Candida tropicalis	5	0	1	3	1	3	2	1	0	0	1	1	1	0
Candida pseudotropicalis	3	0	0	3	2	1	1	3	0	1	2	1	1	0
Candida dubliniesis	2	0	0	2	1	0	0	2	0	0	1	0	1	0
Candida spp	2	0	0	2	1	1	1	0	0	0	0	0	0	0
Candida famata	0	0	0	0	0	0	0	2	0	0	0	0	1	0
Total	48							33						

Table 3. Distribution of virulence factors among *Candida* isolates recovered from the oropharynx of HIV seropositive and seronegative pregnant women

Candida isolates	Virulence factors													
	HIV seropositive							HIV seronegative						
	Total no.	Haemolysin	DNAse	Bile hydrolysis	Coagulase	Phospholipase	Biofilm formation	Total no.	Haemolysin	DNAse	Bile hydrolysis	Coagulase	Phospholipase	Biofilm formation
Candida albicans	6	0	0	4	2	3	4	0	0	0	0	0	0	0
Candida glabrata	9	2	0	6	5	2	3	0	0	0	0	0	0	0
Candida tropicalis	3	0	1	0	0	2	1	0	0	0	0	0	0	0
Candida pseudotropicalis	3	0	0	3	2	1	1	0	0	0	0	0	0	0
Candida famata	0	0	0	0	0	0	0	2	0	0	0	0	1	0

Bile is produced by the liver and secreted into human intestinal tract where it is utililized for the emulsification and solubilization of fats and lipids. Bile salts can cause haemolysis of bacterial membranes by dissolving membrane phospholipids and dissociation of integral membrane proteins. It can also induce DNA damage and cause oxidative stress by generating oxygen radicals [23,24]. The results in Table 2 show that 24 (77.4%) of the 31 isolates and 14 (74%) of the 19 isolates of both *C. albicans* and *C. glabrata* that were isolated from the vaginal cultures of HIV seropositive and seronegative pregnant women respectively were positive for bile solubility, indicating that they produce bile salt hydrolases. These results show that *C. albicans* and *C. glabrata* can negate the antimicrobial effects of bile by production of bile salt hydrolases, suggesting that bile solubility by these organisms can serve as an important virulence factor. While earlier studies by Begley et al. [23] had reported bile solubility by *Candida* organisms, our's is the first to document production of bile solubility enzymes by vaginal culture isolates of *C. albicans* and *C. glabrata* in both HIV seropositive and HIV seronegative pregnant women. Coagulase is another virulence factor that is produced by *C. albicans* [22,16]. The enzyme has been extensively studied in *Staphylococcus aureus* bacteria. It converts fibrinogen to fibrin by first reacting with prothrombin to form a complex called staphylothrombin, which enables a protease enzyme to convert fibrinogen to fibrin. Fibrin forms clots in the blood circulation and can also bind to bacterial surface to inhibit phagocytosis. Our results in Table 2 show that 17 (55%) and 37% of vaginal isolates of *C. albicans* and *C. glabrata* from HIV seropositive and HIV seronegative pregnant respectively produce coagulase enzyme which undoubtedly plays the same role as has been demonstrated for coagulase positive bacteria. Phospholipases are produced and used by microbial pathogens for adherence, tissue invasion and destruction of host tissues [25]. Other studies have reported on the production and role of aspartyl proteinases in *Candida* pathogenesis [26,27]. Interestingly, our results show that 34.4% of *C. albicans* isolates from HIV seropositive patients produced phospholipase as compared to 12.5% of isolates from HIV seronegative controls. Our results also show that a significant percentage of *C. albicans* and *C. glabrata* isolates produced biofilm which is known to increase resistance to anti-fungal drugs. Biofilms are described as communities of microorganisms that are embedded in a self-created extracellular matrix and which serve as a diffusion barrier to limit permeability of drugs [28].

5. CONCLUSION

In conclusion, the degree of colonization of *Candida* isolates recovered from the two anatomical sites was higher in HIV seropositive patients (59.7%), compared to 40.3% in HIV seronegative patients. This finding demonstrates that HIV seropositive patients may have a higher burden of colonization because of their immunocompromised status. *C. albicans* and *C. glabrata* were the two most predominant isolates that were recovered from both HIV seropositive and HIV seronegative women in our study. The significant higher rate of colonization by *Candida* species in the oropharynx of the HIV seropositive patients (91.3%) in contrast to 8.7% in HIV seronegative patients again underscores the potential burden of immunosuppression in HIV seropositive patients. It is conceivable that the elaboration of virulence factors as phospholipase, coagulase and biofilm formation could result in adverse pregnancy outcomes. There is therefore an urgent need to study the clinical impact of *Candida* infections in HIV seropositive pregnant women.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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