Original Research Article

Journal of Biological Research & Biotechnology

Bio-Research Vol. 16 No.1; pp. 1025-1032 (2018). ISSN (print): 1596-7409; eISSN (online):2705-3822

Proteinase activities of *Candida* spp. isolated from different anatomical sites of healthy women

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Abstract

Superficial and systemic fungal infections caused by *Candida* have been increasingly reported in recent times. Hydrolytic enzyme production is an important process in fungal pathogenesis and proteases have been identified as important virulence attributes in *Candida* species. The aim of the study was to determine and compare the *in vitro* proteinase activity in sixty *Candida* spp isolated from three different anatomical sites (vagina, oral cavity and skin) of healthy women. Twenty samples per sample source were collected from apparently healthy female subjects. The recovered *Candida* isolates were properly identified and screened for proteolytic activity using established procedures. Overall, the recovery rate of *Candida albicans* was 66.7%, while the non-albicans *Candida* species represent 25% of the positive samples. *Candida albicans* recovered from the oral cavity exhibited the highest proteolytic activity (Pz range = 0.41±0.02- 0.65 ± 0.04), followed by skin isolates (Pz = 0.50 ± 0.05 – 0.79 ± 0.06). Isolates from the vagina had the least proteolytic activity (Pz = 0.57 ± 0.03 - 0.95 ± 0.08). The difference in proteolysis was significant between oral and vagina isolates (p = 0.0042), as well as skin and vaginal isolates (p = 0.0364). This study indicates that *C. albicans* remains the most prevalent species in all the anatomical body sites investigated. Moreover, the secretion of proteases could prove a potent virulence factor during the pathogenesis of the organism in an otherwise immunocompetent host.

Key words: Proteinase, *Candida albicans*, women, body sites, non-albicans *Candida*

https://dx.doi.org/10.4314/br.v16i1.4
Journal Homepage: ***http://www.bioresearch.com.ng.***
Publisher***: Faculty of Biological Sciences, University of Nigeria, Nsukka, Nigeria.***

INTRODUCTION

The incidence of fungal infections caused by *Candida* has been increasing in recent times. Hydrolytic enzyme production is an important process in fungal pathogenesis and proteases have been identified as important virulence attributes in *Candida* species. *Candida* species can colonize humans either as commensals or opportunistic yeast - like organisms depending on the immune status of the host. This feature placed them among the clinically important etiologic agents of mycotic infections. *C. albicans* for instance, is present in the oral cavity of both immunocompetent and immunocompromised

individuals with children and young adults representing the more susceptible group. Therefore, further investigation of their occurrence at a particular site especially in immunocompetent individuals is relevant to have an idea of the potential threat it poses to the health of the individual since yeast infections are often caused by endogenous species. Furthermore, it enhances the ability to draw a line between commensal carriage and infectious phase of the organism. Generally, among other factors, gender is one of the specific risk factors for *C. albicans,* with higher prevalence reported in females than male population irrespective of the ecological niche (Angebault *et al*., 2018). Also, drug therapy, malignancy, immunologic disorders, and salivary changes have been identified as important predisposing factors especially in oral *Candida* colonization (Farah *et al*., 2010). Although *C. albicans* is the species mostly involved in the clinical infections, growing profile of infections due to other non-*albicans* species have been reported in recent times especially in apparently immunocompetent persons (Byadarahally and Rajappa, 2011; Matic *et al*., 2019). As opportunistic fungal pathogens, *Candida* spp. particularly *C. albicans* secretes and expresses wide array of virulence factors facilitating its adhesion, colonization, invasion, and spread to adjacent tissues and deeper organs. Among these virulence factors, hydrolytic enzymes including secreted aspartyl proteases, play significant role in the pathogenesis of *Candida* species (Staniszewska *et al*., 2012). This study therefore focused on extracellular proteinase activities in different *Candida* spp. isolated from different anatomical sites in apparently healthy female subjects.

MATERIALS AND METHODS

Study population

The study group consisted of healthy (non-immunocompromised) subjects who were not on antifungal antibiotics, have not been recently treated with antifungal antibiotics, and were not on hospitalization**.** The carriage of *Candida* species in three common human anatomical sites viz: oral, skin, and vagina was respectively investigated in 20 apparently healthy women volunteers, following a method previously described by Szymanska *et al* (2016). A total of 60 samples (20 samples from each anatomical site) were collected after obtaining informed consent, using sterile swab sticks and inoculated onto the surface of Sabouraud dextrose agar plates containing chloramphenicol and gentamycin and cultured with a medium selective for yeast growth. Multiple colonies were picked from the primary isolation plates for the identification of *Candida* spp. None of the plates contained more than one *Candida* spp. All isolates were identified by germ tube test in human serum, chlamydospore formation and morphology on cornmeal agar. The non-albicans *Candida* spp were further identified by carbohydrate assimilation patterns. All isolates were cultured on and identified based on their characteristic colour on Chromagar *Candida* medium (CHROMagar Co., Paris, France).

Detection of proteolytic activity of *Candida albicans*.

Proteinase assay was performed using bovine serum albumin (BSA) as described by Lahkar *et al* (2017). A basic medium containing dextrose (2%), KH2PO4 (0.1%), MgSO4 (0.05%), and agar (2%) was autoclaved and mixed with 1% BSA solution after cooling to 50°C. About 20ml of the medium was dispensed into each Petri dish. Wells were made on the solidified agar plate after which about 10µl aliquots of the *Candida albicans* suspension (approximately 1 x10^6 yeast cells/ml) was inoculated. The plates were incubated at 37°C and observed daily for 2-5 days. Extracellular protease detection was done after fixing the plates with 20% trichloroacetic acid staining with 1.25% Amido black in methanol-acetic acid-water in the ratio of 30:10:60 (v/v/v) for 1hr at 28°C. Clear zones around the wells were recorded as evidence of enzymatic hydrolysis of the substrates. Pz value was determined as the ratio of the diameter of the colony plus the precipitation zone. The study was repeated twice for each isolate and Pz value was taken as the average of the two measurements. A Pz value of 1.0 indicates no activity, while Pz <1 indicates proteinase activity. The lower the Pz value, the higher the enzymatic activity. Each experiment was repeated twice on different days.

Statistical analysis

Two tailed student's t-test was used for statistical assessment and values of $P < 0.05$ were accepted as significant.

RESULTS

Distribution of *Candida* species in the anatomical sites

A total of 40 isolates of *Candida species* were recovered from the 60 samples giving the

isolation rate of 66.7%. Distribution of the isolates according to the anatomical sites showed that *Candida albicans* is the predominant species (75%), while the non-albicans *Candida* species represent 25% of the positive samples (Table 1.0).

Identification *Candida species*

The recovered yeast - like colonies were observed as smooth, white-cream colonies consistent with morphological features of *Candida* species on Sabouraud dextrose agar (SDA*)* plates (Figure 1). The isolates exhibited expected colour changes on chromagar (Figure 2) Three species of *Candida*, including *C. albicans, C. tropicalis*, and *C. parapsilosis* were presumptively identified based on their peculiar pigmentations on the chromagar.

Proteolytic activity

Opaqueness of the agar, corresponding to the zone of proteolysis around the wells of inoculation, that could not be stained by amido black indicated degradation of the protein (Figures 3 and 4). The proteinase activity (Pz) was determined in terms of the ratio of the diameter of the well to the diameter of the proteolysis.

Table 1.0: Distribution of the isolates according to body sites

Candida species	Skin	Oral	Vagina	Total
C. albicans	5	11	14	30
C. tropicalis	-	2	3	5
C. parapsilopsis	2	2	1	5
Total	7	15	18	40

DISCUSSION

The present study focused on determining the *in vitro* proteinase activities in fourty isolates recovered from in three different anatomical sites (oral cavity, vagina and skin) of apparently healthy subjects. Three different *Candida* species were recovered in the study which indicates that *Candida albicans* remains a common normal flora among immunocompetent individual. Most of the isolates were recovered from the oral cavity compared to the vagina and skin. Despite the predominance of *C. albicans* in all anatomical sites, Rafat *et al* (2017) reported a higher frequency of *C. parapsilosis* than other species in all the groups. The authors did not consider sex as an epidemiological factor of the infection (Rafat *et al*., 2017). *C. albicans* is a member of over 700 microbial species estimated to colonize the oral cavity of humans, and this large microbial community occupying the oral niche could be due to neutral saliva pH in addition to other microenvironmental factors (Montelongo-Jauregui and Lopez - Ribot, 2018). Oral carriage of *Candida* species is common in immunocompetent persons as commensals while among the immunocompromised individuals, it presents as opportunistic infection that is frequently invasive, affecting deeper tissues and organs by systemic spread. Singh *et al* (2014), observed that this commensal state of *Candida* species in the oral cavity may be due to the presence of salivary antimicrobial secretions and polypeptides, including lytic enzymes specific antibodies against *C. albicans*. These constitute colonization and invasion barriers against the fungal cell thereby checking overgrowth of the commensal organism under normal conditions. It was shown that the optimal environment for colonization by microorganisms such as nutrient, temperature, and water activity is facilitated by the oral cavity supporting the growth of diversity of microorganisms (Jenkinson and Douglas, 2002). Studies by Nejad *et al*. (2011) showed the distribution of *Candida* spp in the oral cavity to include *C. albicans* (75%), *C. glabrata* (12.5%), and *C. tropicalis* (6.5%). Similarly, the recovery rate of *Candida* species in oral cavity investigated by Sato *et al* (2017) showed that *C. albicans* maintained the lead in predominance (53.4%), while the non - albicans species were lower (23.7%) in the oral cavity A previous study on the prevalence of *Candida* species in oral cavity of the oral cancer patients observed a comparatively higher prevalence of *C. albicans*

Figure 1.0: *Candida* spp on SDA culture plate

Fig 2.0: showing results of *Candida* spp growing on chrome agar

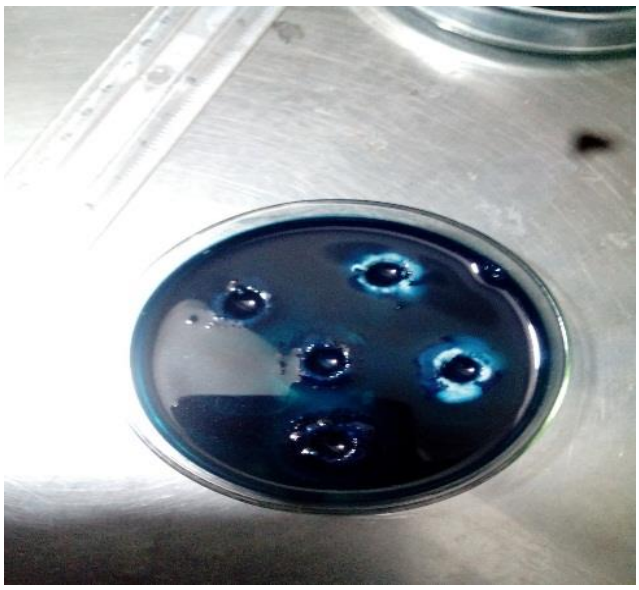

Figure 3.0: Culture plate flooded with amido black for detection of proteinase activity.

Figure 4.0: Proteolytic activity of *Candida albicans.* The zones of clearance correspond to the proteolytic activity of *Candida albicans*

Table 2.0: Proteolytic activity of the isolates of *Candida albicans* as analyzed by their Pz values

sample source	Pz values	Sample source	Pz values	Sample source	Pz values
O1	0.65 ± 0.04	S1	0.50 ± 0.05	V1	0.95 ± 0.08
O2	0.50 ± 0.05	S2	0.57 ± 0.03	V2	0.91 ± 0.08
O3	0.58 ± 0.03	S3	0.79 ± 0.06	V3	0.91 ± 0.08
O4	0.41 ± 0.02	S4	0.57 ± 0.03	V4	0.57 ± 0.03
O5	0.47 ± 0.03	S5	0.72 ± 0.05	V5	0.95 ± 0.08

Key: O = oral, S = skin, V = vagina; Pz < 0.5 = high proteolytic activity; 0.6 - 0.74 = moderate proteolytic activity; 0.75 - 0.89 low proteolytic activity; 0.9 - 1.0 = no proteolytic activity.

(61.1%) than other non-*albicans* species with respective prevalence of 20.4%, 11.1%, and 7.4% (*C. tropicalis, C. krusei, C. glabrata*) (Bajracharyya *et al*., 2019).The isolation of the three *Candida* spp. from the oral cavity in our study is invariably similar to the findings of Matic *et al* (2019) who also reported comparable trends in the fungal oral carriage where *C. albicans* is the predominant species. Oral *Candida* infection affects all adults irrespective of sex or race (Meira et al., 2017), and therefore, our findings is informative of the oral fungal burden of the studied population. As a commensal, it was shown that *C. albicans* carriage in healthy populations is within the range of 2.0 - 69.1% (Patil *et al*., 2015). The ascending order of prevalence of three important *Candida* spp. in oral cavity are *Candida tropicalis* (7%) and *C. albicans* (75%) as revealed by White *et al* (2004). A study demonstrated that although *C. albicans* remains the predominant species isolated from oral cavity, most of the isolates were found to be genetically diverse which highlights among other features their adaption to recurrent stress in the oral ecological site (Sitterle *et al*., 2019). This diversity could be traced to their interaction with the immune cells as well as other components of the oral microbiota (Montelongo-Jauregui and Lopez-Ribot, 2018).

The human skin is one of the largest organs in the human body and as a result harbours diverse groups of microbiotas. *Candida* spp represent important fungal members of skin commensal microorganisms of healthy individuals. Following their penchant for carbon (iv) oxide and moisture generated by friction, the skin provides an ideal microenvironment for colonization of *C. albicans* and non-albicans species (Kuhbacher *et al.*, 2017).

Colonization of vulvovaginal mucosal surfaces by *Candida* species is common among healthy women, and in many cases, *C. albicans* remains the predominant species associated with the urinogenital tract (Achkar and Fries, 2010). Although the invasion of colonizing *Candida* species into the adjacent tissues is a function of immune status of the host, recurrent vulvovaginal infections are common observation in immunocompetent women. There is also accumulating evidence that interactions of *C. albicans* and the host defense mechanisms in the oral and vaginal anatomical sites modulate its carriage and virulence expression (Cassone *et al*., 2016). This supports the observation that virulence of *Candida* species depends on the body site of isolation. Non-*albicans* species particularly showed significant difference in proteinase activity under aerobic compared to anaerobic condition as indicated by Inci *et al*. (2012), suggesting that more aerated body sites like the skin may be more susceptible to their virulence expression than less exposed body sites. Our findings did not indicate major variation in proteinase activity when isolates from the 3 anatomical sites were compared. Detection of proteolytic activity in immunocompetent persons may suggest that some virulence factors are also important for maintenance of commensalism. Thus, even when proteinase activities varied with anatomical sites, there is paucity of strong association of proteolytic activity and site of *Candida* isolation (Oksuz *et al*., 2007).

The mucocutaneous membrane of the female genitalia is a conducive environment where *Candida* species thrive. Consequently, vulvovaginal carriage is high even among the immunocompetent population. Several studies have reported the high recovery rate of *C. albicans* particularly on the vaginal epithelial

surfaces (Ribeiro *et al*., 2001; Nsofor *et al*., 2016; Brandolt *et al*., 2017). The colonization of vulvovaginal cavity was traced to cultural practices and behavioural patterns which placed women in particular as high risk for fungal contamination (Brandolt *et al*., 2017). Hence, reports showed that significant percentage of healthy women harbour species of *Candida* in their genital tract (Makanjuola *et al*., 2018). Other risk factors may include indulgence in sugary, diets, sedentary lifestyle, and abuse of antibiotics (Zeng *et al*., 2018). In their quest for the predilection of *C. albicans* in the vulvovaginal tract, Amabebe and Anumba (2018), suggested that although glycogen accumulation in vagina of post reproductive women population consists in one of the hormonal effects of estrogen, the influence of stress on the pituitary hormone can also, ultimately trigger accumulation of glycogen and susceptibility to colonization by *C. albicans* and subsequent infection in the pre-reproductive women.

Constitutive expression of *Candida* spp. virulence such as proteases following chronic vulvovaginal colonization is an indication of invasive *Candida* infection (Jose *et al*., 2015). A study has demonstrated a link between high *Candida* proteolytic enzyme activity and pre-existing immunosuppressed condition and the strength of its adherence to the mucosal surface of the vagina is mediated by the hydrolytic activity of the enzyme (Mardegan *et al*., 2006). In addition to facilitating adherence to vaginal epithelium, secreted proteinases can induce an increased virulence expression by histolytic and necrotic mechanisms which may result to the release of free nitrogen from peptide compounds (Akcaglar *et al*., 2011). When the proteolytic activity of the isolates was examined, very high activity was recorded among oral *Candida albicans* isolates. This may correlate with the common observation where oral cavity represents the primary site of colonisation and infection including oral thrush. Despite the predominant colonization of mucocutaneous membrane of the vulvovaginal tract by *Candida albicans,* expression of virulent proteinases may be lower than observed in other body sites including the skin epidermal surfaces and oral cavity. This follows the observation that yeast-hypha transition upregulated in low glucose environment such as the skin cutaneous layer is also associated with increased secretion of proteinases (Buu and Chen, 2014). Secreted proteinases have been over expressed in epidermal surfaces suggesting their possible involvement in cutaneous candidiasis (Kuhbacher *et al*., 2017). Strains of *Candida albicans* secrete proteinases in both symptomatic and asymptomatic states of vulvovaginal cavity colonization (Lima *et al.*, 2018). In this study, the difference in proteolytic activity was only significant between the oral and vaginal isolates ($p = 0.0042$), and between isolates from the vagina and skin ($p = 0.0364$). Proteolytic activities of *Candida albicans* isolates recovered from oral and skin samples were similar ($p = 0.1563$).

Variation in pH of anatomical body sites may explain the difference in proteolytic activities of the isolates. This is because specific group of proteinases are secreted at lower pH as found in vagina, whereas other strains adapted at a higher pH can function optimally in the oral and skin microenvironments (Galocha *et al*., 2019).

Previous observation had also reported consistency of strains from different body sites with reference to their physiological features such as proteinase activities (Bradford and Ravel, 2017). Presumptively, the activity of proteinase enzymes varies with the immune integrity of the host colonized site where higher secretion is common with isolates from diseased tissue than those recovered from intact tissue membranes (Modrzewska *et al*., 2016). More so, the results further emphasized the possible role of extracellular proteases in the pathogenicity of *Candida* species. One of the shortcomings of our study is the sample size. In future studies, there may be need to not only include samples from men but also increase the sample size and expand the sample types from oral, skin and vagina to other anatomical positions of the body.

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