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

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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 x 10⁸ 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-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. parapsilosis* | 2   | 2    | 1      | 5     |
| **Total**       | 7    | 15   | 18     | 40    |

**DISCUSSION**

The present study focused on determining the *in vitro* proteinase activities in forty 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

Figure 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*.
The human skin is one of the largest organs in the human body and as a result harbours diverse groups of microorganisms. *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

| 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.
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 (Akcaoglar 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-hyppha 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 (Kühbacher 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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