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Ultrastructural Analysis of *in Vitro* Adherence and Production of Acid Proteases by Clinical Isolates of *Candida parapsilosis* Sensu Stricto Following Growth in the Presence of Keratinous Substrates from Human Source

Ana Flávia L. Specian[1], Luciana Furlaneto-Maia[2], Célia G. T. J. Andrade[3], Marcia Cristina Furlaneto[1*]

[1]Department of Microbiology, Paraná State University at Londrina, Londrina, Brazil
[2]Technological Federal University of Paraná, Londrina, Brazil
[3]Electronic Microscopy and Microanalysis Laboratory, Paraná State University at Londrina, Londrina, Brazil
Email: [*]furlaneto@uel.br

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ABSTRACT

Candida parapsilosis is an increasingly important human pathogen. However, little is known about its potential to cause disease. The aims of the present study were to analyse the production of acid proteinases by clinical isolates of *C. parapsilosis* in the presence of different keratinous substrates from human sources (stratum corneum, nail and hair) and to verify the capability of yeast cells to adhere and grow as biofilm on these substrates. By scanning electron microscopy, it was observed that all *C. parapsilosis* sensu stricto isolates adhered to the keratinous substrates. For the isolate recovered from onychomycosis, the cell population attached to stratum corneum and hair keratin consisted mainly of blastoconidia. Differently, on nail keratin, pseudohyphae production was observed. Overall, there was a loose association between yeast cells and keratinous substrates. However, on stratum corneum, flocculent extracellular material was seen evolving cells from the onychomycosis isolate by forming a biofilm-like structure. The isolates recovered from onychomycosis and cutaneous lesion produced higher amount of acid proteinases in medium supplemented with nail keratin and stratum corneum keratin, respectively, than that in salt medium (absence of keratin). Furthermore, no differences were observed in the amount of acid proteinases produced by the isolate recovered from tracheal secretion in the media tested (absence and presence of keratin substrates). The information derived from this study will further our understanding of acid proteinase production by *C. parapsilosis* isolates and provide an insight into pathogenic mechanisms in *C. parapsilosis* particularly from isolates recovered from superficial mycoses.

Keywords: Adherence; Keratinic Substrates; SEM; Acid Proteinase

1. Introduction

Candida parapsilosis is an opportunistic yeast pathogen that colonizes human skin and can spread nosocomially through hand carriage [1,2]. Over the past decade, the incidence of *C. parapsilosis* has dramatically increased. The yeast can cause candidiasis that can vary from relatively mild skin mycoses to life-threatening systemic or disseminated disease (reviewed in van Asbeck *et al*. [3]).

Concerning superficial mycoses, *C. parapsilosis* has gained increasing recognition worldwide as the most common etiological agent causing *Candida* onychomycosis (reviewed in Trofa *et al*. [4]). In Brazil, *C. parapsilosis* is the first or second most common cause of onychomycosis lesions [5-8]. For *C. parapsilosis* the colonized normal skin presumably serves as a reservoir of infection for the nails. Recently, we showed the capability of *C. parapsilosis* isolates exhibiting distinct phenotypes to grow as biofilm on human nail surfaces [9].

Several virulence factors of *C. parapsilosis* have been proposed, including adhesion, biofilm formation and secretion of hydrolases such as secreted aspartic proteinases (Saps) (reviewed in Trofa *et al*. [4]). We previ-

[*]Corresponding author.

ously reported the production of proteinases and haemolytic factor by isolates of *C. parapsilosis* obtained from distinct clinical sources [10]. More recently, we have demonstrated that *C. parapsilosis* sensu stricto secretes a hemolytic factor into culture medium [11].

However, in contrast to the species *Candida albicans*, virulence traits of *C. parapsilosis* have not been extensively studied. Few studies have been undertaken to evaluate the adhesion ability of clinical strains of *C. parapsilosis* [9,12-14]. Furthermore, the relationship between *C. parapsilosis* virulence and proteinase phenotype is still unclear.

In this study, we investigated for the first time the *in vitro* adherence pattern and production of acid proteases by clinical isolates of *C. parapsilosis* in the presence of keratinous substrates from human source, e.g., stratum corneum, nail and hair.

2. Materials and Methods

2.1. *Candida* Isolates and Identification

Isolates of *C. parapsilosis* sensu stricto included in this study were recovered from fingernail onychomycosis (isolate 150.06), cutaneous candidiasis (isolate 220.07) and tracheal secretion (isolate 205.06) [15]. The identity of isolates was determined by the PCR technique as described by França *et al*. [10] using *C. parapsilosis* (formerly *C. parapsilosis* group I) specific primers for *URA*3 gene (orotidine-5'-phosphate decarboxylase) [16] that allows to distinguish *C. parapsilosis* sensu stricto from the cryptospecies belonging to the *C. parapsilosis* complex.

2.2. Preparation of Keratin Substrates

For the substrate stratum corneum, fragments of human sole from healthy volunteer were prepared as described previously [17] with modifications. The fragments were soaked in ethanol for 96 h following washes with sterilized distillated water until a clean solution was obtained. The fragments were dried at 50˚C, grounded to a powder in liquid nitrogen and dried again at 50˚C.

The substrates nail and hair were prepared as described previously [18] with modifications. Human's hair and nail from healthy volunteer were cut to obtain small fragments ranging from 0.5 to 1 cm of size. The fragments were defatted by soaking for 4 d in chloroform-methanol 1:1 (v/v). The solvent was changed once a day. The fragments were then thoroughly washed with sterilized distillated water and dried for 3 d at 50˚C. The nails fragments were grounded to a powder in presence of liquid nitrogen and dried again at 50˚C. The prepared substrates were autoclaved for 5 min at 115˚C and then added to previously sterilized basal minimal medium.

2.3. Growth Profiles Determination and Proteinase Production

The isolates were pre-cultured in Sabouraud dextrose (4% dextrose, 1% peptone, 1% yeast extract) liquid medium and grown in submerged culture (180 rpm) at 37˚C for 18 h. For enzyme production, cell suspensions (10^4 cells/ml) were inoculated in basal minimal medium (MM) [19], with modifications, containing (g/l): K_2HPO_4—1.5 g; $MgSO_4 \cdot 7H_2O$—0.025 g; $CaCl_2$—0.025 g; $FeSO_4 \cdot 7H_2O$—0.015 g, $ZnSO_4 \cdot 7H_2O$—0.005 g and 1% glucose, and MM amended with either 0.5% (w/v) stratum corneum, hair and nail (MM + SC, MM + H and MM + N, respectively) and grown in submerged culture (180 rpm) at 37˚C for up to 10 days. Media were buffered using a citrate buffer (0.1 M citric acid; 0.1 M sodium citrate), pH 5.0. Following growth, yeast cells were harvested by centrifugation at 8000 g for 20 min at 4˚C. The supernatants obtained were stored at −20˚C and assayed for protease activity. For growth rate assessment samples were removed at 12-h intervals. The growth rate was determined by haemocytometric counts. All experiments were repeated three times, and the results represent mean values ± SD.

2.4. Proteinase Assay

Proteolytic activity was determined as previously described [20] with modifications, using haemoglobin (Sigma; St. Louis, MO) as substrate. Each assay included 50 µl of culture supernatant and 0.2 ml of 20 mM citrate buffer, pH 4.0, containing haemoglobin (0.5 mg/ml). After incubation at 37˚C for 2 h, the reaction was stopped with trichloroacetic acid (TCA) 5% on ice, following incubation at 4˚C for 1 h. The mixture was centrifuged at 4000 g for 10 min at 4˚C. After this, three aliquots (150 µl each) of the reaction mixture were transferred to wells on a microtiter plate containing 100 µl of a Coomassie solution (0.025% Coomassie brilliant blue G-250, 11.75% ethanol, and 21.25% phosphoric acid). After 10 min to allow dye binding, the plate was read on an Asys HiTech UVM 340 microplate reader at an absorbance of 595 nm. Protease activity was calculated based on the absorbance difference between samples and controls. The control samples were added supernatants that were immediately treated with TCA. One unit (U) of proteolytic activity was defined as the amount of enzyme that caused an increase of 0.001 in absorbance unit, under standard assay conditions. The proteolytic activity is expressed as U/ml.

2.5. Scanning Electron Microscopy

To verify the adhesion pattern of yeast cells with human keratin substrates, samples were fixed in 2.5% glutaral-

dehyde (Electron Microscopy Sciences) in 0.1 M phosphate buffer, pH 7.2, followed by incubation at 4˚C for 18 h. Then, the samples were carefully washed with 0.1 M phosphate buffer, pH 7.2. Post-fixation was carried out for 1 h at 25˚C with 1% osmium tetroxide in 0.1 M phosphate buffer. Samples were gently dehydrated in graded ethanol, critical point-dried in CO_2 (BALTEC DCP 030 Critical Point Dryer), coated with gold (BALTEC SDC 050 Sputter Coater) and viewed in a FEI Quanta 200 Scanning Electron Microscope.

2.6. Statistical Analysis

All the experiments were repeated three times and assay was performed in duplicate. Tukey test was used to determine statistical significance. $P < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. *In Vitro* Adherence Pattern of *C. parapsilsois* Cells to Human Keratinous Substrates

Yeast pathogenicity arises through complex interactions between the organism's virulence characteristics and the host's response. Compared with *C. albicans*, little is specifically known regarding virulence factors in *C. parapsilosis* sensu stricto.

Adherence is essential for members of the genus *Candida* to develop their pathogenic potential since it triggers the process that leads to colonization and allows their persistence in the host. For instance, more pathogenic *Candida* isolates showed higher adherence capacity on human oral and epithelial cells [21]. According to these authors, epithelial cell variability played a critical role in the adherence phenomenon [21].

In our study, we analysed the *in vitro* pattern of adherence of *C. parapsilosis* sensu stricto cells to human keratinised substrates, *i.e.*, on soft keratin (cutaneous stratum corneum—the outermost layer of skin) and hard keratin (nail and hair). By scanning electron microscopy it was observed that all *C. parapsilosis* isolates adhered to the keratinous substrates (**Figure 1**). On hair fragments, the adherent cells are seen only within the follicle cortex and the number of cells adhered to this substrate varied among isolates (**Figure 1(c)**).

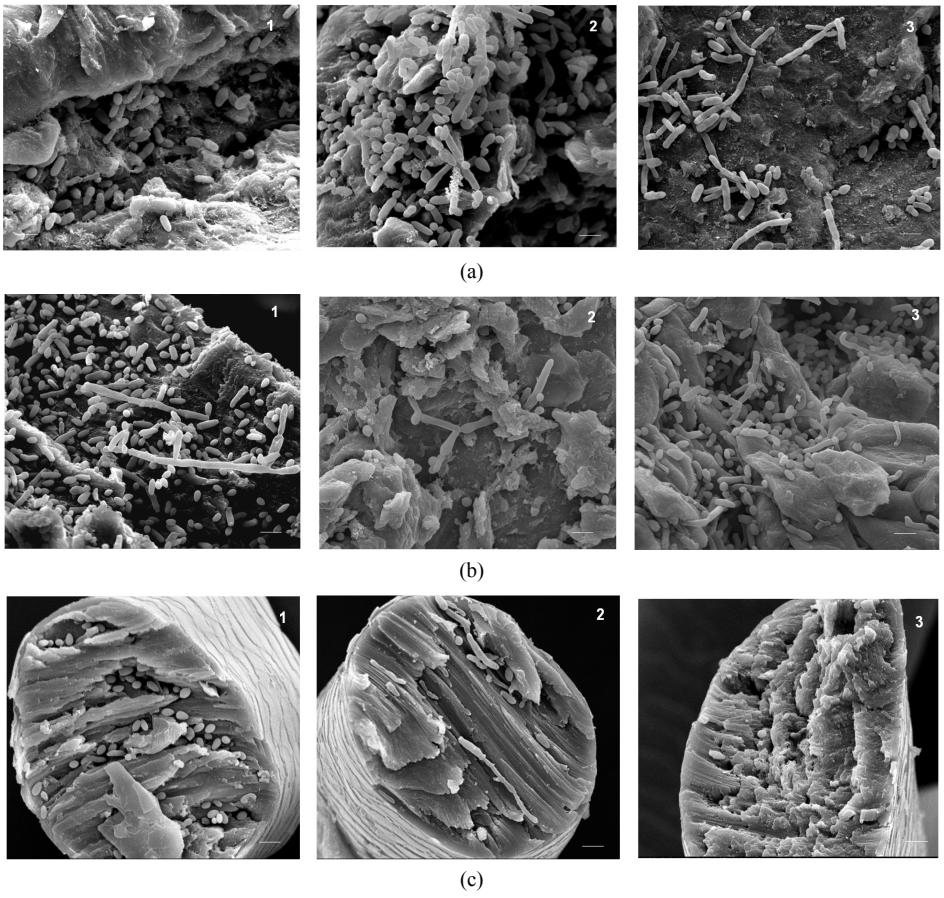

Figure 1. Electron micrographs showing the *in vitro* adherence pattern of *Candida parapsilsois* cells to human keratinous substrates. (a) Stratum corneum, (b) Nail and (c) Hair. (1) Isolate 150.06, (2) Isolate 220.07 and (3) Isolate 205.06. Magnification of 3000×. Extracellular material (arrows) is seeing evolving cells from the isolate 150.06 (a1).

SEM analysis revealed that the onychomycoses isolate presented different morphological pattern according to the substrate that they were in contact. For instance, cells adhered to stratum corneum and hair keratin consisted mainly of cells in the budding-yeast phase of growth (blastoconidia) (**Figures 1(a1)** and **(c1)**). Differently, on nail keratin pseudohyphae production was observed (**Figure 1(b1)**), a pattern that could indicate that this situation favours cellular morphologies with capacity for tissue invasion. This data extend our previous observation that different profiles of biofilm formation by *C. parapsilosis* occurred as function of the keratinous substrate [9].

For the other isolates the cellular population consisted of blastoconidia and pseudohyphae on all substrates analysed, with exception of the isolate 205.06 (tracheal secretion) on hair keratin (**Figure 1(c3)**). Intraspecific differences in adherence to polystyrene have been described among clinical isolates of *C. parapsilosis* obtained from distinct body sites [12]. More recently, reconstituted human epithelium (RHE) has been used to study *in vitro* colonization by *C. parapsilosis* complex [13,14]. According to these authors, the extent of surface colonization on RHE by *C. parapsilosis* was strain dependent.

Overall, there was a loose association between yeast cells and keratinous substrates. However, on stratum corneum flocculent extracellular material was seen evolving cells from the onychomycoses isolate by forming a biofilm-like structure (**Figure 1(a1)**). This feature was not observed on the other two sources of human keratin (nail and hair).

It has been showed that adhesion [22] and ability to grow as biofilms [23] on abiotic surfaces are especially important for outbreaks of *C. parapsilosis* infections (reviewed in Trofa *et al*. [4]). This is the first report of ultrastructural features related to adhesion of *C. parapsilosis* isolates associated with skin and nail infections to distinct keratinised substrates from human source.

3.2. Acid Proteinases Production

In this study, we evaluated for the first time the growth profile and the production of acid proteinases by *C. parapsilosis* using human keratin as sole source of nitrogen. The isolates tested presented similar trend of growth rate in keratin-supplemented media. However, the fungal growths were more profusely in stratum corneum-supplemented medium than in hair- and nail-supplemented medium (cell density reached 10^8 cells/ml and 10^7 cells/ml, respectively), probably due to differences in keratin structure and the degree of cross-linkages by disulfide and hydrogen bonds.

The results obtained (**Figure 2**) showed that *C. parapsilosis* sensu stricto isolates produced proteinases in all tested media. The isolate recovered from onychomycosis

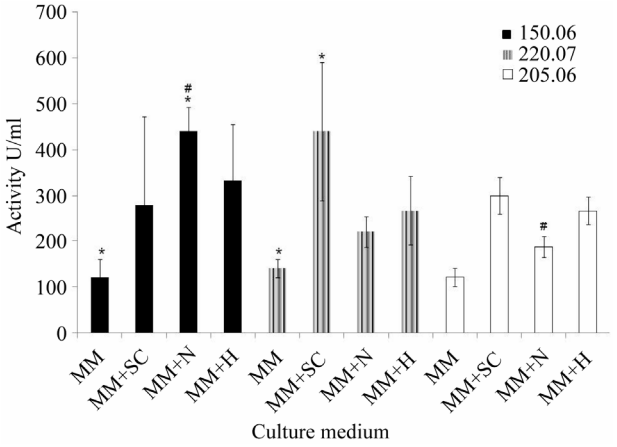

Figure 2. Measurement of secreted acid proteinase activity on haemoglobin in clinical isolates of *Candida parapsilosis* obtained from onychomycosis (150.06), cutaneous candidiasis (220.07) and tracheal secretion (205.06). After growth in basal minimal medium (MM) and MM supplemented with stratum corneum (MM + SC), MM supplemented with hair (M + H) and MM supplemented with nail (MM + N) for 10 d at 37˚C, the cultures were harvested and the spent culture media were then tested to degrade soluble haemoglobin. The proteolytic activity was determined as described in Material and methods and is reported as arbitrary units (U/ml). Standard errors of the means for three measurements are presented as bars. *P < 0.05 for MM + N vs MM (isolate 150.06) and MM + SC vs MM (isolate 220.07). #P < 0.05 for isolate 150.06 vs 205.06 (MM + N medium).

produced higher amount of acid proteinases ($P < 0.05$) in medium supplemented with nail keratin (440 U/ml) than in salt medium (absence of keratin) (120 U/ml). No differences ($P > 0.05$) were observed on proteinase production in the presence of the other two sources of keratin (stratum corneum and hair). For the isolate 220.07 (cutaneous lesion) the production of acid proteinases was higher in medium supplemented with stratum corneum keratin (440 U/ml) than in salt medium (120 U/ml) (**Figure 2**). These data suggest that the source of keratin seems to be correlated to the induction of acid proteinases in an isolate dependent manner.

Differently, no differences were observed in the amount of acid proteinases produced by the isolate recovered from tracheal secretion in the media tested (absence and presence of keratin substrates) (**Figure 2**). Furthermore, when compared proteinase production by *Candida* isolates after growth in the same source of keratin, e.g., in nail keratin medium, the isolate recovered from onychomycosis exhibited higher proteinase activity ($P < 0.05$) than isolate obtained from tracheal secretion, suggesting that the potential of *C. parapsilosis* nail isolate to cause onychomycoses may be associated with acid proteinase production.

It has been reported that the expression of genes en-

coding aspartic proteinases (Saps) varied among different clinical isolates of *C. parapsilosis* complex when grown in contact with human oral epithelium [14]. According to these authors there is a trend relating Sap production and site of isolates recovering.

In *C. albicans*, the most discussed hydrolytic enzymes are secreted aspartic proteinases (Saps), which are one of the well-known virulence factors of this species (reviewed in Schaller *et al*. [24]). According to Monod and Borg-von [25] Saps play a role in fungal adherence and invasion of skin by *C. albicans*. The occurrence of Saps has been previously demonstrated in *C. parapsilosis* isolates obtained from distinct clinical samples [10,26-28] and it has being suggested that Saps are associated in superficial, but not with systemic invasion, caused by *C. parapsilosis* [26]. They observed the induction of Saps from *C. parapsilosis* cultivated in media containing bovine serum albumin (BSA) as a nitrogen source. Considering the clinical point view, it is questionable whether these enzymes could have the required function of digesting keratinised tissue for parasitism. Recently we showed that the production of Saps, in BSA inducing medium, by *C. parapsilosis* isolates obtained from nail and skin was less expressive compared to isolates obtained from blood and tracheal secretion [10]. Although it has been established that *C. parapsilosis* is an opportunistic pathogen related to the skin surface, and is also emerging as an important cause of onychomycosis, as far we know, in the present study we describe for the first time the production of acid proteases by isolates of *C. parapsilosis* recovered from superficial mycoses in the presence of keratinous substrates obtained from human sources. Nevertheless, the properties of the individual proteins that presumably account for the virulence of *C. parapsilosis* have not yet been elucidated to date.

4. Conclusion

In conclusion, ultrastructural investigations of the interface of *C. parapsilosis* and the keratinised substrates from human source reveal important features, which may help to clarify the pathogenesis of superficial candidiasis. Our findings indicate the need for investigation of a possible involvement of acid proteinase in the onychomycosis and cutaneous lesion due to this species.

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