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ENZYMATIC AND HEMOLYTIC ACTIVITIES OF *Candida dubliniensis* STRAINS

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SUMMARY

Candida dubliniensis is an opportunistic yeast that has been recovered from several body sites in many populations; it is most often recovered from the oral cavities of human immunodeficiency virus-infected patients. Although extensive studies on epidemiology and phylogeny of *C. dubliniensis* have been performed, little is known about virulence factors such as exoenzymatic and hemolytic activities. In this study we compared proteinase, hyaluronidase, chondroitin sulphatase and hemolytic activities in 18 *C. dubliniensis* and 30 *C. albicans* strains isolated from AIDS patients. *C. albicans* isolates produced higher amounts of proteinase than *C. dubliniensis* ($p < 0.05$). All the tested *C. dubliniensis* strains expressed hyaluronidase and chondroitin sulphatase activities, but none of them were significantly different from those observed with *C. albicans* ($p > 0.05$). Hemolytic activity was affected by $CaCl_2$; when this component was absent, we did not notice any significant difference between *C. albicans* and *C. dubliniensis* hemolytic activities. On the contrary, when we added 2.5 g% $CaCl_2$, the hemolytic activity was reduced on *C. dubliniensis* and stimulated on *C. albicans* tested strains ($p < 0.05$).

KEYWORDS: *Candida dubliniensis;* Proteinase; Hyaluronidase; Chondroitin sulphatase; Hemolytic activity.

INTRODUCTION

Candida dubliniensis has recently been added up to the growing list of potential opportunistic pathogen yeasts. This species shares many phenotypic characteristics with *C. albicans*, such as production of chlamydospores and germ tube[17]. The pathogenesis of diseases caused by this species is partially known and then, well-studied virulence factors in C. *albicans* must also be assessed in *C. dubliniensis*. In *C. albicans* the widely advocated virulence traits include dimorphism, adherence, enzyme production, rapid phenotypic switch, antigenic variation and other several immunoevasion mechanisms[4]. Regarding enzyme production, hydrolytic enzymes such as proteinase, phospholipase, hyaluronidase and chondroitin-sulphatase are putative virulence factors that help *C. albicans* to invade tissues[4].

Secreted aspartic proteinases (Saps), encoded by the *SAP* gene family, appear to play a major role in *C. albicans* virulence[7]. Seven homologues genes (*SAP*) were also detected in *C. dubliniensis* by Southern analysis[5] but scarce studies were performed focusing proteinase activity in this species[6,10]. Hyaluronidase and chondroitin-sulphatase are considered to be important virulence factors in oral bacteria that cause oral infectious diseases. SHIMIZU *et al.*[15] were the first researchers that described these exoenzymes in *Candida* species; as far as we know, these enzymes have not yet been studied in *C. dubliniensis* isolates.

Hemolytic activity is another virulence factor exhibited by pathogenic microorganisms which permits growth in the host using several iron-binding proteins as a source of iron. Hemoglobin is an important iron-source for pathogenic microorganisms, and the hemolytic activity and the hemoglobin utilization have been considered as a pathogenic factor[8,19]. Studies to evaluate this virulence factor in *C. dubliniensis* have not been carried out yet.

The purpose of the present study was to determine whether there are differences in the expression of proteinase, hyaluronidase, chondroitin sulphatase and hemolytic activity between *C. albicans* and *C. dubliniensis*. The interference of $CaCl_2$ in the hemolytic activity was also evaluated.

MATERIAL AND METHODS

Candida isolates: We have studied eighteen clinical strains of *C. dubliniensis* and thirty of *C. albicans*, both recovered from oral candidiasis of AIDS patients. Phenotypic identification tests of *C. dubliniensis* were confirmed by genotypic methods as randomly amplified polymorphic DNA (RAPD) using the primers CDU (5' GCGATCCCC3')[17] and B-14 (5' GATCAAGTC3')[2]. *C. albicans* isolates were identified by classical methods[11]. All the cultures were maintained at -80 °C as stock collection of Laboratório de Pesquisas Micológicas, Universidade Federal de Santa Maria, Rio Grande do Sul, Brazil.

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Table 1
Production of proteinase, hyaluronidase and chondroitin sulphatase by *C. dubliniensis* and *C. albicans*

Species	Proteinase (Pz)*	Hyaluronidase (Hz)*	Condroitin sulphatase (Cz)*
C. dubliniensis	0.61 - 0.84[a]	0.70 - 0.85[c]	0.55 - 0.81[e]
(n = 18)	mean = 0.75	mean = 0.79	mean = 0.72
C. albicans	0.60 - 0.79[b]	0.80 - 0.86[d]	0.60 - 0.92[f]
(n = 30)	mean = 0.68	mean = 0.81	mean = 0.78

*Value obtained by dividing the diameter of the colony by the total diameter of the colony including precipitation or clear zone. a < b ($p < 0.02$)

Exoenzymes: The production of four functional enzyme categories by *C. dubliniensis* was investigated by employing the culture media and techniques as described in order to check the production of proteinase[14], hyaluronidase and chondroitin sulphatase[15] by *C. albicans*. Plates were incubated and readings were taken after 48 h at 37 °C for proteinase and after four days at 37 °C for hyaluronidase and chondroitin sulphatase[15]. A clear zone around the colonies was considered to indicate proteinase, hyaluronidase and chondroitin sulphatase activities. Enzymatic activities of proteinase (Pz), hyaluronidase (Hz) and chondroitin sulphatase (Cz) were measured by dividing the colony diameter by the clear or precipitation zone plus colony diameter. *C. albicans* CBS 2730, *C. albicans* 2630 and *C. dubliniensis* CBS 7987 were included as control.

Hemolytic activity[8]: *C. dubliniensis* and *C. albicans* strains were streaked onto Sabouraud dextrose agar and incubated at 37 °C for 18 h. The resulting cultures were harvested and washed with sterile saline, and yeast suspensions with an inoculum sized 1 x 10^8 cells/mL were prepared using hemocytometric counts. Ten microliters of this suspension were spot on Sabouraud dextrose agar supplemented with 3% glucose and fresh sheep blood (7%). Plates were incubated at 37 °C in 5% CO_2 for 48 h. The presence of a distinctive translucent halo around the inoculum site indicated positive hemolytic activity. The ratio obtained by dividing the diameter of the colony by the total diameter of the colony plus the translucent halo was used as a hemolytic index (Hi) representing the intensity of the hemolysin production by *C. dubliniensis* and *C. albicans*.

Statistical analysis: enzymatic production and hemolytic activities in the two groups were compared by the Mann-Whitney test.

RESULTS

All analyzed strains showed proteinase, hyaluronidase and chondroitin sulphatase activities.

Proteinase activity of *C. dubliniensis* resulted in Pz range from 0.61 to 0.84 (mean = 0.75) and 0.60 - 0.79 for *C. albicans* (mean = 0.68); these differences were significant ($p < 0.02$) (Table 1).

Hyaluronidase and chondroitin sulphatase activities in *C. dubliniensis* and *C. albicans* are shown in Table 1 and no statistical differences were detected when we compared both species.

In vitro hemolytic activities of *C. dubliniensis*, expressed as hemolysis index (Hi), changed from 0.55 (well-defined hemolysis zone) to 1.0 (absence of hemolytic activity). Only two *C. dubliniensis* isolates showed Hi = 1.0. In *C. albicans*, the Hi changed from 0.5 to 0.85 (Table 2). No significant differences were observed between both species ($p > 0.05$).

Adding $CaCl_2$ 2.5% to Sabouraud glucose agar supplemented with sheep blood, the hemolytic activities declined in *C. dubliniensis* and rose in *C. albicans*. Hi in the *C. albicans* group, was significantly more expressive than that obtained for *C. dubliniensis* ($p < 0.01$). Furthermore, when we compared the *C. albicans* Hi between the results of the two media (with and without $CaCl_2$), we detected hemolytic activity of *C. albicans* was better expressed by $CaCl_2$ addition ($p < 0.01$) (Table 2).

Table 2
Hemolyic activity of *C. dubliniensis* and *C. albicans* considering the effect of $CaCl_2$

Species	Hemolysis index (Hi)*	
	Without $CaCl_2$	With 2.5% $CaCl_2$
C. dubliniensis	0.55 - 1.0	0.78 - 1.0
(n = 18)	mean = 0.71[a]	mean = 0.96[b]
C. albicans	0.5 - 0.85	0.44 - 0.63
(n = 30)	mean = 0.65[c]	mean = 0.51[d]

*Value obtained by dividing the diameter of the colony by the total diameter of the colony plus the translucent halo. d > b ($p < 0.01$). d > c ($p < 0.01$)

DISCUSSION

The most studied virulence factors of *C. dubliniensis* have been hydrophobicity, adhesion and those observed by experimental infections[20].

We noticed that *C. albicans* produced higher amounts of proteinases than *C. dubliniensis*. This finding is divergent from that reported by McCULLOUGH *et al*.[10] which suggested that *C. dubliniensis* produced higher levels of proteinase activity than *C. albicans* reference isolates. When studying phylogeny and putative virulence factors of *C. dubliniensis,* GILFILLAN *et al*.[5] detected seven genes for secretory aspartyl proteinase (SAP), as occurs in *C. albicans*, but they did not confirm the findings of McCULLOUGH *et al*.[10].

Among the 18 *C. dubliniensis* isolates tested all produced hyaluronidase and chondroitin sulphatase, but these activities were not

different from those observed with *C. albicans*. Hyaluronidase and chondroitin sulphatase are involved in bacterial virulence and the substracts of these enzymes are among the major constituents of connective tissue and gingival epithelium[15]. Hyaluronidase and chondroitin sulphatase can affect the permeability of epithelium in the intercellular spaces by attacking the intercellular cementing substances of tissue[18]. Because *C. dubliniensis* has been isolated mainly from mouth, we judged important to evaluate these exoenzymes. As far as we know, hyaluronidase and chondroitin sulphatase activities from *C. dubliniensis* are here reported by the first time.

Since there is essentially no free iron in the human host, most pathogens acquire this indirectly from commonly available iron-containing compounds such as hemoglobin. The enzymes involved in this activity are classified as hemolysins[3]. We have found that onto blood agar medium without $CaCl_2$, the hemolytic activity of *C. dubliniensis* was similar to that of *C. albicans*. However, when $CaCl_2$ 2.5% was added, the hemolytic activity of *C. dubliniensis* decreased while it increased for *C. albicans*. The hemolytic activity of medically important yeasts like genus *Candida* and *Cryptococcus* has been scarcely explored. A complement-mediated hemolysis induced by *C. albicans* was reported by MANNS *et al*.[9] ; LUO *et al*.[8] studying 80 *Candida* isolates representing 14 species reported that *C. albicans* and *C. dubliniensis* among others showed alpha and beta hemolysis; this was the first study to demonstrate the variable expression profiles of hemolysins by different *Candida* species. However, LUO *et al*.[8] have studied only two isolates of *C. dubliniensis*. The $CaCl_2$ has been included in culture media as Calcium donor in the media proposed by PRICE *et al*.[13] and more recently in the media proposed by SLIFKIN[16], named Tween 80 opacity test. In both methods, after enzymatic activity action on distinct substrates, fatty acids are released and the formation of a calcium complex occurs producing a distinct, well-defined, dense white zone of precipitation around the colony. In the Tween 80 opacity test none of the *Candida* species showed a halo response when $CaCl_2$ was omitted from the medium[16]. Based on these facts, we supplemented the sugar-enriched sheep blood agar medium with growing $CaCl_2$ concentrations in order to obtain a better reading of the hemolytic activity. In a previous assay we have observed that $CaCl_2$ concentrations > 2.5 g% were step by step inhibitory for *C. dubliniensis* (data not shown). So, we established $CaCl_2$ 2.5 g% as the more elevated concentration that did not inhibit the growth of *C. dubliniensis* and *C. albicans*. In general, our results showed that hemolytic activity of *C. dubliniensis* was inhibited by $CaCl_2$ 2.5 g% but the same concentration stimulated the hemolytic activity of *C. albicans* (Table 2). This finding is consistent with previous studies relating *C. dubliniensis* strains as more susceptible to physical and chemical agents than *C. albicans* [1,12,17]. On the other hand, it was not possible to apply this finding as a screening test for differentiation between *C. albicans* and *C. dubliniensis*, because the more elevated *C. albicans* hemolytic activity was not absolute and some strains also showed inhibition.

In conclusion, our results suggest that *C. dubliniensis* seems to be less virulent than *C. albicans* because proteinase, the major putative virulence factors, was less expressed or absent. In addition, hyaluronidase, chondroitin sulphatase as well as hemolytic activity are virulence factors less studied and their importance requires new and more rigorous studies.

RESUMO

Atividade enzimática e hemolítica de *Candida dubliniensis*

C. dubliniensis é uma levedura oportunista que, embora já tenha sido isolada de vários sítios anatômicos é, com maior frequência, encontrada na boca de pacientes infectados pelo HIV. Embora tenham sido realizados numerosos estudos sobre a epidemiologia e filogenia, seus fatores de virulência como atividade exoenzimática e atividade hemolítica, são, ainda, pouco conhecidos. Neste estudo comparou-se a atividade *in vitro* de proteinase, hialuronidase, condroitin sulfatase e atividade hemolítica de 18 cultivos de *C. dubliniensis* com 30 cultivos de *C. albicans*, todos isolados de pacientes com SIDA. Foi evidenciada maior atividade de proteinase em *C. albicans* em relação a *C. dubliniensis* ($p < 0,05$). Todos os isolados de *C. dubliniensis* evidenciaram atividade de hialuronidase e condroitin-sulfatase de forma similar ao observado com *C. albicans* ($p > 0,05$). Constatou-se que a atividade hemolítica foi influenciada pelo $CaCl_2$; em sua ausência não foram observadas diferenças na atividade hemolítica das duas espécies; todavia, ao se agregar 2,5% de $CaCl_2$, a atividade hemolítica de *C. dubliniensis* foi reduzida enquanto a de *C. albicans,* estimulada ($p < 0,05$).

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