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SHORT COMMUNICATION/COURTE COMMUNICATION

Characterization of virulence factors of vaginal and anal isolates of *Candida albicans* sequentially obtained from patients with vulvovaginal candidiasis in north-east Brazil

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Summary In order to better understand the pathogenesis of VVC, focusing on the role of *C. albicans* virulence factors in triggering this infirmity; we evaluated four virulence factors of 62 clinical isolates of *C. albicans* sequentially obtained from the vagina and anus of patients with sporadic and recurrent VVC. Virulence factors were phenotypically evaluated in vitro, including: adhesion capacity to epithelial cells obtained from healthy individuals, morphogenesis in the presence of fetal bovine serum, biofilm formation in polystyrene microtiter plates and proteinase activity using bovine serum albumin. Colonizing anal isolates were as able as infecting vaginal isolates to express the virulence factors evaluated in vitro. It was observed an association between the expression of virulence factors studied and the signs and symptoms of VVC presented by the patients. No statistically significant difference was observed in the expression of virulence factors between vaginal isolates of *C. albicans* obtained from patients with sporadic VVC and those obtained from patients with recurrent VVC. Our results suggest that the ability to express virulence factors is important for the pathogenesis of VVC, but it seems not to be crucial for the transition from colonization to infection.

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Introduction

Candida albicans is considered the most virulent species of the genus Candida and presents a set of virulence attributes, which contribute for pathogenicity [1]. This include: the ability to adhere to human epithelial and endothelial cells, yeast to hyphae transition (morphogenesis), production of extracellular hydrolytic enzymes such as proteinases and phospholipases, phenotypic variability (''phenotypic switching'') and the ability to form biofilm in both implanted materials and living tissues [2].

Vulvovaginal candidiasis (VVC) causes great discomfort, impairs sexual relationships and damages work performance, mainly in women that present recurrent VVC (three or more episodes per year) [3,4]. Recently, this infection has been considered a global public health problem, affecting millions of women of all social strata [4]. Candida albicans is still the main etiological agent of VVC, although is remarkable the increased prevalence of non-*C. albicans* Candida species [5].

Endogenous transmission through the gastrointestinal tract has been considered the main source of vaginal yeasts [3]. In a previous work performed by our group, we observed that patients colonized by *C. albicans* in the anus are about five times more likely to develop VVC [6]. Several virulence attributes are proposed in VVC, but they have been poorly investigated.

In this study, we aimed to investigate the virulence attributes of clinical isolates of *C. albicans* sequentially obtained from the anus and the vagina of patients with VVC, investigating a possible correlation with clinical manifestations of the patients. This study will contribute for a better understanding of the pathogenesis of VVC, as well as *C. albicans* natural history during the transition from commensalism to infection.

Materials and methods

Study population and *Candida albicans* isolates

We selected 62 clinical isolates of *C. albicans* stored at the culture collection belonging to the laboratory of medical and molecular mycology, from the Federal University of Rio Grande do Norte. Sampling, culturing procedures and identification of yeastswere performed according to Medeiros et al. [7].

The isolates of *C. albicans* included in the study were obtained from the vagina and anus of 21 patients with clinical symptoms of VVC (vaginal discharge, itching, burning, dysuria, edema, erythema and/or dyspareunia) and no symptoms on the anus or perianal region, aged between 20 and 47 years. Seven patients had sporadic VVC, while 14 women had the recurrent condition, defined as three or more episodes of VVC per year.

Thirty-six isolates were obtained from vaginal secretion (16 from patients with sporadic VVC and 20 from patients with RVVC) while 26 were isolated from the anus of the patients. It is worth mentioning that more than one vaginal and/or anal isolate was sequentially obtained from 10 patients (weekly for patients with sporadic VVC and monthly for patients with RVVC, up to six time points of collection).

Of note, antifungal treatment started after clinical and laboratorial diagnosis of VVC, at the first time point of sample collections (topical administration of clotrimazole or miconaloze vaginal cream during seven days, topical administration of nystatin vaginal cream during 14 days or oral administration of fluconazole 150 mg tablet, single dose).

Virulence factors assays

Cultures recently grown on SDA were used for all virulence assays and yeast inoculum was standardized after growth in NGY medium (0.1% Neopeptone [Difco®], 0.4% glucose and 0.1% yeast extract [Difco®]) [8]. Of note, for biofilm formation assay, YNB medium (Yeast Nitrogen Base; Difco®) supplemented with 50 mol L^{-1} glucose was used to standardize the inoculum. After the overnight incubation of the isolates in the respective media at 30 °C, 200 rpm, the optical density (OD) at 600 nm was measured (Biochrom Libra S32). *C. albicans* ATCC 90028 and SC5314 were used as reference controls.

Candida albicans adherence to human buccal epithelial cells (HBECs)

C. albicans cells grown in NGY were mixed with HBECs from healthy volunteers at a ratio of 10 yeast cells per HBEC. The mixtures were incubated at 37 °C for 1 h with shaking; then cells were vortexed, formalin-fixed and transferred to a microscope slide. The number of *C. albicans* cells adhering to 150 HBEC was determined with the operator blinded to the nature of the material on the slide. Tests were done in triplicate [8].

Candida albicans morphogenesis

An initial amount of 106 *C. albicans* cells/mL in YPD (10 g L^{-1} Yeast Extract, 20 g L^{-1} glucose and 20 g L^{-1} peptone) + 20% FBS (Fetal Bovine Serum, Sigma-Aldrich) was incubated at 37 °C, 200 rpm. After 1- and 3-h incubation, samples of the cultures were formalin-fixed. The sample at 1 h was examined microscopically for determination of the percentage of cells bearing evaginations. The 3-h sample was examined for determination of the morphological index (MI) [8]. Values close to 1 indicate a population of spheroidal yeast cells and values close to 4 indicate a population of true hyphal cells, with values between 2.5 and 3.4 indicating mixed or pseudohyphal morphologies.

Candida albicans biofilm formation

Biofilms were performed according to Meloet al. [9]. At first, aliquots of a standardized *C. albicans* cell suspension (107cells mL^{-1}) were transferred to flat-bottom 96-well microtiter plates and incubated for 1.5 h at 37 °C at 75 rpm, including eight wells as controls. After adhesion phase, cell suspensions were aspirated, the wells were washed with PBS and YNB medium supplemented with 50 mol L^{-1} glucose was added to each well. The plates were incubated for 66 h at 37 °C, 75 rpm, and then biofilm was quantified by the crystal violet assay.

Candida albicans proteinase activity

Proteinase activity was determined by a method of Chaves et al. [8]. Samples were grown in 5 mL YCB + BSA medium (11.7 g L^{-1} Yeast Carbon Base [Difco®]; 10 g L^{-1} glucose; 5 g