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Candida species from oral cavity of HIV-infected children exhibit reduced virulence factors in the HAART era

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ABSTRACT

This study aimed to assess, *in vitro*, the biofilm viability and the phospholipase and protease production of *Candida* spp. from the saliva of HIV infected children and healthy controls, and to correlate the results with the use of medical data. A total of 79 isolates were analyzed: 48 *Candida albicans* isolates (33/15) and 20 *Candida parapsilosis sensu lato complex* isolates (12/8) (from HIV/control patients, respectively), and 8 *Candida krusei*, 1 *Candida tropicalis*, 1 *Candida dubliniensis* and 1 *Candida guilliermondii* from HIV patients. The XTT (2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-Carboxanilide) reduction assay analyzed the biofilm viability. Phospholipase and protease assays were performed using the egg yolk and Bovine Serum Albumin agar plate methods, respectively. All isolates were able to form biofilm with cell viability. Quantitatively, *Candida* isolates from both groups presented a similar ability to form biofilm ($p > 0.05$). The biofilm viability activity was higher in *C. albicans* isolates than in non-*albicans Candida* isolates ($p < 0.05$) for both groups. Phospholipase activity was detected in 32 isolates (40.5%) and it was significantly higher in the HIV group ($p = 0.006$). Protease activity was detected in 66 isolates (84.8%) and most of them were relatively/very strong producers. No statistical association with medical data was found in the HIV group. Although *Candida* spp. isolates from HIV-positive children presented higher phospholipase production, *in vitro* they exhibited reduced virulence factors compared to isolates from healthy individuals. This finding may enlighten the role played by immunosuppression in the modulation of *Candida* virulence attributes.

1. Introduction

Candida spp. are fungi that are usually present in the oral cavity [1,2] of healthy individuals [3] and *Candida albicans* is the most prevalent specie [2]. *C. tropicalis*, *C. glabrata*, *C. dubliniensis* and *C. krusei* are also important agents that may lead superficial infections to disseminated infections [3,4] and the complex *C. parapsilosis sensu lato* has been associated to nosocomial infections [5]. In immunocompromised individuals, such as those infected with HIV, these microorganisms can lead to infections affecting the oral cavity (oral candidiasis), oropharynx, esophagus [6] and can be disseminate to the bloodstream [4]. The transition from commensal to opportunistic pathogen is supported by a variety of mechanisms to overcome host defense mechanisms, such as the adhesion to host cells, biofilm formation, phenotypic switching and the production of extracellular hydrolytic enzymes such as phospholipase and protease. These attributes are called virulence factors [4,6,7].

When organized in biofilms, the cells are, in general, embedded in a self-produced extracellular matrix containing proteins, carbohydrates, phosphorus and hexosamines [8]. This biofilm provides protection against drugs and microorganisms; removes harmful metabolic products; favors the acquisition and processing of nutrients [9]; and enables communication through the secretion of signaling molecules in a delimited population - Quorum sensing [10]. Phospholipases are a group of enzymes that are involved in

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cell maintenance, membrane remodeling and triggering of the inflammatory process in the arachidonic acid cascade [11]. Moreover, *Candida* spp. presents the ability to produce and secret proteases in order to acquire nutrients. However, this skill is also important during the development of the infectious process, promoting the degradation of host proteins [12].

Although the prevalence of oral candidiasis decreased in HIV-infected children after the introduction of the High Active Antiretroviral Therapy (HAART), oral colonization by *Candida* spp. still remains high when compared to healthy patients [2,13]. Additionally, when present in HIV patients, the oral lesions tend to be more resistant to antifungal therapy and more invasive [14]. Therefore a better understanding of the pathogenesis of this infection is necessary in order to choose the best form of treatment and prevention. The aim of this study was to analyze some virulence factors such as the metabolic activity of biofilm formation, the phospholipase and protease production of *Candida* spp. isolated from the oral cavity of HIV-positive children compared with healthy children from Rio de Janeiro, Brazil.

2. Materials and methods

This cross-sectional, clinical and laboratorial, blinded and controlled study was approved by the Ethics Committee (CEP/IPPMG-RJ 63/11) and informed consent was obtained for each child from their legal guardians.

2.1. Clinical isolates

A total of 79 *Candida* spp. isolates from 60 pediatric individuals (43 HIV infected children and 17 healthy children) previously obtained by ALVES et al. [15] were assessed in this study. Both groups attended the Pediatric Dentistry Department at the Universidade Federal do Rio de Janeiro (UFRJ), Brazil. The recruitment criteria, saliva collection and the distribution and identification of *Candida* species were previously published elsewhere [15]. In brief, children who were under antifungal therapy in the previous three months or who were using oropharyngeal antimicrobial drugs or children with a salivary flow less than 0.5 ml/min were excluded from this study. Medical information of the HIV group regarding antiretroviral therapy (HAART) and immunosuppression status (AIDS diagnosis, CD4 count and viral load) were collected from their medical records and are shown in Table 1. Data about the presence and/or history of oral candidiasis was also recorded.

The children were requested not to eat anything or brush their teeth for a minimum of one hour before the whole saliva samples were collected between 1:00–2:00 p.m. After that, an oral exam was performed. The salivary samples were cultured (CHROMagar Candida® - PROBAC, São Paulo, Brazil) and the *Candida* spp. colonies counted and then identified by sugar assimilation and fermentation (API 20C system® - BioMeriéux, Marcy l'Etoile, France). From the 79 *Candida* spp. isolates, 56 were from the HIV group (33 *C. albicans,* 12 *C. parapsilosis,* 8 *C. krusei*, 1 *C. tropicalis*, 1 *C. dubliniensis* and 1 *C. guilliermondii*) and 23 from the control group (15 *C. albicans and* 8 *C. parapsilosis*). Since molecular methods were not used to identify the *C. parapsilosis sensu lato* complex species (*C. orthopsilosis*, *C. metapsilosis* and *C. parapsilosis sensu strictu*), they were referred to in this study as *Candida parapsilosis* [16].

2.2. Biofilm formation and metabolic assay

The biofilm formation was performed according to the methodology described by Thein et al. [17]. The biofilm was measured by the cell metabolic assay using the XTT reduction reaction [18].

Briefly, the microorganisms were grown in Brain Heart Infusion (BHI - BD Difco™, Maryland, USA) for 48 h at 37 °C under agitation and suspensions of each strain were prepared using Yeast Nitrogen Base medium (YNB - BD Difco™, Maryland, USA) with 100 mM glucose. Each strain had 100 μl of cell suspension (10^7 cell/ml) added to each well in 96 wells plate in triplicate. The system was kept under agitation (75 rpm) at 37 °C for 1½ hours. After this time, the supernatant was removed and washed twice with Phosphate Buffer Solution (PBS – pH 7.2) in order to remove the loosely adherent cells. Then 200 μl of fresh YNB was added to each well and incubated at 37 °C for 48 h without agitation. The biofilm metabolic assay (biofilm viability) was determined by the addition of XTT (2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-Carboxanilide - Invitrogen™, Life Technologies, California, USA) with menadione (1:20 proportion) in each well. The tetrazolium salt was incubated for 3 h at 37 °C. After this process, 100 μl of the supernatant was collected, stored in a new plate and read in a spectrophotometer at 492 nm. The values from the XTT reduction technique were categorized into weak, moderate and strong producers, according to Sanchéz-Vargas et al. [18].

Table 1
Medical and personal data from HIV and Control Groups.

Personal Data	HIV group (n = 43)	Control group (n = 17)
Gender	Male - 20 (46.52%)	Male - 9 (52.95%)
	Female - 23 (53.48%)	Female - 8 (47.05%)
Age (years)	9.8 (±2.76)	8.9 (±2.40)
History of Oral Manifestation		
Oral Candidiasis	23 (53.48%)	3 (17.64%)
Gingival Erythema Linear	12 (27.90%)	3 (17.64%)
Simplex Herpes	12 (27.90%)	–
Parotid Hypertrophy	13 (30.23%)	–
Medical Data	HIV Group (n = 43)	
HAART use[a]	Yes - 31 (72.09%)	
	No - 12 (27.91%)	
Immunological classification[b]	1–26 (60%)	
	2–15 (35%)	
	3 - 2 (5%)	
AIDS[c]	Yes - 2 (5%)	
	No - 41 (95%)	
Viral Load[d]	Detectable - 12 (28%)	
	Undetectable - 31 (72%)	

[a] Note: Use of three or more antiretroviral drugs (CDC, 1994).
[b] 1994 revised classification system for human Immunodeficiency Virus Infection in children less than 13 years old (CDC): 1-absence of immunosuppression (CD4% > 25); 2-moderate immunosuppression (CD4% = 15–24); 3- severe immunosuppression (CD4% < 15).
[c] CD4 < 15%.
[d] Viral Load detectable when <400 copies/ml.

2.3. Confocal laser scanning microscopy (CLSM)

One representative isolate of each *Candida* species from the HIV group was selected to undergo CLSM for better visualization of its biofilm morphology and architecture. The selection criteria was the need to be a strong biofilm producer [18]. Isolates were prepared according to the biofilm formation assay steps previously described and were incubated for 48 h. The staining process was performed as described by Chandra et al. [19]. The dye mix was composed of the fluorescent stains Concanavalin A, Alexa Fluor 488 conjugate conA and FUN-1. FUN-1 is converted to orange-red by metabolically active cells while Alexa Fluor-ConA turns into fluorescent green which binds to mannose and glucose residues of cell wall and extracellular matrix polysaccharides. Images were captured by the confocal microscope Leica TCS SP5 software LAS-AF 4.1.2 and processed using Fiji ImageJ 1.48s (National Institute of Health, USA).