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Candida species biotypes in the oral cavity of infants and children with orofacial clefts under surgical rehabilitation

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ABSTRACT

Patients with orofacial clefts present various risk factors for oral infectious diseases, resulting from anatomical and physiological changes and those resulting from rehabilitating therapeutic interventions. The incidence of *Candida* species in groups of babies and children with orofacial clefts, during pre- and post-operative periods and until return to first consultation, and the profiles for antifungal sensitivity and virulence *in vitro* were investigated. Oral samples were collected at different times over the surgical procedures and post-surgical clinical consultation and seeded in chromogenic culture media CHROMagar Candida®. *Candida* biotypes were identified by accessing species-specific genomic DNA sequences by PCR techniques and electrophoretic procedures. Antifungal susceptibility testing was performed by the method of microdilution in broth using the antifungals amphotericin B (AP), nystatin (NYS) and fluconazole (FLC). SAP and PL exoenzyme activities were determined by classical microbiological methods. Some orofacial clefts occurred preferentially in male or female. Low incidence (39.1%) of oral colonization by *Candida* species (*C. albicans*, *C. krusei*, *C. tropicalis* and *Candida* spp.) was reported in patient admission to surgical ward, with no correlation to orofacial cleft types or surgical history. Significant reduction in frequencies of *Candida* and changes of species, over sampling periods, showed dynamic patterns of oral colonization: elimination, maintenance or neocolonization of the biotypes. These biotypes showed sensitivity to AP (100%), partial resistance to FLC (< 10%) and variable MICs for NYS (0.125–4 μg/mL), in addition to strong exoenzyme activities, especially for SAP. Clinical and therapeutic conducts for surgical rehabilitation, anatomical and physiological characteristics of patients with orofacial clefts, and cultural behavior and regionalism of the patient population served could influence the frequencies and dynamics of oral colonization by *Candida* species. The data showed *Candida* biotypes resistant to FLC and sensitive (AP) or clinically compatible (NYS) to polyenes, especially *C. albicans*, in the oral cavity of patients predisposed to oral colonization and candidiases, contributing to clinical conducts in possible antifungal therapies. These biotypes were considered potentially virulent and able to partially modulate their virulence factors, especially SAP, under the conditions favored by host.

1. Introduction

Congenital orofacial malformations affect the structure and functions of the oral cavity, significantly modifying its characteristics. As a result, such malformations can have influence on the microbiota of the environment. Orofacial clefts are congenital malformations of the middle third of the face that present varying degrees of severity. They are the most common congenital developmental malformations of the oral cavity. This condition adversely affects natural suction or impairs the ability to swallow food. The treatment of these patients is a process that begins at birth and continues into adulthood, involving a multidisciplinary team in order to promote the rehabilitation of the patient. The etiology of these malformations is a controversial topic, possibly multifactorial, on which genetic and environmental factors can act in

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isolation or in association [1].

Patients with orofacial clefts present various risk factors for oral infectious diseases, resulting from anatomical changes of the maxillary segments, generated by deficient fusion of the facial embryonic processes. This fact is related to poor dental positioning, nasal septum deviation or nostril stenosis, which leads to mouth breathing. Other changes include those resulting from rehabilitating therapeutic interventions, such as scar fibrosis resulting from surgical repair, the use of orthodontic appliances and/or dental prostheses, which modify the ecological environment of the oral microbiota in patients with cleft lip and palate, and may encourage the colonization of the oral cavity by pathogenic microorganisms or make pathogenic the commensal members of this microbiota, including among these the *Candida* species [2].

Candida is commonly present in the normal oral microbiota of healthy individuals. Its presence is estimated in 45–65% of healthy babies and in 30–55% of healthy adults. In humans, the most common *Candida* species found in the oral cavity is *C. albicans* due to its adhesion properties and greater degree of pathogenicity. *C. albicans* is a dimorphic yeast, which can exist both in forms of yeasts and of hyphae, depending on the environment. *C. albicans* has been isolated in more than 80% of oral lesions. Other species that are incident to oral infections include *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. kefyr*, *C. parapsilosis*, *C. stellatoidea* and *C. tropicalis* [3].

A variety of systemic and local factors can cause overgrowth of *Candida* species in buccal mucosa, leading commensal *Candida* species to become pathogenic, making oral candidiasis (OC) an important oral dermatologic disease. The factors include the use of dentures, corticosteroid and xerostomia inhalers, while systemic factors include immunosuppressed states, such as the human immunodeficiency virus (HIV), leukemia, malnutrition, impaired immunity related to aging, endocrine dysfunction such as diabetes, anatomical changes, chemotherapy, radiation therapy, and the use of systemic corticosteroids, immunomodulatory drugs, xerogenic drugs and broad-spectrum antimicrobials [3].

The transition from amphibiont to pathogenic form in *Candida* spp. has been attributed to the selective expression of various virulence factors, which act synergistically, under favorable predisposing conditions [4]. Thus, the type, stage and site of the infection, as well as the nature of individual immune response, cause the yeast to express one or more virulence factors [5]. Among the virulence factors, extracellular enzyme activity, proteolytic or lipolytic, plays important role in the pathogenicity of *C. albicans* [6,7]. Lipolytic enzymes have an active role in the invasion of lesions in the host tissue, since these enzymes cause rupture of the epithelial cell membrane and allow fungi cell to penetrate the cytoplasm. While proteolytic enzymes induce the degradation of a wide variety of host proteins, facilitating fungi penetration into the tissues [8].

There are several classes of compounds that constitute the arsenal against numerous *Candida* infections. Polyenes, azoles, echinocandins and alilamins are used, varying according to the type of infection and the sensitivity of the *Candida* species involved [9]. The antifungal prescription most often employed against infections caused by *C. albicans* has been fluconazole, a member of the class of azoles. However, cases of infections by fluconazole-resistant *Candida* species have also been reported. Clinical isolates of *C. albicans* from candidemic patients have the lowest incidence of resistance to azoles (0–5%) among the Candida species [9]. Azoles are fungistatic against *Candida* spp. and act by binding and inhibiting the intracellular target enzyme ERG11p, involved in ergosterol biosynthesis. More than 140 alterations were described in target gene ERG11, some of which were found exclusively in azole-resistant isolates, while others were also found in sensitive isolates. Moreover, efflux pumps contribute significantly to azole resistance in *Candida* spp [10].

Various systemic and topical agents are currently available for the treatment of oral candidiasis. Systemic antifungal agents, including fluconazole and itraconazole, are suitable for patients who do not respond to or are intolerant to topical treatment and who have high risk of developing systemic infections. However, numerous drug interactions and the decrease in sensitivity limit the application of systemic antifungal agents. Topical antifungal agents, such as nystatin and amphotericin B, are typically recommended for first-line treatment for cases of oral candidiasis [11]. Amphotericin B has fungicidal activity through its binding to ergosterol, present in the fungal cell membrane. This association results in the formation of pores in the membrane and the loss of intracellular compounds and cell death. *Candida* spp. mutation mechanisms resulting in reduced binding between ergosterol and amphotericin B establish antifungal resistance. However, the mechanisms that establish resistance to amphotericin B and nystatin are considered rare [10]. Therefore, the detection of pathogenic fungal strains resistant to antifungal therapy in patients predisposed to colonization or infection processes, such as patients with orofacial clefts, is essential for effective treatment, clinical health and welfare of patients [12].

Based on data from the available literature and aiming to contribute to studies on the epidemiology of *Candida* species and their intrinsic characteristics of pathogenicity, this research investigated (*i*) the incidence oral clinical *Candida* species from babies and children with orofacial clefts, before, during and after surgical rehabilitation procedures, and their possible epidemiological and clinical correlations, (*ii*) the characteristics of virulence *in vitro* of *Candida* species, especially the hydrolytic enzymes secreted aspartyl proteinases (SAP) and phospholipases (PL), and (*iii*) the antifungal sensitivity and resistance patterns; the minimum inhibitory concentration (MIC) *in vitro* of the antifungal agents amphotericin B, nystatin and fluconazole.

2. Material and methods

2.1. Subjects

The study involved 46 patients, aged between 0 and < 12 years, both male and female, with orofacial clefts, indicated for surgical rehabilitation, under medical and dental follow-up in the Clinics of the School of Dentistry of the José do Rosário Vellano University (UNIFENAS) — Centro Pró-Sorriso aos Portadores de Fissuras Labial e Palatina —, of the municipality of Alfenas, state of Minas Gerais, Brazil. Patients showing different types of orofacial clefts [13] with and without surgical history were subdivided into two main groups: Babies ($n = 27$; mean of 11.2 ± 6.6 months of age) and children ($n = 19$; mean of 7.2 ± 3.2 years of age) (Fig. 1 and Table 1). This research was conducted in accordance with Resolution No. 466/2012 of the National Health Council and approved by the Research Ethics Committee of the FOP/UNICAMP (Protocol No. 093/2014).

2.2. Sampling

Microbiological samples were obtained using the method described previously by Samaranayake et al. (1986) [14], with some adaptations. For each patient with orofacial cleft, the samples were collected (pre-surgery: orofacial clefts; post-surgery: oral cavity rehabilitated surgically) using a sterile swab, in the presence of a physician, and maintained in 50 mL polypropylene tubes containing 10 mL of sterile PBS solution (100 mM NaCl, 100 mM NaH_2PO_4, pH 7.2). Then, these samples were properly transported (4 °C) to the Laboratory of Oral Microbiology and Immunology, School of Dentistry of Piracicaba, State University of Campinas (FOP/UNICAMP). These tubes were centrifuged at 1700 × g for 10 min, the supernatant was discarded and the sediments were resuspended in 1 mL of sterile PBS solution (concentrated sample 10 ×). Soon after, the sediments were transferred to 2 mL microtubes and shaken in vortex for 0.5 min [14,15]. Then, 100 µL aliquots of each sample were inoculated on plates containing CHROMagar Candida® (1.5% agar, 1.02% peptone, 2.2%, chromogenic mixture, 0.5% chloramphenicol) [16,17] and aerobically incubated at 37 °C for 48 h [18,19]. For each patient with orofacial cleft microbiological

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