PHENOTYPIC ASPECTS OF ORAL STRAINS OF *Candida albicans* IN CHILDREN WITH DOWN'S SYNDROME

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ABSTRACT

The aim of this article is to characterize the biological aspects of oral strains of *C. albicans* in children with Down's syndrome. These yeasts were analyzed as to their macromorphological and enzymatic aspects and were tested as to their *in vitro* susceptibility to antifungal drugs using broth microdilution to determine the minimum inhibitory concentration (MIC). The morphotyping revealed that all oral *C. albicans* isolates from children with Down's syndrome promoted the formation of fringes regardless of size, while the control group presented smaller fringes. All oral *C. albicans* strains produced proteinase, but those with phospholipolytic activity showed greater enzyme capacity in the test group. *In vitro* susceptibility showed that all oral *C. albicans* isolates were sensitive to the drugs used.

Keywords: Phenotyping, *Candida albicans,* Down's syndrome.

RESUMO

Aspectos fenotípicos de cepas de *Candida albicans* orais em crianças com síndrome de down

O objetivo deste artigo foi caracterizar os aspectos biológicos de cepas de *C. albicans* orais em crianças com síndrome de Down. Estas leveduras foram analisadas quanto aos seus aspectos macromorfológicos e enzimáticos e foram testadas quanto a sua suscetibilidade *in vitro* a drogas antifúngicas, usando a microdiluição em caldo para a determinação da concentração inibitória mínima (CIM). A morfotipagem revelou que todos os isolados de *C. albicans* orais de crianças com síndrome de Down induziram à formação de franjas independente do tamanho, enquanto o grupo controle teve franjas menores. Todas as cepas de *C. albicans* orais produziram proteinase, mas aquelas com atividade fosfolipidolítica mostraram maior capacidade enzimática no grupo teste. A suscetibilidade *in vitro* mostrou que todos os isolados de *C. albicans* orais foram sensíveis a drogas empregadas.

Palavras-chave: fenotipagem*, Candida albicans,* síndrome de Down.

INTRODUCTION

The mouth is one of the most diversified sources of microorganisms, where yeasts of the genus *Candida* play a relevant role due to their presence from the initial development of the oral microbiota to the definitive one (Marcantoni, 1999). In children with Down's syndrome, pathoanatomical disorders predispose them to the proliferation of *Candida* strains, completely populating the floor of the mouth with this yeast-like fungus, which often causes candidiasis (Campos, 2001).

The onset of the infectious process by *Candida* in the mouth of children with Down's syndrome, in addition to abnormal innate and acquired

immunological conditions, is also influenced by the pathogenic capacity of these yeasts, initially acting as colonizers and later on as infectious agents due to the alteration of the oral microbiota produced by microbiological, chemical and physical factors, such as chewing and/or poor mouth cleaning (Campos, 2001; Ribeiro *et al.*, 2002).

The methods for typing *Candida* cultures have been used to better characterize the biological aspects presented by these yeasts. Therefore, the phenotypic characterization of yeast-like fungi enables us to assess macroscopically how these microorganisms behave as a constituent part of the topical microbiota and/or as the triggering mechanism of candidiasis regarding morphological, serological, biological, and antifungal parameters (Prince *et al.*, 1982; Ghannoum & Abu-Elteen, 1990; Odds, 1998; Cândido *et al.*, 2000; Pinto, 2003).

The aim of this article is to establish the phenotypic profile of oral *Candida* yeasts in children with Down's syndrome given the morphotyping of fringes and the fungal topography of colonies, production of exoenzymes (proteinase and phospholipase) and antifungaltyping.

MATERIAL AND METHODS

Strains of C. albicans

The 35 strains of *C. albicans* used in this work were due to a case-control study involving oral yeasts of *Candida* isolated from children with and without trisomy of the 21st chromosome. There were twenty five (25/30) (83.3%) oral strains of *C. albicans* from children with Down's syndrome and ten (10/60) (16.7%) from the control group (children without the syndrome) treated at the Pediatric Dental Clinic of the School of Dentistry at the Universidade Federal de Goiás (CO/FO/UFG) in Goiânia, state of Goiás, Brazil. The yeasts used in this study were isolated and identified according to Kreegen-van Rij (1984). These strains of *C. albicans* were obtained from individuals between 0 and 10 years old with an intact oral mucosa and who were not on any type of drug therapy. The present study was previously accepted by the Committee of Ethics in the Human Medical Investigation and Animal of the Clinics Hospital at the Universidade Federal de Goiás (HC/UFG) and the parents or guardians of the children gave their consent to the investigators.

Phenotyping

Morphological, enzyme and antifungal phenotyping used in the present study aims at establishing an epidemiological correlation between the *Candida* strains analyzed herein.

Morphotyping

A phenotypic marker was detected in malt extract agar with the seeding of two strains of *Candida* (inoculum with turbidity adjusted in 3 on Mc Farland scale) on each Petri dish with sterilized swabs. After the incubation of the dishes at 25 °C for 10 days, the reading was based on the macromorphological aspects of the fringes and on the topography of the colony by way of the model proposed by Hunter *et al.* (1989) (Table 1).

Enzymetyping

The production of extracellular enzymes by *Candida* species was detected for proteinase in a basic medium enriched with albumin and vitamins (Rüchel *et al.*, 1982) and for phospholipase in Sabouraud dextrose agar (SDA) and egg yolk (Prince *et al.*, 1982). After incubation in a greenhouse at 37 °C for 48 h for proteinase and for 96 h for phospholipase, the reading of test isolates and of the standard strain (*Candida albicans* CBS 562) granted by the mycological collection of the Institute of Biomedical Sciences at the Universidade de São Paulo (ICB/USP), Brazil was performed based on the production of the precipitation zone around the inoculation point of *Candida* isolates. The enzyme activity (P_z) resulted from the relation between the diameter of the colony (dc) and the diameter of the colony and precipitation zone (dcp). The results were classified as negative (Pz = 1 cm), positive (0.64 cm $\geq$ Pz $<$ 1 cm) and strongly positive (Pz $<$ 0.64 cm).

Antifungaltyping

The *in vitro* susceptibility test was carried out by broth microdilution, standardized by the *National Committee for Clinical Laboratory Standards* (NCCLS) (Atlanta, USA) to determine the minimum inhibitory concentration (MIC). Amphotericin B and 5–fluorocytosine were obtained from Sigma Chemical Company. Fluconazole and itraconazole were used in their commercial formulation available in Brazil: 150 mg and 100 mg respectively. To prepare

TABLE 1
Model proposed by Hunter *et al.* (1989).

Order of the digits		Values
1º Fringe - Distribution	Absent	0
	Discontinuous (≤ 20% of margin)	1
	Discontinuous (20 a 50% of margin)	2
	Discontinuous (60 a 90% of margin)	3
	Continuous at periphery only, or strands conspicuously fan-shaped	5
	Continuous: filamentous outgrowths uniformly parallel	7
2º Fringe - Width	Absent	0
	2 mm or less	2
	3-5 mm	3
	Greater than 6 mm	5
3º Fringe - Texture	Absent	0
	Very coarse	1
	Coarse	2
	Intermediate	3
	Fine	4
4º Topographical aspect of colony	Smooth	0
	Nodular	1
	Pitted	2
	Crateriform	4
	Crateriform plus wrinkles or folds	5
	Wrinkles or folds	6
	Hairy	8

the standard solution, 5 mg of each drug were weighed, yielding concentrations of 640 µg/mL and 1280 µg/mL for amphotericin B and itraconazole, respectively, which were solubilized in dimethyl sulfoxide (DMSO) at the concentration of 1% of the volume of the standard solution. Both water-soluble fluconazole and 5–fluorocytosine yielded concentrations of 1.250 µg/mL and 1.000 µg/mL, respectively. From these solutions, serial dilutions of the drugs were prepared in MOPS (morpholinepropanesulfonic acid)-buffered RPMI-1640 (pH 7.0) in order to obtain the desired concentrations of antifungals. The following concentrations were used: 0.02 at 16.0 µg/mL for amphotericin B, 0.012 at 12.5 µg/mL for 5-fluorocytosine, 0.04 at 32.0 µg/mL for itraconazole and 0.08 at 64.0 µg/mL for fluconazole.

The inoculum of *Candida* strains was grown in Sabouraud dextrose agar (SDA) for 48 h at 35 °C. This solution was prepared in 5 mL of deionized and sterilized water and the cellular density was adjusted on a spectrophotometer for an 85% transmittance reading in the 530 nm wavelength, resulting in a solution with one inoculum between 0.5×10^2 and 2.5×10^3 CFU/mL (colony forming units per milliliter).

Covered acrylic dishes were used for microdilution, with 96 wells (Difco, Detroit, USA). Each well received 100 µg/mL of the antifungal drug concentration and 100 µg/mL of the test suspension of *Candida* strain. The dishes were incubated at 37 °C for 48 h. The MIC for azole drugs was defined as the first well with a 50% reduction in yeast cell growth, comparatively to the positive control (drug-free medium). For amphotericin B and 5-fluorocytosine, the MIC was defined as the lowest concentration able to inhibit any visible growth.

RESULTS

Morphotyping

The morphotyping of 35 *C. albicans* strains from the oral mucosa of children with and without Down's syndrome enabled us to detect nine different morphotypes; the test group (children with Down's syndrome) showed a predominance of morphotype 5530 (colony with continuous fanlike fringes, equal to or greater than 6 mm, with

an intermediate texture and smooth topographical aspect). In the group of children without Down's syndrome, a higher incidence of morphotype 5240 (colony with continuous fanlike fringes, equal to or greater than 2 mm, fine texture and smooth topographical aspect) was observed (Table 2).

Enzymetyping

Proteinase production was detected in all oral strains of *C. albicans* obtained from the mouth of children with and without Down's syndrome, whereas the test group (children with Down's syndrome) was highly proteolytic. As for phospholipase, 88.0% (22/25) of oral *C. albicans* strains in the group of children with Down's syndrome produced this exoenzyme in remarkable amounts, while in the group of children without the syndrome, only 80.0% (8/10) of the oral strains showed enzymatic capacity (Table 3).

Antifungaltyping

All oral strains of *C. albicans* in the test and control groups (children with and without Down's syndrome) revealed *in vitro* sensitivity to the antifungal drugs used in broth microdilution (Table 4).

DISCUSSION

A deeper understanding concerning the phenotypic behavior of *Candida* cultures analysing each sample, either as colonizer and/or pathogen, enables us to evaluate the biological diversity of existing strains and how such characteristics express themselves in the sequencing of the fungal process. Yeast-like aspects of *Candida* observed in genotypic characteristics through molecular biology by way of one-dimensional electrophoretic karyotyping and pulsed field electrophoresis with DNA probes, polymerase chain reaction (PCR), or also the combination of these techniques enables us to differentiate phenotypically undistinguishable strains, finding the genetic mapping sequence of these yeasts and showing the phenotypic aspect of each gene of the fungus (Fukazawa *et al*., 1997; Radford *et al.*, 1997; López-Ribot *et al.,* 2000; Melo *et al*., 2003).

The morphological aspect of *C. albicans* colonies showed that the predominance of a smooth surface does not depend on the analyzed group (children with and without Down's syndrome). As for the disposition of the colonies in fringes, 84.0% (21/25) of the strains isolated from the oral mucosa of children with Down's syndrome revealed fringes throughout the periphery of the fungal colony, whose length was greater than or equal to 6 mm in 48.0% (12/25) of the strains, and rough structure in 68.0% (17/25). Concerning the control group (children without the syndrome), fringes were present in 80.0% (8/10) of *C. albicans* strains on the entire colony surface, but with a length shorter than 5 mm in all strains and rough texture in approximately 60.0% (6/10) of the fringes. This phenotypic aspect thus showed that the adherence capacity of oral strains of *C. albicans* in the test

TABLE 2
Morphotypes observed in *C. albicans* strains isolated from the mouth of children with and without Down's syndrome, treated at the Pediatric Dental Clinic of the School of Dentistry of Universidade Federal de Goiás, Goiânia (GO)/Brazil, 2005.

Morphotypes	Children with Down's syndrome n (%)	Children without Down's syndrome n (%)
2,330	-	02 (20.00)
3,240	02 (8.00)	-
3,340	02 (8.00)	-
5,240	-	04 (40.00)
5,320	02 (8.00)	02 (20.00)
5,330	05 (20.00)	02 (20.00)
5,340	02 (8.00)	-
5,530	10 (40.00)	-
5,540	02 (8.00)	-
Total	25 (100.00)	10 (100.00)

Model proposed by Hunter *et al.* (1989).

TABLE 3
Production of proteinase and phospholipase by oral strains of *C. albicans* in children with and without Down's syndrome, treated at the Pediatric Dental Clinic of the School of Dentistry of Universidade Federal de Goiás, Goiânia (GO)/Brazil, 2005.

	Proteinase*			Phospholipase*		
Children	**Pz = 1 (%)**	**(0.64 ≥ Pz < 1)**	**(Pz < 0.64)**	**Pz = 1 (%)**	**(0.64 ≥ Pz < 1)**	**(Pz < 0.64)**
w/ Down's syndrome	-	01 (4.0)	24 (96.0)	01 (4.0)	02 (8.0)	22 (88.0)
w/o Down's syndrome	-	06 (60.0)	04 (40.0)	02 (20.0)	-	08 (80.0)
Total	-	07 (20.0)	28 (80.0)	03 (8.6)	02 (5.7)	30 (85.7)

*(Pz = 1 cm) negative;
(0.64 cm ≥ Pz < 1 cm) positive; and
(Pz < 0.64 cm) strongly positive.

TABLE 4
Minimum inhibitory concentration (MIC) of antifungals in relation to the *C. albicans* strains isolated from the mouth of children treated at the Pediatric Dental Clinic of the School of Dentistry of Universidade Federal de Goiás, Brazil, 2005.

Antifungals MIC	**Children w/ Down's syndrome**		**Children w/o Down's syndrome**	
	MIC threshold (μg/mL)	**CI50 (μg/mL)**	**MIC threshold (μg/mL)**	**CI50 (μg/mL)**
Amphotericin B	0.08 - 1.00	0.25	0.04 - 1.00	0.25
Fluconazole	0.25 - 4.00	1.00	0.50 - 4.00	2.00
Itraconazole	0.08 - 1.00	0.50	0.04 - 1.00	0.50
5-fluorocytosine	0.024 - 1.56	0.39	0.049 - 1.56	0.39

MIC – minimum inhibitory concentration; and
CI_{50} – concentration of the drug able to inhibit the growth of *C. albicans* strains by 50%.

group (children with Down's syndrome) is more exacerbated *in vitro* than in the control group, although all these yeasts are of colonizing type and originate from the oral mucosa of children with or without this chromosome disorder. Therefore, the presence of fringes in yeasts of *Candida* obtained from the mouth is possibly attributed to the virulence of this fungus, thus showing a higher tendency towards the development of the mycelial form by the yeast and favoring the adaptation to the oral cavity (Melhem, 1997; Radford *et al*., 1997; Melo *et al*., 2003). Patients with neoplasms or with AIDS have shown oral morphotypes of *C. albicans* with fringes more often than those obtained from the mouth of healthy individuals, which reinforces its association with yeast virulence (Melhem, 1997).

The production of extracellular enzymes by *Candida* yeasts is another capacity that this fungus has to establish itself as a colonizing and/or infectious microorganism in man. Proteinase favors the adherence of *Candida* isolates to the surface of infected mucosa due to the penetration of germ tubes into the infected tissue (Odds, 1998). However, phospholipase induces the control of fungal growth by its presence in the extremities and destruction of lipids that constitute the cell membrane (Ribeiro *et al.*, 2002). Approximately 96.0% (24/25) of oral strains of *C. albicans* from children with Down's syndrome were highly proteolytic, produced phospholipase and were highly phospholipidolytic, while 80.0% (8/10) of the yeasts in the control group (children with Down's syndrome) showed to produce both enzymes. Nevertheless, the individual analysis of the productive enzyme capacity of each strain of *C. albicans* revealed that strains from the mouth of children with Down's syndrome showed enhanced production. Cândido *et al.* (2000) studied 79 *Candida* strains isolated from the mouth of patients with lesions characteristic of candidiasis and from individuals with clinically normal oral cavity treated at the School of Dentistry in Ribeirão Preto, Universidade de São Paulo (FO/RP/USP), Brazil, and found out that 83.3% and 66.7% of *C. albicans* strains from

oral lesions produced phospholipase and proteinase, respectively. However, the strains of this yeast obtained from the mouth of individuals with no lesions produced phospholipase in 71.9% and proteinase in 68.7% of the cases. Among the strains of *C. albicans* in both groups, enzymetype 22 (positive phospholipase and weakly positive proteinase) was the most prevalent. This showed that the phospholipidolytic capacity of oral strains of *C. albicans* is a preponderating factor for candidiasis (Cândido *et al.*, 2000; Ribeiro *et al.,* 2002). Ghannoum & Abu-Elteen (1990) and Cândido *et al.* (2000) affirm that these hydrolytic enzymes produced by *Candida* yeasts are virulence factors for this yeast-like fungus.

In vitro tests for sensitivity of oral yeasts of *C. albicans* in children with or without Down's syndrome regarding the antifungal agents used (amphotericin B, 5-fluorocytosine, fluconazole and itraconazole) showed to be susceptible to all antifungals. The strains of *C. albicans* obtained from the mouth of children with this chromosome disorder revealed a CI_{50} (concentration index able to inhibit 50% of the strains) equal to 0.25 µg/mL for amphotericin B, CI_{50} = 0.39 µg/mL for 5-flourocytosine, IC_{50} = 1.00 µg/mL for fluconazole and IC_{50} = 0.50 µg/mL for itraconazole. The control group (children without Down's syndrome) showed a CI_{50} = 0.25 µg/mL for amphotericin B, CI_{50} = 0.39 µg/mL for 5-fluorocytosine, CI_{50} = 2.00 µg/mL for fluconazole and CI_{50} = 0.50 µg/mL for itraconazole. Batista *et al.* (1999) observed the susceptibility of 19 strains of *C. albicans*, isolated from patients with protein-induced stomatitis, and found that polyene antibiotics had a lower minimal inhibitory concentration than azole derivatives, although their *in vitro* antifungal action fell within the serum blood levels reached by these drugs in man. Pinto (2003) observed that the *C. albicans* strains obtained from patients with HIV, cancer, diabetes and other diseases, also showed *in vitro* susceptibility to 5-fluorocytosine.

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