

Yeasts from the oral cavity of children with AIDS: exoenzyme production and antifungal resistance

Leveduras da cavidade oral de crianças com AIDS: produção de exoenzimas e resistência antifúngica

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ABSTRACT: The oral fungal microbiota of 30 children with AIDS, of both genders, aged from two to six years, receiving outpatient treatment, was evaluated and compared with that of a control group composed of 30 healthy subjects with matching ages and genders. Virulence factors, such as exoenzyme production, and susceptibility to five antifungal agents using an E-Test kit were evaluated. *C. albicans* predominated over other species in the AIDS group, showing a higher production of proteinase and phospholipase when compared with that observed in the control group. In this study few clinical manifestations of and low selectivity for *C. albicans* (23.3%) were observed in the AIDS group. The enzymatic studies showed that 53.8% of the AIDS strains were strongly positive whereas only 33.3% of the non-AIDS strains were positive. Amphotericin B was the most effective drug among the antifungal agents tested against *C. albicans*. The frequency, selectivity and level of exoenzyme production by *C. albicans* suggest a higher pathogenicity in the AIDS children than in the control children.

DESCRIPTORS: Acquired immunodeficiency syndrome; Child; Antifungal agents.

RESUMO: A microbiota fúngica bucal de 30 crianças com AIDS, de ambos os gêneros, com idades entre dois e seis anos, recebendo atendimento ambulatorial, foi avaliada e comparada com a de um grupo controle constituído de 30 indivíduos saudáveis com idades e gêneros equivalentes. Fatores de virulência, tais como a produção de exoenzimas, e a suscetibilidade dos microorganismos a cinco agentes antifúngicos, medida por meio de um kit E-Test, foram avaliados. O microorganismo *C. albicans* predominou sobre outras espécies no grupo com AIDS, demonstrando uma maior produção de proteinase e fosfolipase quando comparada com a produção observada no grupo controle. No grupo com AIDS, foram observadas poucas manifestações clínicas de *C. albicans*, além de uma baixa seletividade para o microorganismo (23,3%). Os estudos enzimáticos mostraram que 53,8% das linhagens no grupo com AIDS foram fortemente positivas, ao passo que apenas 33,3% o foram no grupo controle. Dentre os agentes antifúngicos testados, a anfotericina B foi a droga mais efetiva contra *C. albicans*. A frequência, a seletividade e o nível de produção de exoenzimas pelo microorganismo sugerem a ocorrência de uma maior patogenicidade nas crianças com AIDS do que nas crianças do grupo controle.

DESCRIPTORIOS: Síndrome de imunodeficiência adquirida; Criança; Antimicóticos.

INTRODUCTION

The infection caused by HIV in children leads to deep immunosuppression, has a shorter incubation period than in adults, and produces various manifestations that include oral symptoms. These occur earlier in children and facilitate the development of opportunistic infections like fungal infections. The disease progression is faster and more severe in children, as they possess an immature immune system and are still undergoing development^{5,8,9,13,23}. The early diagnosis of lesions and

their complications is an important component in the treatment of children, particularly considering that candidiasis is one of the first signs of viral disease progression^{4,11,15}. Pseudomembranous candidiasis has a close correlation with the immune status of the patient, especially in symptomatic children, in whom reduced CD4+ leukocyte counts lead to an increase in the frequency and severity of AIDS-associated manifestations^{1,2,6,10}.

Among the first reports concerning AIDS are cases of children exhibiting early and unexplained immunodeficiencies associated with opportunistic

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infections, favored by intrauterine or post-birth transmission^{10,16}. Oral candidiasis and parotid enlargement are considered to be important signs of disease progression, guiding decisions during the treatment of children with AIDS^{2,3,8,9,24}.

In the present study, the frequency of yeasts, their characteristics and levels of proteinase and phospholipase production in children with AIDS were evaluated and compared to those of 30 clinically healthy control individuals without associated risk factors. The susceptibility of *C. albicans* to five antifungal agents was also analyzed using a commercial kit (E-Test).

MATERIAL AND METHODS

Patients

Among the patients treated and followed up by the Section of Infectology, Santa Casa de Misericórdia, São Paulo, SP, Brazil, we evaluated male and female children aged between two and six years, born to mothers with AIDS. The children exhibited anti-HIV antibodies, which were detected using the ELISA and Western Blot tests at 15 months of age. The results of recent laboratory exams as well as past and present medical histories were obtained from the hospital records. Using these criteria, 30 symptomatic children with AIDS receiving regular treatment with drugs like sulfamethoxazol + trimethoprim (Bactrin), and antiretroviral agents such as zidovudine (AZT), dideoxynosine (ddI), and/or 2-deoxy-3-thiactidine (3TC or Epivir) were selected. A control group composed of 30 children, healthy at examination and with matching ages and genders, was evaluated and compared as to intraoral lesions. These children had not received any blood transfusion and had no history of associated risk factors. The ELISA and Western Blot tests were negative.

The Ethics Committee of the Santa Casa de Misericórdia of São Paulo, and that of the University of São Paulo approved the procedures adopted in the present study, which was initiated only after the agreement and informed consent was obtained from the parents and/or guardians of both groups of children. They were examined under artificial illumination. The extraoral examination consisted of visual inspection and cervico-facial palpation, performed initially to detect lymphadenitis and/or enlarged parotid glands, as well as any facial lesions. The intraoral examination was carried out using wooden spatulas for lip retraction. The characteristics and probable clinical diagnoses of all

oral manifestations were recorded. Protective measures were taken against infection dissemination between patients.

Sampling of biological material

Sterile, alginate swabs (Cefar, Brazil), previously moistened in saline solution were used to perform circular and rotatory movements at the base of the upper and lower vestibules, on the floor of the mouth, and/or on the tongue of each child to collect material for yeast analysis. The material was inoculated onto Petri dishes containing Sabouraud-dextrose agar (Difco, Detroit, USA) and 100 µg/ml chloramphenicol (Park Davis, São Paulo, Brazil).

Yeast isolation and identification

Inoculated plates were incubated at 25°C up to 15 days. Subsequently, colonies were selected by color, size, consistency, surface texture, borders, brightness and topography, and the strains were subcultured in test tubes containing the same culture medium. Isolated colonies were identified employing germ tube test, clamidoconidia formation, micromorphological aspects, carbon and nitrogen assimilation and sugar fermentation¹².

Exoenzyme production

Exoenzyme (proteinase and phospholipase) production tests and determination of their levels were performed according to Price et al.¹⁷ and Ruchel et al.²¹ The standard strains (*C. albicans* ICB-12A) were also used for comparison.

Susceptibility to antifungal agents

Candida strains were analyzed with regard to their susceptibility to five antifungal agents, based on minimum inhibitory concentrations (MIC) in the E-Test⁷ (Biodesk, Solna, Sweden) performed according to the manufacturers' instructions. The susceptibility to antifungal agents was based on the criteria of Thornsberry, Sabath²² and Richardson, Warnock²⁰ (seric levels). The five agents used were: amphoterin B, 5-fluorocytosine, fluconazole, itraconazole and ketoconazole. Standard strains CBS (Central Bureau voor Schimmelcultures) were used as controls.

Statistical analysis

Statistical analysis was performed on the data for isolated yeasts using the chi-square test, the Mann-Whitney test for proteinases and phospholi-

pases, and the Kruskal-Wallis test for antifungal evaluation.

RESULTS

Table 1 shows the distribution of children with AIDS according to age and CD4+ cell count. There were no children included in the range of no suppression ($\geq 1,000$ cell/mm³); a percentage of 33.3% (10/30) showed a moderate suppression (500-999 cell/mm³) and 66.6% (20/30) presented a severe suppression (< 500 cell/mm³), although differences were not statistically significant ($\chi^2 = 2.11$). The cultures were positive in all of the children (7/30) with confirmed symptoms.

There was an association between candidiasis and moderate suppression in only two of the seven children (28.7%), while candidiasis and severe suppression were associated in five out of the seven children (71.4%).

With regard to yeast isolation, 83.3% (25/30) of the samples collected from children with AIDS gave positive results. Twenty-four samples were positive for *Candida albicans*, one for *Candida parapsilosis* and one for *Candida tropicalis*, which was associated with *C. albicans* (Graph 1).

The distribution of *Candida* species in children with AIDS according to CD4+ cell count is shown on Table 2. Positive cultures for *Candida* were found in 34.6% (9/26) of the children with moderate suppression, and in 57.6% (15/26) of those with severe suppression. Among severely immunosuppressed children, 3.85% (1/26) showed a positive culture for *Candida parapsilosis*, and 3.85% (1/26) for *Candida tropicalis* associated with *C. albicans*. The AIDS group presented fungal selectivity species among the positive samples. There was a strong correlation between the presence of AIDS and yeasts ($\chi^2 = 7.89$, $p = 0.005$).

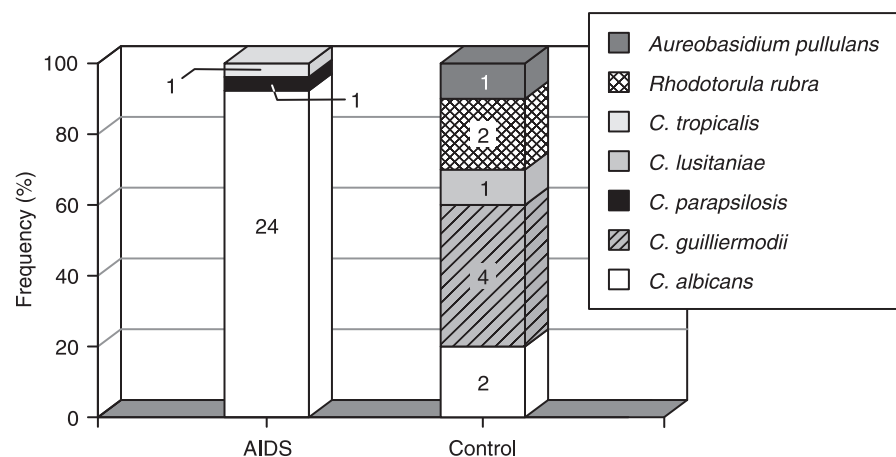
TABLE 1 - Distribution of children with AIDS according to age and CD4+ cell count.

Age	No suppression ($\geq 1,000$ cell/mm ³)	Moderate suppression (500-999 cell/mm ³)	Severe suppression (< 500 cell/mm ³)	Total
2 to 4 years old	-	4	2	6
4 to 6 years old	-	6	18	24
Total	0	10	20	30

TABLE 2 - *Candida* species isolated from the oral cavity of children with AIDS, evaluated by CD4+ cell count.

Species	No suppression ($\geq 1,000$ cell/mm ³)	Moderate suppression (500-999 cell/mm ³)	Severe suppression (< 500 cell/mm ³)
<i>C. albicans</i>	-	9	15
<i>C. parapsilosis</i>	-	-	1
<i>C. tropicalis</i> *	-	-	1

*Associated with *C. albicans*.



GRAPH 1 - Distribution of yeasts isolated from the oral cavity of children with AIDS (one children was positive for *C. tropicalis* associated with *C. albicans*) and from the control group.

TABLE 3 - Susceptibility to antifungal agents of *Candida albicans* strains isolated from the oral cavity of children with AIDS.

Antifungal agent	Sensitive		Resistant	
	n	%	n	%
Amphotericin B	20	76.9	6	23.1
5-Fluorocytosine	16	61.5	10	38.5
Ketoconazole	16	61.5	10	38.5
Fluconazole	5	19.2	21	80.8
Itraconazole	15	57.7	11	42.3

In the control children, 10 samples were positive for yeasts: two for *C. albicans*, four for *Candida guilliermondii*, one for *Candida lusitanae*, two for *Rhodotorula rubra*, and one for *Aureobasidium pullulans* (Graph 1). The enzyme activity study showed that all yeasts isolated from the AIDS group possessed proteinase activity. Fourteen samples (53.8%) were strongly positive while 12 (46.1%) exhibited positive activity. Nearly all samples 96.1% (25/26) exhibited a strongly positive phospholipase activity.

Among *Candida* strains isolated from the control group, 33.3% (3/10) presented a strongly positive proteinase activity, 44.4% (4/10) presented positive activity, and 22.2% (2/10) were proteinase-negative. Phospholipase production, according to the samples in this group, was strongly positive in 66.6%, and only positive in 33.3%.

The Mann-Whitney test detected no statistically significant differences in proteinase activity between the control and the AIDS groups ($U = 149.5$; $U' = 8$; $p = 0.2275$), but revealed higher phospholipase levels in the control group ($U = 205$; $U' = -47.5$; $p = 0.0004$).

The antifungal susceptibility analysis demonstrated that some strains were resistant as the characteristic formation of the inhibition was not observed. The minimum inhibitory concentration test was 0.38 to 2.0 $\mu\text{g/ml}$ for amphotericin B; 0.5 to 12.0 $\mu\text{g/ml}$ for 5-fluorocytosine; 0.019 to 0.064 $\mu\text{g/ml}$ for ketoconazole; 2.0 to 4.80 $\mu\text{g/ml}$ for fluconazole and 0.012 to 8.0 $\mu\text{g/ml}$ for itraconazole. Considering that the main objective of this study was to evaluate the antifungal susceptibility of *Candida* species isolated from children with AIDS, the E-Test was not performed with samples from the control group.

Candida was more resistant to fluconazole (80.76%), followed by itraconazole (42.30%), 5-flu-

orocytosine and ketoconazole (38.46%), and amphotericin B (23.07%), with only six samples showing resistance to the latter (Table 3).

Statistically significant differences were found using the Kruskal-Wallis test when individual comparisons were made among the antifungal agents ($t = 19.33$; $p = 0.0007$). In these individual comparisons ($p < 0.01$), the error rate was less than 1%, indicating activity differences between the antifungal agents.

DISCUSSION

The known number of pediatric AIDS cases does not reflect the real impact of the epidemic. The disease continues to grow and will soon be one of the five major causes of child death, although new drugs may revert this prediction. Since the mouth is easily accessible for clinical examination, it is one of the first areas to show signs of AIDS. Clinical manifestations and various other aspects must be examined, not only for diagnostic purposes, but also to delineate an adequate treatment plan and prognosis^{1,3,5}.

In our subjects, candidiasis was present with a frequency similar to that reported by other researchers^{6,18}. Pseudomembraneous candidiasis predominated over the erythematous type, both affecting the children with moderate and severe suppression. Candidiasis is considered to be a prognostic factor and may indicate rapid disease progression in children, differently from parotid enlargement which is associated with a slower progression¹⁰.

Pseudomembraneous and erythematous candidiasis were detected on the tongue and/or palate, with a higher frequency of the former, corroborating literature data^{5,18}. Although some hypotheses associate the presence of linear gingivitis with erythematous candidiasis, this was not a finding in

the group studied here²⁵. The frequency of candidiasis has an important relationship with extrinsic factors such as treatment and hospitalization. Both may lead to low rates of clinical manifestations, which was demonstrated by the treatment given to our patients and their subsequent outpatient control.

Candida albicans was the predominant yeast isolated from the oral cavity of the children with AIDS. A statistically significant correlation was found between AIDS and the presence of *Candida*, which is important despite the lack of relevant clinical candidiasis manifestations in most children since many yeast infections show no clinical manifestations¹⁶.

A large number of *C. albicans* carriers have been reported in some studies with children, in whom this species is the most prevalent microorganism, both in the oral cavity and in the esophagus. However, a smaller number of *C. albicans* carriers among children, corroborated by our data, reveal the prevalence of erythematous candidiasis¹⁴.

The children with AIDS in the present study exhibited *C. albicans* almost exclusively as the predominant fungus, which may be attributed to the antiviral drugs employed during treatment¹⁹. However, considering the secondary effects of such drugs on the development of *C. albicans*, the control children showed a greater diversity of species.

Evaluation of the production of proteinase and phospholipase, exoenzymes considered to be virulence factors of *Candida* species, is relevant to the expression of pathogenicity considering that *C. albicans* is associated with various factors that require attention, especially when related to AIDS¹¹. High levels of phospholipase and proteinase, in addition to the selectivity of species present in the AIDS group, provide conditions for greater adherence, colonization and development, facilitating yeast multiplication. Proteinase expression seems to be more active in isolates from HIV-infected patients, a relevant aspect considering that antiretroviral agents may reduce exoenzyme production¹³, a fact not confirmed in this study.

Inhibitors present in the saliva, and certain antibodies (IgA) inhibit fungal adherence, and immunodepression seems to favor greater pathogenicity, especially of *C. albicans*, thus allowing its clinical manifestation^{9,16}. Few studies have examined these aspects and they will not be discussed further in the present study since different time periods,

drugs and conditions seen in adult AIDS groups should also be considered.

Drugs effective against *C. albicans* strains were studied using a commercial E-Test kit and the criteria for susceptible resistance were followed according to Richardson, Warrnock²⁰ and Thornsberry, Sabath²². In this study amphotericin B seems to be the most effective. However, we point out the secondary effects, contra-indications and other problems regarding intravenous use to be limitations to the use of this drug, in addition to the lack of a topical preparation for dental use. Among the azoles, itraconazol and ketoconazole are noteworthy; fluconazole was the least effective due to high resistance and the need for higher titers to inhibit fungal growth. *In vitro* tests are important markers, although *in vivo* studies, when performed, deserve greater consideration. The clinical use of antifungal agents, in association with *in vitro* tests, may contribute to a more effective treatment of candidiasis, not always achieved with empirical treatments or even with those based on well controlled tests. The range of available drugs is still very small and so the use of certain agents cannot be avoided.

The development and use of new drugs and an early diagnosis, associated to more detailed knowledge about infants with AIDS, have demonstrated the effectiveness of certain treatments which have resulted in a lower frequency of oral/facial and bacterial manifestations. Progress in studies on yeasts has been increasing, although new and more effective therapeutics must be developed.

Our results show that the yeasts found in the AIDS group were predominantly *Candida albicans* while the control group exhibited a more diversity of yeast species.

CONCLUSIONS

Proteinase and phospholipase production was strongly positive and with a high activity in the AIDS group, suggesting an increased pathogenicity in that group when compared to the control group. According to the E-Test carried out in this study, strains obtained from children with AIDS were more susceptible to amphotericin B, followed by 5-fluorocytosine and ketoconazole; however, they were less susceptible to itraconazole and fluconazole. Differences were observed between the AIDS and the control groups, although the drugs used during ongoing treatment may have led to changes in the behavior of the yeasts evaluated.

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