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| ORIGINAL

Candida on oral cavity of pediatric individuals with ALL and its susceptibility to nystatin and amphotericin B

Candida na cavidade oral de indivíduos pediátricos com LLA e sua susceptibilidade à nistatina e à anfotericina B

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ABSTRACT

Objective: The aim of this study was to evaluate the prevalence of Candida colonizationon oral cavity of pediatric individuals with acute lymphocytic leukemia (ALL) and its susceptibility/resistance to nystatin and amphotericin B. **Methods**: This was a cross sectional study with observational, descriptive and analytic approach. Saliva was collected from40 individuals diagnosed with ALL and from40 healthy subjects, as a comparative group, matched by age and gender with ALL group. The mean age for both groups were 8 years-old. The isolation and identification of the Candidaspecies were performed using the CHROMagarCandidaTM and confirmed by polymerase chain reaction. The samples were subjected to antifungal susceptibility by microdilution assay for nystatin and amphotericin B. Salivary alterations and chemotherapy-induced oralmucositis were evaluated using modifiedOral Assessment Guide. **Results**: The positivity to Candida was higher inALL individuals (32.5%,13/40)than in a comparative group(2.5%, 1/40) (p<0.001). Candida albicans was the most prevalent strain (86.6%). The mucositis was directly associated with positive Candidacolonization (p=0.017) in the ALL group but not related with salivary alterations (p= 0.479). Six strains of C. albicans (54.5%), on ALL group, were resistant to nystatin

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and all strains were not susceptible to amphotericin B. **Conclusion**: Candida colonization was associated with ALL condition and with oral mucositis in these individuals. C. albicans was the prevalent strain and most samples were resistant to antifungal agents tested, nystatin and amphotericin B.

Keywords: Candida. Precursor cell lymphoblastic leukemia-lymphoma. Prevalence.

RESUMO

***Objetivo**: o objetivo deste estudo foi avaliar a prevalência e colonização de Candida na cavidade oral de indivíduos pediátricos com leucemia linfocítica aguda (LLA) e sua susceptibilidade/resistência à nistatina e à anfotericina B. **Métodos**: estudo transversal observacional com abordagem descritiva e analítica. A saliva foi coletada de 40 indivíduos diagnosticados com LLA e de 40 indivíduos saudáveis, como grupo comparativo, combinados por idade e sexo com o grupo LLA. A idade média para ambos os grupos foi de 8 anos de idade. O isolamento e a identificação das espécies de Candida foram realizados utilizando o CHROMagarCandidaTM e confirmados pela reação em cadeia da polimerase. As amostras foram submetidas a susceptibilidade antifúngica por meio de ensaio de microdiluição para nistatina e anfotericina B. As alterações salivares e a mucosite oral induzida por quimioterapia foram avaliadas utilizando o Guia de avaliação modificada. **Resultados**: A positividade para Candida foi superior aos indivíduos in situ (32,5%, 13/40) do que em um grupo comparativo (2,5%, 1/40) (p <0,001). Candida albicans foi a cepa mais prevalente (86,6%). A mucosite foi diretamente associada à colonização positive por Candida (p = 0,017) no grupo LLA, mas não relacionada com alterações salivares (p = 0,479). Seis estirpes de C. albicans (54,5%), no grupo LLA, eram resistentes à nistatina e todas as cepas não eram suscetíveis à anfotericina B. **Conclusão**: A colonização por Candida foi associada à condição LLA e à mucosite oral nesses indivíduos. C. albicans era a cepa predominante e a maioria das amostras eram resistentes aos agentes antifúngicos testados, nistatina e anfotericina B.*

***Palavras-chaves**: Candida. Leucemia-linfoma linfoblástico de células precursoras. Prevalência.*

INTRODUCTION

The acute lymphocytic leukemia (ALL) represents 75% of all diagnosed leukemia in oncopediatric individuals and 25% of all malignant childhood illnesses [1,2]. The immunosuppression favoring related to disease, disease profile and myeloablative treatment could be associated with bacterial and fungal infections, including candidosis [2-4] In ALL patients, it could be raised in some part of chemotherapic treatment and be associated with immunosuppresionfavoring nonalbicans species colonization [4]. Other concern could be recurrent infections caused by resistant Candida species [5]. Even with the use of drugs such as nystatin and amphotericin B, it is highly desirable to select better treatment options defined by sensibility tests [6,7]. It is important because candidemia could be a critical problem in ALL patients with bad outcome and worsening of overall prognosis [3].

Highlighting the importance of knowing the profile of *Candida* colonization in ALL pediatric individuals and its resistance to usual treatment, the purpose of this study was to evaluate the prevalence of Candida colonizationon oral cavity of pediatric individuals with acute lymphocytic leukemia (ALL) and its susceptibility/resistance to nystatin and amphotericin B.

METHODS

The study was cross-sectional, with dual observational and descriptive characteristics approved by ethic committee of Health Centre of Federal University of Paraiba. For this study, we studied two groups. The first was ALL group, formed by 40 individuals, enrolled by census approach during 2014 and 2015. Individuals with ages ranged from 1 to 19 years old were diagnosed with ALL, at a reference oncopediatric unit located in João Pessoa, Paraíba, Brazil. The inclusion criteria were determined as ALL diagnosis and being submitted to chemotherapy or with recent history of chemotherapic treatment.

Data about hemogram with differential count, chemotherapy employed, antimicrobial drugs used, oral mucositis, xerostomia and DMFT/dmft (Decayed/Missing/Filled Teeth) were collected from medical and dental records. Last hematological exams performed close to the salivary collection date, during the same week, were considered.

The indexes of mucositis and xerostomia were obtained from OAG (Oral Assessment Guide), modified by Cheng et al. [8] and made by calibrated examiners being dichotomized in presence/absence. Values above 9 points indicated presence of mucositis and scores 2 and 3 on saliva item, salivary alterations. The table 1 demonstrated this modified guide used in this research.

Clinical candidosis was evaluated by calibrated examiners seeking for symptomatic white removable plaques (pseudomembranous form) over a bleeding or erythematous layer or reddish areas on mucosa associated with pain/sensibility complains (erythematous form). In cases of clinical doubt between erythematous candidosis and oral mucositis, salivary smears could be used to confirm or dismiss *Candida* presence in culture.

Of 40 ALL, 19 were in induction therapy, 3 in reinduction, 3 in consolidation and 15 in maintenance phases. These phases were reclassified in induction (induction/reinduction/consolidation) and maintenance phases for statistical analysis due to similarities among them. The standard chemotherapeutical protocol was BMF (Berlin-Frankfurt-Munich), 2002 [9].

A comparative group was formed by 40 individuals matched by age and gender with the ALL group. This group was formed by healthy individuals, without systemic alterations and other diseases/drugs associated to bacterial/fungal infections or salivary complaints. Clinical candidosis was evaluated by calibrated examiners following the same criteria for ALL group. The Kappa results for the calibration of oral diagnosis were 0.877 to 1.0 (individually) and 0.844 between the evaluators [10].

Isolation, Culture and Identification of *Candida species*

Salivary collection was made with a sterile swab on the buccal mucosa (right and left) and tongue, in order from top to bottom, and back to forward. A standard collection using swabs with Stuart medium (Global Transport Medium Swabs, Global Trade Technology, Brazil) was made in three volunteers. Swabs were weighed before and after the salivary collection. Mean volume of salivary collection was 0.03 mL.

After the collection transportation, the swabs were immediately seeded into plates with CHROMagar*Candida*™ (BD™ CHROMAGAR™ *Candida*, France). After seeding, plates were placed on incubator at 370C for 48 hours. The mean of CFU/mL was obtained by the counting of positive growth of *Candida*. The CFUs were presumptively identified according to color and texture patterns. These colonies were stored at -2 °C into Sabouraud Dextrose Broth (SDB) (KASVI®, Curitiba, Brazil) supplemented by glycerol (40% v/v), into cryotubes (2 mL).

Polymerase Chain Reaction (PCR) identification

PureLink™Genomic DNA extraction kit (Invitrogen, USA) was used to extract DNA from samples. The amplification reaction was performed with a final volume of 50mL containing 100ng of DNA, 1X buffer, 5mM MgCl2, 1mM dNTP's, 1µL of each primer and 5U Taq platinum DNA polymerase (Invitrogen, Carlsbad, CA, USA). Strain identification was determined from the primer ACT1-f(TGCTGAACGTATGCAAAAGG) and ACT1-r (TGAACAATGGATGGACCAGA) (Exxtend Biotechnology Ltd., Brazil), which is a constitutive primer

Table 1. Modified OAG index used in this study (8).

	Score		
Item	1	2	3
Voice	Normal	Deeper or raspy	Difficulty talking or painful
Swallow	Normal	Some pai non swallowing	Unableto swallow
Lips	Smooth and pink and moist	Dry or cracked	Ulcerated bleeding
Tongue	Pink and moist and papillae present	Coated orloss of papillae with shiny appearance withor without redness	Blistered or cracked
Saliva	Watery	Thickorropy	Absent
Mucousmembrane Palate	Pink and moist	Reddened or coated Without ulceration	Ulceration with or without bleeding
Labial mucosa	Pink and moist	Reddened or coated Without ulceration	Ulceration with or without bleeding
Gingiva	Pink and stippled and firm	Edematous withor without redness	Spontaneous bleeding or bleeding with pressure

specific for *Candida albicans* and from the primer HWP1-f (TCTACTGCTCCAGCCACTGA) and HWP1-r (GTGGAATGGAAGCTTCTGGA) (Exxtend Biotechnology Ltd, Brazil), which is for non albicans(11). PCR assays 186pb generate amplification products for *C. albicans* (ACT1) and for non *C. albicans* (226pb) (HWP1).

Thermocycling phase consisted of an initial denaturation cycle at 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds, primer annealing at 58 °C for 30 seconds, and primer extension at 72°C for 30 seconds. A final primer extension at 72°C for 2 minutes was performed, followed by cooling to 4°C. The samples ran on agarose gel prepared with 2% Tris-base buffer + Boric acid + EDTA (TBE) diluted in water with adjusted pH. The run was carried out for 50 minutes at 80W.

Antifungal susceptibility

The samples of each identified species on ALL group were activated in Falcon sterile tubes containing 5mL of SBS and cultivated at 37°C for 48 hours. The concentration of *Candida* employed was 5 x 10 [6] UFC/mL [12]. *Candida* inoculums were diluted to a final concentration of 5.0X10 [3] UFC/mL and placed into 96 well microplates (Global Plast®, Monte Alto-SP, Brazil). Amphotericin B (Sigma Aldrich®, Saint Louis, USA) (320µg/mL) was diluted in filtered DMSO and nystatin (Dilecta, João Pessoa-PB, Brazil) (256µg/mL) on distilled water to achieve the initial concentrations.

In each well microplate, 100µL of Sabouraud Dextrose Broth (SDB) (KASVI®, Curitiba, Brazil) were placed. In the next step, 100µL of evaluated antifungal drugs were added to the first well microplate and serially diluted. The microplates were incubated at 37°C for 48 hours. Focusing cellular viability, 50µL of TCT (Triphenyl Tetrazolium Chloride) dye (Sigma-Aldrich®, Saint Louis, USA) were dropped in each well microplate [13]. The reference values to MIC for nystatin were ≤ 4 µg/mL for sensitive isolates, and ≥ 64µg/mL for resistant isolates Fornari et al. [14]. For amphotericin B, susceptibility was found to be < 1µg/mL, and resistance > 2µg/mL [14]. *C. albicans* (ATCC 10221), C. *tropicalis* (ATCC 750), *C. glabrata* (ATCC 2001) and C. krusei (ATCC 34135) were used as reference strains. The results of the MIC obtained from the reference samples was MIC < 4□g/mL for nystatin, and CIM ≤ 1mg/mL, for amphotericin B.

Data analysis

Proportion tests (Chi-Square Pearson and Fisher Exacttests) for evaluation of distribution differences were made to *Candida* colonization and variables of interest on ALL group. The analysis was performed using IBM SPSS (22.0) software, at significance level of 5%.

Ethical aspects

This research was approved by the Ethics and Research Committee of the Health Sciences Center of the Federal University of Paraíba with the number of opinion 706.409.

RESULTS

The mean age of ALL group was 8.1 (± 5.11) and the comparative group was 8.2 (±4.58). The mean DMFT of both groups was 2.04 and dmft was 1.25. The matched and other variables evaluated in both groups are placed on table 2. The positivity to *Candida* was higher in the ALL group compared with the comparative one (p=0.001). In both groups, clinical lesions suggesting oral candidosis were not found and apparently none clinical overlaying with oral mucositis on ALL group.

The descriptive analysis of *Candida* colonization of groups is detached on table 3. CHROMagar*Candida* suggested the isolation of 8 strains as being C. *albicans* (61.5%), being one collected from the comparative group, followed by *C. glabratain* five strains (35.7%), **C.** *krusei* on one ALL individual and on one comparative subject (14.3%) and one strain of *C. tropicalis* (7.1%). According to PCR identification, *C. albicans* colonies were detected in 13 samples (86.6%), while only on 2 (13.4%) samples was identified as being *non albicans*. Of these positive individuals to *Candida*, 7 (53.8%) were in induction/reinduction/consolidation therapy and 6 (46.2%) in maintenance phase. Eight (61.5%) ALL individuals with positive *Candida* detection were under the use of systemic steroids at the moment of sampling. Moreover, 2 (15.0%) individuals were under the use of nystatin and/or amphotericin B to systemic candidosis but without any oral signs of infection. All individuals who had 10^3 CFU/mL counts were on the induction phase of chemotherapy.

Considering age strata, gender, treatment phase, hematological variables and OAG indicators, the association of presence of oral mucositis and positivity to *Candida* was evident (p=0.017). Positive individuals colonized to *Candida* presented mucositis indexes mainly ranging from 10 to 14 points. The results of these associations or the lack

Table 2. Descriptive analysis of ALL and comparative groups according some variables (n=80).

Variables	Categories	ALL (n = 40)	Comparative (n = 40)	p-value
Stratified age	Upto 6 yrs	19 (46.3%)	22 (53.7%)	0.502[a]
	Above 6 yrs	21 (53.8%)	18 (46.2%)	
Gender	Male	17 (50.0%)	17 (50.0%)	1.000[a]
	Female	23 (50.0%)	23 (50.0%)	
DMFT / dmft	Under reference	34 (58.6%)	24 (41.4%)	0.012[a]
	Over reference	6 (27.3%)	16 (72.7%)	
***Candida* Colonization**	Negative	27 (67.5%)	39 (32.5%)	0.001[B]
	Positive	13 (92.9%)	1 (7.1%)	

[*] Epidemiologic Research SB Brasil, 2010; [a]Chi- square Pearson Test; [b]Fisher Exact Test; DMFT / dmf – Decayed/Missing/FilledTeeth

Table 3. Description of quantification and molecular identification of species found on ALL and comparative groups.

PatientCode	ChemotherapyPhase	Quantification(CFU/mL)	Strain
1	Induction	2.2 x 10^3	*Candida albicans*
2	Induction	66	*non albicans*
3	Maintenance	400	*Candidaalbicans*
8	Maintenance	33	*Candidaalbicans*
12	Maintenance	33	*nonalbicans*
19	Induction	233	*Candidaalbicans*
21	Maintenance	66	*Candidaalbicans*
22	Induction	1.4 x 10^3	*Candidaalbicans*
30	Induction	1.3 x 10^3	*Candidaalbicans*
34	Maintenance	300	*Candidaalbicans*
36	Maintenance	33	*Candidaalbicans*
39	Induction	766	*Candidaalbicans*
40	Induction	6.6 x 10^3	*Candidaalbicans*
1[*]	Comparative Group	33	*Candidaalbicans*

of them were placed on table 4. The table 5 describes the results of microdilution assay and resistance/susceptibility protocol to the employed antifungal drugs. It was found that six out of 11 strains of *C. albicans* (54.5%) on ALL group were resistant to nystatin and all species were not susceptible to amphotericin B. All reference strains (ATCC) were susceptible to nystatin and amphotericin B at the same experimental conditions.

Table 4. Evaluation of crossing between positiveness condition to *Candida* colonization and some variables on ALL group (n=40).

Variable	Categories	Positive	Negative	p-value
Stratified age	Upto 6 yrs	8 (42.1%)	11 (57.9%)	0.217[a]
	Above 6 yrs	5 (23.8%)	16 (76.2%)	
Gender	Male	7 (41.2%)	10 (58.8%)	0.314[a]
	Female	6 (26.1%)	17 (73.9%)	
Erythrocytes	under reference (< 4.4X10 ^{6} cells /mm³)	12 (35.3%)	22 (64.7%)	0.351[b]
	reference or over (> 4.4 X10^{6} cells /mm³)	1 (16.7%)	5 (83.3%)	
Leukocytes	under reference (< 6,000 cells/ mm³)	5 (22.7%)	17 (77.3%)	0.145[a]
	reference or over (> 6,000 cells/ mm³)	8(44.4%)	10(55.6%)	
Neutrophils	under reference (< 3,480 cells/ mm³)	9 (34.6%)	17 (65.4%)	0.491[b]
	reference or over (> 3,480 cells/mm³)	4(28.6%)	10 (71.4%)	
Lymphocyte	underreference (<1,200 cells /mm³)	9 (39.1%)	14 (60.9%)	0.244[a]
	reference or over (>1,200 cells/mm³)	4(23.5%)	13 (76.5%)	
Platelets	under reference (<150,000 units/ mm³)	5 (35.7%)	9 (64.3%)	0.750[a]
	reference or over (>150,000 units/mm³)	8 (30.8%)	18 (69.2%)	
Mucositis	Absence	2(11.8%)	15(88.2%)	0.017[b]
	Presence	11 (47.8%)	12 (52.2%)	
Salivaryalteration	Absence	2 (25.0%)	6 (75.0%)	0.479[b]
	Presence	11 (34.4%)	21 (65.6%)	
Chemotherapyphase	Induction	7 (29.2%)	17 (70.8%)	0.581[a]
	Maintenance	6 (37.5%)	10 (62.5%)	
DMFT / dmft	Underreference* (< 2.5)	10 (29.4%)	24(70.6%)	0.293[b]
	Over reference* (> 2.5)	3 (50.0%)	3 (50.0%)	

*Epidemiologic Research SB Brasil, 2010; [a]Chi- square Pearson Test; [b]Fisher Exact Test; DMFT / dmf – Decayed/Missing/FilledTeeth.

Table 5. MIC values found on molecular identified species of *Candida* on ALL group(n=13).

Drug	Concentration	Candidaalbicans	Non albicans
Nystatin	>64 µg/mL	5	1
	64 µg/mL	1	--
	32 µg/mL	3	--
	16 µg/mL	1	1
	8 µg/mL	1	--
AmphotericinB	>80 µg/mL	--	--
	80 µg/mL	--	--
	40 µg/mL	–	--
	20 µg/mL	5	1
	10 µg/mL	3	--
	5 µg/mL	1	--
	2.5 µg/mL	2	1

DISCUSSION

The ALL affects children of all ages, with peak incidence normally happening between two and five years old, with a slight predominance of males corroborating our data with previous distribution found in the literature [15,16]. It is extremely important to undergo the dental treatment in ALL individuals and to be screened to oral infections [17,18]. Curiously,ALL individuals could present good dental condition compared with other populations as mentioned in our study [19]. In our study both groups had similar access to dental care without any considerable sociodemographic differences [20].

Candidemia in ALL patients could be associated with relapses, prolonged neutropenia and antibiotic administration [3,21]. Curiously, our study showed a lack of association between *Candida* colonization and hematological parameters. However, considering different types of variables including hematological counts, supportive and treatment conditions could be more relevant [21].CHROMagar*Candida*could be used to presumptively identify *Candida* species. Good compliance between CHROMagar*Candida*and other methods of identification was shown but without perfect concordance [22]. In fact, the use of nonmolecular identification methods could be markedly influenced by personal assessment [23]. In fact, molecular identification is an important addition to the conventional identification of *Candida* species [24].

C. Albicans was the most prevalent strain in the ALL group. A study which evaluated the oral cavity of 111 positive HIV individuals found *C. Albicans* was the most isolated species (83.5%) whereas non albicans species were isolated from 16.5% of colonized individuals [25]. Previously, our group showed increasing of nonalbicans species but on elderly irradiated individuals on head and neck [26].

Other interesting result was the higher counting of CFU of *Candida* associated with the induction phase of chemotherapy. Probably, induction phase reduces inflammatory response, even on salivary level, favoring *Candida* colonization. As previously showed, the induction phase had more association of oral complications [19] but them could remain on maintenance phase. The association between the absence of candidosis and mucositis was evident. Previously, it was observed correlation between mucositis and the presence of *Candida* on ALL patients [27,28].

It is important to carry out antifungal susceptibility testing because of the resistance of *Candida* strains to certain drugs. Curiously, a previous study found 95% of their isolates in HIV patients were inhibited by nystatin and amphotericin B [29]. However, emerging resistance of *Candida* species to antifungal drugs is a real problem. For example, a study with six out of nine children with ALL, fungal infection was progressive despite intravenous

antifungals. The high percentage (21%) of death from invasive fungal infection among lethal infections in pediatric ALL individuals illustrates the relevance of fungi in this group of individuals [30].

CONCLUSION

In conclusion, *Candida* colonization was associated with ALL probably due to its relation to mucositis events being the higher colony counts found during the induction phase of chemotherapy. *Candida Albicans* was the prevalent strain and resistance to nystatin and amphotericin B was found. PCR fingerprinting could be used as definitive method to identify *Candida* species in addition to presumptive identification

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Collaborators

LC MONTEIRO, conceptualization; data curation; investigation; methodology; visualization; writing-original draft. ILA RIBEIRO, RFB BONAN and AMG VALENÇA, data curation; formal analysis. PP MACIEL, data curation; formal analysis; investigation; methodology; visualization. ACB DULGHEROFF, JR SOUZA and LRC CASTELLANO, data curation; investigation; methodology; visualization; supervision; validation; writing-review & editing. Y WANDERLEY, conceptualization; data curation; visualization; supervision; validation. PRF BONAN, conceptualization; data curation; funding acquisition; project administration; resources; software; visualization; supervision; validation; writing-review & editing.

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