Objectives: Prospective evaluation of the buccal mucosa of pediatric patients with acute lymphocytic leukemia (ALL) regarding its clinical aspect and expression of members from the HHV family.

Methods: From September 2014 to January 2015, a series of nine consecutive patients was evaluated, with ages ranging from 2 to 14 years, in treatment at the Amazon Hematology and Hemotherapy Foundation (HEMOAM). The buccal mucosa of those patients was clinically evaluated, screened for alterations and analyzed by PCR, to search DNA from HSV-1, CMV and EBV during four moments of the pre-phase/induction phase of the chemotherapy treatment (P/I) – D0/D1, D8, D15 and D35, as well as four moments of the consolidation of the remission phase (CR) – D1, D15, D29 and D50. The protocol proposed by the Brazilian Cooperative Group for Treatment of Childhood Lymphocytic Leukemia (GBTLI ALL-2009) was followed.

Results: A prevalence of 2-year-old children (66.7%, n=6) with a diagnose of B-cell ALL (88.9%, n=8) was observed. Buccal alterations were observed in 33.3% (n=3) of the patients: erythema (D35 P/I), dry lips (D8 and D15 P/I) and an episode of xerostomia (D15 P/I). None of the samples was positive for HSV-1 and CMV, but 33.3% (n=3) of the cases expressed EBV (D8 and D15 P/I).

Conclusions: Buccal alterations and the presence of HSV-1, CMV and EBV in patients with ALL was inexpressive, with most of the patients being sound throughout the treatment. Thus, it cannot be affirmed that the analyzed viruses are part of the microbiome of those patients. However, it has been suggested that the presence of EBV is more expected than HSV-1 and CMV. (Rev Port Estomatol Med Dent Cir Maxilofac. 2019;60(4):163-168)
Resumo

Avaliação da mucosa bucal de pacientes com leucemia linfocítica aguda: Estudo de série de casos

Objetivo: Avaliar prospectivamente a mucosa bucal de pacientes pediátricos com Leucemia Linfoblástica Aguda (LLA) quanto ao seu aspecto clínico e expressão de membros da família HHV.

Métodos: No período de setembro de 2014 a janeiro de 2015, foram avaliados consecutivamente, uma série de nove pacientes com idades entre 2 e 14 anos atendidos na Fundação de Hematologia e Hemoterapia do Amazonas (HEMOAM). A mucosa bucal desses pacientes foi avaliada clinicamente quanto a alterações bucais e por meio de PCR para detecção do DNA do HSV-1, CMV e EBV em 4 momentos da pré-fase/indução (P/I), sendo eles D0/D1, D8, D15 e D35, assim como em 4 momentos da consolidação da remissão (CR), sendo eles D1, D15, D29 e D50 pelo protocolo do Grupo Brasileiro de Leucemias na infância (GBTLI LLA 2009).

Resultados: Foi observada maior prevalência de crianças com 2 anos de idade (66,7%, n=6), do sexo masculino (66,7%, n=6) e com diagnóstico de LLA de células B (88,9%, n=8). Alterações bucais foram observadas somente em 33,3% (n=3) dos pacientes, sendo elas eritema (D35 P/I), lábios ressecados (D8 e D15 P/I) e relato de xerostomia (D15 P/I). Nenhuma das amostras foi positiva para HSV-1 e CMV, no entanto 33,3% (n= 3) dos casos apresentaram expressão do EBV (D8 e D15 P/I).

Conclusões: Alterações bucais e presença dos vírus HSV-1, CMV e EBV nos pacientes com LLA avaliados mostrou-se inexpressiva, estando a maioria dos pacientes com mucosa hígida ao longo do tratamento. Não se pode afirmar que os vírus analisados integrem a microbiota desses pacientes, no entanto, há indícios de que a presença do EBV seja mais esperada que o HSV-1 e CMV. (Rev Port Estomatol Med Dent Cir Maxilofac. 2019;60(4):163-168)

Palavras-chave: Leucemia linfocítica aguda Herpesviridae Mucosite oral Reação em cadeia da polimerase

Introduction

Acute lymphocytic leukemia (ALL) is the most common malignant neoplasm in childhood and adolescence with a prevalence peak at 2 to 5 years of age.1-3 It is frequently sensible to chemotherapy, having an event-free survival rate of 90% when the ideal therapeutic regimen, based on subgroup stratification, is adopted.4 This regimen’s treatment strategies comprehend phases, such as the induction, consolidation and maintenance phases.5

Secondary complications, such as mucocutaneous pallor, fever, asthenia, lymphadenopathy, xerostomia, cushingoid facies, hemorrhage, mucositis, candidiasis, ulcers and alterations on the saliva constituents with swallowing impairment may arise as a consequence of the chemotherapy treatment.6-9 The presence of microorganisms is an aggravating factor of those effects.10

The type-1 herpes simplex virus (HSV-1) has an important role on the severity of chemotherapy-induced buccal mucositis in patients with hematological neoplasms,10-12 as do the Epstein-Barr virus (EBV) and the cytomegalovirus (CMV), representing a threat to immunocompromised patients.13,14

In the ‘80s, the first multicenter protocol for the treatment of childhood ALL (GBTLI-80) was created in Brazil and it is currently in its sixth version (GBTLI-2009). This protocol has been used in about 14 treatment centers in Brazil, one of which is the Amazon Hematology and Hemotherapy Foundation (HE- MOAM). The HEMOAM is the only public reference service for treatment of childhood leukemia in the Northern region, with an annual demand of approximately 40 new ALL cases.25 Due to the lack of publications related to the profile of ALL patients in treatment with GBTLI ALL-2009,3 this study aims to evaluate the buccal mucosa of such patients regarding its clinical aspect and expression of the Herpesviridae (HHV) family members in several moments of the adopted chemotherapy protocol.

Material and methods

This research represents a case series study composed of nine consecutive cases of children and adolescents diagnosed with ALL, of both genders, with ages ranging from 2 to 14 years, from September 2014 to January 2015 in the HEMOAM Foundation. The procedures of this study follow the guidelines established by the ethics committee of the Federal University of Amazonas (UFAM protocol: 30934514.9.0000.5020) and HEMOAM (Protocol: 30934514.9.3001.0009).

A clinical analysis was performed to search for possible alterations of the lips, cheek mucosa, tongue, floor of the mouth, hard and soft palate, oropharynx, gums and surrounding regions, using a mouth mirror, a wooden spatula, gauze and proper biosecurity equipment. When buccal mucositis was observed, it was graded according to the Oral Toxicity
Scale of the World Health Organization (WHO),\textsuperscript{16} where grades 1 and 2 correspond to moderate mucositis and grades 3 and 4 to severe mucositis.\textsuperscript{17}

For the evaluation of HSV-1, EBV and CMV, samples were collected from the buccal mucosa and submitted to PCR analysis regardless of the presence of buccal mucositis. The patient’s mouth rinsed with sterile water and then the samples were obtained via oral swabs from the cheek mucosa (from molars to incisors), bilaterally, after brushing the mucosa with sterile cervi-

Table 1. PCR conditions, regarding primers’ sequence of housekeeping gene and viruses, and their respective fragment size (bp, base pairs)

<table>
<thead>
<tr>
<th>Virus</th>
<th>PCR Conditions</th>
<th>Primers (5’-3’)</th>
<th>Sense</th>
<th>Antisense</th>
<th>Fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-globin</td>
<td>94°C/ 2 min, 94°C/ 30 sec (40x), 60°C/ 45 sec, 72°C/ 1 min, 72°C/ 5 min.</td>
<td>CTTCTGACACAATGTTGTCACTAGC</td>
<td></td>
<td>TCACCACCAACTTCCAGCTTACC</td>
<td>123</td>
</tr>
<tr>
<td>HSV-1</td>
<td>94°C/ 2 min, 94°C/ 30 sec (40x), 60°C/ 45 sec, 68°C/ 30 sec, 72°C/ 5 min.</td>
<td>GGAGGACACATGCTCCTTGG</td>
<td></td>
<td>ACCCTTAGCACGTCTGTATCTTACC</td>
<td>147</td>
</tr>
<tr>
<td>HSV-2</td>
<td>94°C/ 2 min, 94°C/ 30 sec (40x), 60°C/ 45 sec, 68°C/ 30 sec, 72°C/ 5 min.</td>
<td>CGTTTCATCATGGGC</td>
<td></td>
<td>GTACAGACCTCGGAGG</td>
<td>227</td>
</tr>
<tr>
<td>EBV</td>
<td>94°C/ 2 min, 94°C/ 30 sec (40x), 60°C/ 45 sec, 68°C/ 30 sec, 72°C/ 5 min.</td>
<td>GTTTTCGACTTTGCCAGGCCTTAC</td>
<td></td>
<td>ACTCGTGACGTGTCCTCTTTAC</td>
<td>176</td>
</tr>
<tr>
<td>CMV</td>
<td>94°C/ 2 min, 94°C/ 30 sec (40x), 60°C/ 45 sec, 68°C/ 30 sec, 72°C/ 5 min.</td>
<td>GTTTTCGACTTTGCCAGGCCTTAC</td>
<td></td>
<td>TTGACACTCGGCGATCGATTC</td>
<td>242</td>
</tr>
</tbody>
</table>

The samples were transferred to 1.5 mL microtubes with 500 μL of TE buffer (10 mM Tris HCl and 1 mM EDTA, pH 8.0) and stored at -20°C. For the DNA extraction, 500 μL of TPK (10 mg/mL proteinase K, plus TE buffer and Tween 20%) were added to the tubes. The microtubes were briefly submitted to a vortex to homogenize the mix and then were incubated at 56°C for an hour, followed by ten more minutes at 100°C to activate the proteinase K. Afterwards, the DNA concentration was obtained via absorbance reading at 260 nm. The microtubes were stored at -20°C.

Specific sets of initiation pairs were used for the amplification and detection of HSV-1, EBV and CMV via PCR analysis.\textsuperscript{19} The same samples were submitted to PCR for the β-globin constitutive gene to control the occurrence of false-positive results. The positive controls for HSV-1, EBV and CMV were provided by the Tropical Medicine Foundation of Amazonas. Ultrapure water associated with the reaction’s reagents was used for negative controls.

The PCR reactions for β-globin, HSV-1, EBV and CMV were performed in PCR conditions and primers according to Table 1. All of the PCR reactions were performed in Thermal Cycler (Applied Biosystems® Veriti® Thermal Cycler). The amplicons were analyzed by electrophoresis in 2% agarose gel, stained with 0.8 μL of ethidium bromide (1μg/μL), in TBE 1X (tris + boric acid 0.089 M and EDTA 0.02 M) and visualized in a transilluminator under UV light, under electrical tension of 80 to 100 Volts. The size of amplified PCR fragments was determined by comparison with molecular weight markers (100 bp ready-to-use DNA Ladder, Bioron®).

Results

From September 2014 to January 2015, nine consecutive ALL cases of children and adolescents were analyzed, with a prevalence peak at around 2 years of age (66.7%), in males (66.7%) and with B-cell ALL diagnosis (55.6%). Clinical data regarding gender, age and subtype of ALL from the nine cases can be found in Table 2.

Table 2. Clinical profile of ALL patients in treatment at HEMOAM from September 2014 to January 2015

<table>
<thead>
<tr>
<th>Clinical Characteristics</th>
<th>Number of cases (n=9)</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6</td>
<td>66.7%</td>
</tr>
<tr>
<td>Female</td>
<td>3</td>
<td>33.3%</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 years</td>
<td>6</td>
<td>66.7%</td>
</tr>
<tr>
<td>7 years</td>
<td>1</td>
<td>11.1%</td>
</tr>
<tr>
<td>9 years</td>
<td>1</td>
<td>11.1%</td>
</tr>
<tr>
<td>14 years</td>
<td>1</td>
<td>11.1%</td>
</tr>
<tr>
<td>ALL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Risk of Recurrence B-cell</td>
<td>5</td>
<td>55.6%</td>
</tr>
<tr>
<td>High Risk of Recurrence B-cell</td>
<td>3</td>
<td>33.3%</td>
</tr>
<tr>
<td>T-cell</td>
<td>1</td>
<td>11.1%</td>
</tr>
<tr>
<td>PH+</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>

ALL PH+: Acute lymphocytic leukemia positive for Philadelphia chromosome.
None of the patients with ALL presented buccal mucositis, but two patients presented dry lips on the 8th and 15th days of the induction phase and one of them also complained of xerostomia. A third patient presented erythema on the floor of the mouth during the induction phase and another presented erythema on the floor of the mouth on the 35th day of the same chemotherapy phase. None of the 23 samples gathered for the screening of HSV-1 and CMV were positive. On the other hand, three samples (33.3%) showed positive DNA amplification for EBV. The results of this study are assembled in Table 3.

### Discussion

ALL is the most common malignant neoplasm in childhood and adolescence.1-3 It derives from hematopoietic cells and is characterized by increased malignant proliferation of lymphoid progenitor cells.20 Leukemia can be subclassified as acute or chronic. In its acute form, there is a fast increase in the number of immature cells circulating, impairing the bone marrow’s production of healthy blood cells. In its chronic form, there is an excessive number of mature but abnormal white blood cells in the blood.21 It is frequently chemotherapeutically sensitive, but the chemotherapy may cause stomatological side effects that can be eased by oral monitoring of the patients throughout the entire treatment.2

The nine patients evaluated had 2 to 14 years of age, with a prevalence peak at 2-year-old male kids (66.7%), which confirms the literature that ALL affects children and adults, with a prevalence peak at 2 to 5 years of age.1 In one of the published researches, 24 men and 19 women were evaluated and, of those, 23 (53.5%) patients ranged from 3 to 6 years of age, with the most prevalent patients being females with 6 to 9 years of age.9 Another study with 42 patients pointed to 54.8% (n=23) female patients, with a mean age of 7.1 (± 4.7) years (median 5, minimum 2 and maximum 18 years), and distributed the patients regarding the age at diagnosis in groups of 2 (n=7; 16.7%), 4 (n=8; 19.0%) and 5 (n=4; 9.5%) years.8 The most affected gender in the present study counteracts the literature. Regarding age, some authors are unanimous when affirming that the age at diagnosis has a strong effect over the prognosis, with more favorable outcomes for younger patients.4,22

Previous studies investigating the buccal mucosa of patients in treatment for ALL highlighted secondary buccal complications commonly occurring in the induction phase of the chemotherapy treatment, with a gradual decline over the following weeks.23 The most common complications are mucositis, candidiasis, periodontitis, gingivitis, ulcers, petechiae, ecchymosis, erythema, gingival bleeding, xerostomia, mucosal pallor and leukemic gingival enlargement.7-9,23 However, in a prospective evaluation from diagnosis to the 10th week of treatment, alterations in saliva and lip mucosa were observed, because of the occurrence of severe buccal mucositis throughout almost all of the evaluation weeks.8 In the present study, only a few patients presented buccal alterations, contrasting with previous reports. The clinical findings of erythema (D35 P/I), dry lips (D8 and D15 P/I) and xerostomia (D15 P/I) are reported by most of the literature.5-9,23 Although buccal mucositis is a common side effect of chemotherapy,10,18,23 it was not observed in the present case series, probably because of the small number of cases studied.

The relationship between HHV and hematological malignancies has been extensively studied, considering that it can be reactivated under immunosuppression.10,24,25 Studies have reported the seroprevalence of HSV-1, EBV and CMV25,26 as well as the detection of DNA from those viruses in the buccal mucosa of ALL patients, which suggest that HSV-1 may be related to the severity of chemotherapy-induced buccal mucositis,10-12 and that EBV may be associated to many kinds of epithelial and lymphoid malignancies.24 However, a research that evaluated the role of CMV and EBV in childhood B- and T-cell ALL pathogenesis reported that less than 20% of the samples were positive for at least one of the tested viruses and that the positive samples showed low infection levels;19 nevertheless, both represent a threat to immunocompromised patients, leading to morbidity and mortality among this group.13,14 In comparison, in this study, after evaluation by qualitative PCR to search

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**Table 3. Results of the clinical evaluations and HHV viral expression on the buccal mucosa of ALL patients in treatment at HEMOAM from September 2014 to January 2015**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Clinical Status of the buccal mucosa/Type of alteration</th>
<th>Phase</th>
<th>HHV</th>
<th>Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>14</td>
<td>B-cell ALL</td>
<td>Dry lips; Xerostomia</td>
<td>D8 e D15 (P/I)</td>
<td>NA</td>
<td>EBV</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>2</td>
<td>B-cell ALL</td>
<td>Erythema on the floor of the mouth</td>
<td>D35 (P/I)</td>
<td>NA</td>
<td>EBV</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>2</td>
<td>B-cell ALL</td>
<td>Sound</td>
<td>NA</td>
<td>EBV</td>
<td>D8 (P/I) and D15 (P/I)</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>2</td>
<td>B-cell ALL</td>
<td>Sound</td>
<td>NA</td>
<td>EBV</td>
<td>D8 (P/I) and D15 (P/I)</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>2</td>
<td>B-cell ALL</td>
<td>Dry lips</td>
<td>D8 (P/I) and D15 (P/I)</td>
<td>EBV</td>
<td>D8 (P/I) and D15 (P/I)</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>2</td>
<td>B-cell ALL</td>
<td>Sound</td>
<td>NA</td>
<td>EBV</td>
<td>D8 (P/I) and D15 (P/I)</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>7</td>
<td>B-cell ALL</td>
<td>Sound</td>
<td>NA</td>
<td>EBV</td>
<td>D8 (P/I) and D15 (P/I)</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>9</td>
<td>B-cell ALL</td>
<td>Sound</td>
<td>NA</td>
<td>EBV</td>
<td>D8 (P/I) and D15 (P/I)</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>2</td>
<td>T-cell ALL</td>
<td>Sound</td>
<td>NA</td>
<td>EBV</td>
<td>D8 (P/I) and D15 (P/I)</td>
</tr>
</tbody>
</table>

**B-cell ALL:** B-cell acute lymphocytic leukemia; **T-cell ALL:** T-cell acute lymphocytic leukemia; **D8:** Day 8; **D15:** Day 15; **D35:** Day 35; **P/I:** Prephase and induction; **NA:** No alterations.
for HSV-1, CMV and EBV, no samples were positive for HSV-1 and CMV and three presented DNA amplification for EBV. These results highlight the literature statements that EBV occurs later in life in developed countries, but mainly during childhood in developing countries. For more precise results, real-time PCR, as well as more advanced technologies, could aid in detecting those viruses more accurately, because some may remain in a latent state of the infection with low levels of viral genic expression, being barely detectable. Despite the limitation of access to molecular biology techniques, more studies need to be performed for a deeper analysis and more cases need to be prospectively followed-up, with constant monitoring, to gather more expressive data.

Conclusions

Taking into account the limitations of this case series, it can be concluded that the buccal alterations and the presence of HSV-1, CMV and EBV in the evaluated ALL patients were inexpressive, with most of the patients presenting sound mucosa throughout the treatment, without occurrences of mucositis. It cannot be confirmed that the analyzed viruses integrate the microbiome of those patients. However, there are indications that the presence of EBV is more likely than HSV-1 and CMV, especially among child populations in developing countries, such as Brazil.

Acknowledgements

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Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

Conflict of interest

The authors have no conflicts of interest to declare.

REFERENCES


