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Distinctive EBV infection characteristics in children from a developing country

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ABSTRACT

Background: In developing countries, Epstein–Barr virus (EBV) infection is mostly asymptomatic in early childhood. EBV persistence may lead to different malignancies, such as B cell derived lymphomas. In Argentina, most children are seropositive at three years and an increased association between EBV and lymphoma was proved in children under 10 years old by our group.
Objective: Our aim was to characterize EBV infection at the site of entry and reactivation of viral infection —the tonsils— in order to better understand the mechanism of viral persistence in pediatric patients.
Methods: A cohort of 54 patients was described. We assessed specific antibodies profiles in sera; viral proteins presence by IHC on FFPE samples and EBV type from fresh tissue.
Results: EBV type 1 was prevalent, mostly in the youngest patients. Asymptomatic primary infected patients presented higher viral loads and Latency 0/I or II patterns, whereas the Latency III pattern was observed mostly in healthy carriers. There were no differences between groups in the expression of viral lytic antigens. This study discloses new features in patients undergoing primary infection from a developing population. Low viral inoculum and restricted viral antigen expression may be responsible for the lack of symptoms in children from our country.

Introduction

Epstein–Barr virus (EBV) is a member of the Gamma Herpesvirus family, which infects almost 90% of the people worldwide. Its primary target is human B lymphocytes, where it establishes a lifelong persistent infection, mostly asymptomatic (Hatton et al., 2014).

The mechanism of the EBV persistence, like all of the herpesvirus family, consists of two phases: the latent cycle, in which viral antigen expression is restricted to a minimum in order to avoid immune surveillance; and the lytic cycle, which creates new infectious virions that are spread by the saliva (Szymula et al., 2018).

The greater disease burden produced by EBV has been highly associated with several types of epithelial cell carcinoma, such as nasopharyngeal and gastric carcinoma, and B cell lymphoma, including Burkitt lymphoma, posttransplant lymphoproliferative disorder, and Hodgkin and non-Hodgkin lymphoma (Szymula et al., 2018). In this regard, EBV-associated neoplasias are related to the expression of different latency programs(Thorley-Lawson et al., 2008) that alternatively express viral proteins with oncogenic properties(Young and Murray, 2003; Young et al., 2016).

Four latency stages were described on the basis of differential EBV gene antigen expression: Latency 0 (L0), where no viral antigens or only EBERs transcripts are expressed; Latency I (LI), which involves the expression of EBERs and EBNA1; Latency II (LII), when B cells express EBERs, EBNA1 and both transmembrane viral proteins LMP1 and LMP2A/B; and, finally, Latency III (LIII) where all viral antigens are expressed, including EBNA2 and EBNA3s (Kuppers, 2003). Alternatively, Latency IIb was also suggested, when B cells express EBNA2 without latent membrane proteins (Kurth et al., 2003).

The study of the primary infection has proved to be difficult, not only because of the long incubation period before symptom appearance, but also because this period is nearly impossible to

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define (Dunmire et al., 2015). Therefore, the majority of EBV primary infection studies are based in cases of Infectious Mononucleosis (IM) in adolescents or adult patients. The occurrence of IM is significantly higher in developed countries, and is a consequence of the delay of the primary infection until adolescence or young adulthood. IM elicits a strong response of the immune system, characterized for massive CD8+ T cells expansion, mostly EBV-specific (Abbott et al., 2017), and also displays an elevated viral load, detectable in saliva as well as in peripheral blood (Balfour et al., 2015). Moreover, during the early stages of primary infection, EBV shows the complete antigen expression, LIII program(Thorley-Lawson, 2015), which is sequentially silenced, either through the germinal center(GC) reaction or by the generation of a GC-like environment (Shannon-Lowe et al., 2017) once the persistence is accomplished.

Those difficulties are also present in children, mostly because of the lack of symptoms. A recent study, performed with samples from pediatric patients undergoing tonsillectomy, was published (Jud et al., 2017), but it was carried out in a highly developed country and was focused only on the immune response. On the other hand, although there are a few studies of primary infected children from developing countries, such as the Jayasooriya et al. and Piriou et al. reports, that aim to determine the characteristics of primary infection in a cohort of African children (Jayasooriya et al., 2015; Piriou et al., 2012), or the Slyker et al. study (Slyker et al., 2013), which focused on HIV infected children; these results were restricted to peripheral blood samples, which do not accurately reflect the underlying mechanisms of what happens in the tonsils, the site of infection. Thus, deep characterization of primary EBV infection in developing countries like Argentina, in which viral infection occurs almost exclusively in the first years of life, and where the incidence of EBV-associated lymphomas in children younger than 10 years old is higher (Chabay, Preciado, 2013), is needed.

Therefore, in the light of these considerations, our aim was to characterize and compare EBV infection in tonsil tissue from children undergoing either primary infection or viral reactivation and healthy carriers, as a way to better understand viral infection stages.

Results

Serological status and viral load

In order to establish the EBV serological status, the presence of VCA-IgM, VCA-IgG, Early Antigen (EA)- IgG and EBNA1-IgG was determined in study participants' sera according to previous reports (Klutts et al., 2009). Fifty-four samples were analyzed by this approach, the median age of this group being 5 years old (ranging from 1 to 15) with female predominance (30/54, 55.5%). Four groups were defined based on these results: asymptomatic primary infected patients (PI) identified as VCA-IgM^{+}/VCA-IgG$^{-/+}$/EA-IgG-/ EBNAI-IgG-; healthy carriers (HC) identified as VCA-IgM-/VCA-IgG^{+}/EA-IgG-/EBNAI-IgG^{+}, patients undergoing viral reactivation (R) identified as VCA-IgM$^{+/-}$/VCA-IgG^{+}/EA-IgG^{+}/EBNAI-IgG^{+} and non-infected patients (NI) identified as VCA-IgM-/VCA-IgG-/EA-IgG-/EBNAI-IgG-. Among the studied group 18 turned to be PI, 25 HC, 7 R and 4 NI cases, the median age being 6 years old for HC, 5 years old for R and 4 years old for both PI and NI patients (Table 1). Even though no statistical differences among ages were observed between groups, HC median age was slightly higher. All patients' data is summarized in Table 2.

Viral load at the tonsils was unexpectedly low in all groups, the global mean being 988 viral copies/ug of DNA. When the four groups were analyzed separately, the mean viral load was 4854 viral copies/ug of DNA in API, 339 viral copies/ug of DNA in HC and 345 viral copies/ug of DNA in R group. As expected, API's mean viral load at the tonsils was statistically higher than in the rest of the groups ($p = 0.0051$, Kruskal–Wallis test).

Latency and lytic viral proteins expression

The presence of EBV latent proteins LMP1, LMP2A and EBNA2, along with the lytic cycle protein BMF1 was assessed by IHC (Figure 1A–E). In addition, EBERs transcripts expression was evaluated by ISH. Latency patterns were identified as follows: L0, cases without viral antigen expression; L0/LI, cases with EBERs expression; LII, EBERs together with LMP1 and/or LMP2A; LIII, LII proteins together with EBNA2 expression; LIIb, LI plus EBNA2 expression, without LMPs.

Given the lack of anti-EBNA1 antibodies for FFPE tissue that restrain us from defining L0 accurately, we decided to grouped L0 patients within the LI category in the analysis. Also, we grouped LIIb with LIII cases because the first pattern is considered to be an immediately previous stage of the LIII pattern (Kurth et al., 2003). On the other hand, cells undergoing the viral lytic cycle (LC) were defined by nuclear antigen BMRF1 expression.

A total of 29 patients displayed L0/LI pattern, 12 LII and 9 LIIb/LIII, whereas only 8 children presented cells with lytic cycle protein BMRF1 expression along with cells expressing latent antigens. When divided in groups, 10 PI, 15 HC and 4 R children expressed L0/LI antigens; 8 PI, 2 HC, and 2 R patients showed LII, while LIIb/LIII profile was identified in 8 HC and 1 R children. Unexpectedly, 6 out of the 8 cases with presence of LC antigens turned out to be HC while only 2 were R patients. These results are summarized in Table 3.

When LC and latency patterns were analyzed altogether, we observed that BMRF1 was expressed along with LI antigens in 2 HC and 1 R children, with LII antigens in 3 HC and none R patients, and with LIII antigens in 2 HC and 1 R patients. Non statistically differences were found in LC antigen expression among the three latencies in LC antigen expression ($p > 0.05$, X^2 test).

Table 1
Differentiation of four groups based on their EBV serological profile.

Group	N	Median Age (range)	VCA-IgM	VCA-IgG 1/10	VCA-IgG 1/40	VCA-IgG 1/320	EA-IgG	EBNA1-IgG	Mean VL* (copies/ug DNA) (SEM)
PI	18	4 (2–12)	++	+/–	+/–	+/–	-	-	4854(1788)
HC	25	6 (2–15)	-	+	+	+/–	-	+	339(101)
R	7	5(2–8)	+/–	+	+/–	+/–	+	+	345(56)
NI	4	4(1–7)	-	-	-	-	-	-	-
Total	54	5 (1–15)							988(492)

*Statistical difference ($p = 0{,}0051$). PI: asymptomatic primary infected children, HC: Healthy carriers, R: children undergoing viral reactivation, NI: non-infected children. VL: viral load (copies/ug DNA). N: patients per group. VCA-IgM: IgM Ab against the viral capsid. VCA-IgG: IgG Ab against viral capsid. 1/10; 1/40 and 1/320 were the serum dilutions used in the VCA-IgG titration. EA-IgG: early antigen directed IgG (sera dilution used: 1/10) EBNA1-IgG: EBNA1 directed IgG (sera dilution 1/10). ++: intense positivity. +: positive. +/–: either positive or negative. –: negative.

Table 2
All patient characteristics.

Patient	Age	Sex	Viral Load	EBV type	LMP1	EBNA2	EBERs	Latency	BMRF1
PI									
3S	4	F	0	N/D	-	-	+	LI	-
4S	12	M	1840	1/2	+	-	-	LII	-
5S	3	M	143000	1	-	-	+	LI	-
10S	10	F	0	N/D	-	-	+	LI	-
14S	10	F	0	N/D	-	-	+	LI	-
15S	6	F	0	N/D	+	-	+	LII	-
26S	5	F	41000	1	+	-	+	LII	-
28S	2	M	8410	2	-	-	+	LI	-
32S	8	M	0	N/D	+	-	+	LII	-
33S	4	M	1280	1	-	-	-	L0	-
37S	2	M	3860	1	-	-	+	LI	-
40S	3	M	1450	2	+	-	+	LII	-
41S	3	M	120	1	+	-	-	LII	-
42S	5	F	0	N/D	+	-	+	LII	-
52S	3	M	15200	2	-	-	+	LI	-
53S	4	F	14000	1	-	-	+	LI	-
54S	11	F	540	1	-	-	+	LI	-
60S	5	F	1840	1	+	-	+	LII	-
HC									
11S	10	F	4260	2	-	-	+	LI	+
20S	7	M	100	1	-	-	+	LI	-
25S	9	F	0	N/D	+	-	-	LII	+
29S	8	F	140	1	-	-	-	L0	-
31S	2	M	310	1	-	-	+	LI	-
35S	9	F	0	N/D	-	-	-	L0	-
34S	4	M	240	N/A	-	-	+	LI	-
38S	6	F	28500	1	-	-	+	LI	-
39S	7	F	460	2	-	-	+	LI	-
44S	2	F	22000	1	-	-	+	LI	-
47S	4	M	0	N/D	-	+	+	LIIb	-
56S	6	F	0	N/D	-	+	+	LIIb	-
57S	12	F	950	N/A	+	+	+	LIII	-
58S	8	F	0	N/D	-	-	-	L0	-
59S	15	F	430	2	+	-	+	LI	+
61S	8	M	0	N/D	-	-	-	L0	+
63S	3	F	0	N/D	+	-	+	LII	+
64S	5	F	100	1	-	-	+	LI	-
65S	8	M	0	N/D	-	+	+	LIII	+
66S	2	M	0	N/D	-	+	+	LIIb	-
71S	4	F	570	2	-	-	-	L0	-
72S	4	M	0	N/D	+	+	+	LIII	-
73S	6	M	2880	1	-	+	+	LIII	+
74S	3	F	0	N/D	-	+	+	LIII	-
77S	10	F	0	N/D	-	-	-	L0	-
R									
13S	5	M	290	1	-	-	+	LI	-
19S	8	M	400	1	-	-	+	LI	-
27S	2	M	1250	2	-	+	+	LIII	+
51S	2	M	560	1	-	-	-	L0	-
55S	8	M	220	1	+	-	+	LII	-
62S	5	F	0	N/D	+	-	+	LII	-
69S	3	F	940	2	-	-	+	LI	+
NI									
30S	7	M	0	-	-	-	-	/	-
46S	1	F	0	-	-	-	-	/	-
50S	1	F	0	-	-	-	-	/	-
68S	11	F	0	-	-	-	-	/	-

N/D: Not detectable: EBV typing assay was negative even after performing the semi-nested PCR.
N/A: DNA not available for the test.
Presence of viral antigen was assessed in the complete tissue slide and expressed as positive or negative
L0: Latency 0 profile; LI: Latency I profile; LII: Latency II profile; LIIb: Latency IIb profile; LIII: Latency III profile.

Regarding viral antigen expression, in most cases, EBERs, LMP1 and EBNA2 stained cells were located in the interfollicular zone (IF), except a few cases, where EBERs positive staining was detected in germinal center (GC).

Surprisingly, neither LIII pattern nor cells expressing BMRF1 antigen were observed in children undergoing asymptomatic primary infection. In contrast, we found that full latency antigen repertory (LIII) was proven almost exclusively in HC ($p = 0.0374$, Fisher exact test). Viral load showed no differences when compared among the four latencies patterns ($p > 0.05$, Kruskal–Wallis test), but when LI was compared with LII and LIII grouped together, viral load was significantly higher in LI ($p = 0.028$, Mann–Whitney test). In addition, there were no differences in mean age between the four latencies patterns ($p > 0.05$, Kruskal–Wallis test), which is explained by the lack of age differences between groups of patients.

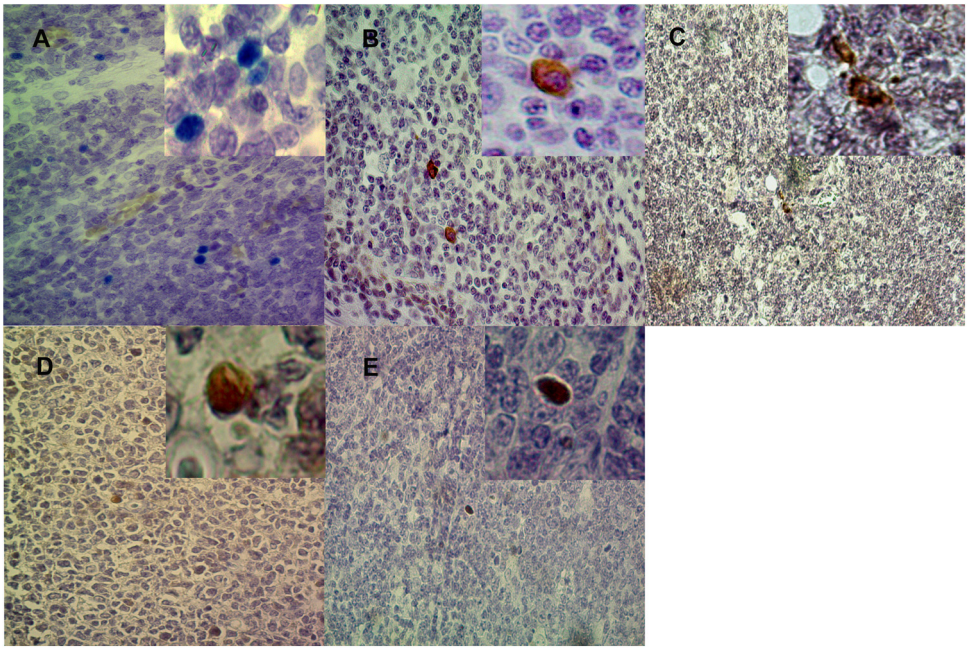

Figure 1. In Situ Hybridization and Immunohistochemistry on FFPE tonsils. (A) EBERs transcripts expressed in cells' nuclei. (B) LMP1 expressed on cells' membrane and cytoplasm. (C) LMP2A expressed in the membrane of the cells. (D) EBNA2 nuclear expression. (E) BMRF1 nuclear expression. Digital images were obtained with an AxioCamErc 5 s (Zeiss) camera and acquired using Digital Axio Vision Rel. 4.8 image acquisition software. Original magnification ×400, insets ×1000.

Table 3
Viral antigen expression.

Group	L0/L I	L II	LIIb/L III*	LC
API	10/18 (55,6)	8/18 (44,4)	0/18 (0)	0/18 (0)
HC	15/25 (60)	2/25 (8)	8/25 (32)	6/25 (24)
R	4/7 (57)	2/7 (29)	1/7 (14)	2/7 (29)
Total	29/50 (58)	12/50 (24)	9/50 (18)	8/50 (16)

*Statistical difference(p = 0,036). Latency I, II, IIb and III patterns and Lytic Cycle antigen expression were defined in each subgroup of patients. The percentages of cases expressing the viral antigens are shown between the parentheses.

EBV typing

EBV is divided in two types, recognized as EBV-1 and EBV-2, which have been distinguished based upon genetic differences in the EBNAs. For this purpose, DNA samples from patients with viral load above the detection limit were selected. Out of 33 patients that met this condition, we had available DNA to be tested from 31: 12 PI, 13 HC and 6 R children. Specific PCR product was proven in 27 patients (age range 2–15 years) in a first round PCR (Figure 2). Nineteen were positive for EBV-1, 7 for EBV-2, and 1 patient was co- infected. When analyzed by serological status, 8 PI patients were positive for EBV-1 and the remaining one was co-infected; in the HC group, 8 were positive for EBV-1 and 5 for EBV-2, and, in the R patients, 3 were positives for EBV-1 and 2 for EBV-2, there were no statistical differences in viral subtypes between groups ($p > 0.05$, X^2 test). To further analyze the remaining 4 patients, we performed a semi-nested PCR and found that the 3 PI children were infected with EBV-2 and the R child was infected with EBV-1 (data not shown).

Given that previous reports (Coleman et al., 2017; Coleman et al., 2018) proposed that the EBV-2 could infect T cells in younger patients, we evaluated the results classifying the patients in two groups according to their ages: those younger than 10 years old and those which were 10 years old or older. Of 26 patients that belong to the first group, 19 were EBV-1 and 7 EBV-2. Regarding the 5 older children, 1 was positive for EBV-1, 3 for EBV-2 and 1 was co-infected. Consistent with these results, EBV1 is statistically

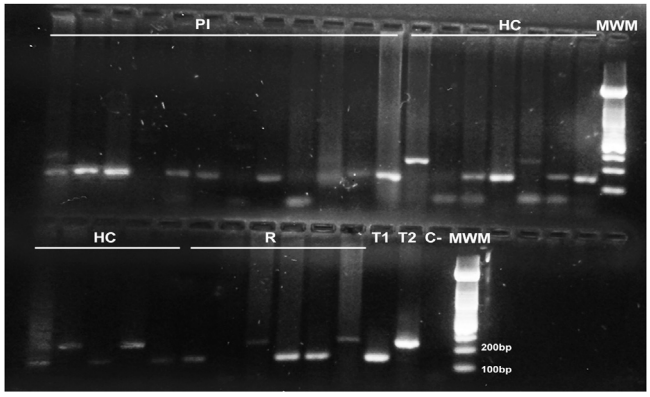

Figure 2. EBV typing by one round PCR from tonsils samples. PI: Asymptomatic primary infected children, HC: Healthy carriers, R: Patients undergoing viral reactivation T1: EBV type 1 positive control (B95.8 cell line) T2: EBV type 2 positive control (P3HR1 cell line) C-: negative control (without DNA input) MWM: molecular weight marker

distributed among younger children ($p = 0.0466$, Fisher exact test). There were no statistical differences between patients infected with EBV-1 or EBV-2 neither in mean viral load, mean age ($P > 0.05$, Mann–Whitney) nor latency profiles ($P > 0.05$, Fisher exact test).

Discussion

EBV primary infection was mostly characterized in adult patients with infectious mononucleosis, whereas little is known about viral load, latent and lytic gene expression at the site of viral entry and reactivation in children undergoing primary infection (Vistarop et al., 2016). Our approach is the first that identifies differential stages of asymptomatic viral infection in a significantly large cohort of pediatric patients outside Africa. In Argentina, children acquire the infection in the first few years of life, and seroconversion is often seen by ages 3–4 years, whereas infection

in developed countries is often delayed until adolescence (Chabay and Preciado, 2013). In this cohort, EBV serology was performed in order to differentiate patients undergoing EBV asymptomatic primary infection (PI), healthy carriers (HC) and children undergoing reactivation of viral infection(R). As far as we know, EBV characterization in this last group was never performed.

Surprisingly, in our series, even though a lower age for children with primary infection was expected, no differences in age among the four groups were demonstrated. Also, the PI, HC and R groups displayed a median age of 4, 6 and 5 years respectively, showing the fact that children, in our population, are infected at a very early age, in line with previous results in children from Africa (Jayasooriya et al., 2015; Piriou et al., 2012). This fact might reflect a combination of age of infection and developing environment, given than in developed populations, the age of primary infection increases (Balfour et al., 2015). Additionally, the low age difference between children undergoing viral reactivation and PI or HC may point out that this event occurs in a relatively short period after the primary infection.

Previously, a higher EBV viral load in plasma among pediatric patients with recent asymptomatic infection has been reported, as well as among patients undergoing IM (Jud et al., 2017), and even in some cases including young adults with no IM symptoms. In contrast, our study revealed that, even though viral load was assessed in tonsils, and it was statistically higher between PI children, all of the infected patients presented lower viral load. These results point out that low viral load observed in this work at the site of viral entry and reactivation, the tonsils, may be responsible, at least in part, for asymptomatic infection characteristics. Furthermore, our findings are similar to those of Seishima et al. (2017), in which the authors used fresh tissue samples from adults and pediatric patients to determine viral load, that was lower that the viral load of our cohort (median 57 copies/ug of DNA).

Regarding the expression of viral proteins, numerous studies reported that, in post-transplanted and IM patients' tonsils, most of the latency antigens were found in the extrafollicular zones with exception of a few cases that expressed EBERs transcripts in the GC (Kurth et al., 2003; Meru et al., 2001; Niedobitek et al., 1997; Roughan et al., 2010). In addition, the frequency of EBV-infected GC B cells in normal persistently infected individuals is very low (Roughan et al., 2010). In contrast, Barros et al described that around 50% of cells were infected by EBV in 16 patients with IM (Barros et al., 2019). In our series, only a few cells located at the GC were infected by EBV as well, without differences among the three groups.

EBV latent protein expression in tonsils so far rendered conflicting results, since in some studies EBV serological status is unknown. LMP1 expression in tonsillar B cells was previously described in 20 EBV infected patients randomly chosen, along with EBNA2 presence in a few cases (Hudnall et al., 2005). In contrast, EBNA2 expression was not described in 6 patients with tonsillectomy (Babcock and Thorley-Lawson, 2000). In our group, an unexpected prevalence of LI and LII was demonstrated in PI patients, which differ with most previous findings in adult patients undergoing primary infection, where the virus displayed the full LIII pattern (Kurth et al., 2000; Thorley-Lawson and Gross, 2004). It was demonstrated that six cases displayed an alternative latency pattern that expressed EBNA2 without LMP1 (Kurth et al., 2003). This LIIb form of latency was proposed as the first latency pattern observed following primary infection of B cells, characterized by EBNA2+/LMP1- gene expression phenotype. Recent work has demonstrated that this latent gene expression state is observed after EBV infection for approximately two weeks before transitioning to the full LIII state in LCLs (Price and Luftig, 2015). Furthermore, we only found LIII in HC, where EBV already established a persistent infection, questioning the previously reported L0 pattern of infection in those patients (Thorley-Lawson et al., 2013).The presence of LIIb and LIII pattern in R and HC, along with the expression of lytic antigens in both groups, may be due to newly infected tonsillar B cells by recently produced viral particles at this histological region. The lack of full latent gene expression in PI children may also explain the asymptomatic infection in this group, reinforced by the absence of lytic antigen expression, as previously observed (Niedobitek et al., 2000). This fact might denote that LC does not play a central role in primary infection (Coleman et al., 2017).

A high viral load in blood and in the oral cavity was described in adults with infectious mononucleosis (Abbott et al., 2017) associated with the LIII pattern, that includes EBERs transcripts, along with LMP1 and EBNA2 latent proteins (Young and Rickinson, 2004). However, when mean viral load was compared between the L0/LI cases and LII/LIII ones, we found that children with higher viral load were the ones that expressed the L0/LI pattern. This was not unexpected, given the fact that most of LI expressing cases were PI children. These findings reinforce our hypothesis that asymptomatic primary infection in children presents differences with IM in adults and adolescents.

It was previously reported, in a cohort of patients from a developing country, that the coexistence of the two EBV types (1 and 2) is possible in those infants and, additionally, EBV type 2 was associated with younger children, in particular on T cells, while type 1 prevailed in B lymphocytes in older children from Kenya (Coleman et al., 2018). In our territory, the circulation of EBV type 2 is lower with respect to EBV type 1, as previously described by our group (Chabay and Preciado, 2013). However, in contrast to previous reports in Kenya, EBV type 1 was prevalent in younger patients, whereas type 2 was associated with the older ones. In addition, even though a statistical difference was not proven, EBV-1 prevailed in PI patients. Coleman et al suggested that EBV-2 may establish a prolonged transient infection in the T cells subsequent to primary infection, that is lost with age (Coleman et al., 2018). Quite the opposite, our findings indicate that EBV type 1 infection in PI children is prevalent, suggesting that EBV-2 may appear in subsequent reinfections at older ages.

This work presents the comparative characteristics of the EBV infection among PI, HC and R patients in our population, at the site of viral entry and reactivation. These findings are remarkable in comparison with those previously described in cohorts from either developed or developing countries, and may explain, at least in part, the asymptomatic viral infection in children from an underdeveloped region, given that the establishment of the viral persistence may be associated with the low viral inoculum and the lack of latency antigen expression in early stages of the primary infection. In addition, our results also challenge the typical LIII pattern described in patients undergoing primary infection.

Materials & methods

Ethics statement

All samples were collected after written consent (patients older than 12 years old and legal guardians of children younger than 12 years old) and assent (7 to 12 years old patients and legal guardians of children older than 12 years old) was obtained; following the national and international ethics standards and under the supervision of the Ethical Committee of the Ricardo Gutiérrez Children's Hospital, in accordance to the Helsinki Declaration of 1975.

Patients and samples

Formalin fixed paraffin embedded (FFPE), −70 °C frozen tonsils tissue and blood samples were collected from 54 children aged

between 1 and 15 years (median 5 years) undergoing tonsillectomy due to non-reactive hyperplasia at the Otorhinolaryngology Division, Ricardo Gutierrez Children's Hospital (Buenos Aires, Argentina). Tonsillar hyperplasia was diagnosed according to international routine protocols for recurrent chronic inflammation. Tonsils were not acutely swollen at the time of removal.

Serological status

Blood samples were incubated 1 hour at 37 °C, and then centrifuged at 3500 rpm for 5 min. The serum was collected and frozen at −20 °C. Serological status was assessed by the presence of VCA-IgM, VCA-IgG titration (diluted 1/10, 1/40 and 1/320), presence of Early Antigen (EA)-IgG and EBNA1-IgG, as previously described (Klutts et al., 2009).

DNA extraction and viral load

In order to assess EBV viral load specifically at the tonsil samples, DNA was extracted from fresh tissue samples using commercial columns (QIAGEN) according to manufacturer's instructions. The extraction product was quantified using NanoDrop One (Thermo Fisher) and its integrity checked by a PCR assay for human ß-globin. The samples were diluted so a total of 100 ng of DNA were used in the qPCR assay.

Viral load was assessed through quantitative PCR in the StepOne (Applied Biosystems) using TaqMan probe (ROX). As previously described (Fellner et al., 2016), this assay amplifies a single copy viral gene, EBNA1.

EBV Typing

PCR directed against EBNA3C gene, which exhibits a deletion in EBV type 1 in comparison with type 2, was performed. This difference in length generates a PCR product for EBV type 1 shorter (157 bp) than that observed in EBV type 2 (246 bp). The PCR assay was performed according to Sample et al. (1990) and, in cases that could not be typified, we performed a semi nested PCR using in the first round a reverse primer against a farther region in the EBNA3C (Rv: 5'-AGCAGTAGCTTGGGAACACC-3') and, in the second round, the primers previously described (Sample et al., 1990).

EBERs In-Situ hybridization *(ISH) & Immunohistochemistry (IHC)*

To determine viral latency, we performed *in-situ hybridization* with ViewRNA ISH Tissue 1-Plex Assay and specific probes (Affymetrix) to detect the presence of EBERs transcripts according manufacturer protocol. Immunohistochemistry (IHC) was performed to detect and localize EBV latent and lytic protein expression, using the following antibodies: mouse Anti-LMP1 (Dako), rat Anti-LMP2A (ABCAM), rat Anti-EBNA2 (supernatant, R3 clone, Kremmer) and mouse Anti-BMRF1 (ABCAM), as previously described (Cohen et al., 2013). The positive controls were performed in FFPE EBV + cell lines Raji (for EBNA2), P3HR1 treated with TPA (12-O-tetradecanoylphorbol-13-acetate, Sigma) to stimulate lytic infection (for BMRF1), FFPE EBV positive diffuse large B-cell lymphoma (for EBERs) and Hodgkin Lymphoma (for LMP1 and 2A) (Figure S1). As negative controls, we performed the same method without the primary antibody. In all cases, we analyze the whole section to assess the presence or absence of immunohistochemical staining.

Statistical analysis

The data was analyzed using GraphPad Prism 5 software. Group normality was tested using Shapiro–Wilks test. Comparison between groups was assessed by 1-way ANOVA or Kruskal–Wallis test according to the normality test results, and correlations were tested using Spearman test. Categorical variables were analyzed with Fisher exact test. Outliers were defined using Robust test to compare data median absolute deviation (Mad) in Excel. All tests were two-tailed, and $p < 0.05$ was considered statistically significant.

Conflict of interest

The authors state that they do not have any conflict of interest regarding this study.

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Author contributions

Conceptualization, Paola A. Chabay, Natalia M FerressiniGerpe. and Aldana G Vistarop.; Methodology, Natalia M FerressiniGerpe and Aldana G Vistarop.; Formal Analysis, Natalia M Ferressini-Gerpe.; Investigation, Natalia M. FerressiniGerpe, Adana G. Vistarop and Agustina Moyano; Resources, Paola A. Chabay and Elena De Matteo.; Data Curation, Natalia M FerressiniGerpe.; Writing-Original Draft Preparation, Natalaia M FerressiniGerpe, Aldana G Vistarop and Paola A Chabay.; Writing-Review & Editing, Paola A Chabay and María Victoria Preciado.; Supervision, Paola A. Chabay.; Project Administration, Paola A Chabay.; Funding Acquisition, Paola A Chabay.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2020.01.044.

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