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Prevalence of Salivary Human Herpesviruses in Pediatric Multiple Sclerosis Cases and Controls

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Abstract

Background—Multiple sclerosis (MS) is a multifactorial disease of unknown origin. The current paradigm is that disease develops in genetically susceptible individuals, influenced by environmental factors. Epstein-Barr virus (EBV) and Human Herpesvirus 6 (HHV-6) have particularly strong associations with the disease. Both viruses are typically acquired during childhood, decades before MS presents. However, in patients with pediatric MS, the temporal window between viral acquisition and disease onset is shortened, which may provide insights into the association of herpesviruses with MS.

Objectives—To compare the frequency of EBV and HHV-6 in the saliva of a cohort of pediatric MS patients and age-matched controls.

Methods—The study enrolled 32 MS pediatric patients and 42 controls, and evaluated saliva for HHV-6 *u57* and EBV *lmp-1* amplification by ddPCR.

Results—MS pediatric patients did not differ from controls in the frequency or magnitude of salivary viral shedding. During the assessment of EBV positivity, distinct profiles emerged that correlated with target amplicon mutations.

Conclusions—None of these mutations were evident in EBV positive samples from MS pediatric patients, whereas they were present in pediatric controls, in addition to MS and control adults, suggesting differential host immune control of EBV in this pediatric MS cohort.

Keywords

Multiple Sclerosis; Pediatric Multiple Sclerosis; Epstein-Barr Virus; Human Herpesvirus 6; herpesvirus; saliva; ddPCR

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Introduction

Multiple sclerosis (MS) is an inflammatory, demyelinating disease that affects the central nervous system (CNS). Though the disease typically presents in young adulthood, approximately 2% to 5% of patients experience symptom onset before 18 years of age{Duquette, 1987 #170;Ghezzi, 1997 #171;Ghezzi, 1997 #3235;Ghezzi, 1997 #3235}, termed pediatric MS[1]. Pediatric MS typically follows a relapsing-remitting course, with a significantly higher annual relapse rate early in the disease course compared to adult-onset MS[2]. Several clinical features are more common among pediatric MS patients, such as a history of preceding infection, cognitive problems, seizures, and brainstem or cerebellar involvement[3]. MS diagnosis in a pediatric population is complicated by other polysymptomatic demyelinating disorders, such as acute disseminated encephalomyelitis (ADEM); approximately 30% of children presenting with an acute demyelinating disorder are eventually diagnosed with pediatric MS[4].

MS is a multifactorial disease, which likely requires both a genetic predisposition and environmental trigger(s) for disease onset. Many molecular and epidemiological studies have identified genetic and environmental factors associated with MS development; viruses have long been considered one possible environmental trigger[5]. Though many studies have examined the association between viral infections and MS risk[6], the mechanisms underlying this association remain unclear. Studying pediatric MS patients provides a singular opportunity to examine the role of environmental factors in disease onset, particularly herpesviruses that are acquired at a young age, as the window between acquisition/exposure and disease onset is much shortened compared to adult-onset MS.

Among the human herpesviruses, EBV and HHV-6 have particularly strong associations with MS risk. At least 90% of the global adult population is seropositive for both viruses, and the vast majority remain asymptomatic after primary infection[7,8]. Only a small subset develop HHV-6 or EBV-associated diseases, which for EBV include infectious mononucleosis (IM) and several forms of cancer[9,10], and for HHV-6 include roseola infantum[11] and encephalitis[12].

Epidemiological studies report a low MS prevalence in countries with an almost universal early acquisition of EBV, and a higher MS prevalence in countries with delayed EBV acquisition presenting as IM[13]. Moreover, studies have noted increased EBV antibody titers in individuals prior to MS onset[14,15]. EBV infection is often asymptomatic in childhood, and more commonly manifests as IM when acquisition is delayed to adolescence or adulthood[16]. As individuals with a history of IM have higher serum concentrations of anti-EBNA antibodies, the risk factors of a history of IM and higher anti-EBV antibodies may be interrelated[17].

There are two distinct species of the ubiquitous human herpesvirus 6, HHV-6A and HHV-6B. HHV-6B is the etiologic agent of the self-limiting childhood illness roseola infantum[11], while HHV-6A has not been definitively linked to any disorder. Both HHV-6A and HHV-6B have been associated with MS, though only a subset of studies has distinguished between the two viruses. Elevated HHV-6 IgG titers have been reported in MS

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patients[18,19], and multiple studies have linked increased titers to clinically active disease. Recently, a large scale study revealed increased IgG levels to an immediate early protein of HHV-6A in both pre-symptomatic and established MS cases compared to controls[20]. HHV-6 viral nucleic acids have also been detected at elevated levels in MS brains, specifically in demyelinated plaques[21].

The pediatric-onset form of MS presents a unique opportunity to study environmental factors associated with the adult-onset form, as there is a much-shortened window of exposure compared to adults. This is particularly relevant for environmental exposures that occur during childhood, such as infection with EBV and HHV-6. There is some suggestion in the literature that pediatric MS may also be associated with human herpesviruses. Similar to findings in adults, multiple groups report increased EBNA antibody titers among pediatric MS patients compared to controls[22,23]. HHV-6 in pediatric MS patients is less well studied; one study reported no difference in the frequency of HHV-6 DNA detection in pediatric MS saliva compared to controls[24].

The present study examined EBV and HHV-6 viral loads in the saliva of pediatric MS patients and age-matched healthy controls utilizing an advanced nucleic acid amplification technology called droplet digital PCR (ddPCR). We compared our previously generated data on HHV-6[25] and EBV viral loads in the saliva of adult MS patients and controls to the pediatric cohorts of the current study. Compared to peripheral blood, saliva contains high viral loads of both EBV and HHV-6[26,27] and is relatively easy to collect from pediatric populations, rendering it an ideal sample type for this study.

Materials and Methods

Study Design

This study was a retrospective cross-sectional study comparing salivary human herpesviruses in MS pediatric patients with controls. All pediatric samples were obtained from the neurology clinic of the University of California at San Francisco (UCSF) Hospital under an institutional review board-approved protocol. Pediatric saliva specimens were shipped to the Viral Immunology Branch at NIH for DNA extraction and ddPCR. Previously published data from MS and control adult cohorts are included[25]. Adult samples were obtained from the NIH Neuroimmunology clinic, under an institutional review board-approved protocol.

Study participants

Pediatric cohorts—Table 1 contains demographic information for the UCSF pediatric cohorts. Controls were excluded if they were 20 years of age or older, known to have MS or another demyelinating disease, had an immediate, biological family (parent/sibling) diagnosed with MS, or had a chronic neurological condition with major disability. A subset of patients was on disease-modifying therapy at the time of saliva collection. There were no significant differences in EBV serostatus between the patient and control cohorts (Table 1).

Adult cohorts—Demographic information about the 59 adult MS patients and 39 healthy adult controls analyzed for HHV-6 has been previously published[25]. None of the controls

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were on treatment or diagnosed with a neurological disorder at the time of saliva collection. *A subset of these patients and controls were used in the evaluation of EBV viral load.*

Pediatric sample collection and processing

Saliva collection was performed as previously described by Hadinoto and colleagues[28]. Briefly, subjects rinsed their mouths, spit and gargled with 5ml purified water, which was frozen at −80°C until use. A volume of 200μl was used for DNA extraction, performed with the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's specifications, and eluted in 100μl buffer AE.

Serologic and PCR assays

EBV serology was performed as previously described[29]. The EBV *lmp-1* and HHV-6 *u57* primer/probe sequences and details of the ddPCR procedure have been previously published[30]. A specimen was considered positive if at least two positive droplets appeared at the expected fluorescence amplitude. One positive droplet represents the lower limit of detection, which in our experience with saliva DNA corresponds to approximately 300 viral copies/ml saliva[31]. Samples with one positive droplet were confirmed by an additional ddPCR run. A housekeeping gene, *rpp30*, was included for each sample. Data are expressed as viral copies per ml saliva.

ddPCR lmp-1 amplicon sequencing

The EBV *lmp-1* amplicon (65 bp) was sub-cloned into a TOPO TA vector (Invitrogen) prior to sequencing. Sequencing was performed by Genewiz using standard M13 forward and reverse primers. Sequence data were analyzed using MacVector software.

Statistical analysis

Chi-squared and McNemar tests compared the frequencies among the demographics (Table 1) and for viral positivity between the cohorts. Fisher's Exact test analyzed any frequency below five cases after stratification by disease status and age group. A non-parametric Mann-Whitney test compared viral load means between MS patients and controls. Data were analyzed using RStudio software version 0.99.486 (RStudio, Inc.) and Prism 6 (GraphPad Software, Inc.). All p-values reported are two-tailed, and p-values smaller than alpha=5% are deemed significant.

Results

Salivary HHV-6 and EBV in pediatric MS patients and controls

All saliva specimens were evaluated by ddPCR for the *lmp-1* region of EBV and the *u57* region of both species of HHV-6 (HHV-6A and HHV-6B). The HHV-6 primers and probes were designed to amplify both viral species with comparable kinetics; the forward and reverse primers are shared, while a three nucleotide difference between probe sequences distinguishes HHV-6A from HHV-6B with high specificity[25].

Among the pediatric MS cohort, 8/32 saliva samples (25%) were positive for EBV. This positivity frequency did not differ from the pediatric controls, as 12/42 (29%) were positive

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for EBV (p=0.7) (Figure 1A). Similar to EBV, the frequency of HHV-6 positivity in saliva did not differ between MS patients and controls (50% versus 52%, respectively, p=0.8). The majority of the HHV-6 positive samples were HHV-6B: 88% of the HHV-6 positive MS samples, and 96% of the HHV-6 positive control samples. This is consistent with our prior observations in adult saliva samples[25].

HHV-6 was detected almost twice as frequently as EBV (50% versus 25% and 52% versus 29%); this difference was significant for both pediatric patients (p=0.04) and pediatric controls (p=0.03) (Figure 1A). Though the frequency of HHV-6 detection was significantly elevated compared to EBV, the viral load means were not different (Figure 1B). Comparing across the pediatric MS and control cohorts, there were no viral load mean differences for either EBV or HHV-6.

In the pediatric MS cohort, two samples showed particularly high levels of HHV-6, each with approximately 100,000 copies per ml saliva. One sample was HHV-6A positive, and the other was HHV-6B positive (Figure 1B). Both HHV-6A and HHV-6B can integrate into host chromosomes, at a frequency of approximately 1/100, leading to high viral loads in all biological samples[32]. We therefore tested these two individuals with unusually high HHV-6 viral loads for chromosomally integrated HHV-6 (ciHHV-6). As ciHHV-6 detection is most accurate from cell-rich biological samples, PBMC DNA was obtained from each individual, and viral load was quantified as a function of copies per cell. From the individual with the high HHV-6B salivary load, HHV-6 was undetected in PBMC. However, from the individual with the high HHV-6A salivary load, HHV-6A was detected at 0.998 copies per cell in PBMC, indicative of chromosomal integration (data not shown). Therefore, the high salivary level of one patient likely reflects active viral shedding, while the high salivary level of the other patient likely reflects chromosomal integration, underscoring the importance of testing for HHV-6 chromosomal integration in individuals with high viral loads.

Higher HHV-6 viral loads in adult versus pediatric cohorts

As shown in Figure 2A, there were no significant differences in salivary EBV viral loads between pediatric and adult cohorts, stratified by disease status. However, there is a trend for higher viral loads in the adult compared to the pediatric cohorts.

By contrast, the HHV-6 viral loads were significantly lower in both MS and control pediatric cohorts compared to the respective adult cohorts[25] (Figure 2B). The adult MS cohort averaged 18,000 copies/ml saliva, which was approximately three-fold higher than the MS pediatric cohort (p=0.01) (Figure 2B). The adult control cohort averaged 13,000 copies/ml saliva, which was approximately 18-fold higher than the pediatric control cohort (p=0.001) (Figure 2B).

Mutations identified in EBV Imp-1 probe binding region

Figure 3A shows a representative two-dimensional ddPCR plot of an EBV-positive saliva sample. The lower left quadrant contains negative droplets, while the lower right quadrant contains droplets positive for the cellular housekeeping gene, *rpp30*. The upper left quadrant contains droplets positive for EBV *Imp-1*, with a fluorescence amplitude of approximately 8,000. In this study, a subset of pediatric saliva samples was positive for EBV at a lower

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fluorescence amplitude, approximately 4,000, as shown in Figure 3B. Such amplitude differences were not observed for HHV-6 positive saliva samples.

Prior studies from our lab have demonstrated that a lower than expected ddPCR fluorescence amplitude may indicate mutations in the target sequence that affects the affinity of the fluorescent probe[33]. We therefore sequenced the *Imp-1* amplicon from a subset of MS saliva samples, along with an EBV B95.8 transformed cell line as reference sequence for an expected fluorescence amplitude. The *Imp-1* amplicon sequence of a saliva sample with a high amplitude EBV population did not differ from this reference sequence (Figure 3A). However, the *Imp-1* amplicon sequence of a saliva sample with a low amplitude EBV population differed in the probe binding region (Figure 3B). The saliva sample with a low fluorescence amplitude contains a point mutation in the probe binding region (indicated in red, Figure 3B).

Reduced frequency of EBV Imp-1 amplicon mutations in pediatric MS patients

Figure 4 shows the frequency of low amplitude (mutated) EBV *Imp-1* as a proportion of the total EBV positive samples within each cohort. Low amplitude populations were detected in approximately two thirds of the adult samples, with no differences between the MS and control groups. The high prevalence of low amplitude EBV in the adult cohorts suggests that mutations in this region of the virus are common. Indeed, *Imp-1* is highly polymorphic, and *Imp-1* nucleotide diversity has been used to define viral variants[34].

Among the pediatric cohorts, low amplitude populations were detected at comparatively reduced frequencies: 5/12 (42%) in the controls and undetected among the MS patients (0/8 (0%)). There was no apparent relationship between EBV viral load and fluorescence amplitude; samples with reduced fluorescence were evenly distributed about the mean (data not shown). No EBV positive samples with mixed amplitude populations were identified.

Discussion

Despite the multitude of studies associating herpesviruses with adult-MS onset and exacerbation, only a few have examined herpesviruses in pediatric MS. Several groups have reported elevated EBV seropositivity in pediatric MS patients[22,23,35], which is reminiscent of data in adult onset MS. These data suggest that EBV, or the immune response to EBV, may be related to MS pathogenesis irrespective of the age of disease onset.

The present study examined EBV and HHV-6 viral DNA levels in saliva samples from 32 pediatric MS patients and 42 age-matched healthy controls. Unlike other reported cohorts, there was no difference in EBV seropositivity between the pediatric MS patients and age matched controls (p=0.2) (Table 1).

No differences in the frequency or average EBV or HHV-6 salivary viral loads were observed when pediatric MS patients were compared with controls. We observed significantly lower levels of HHV-6 in pediatric compared to adult cohorts, suggesting that viral levels may increase with age, perhaps due to re-infection and/or host factors controlling

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viral replication. Though non-significant, there was a similar trend for EBV, which was higher in the adult cohorts than the pediatric cohorts.

In a similar study by Yea and colleagues published several years earlier, pediatric MS patients were found to have higher rates of salivary EBV compared to controls[24]. This difference is perhaps explained by differences in EBV seropositivity between the cohorts; Yea and colleagues reported a two-fold increased EBV seropositivity in their MS patient cohort, whereas there was no such increase in the MS cohort of the present study (Table 1), perhaps reflecting the small sample size of this pilot study.

To understand whether treatment had any effect on salivary viral frequency and loads, we compared treated and untreated patients, but observed no difference for either virus. Several of the disease modifying treatments are forms of interferon, which are known to have anti-viral properties. However, when treated MS pediatric patients were divided into interferon and non-interferon treatments, there were no differences in detection frequency or viral loads for either virus (data not shown).

This study utilized droplet digital PCR (ddPCR) to assess salivary viral loads. In ddPCR, the fluorescence amplitude of a probe reflects its affinity for the target DNA. During the assessment of EBV positivity by digital PCR, distinct fluorescence profiles emerged. The profile with a reduced fluorescent amplitude suggested a variation in probe affinity, which corresponded to mutations in the fluorescent probe-binding region of the target gene *lmp-1*. Our lab has previously correlated reduced fluorescent amplitudes with mutations in the target sequence[33].

EBV *lmp-1* amplicon mutations were present at relatively high frequencies among saliva samples from both adult cohorts, suggesting that EBV *lmp-1* heterogeneity is a normal occurrence, and perhaps common among the general population. The comparatively reduced frequency of these variants in the pediatric control cohort suggests that the extent of viral sequence variation may increase with host age, perhaps reflecting mutations that occur during viral replication, and/or viral adaptation to pressure by the host immune system.

As we become exposed to a wider array of foreign pathogens during aging, maturation and diversification of the host immune system occurs. Pathogens may therefore be under increasing pressure to evade the host immune response. Point mutations, as observed in this study in the EBV *lmp-1* amplicon, are one mechanism that may contribute to viral diversity.

Indeed, a narrow window of environmental exposure in the pediatric population may contribute to reduced host immune pressures that result in less viral diversity and consequently, a lower frequency of mutated EBV. Interestingly, none of these mutations were evident in EBV positive pediatric MS patient saliva, suggesting that individuals in this pediatric MS cohort may differ in viral replication kinetics or host immune regulation of the virus compared to age-matched controls.

Though the numbers of analyzed samples are small, the absence of mutated EBV in EBV positive pediatric MS patients suggests differential (defective?) host immune control of EBV in this particular cohort. This observation should be extended to larger pediatric and adult

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cohorts, with more comprehensive sequencing of EBV, and perhaps other herpesviruses that have been associated with adult onset MS.

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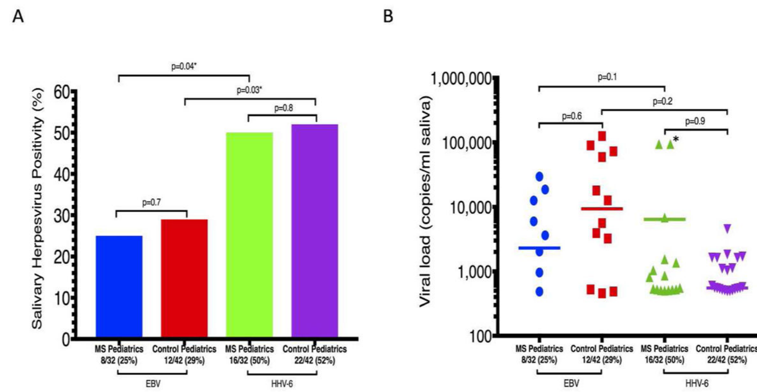

Figure 1. Comparable levels of salivary herpesviruses in a cross-sectional study of pediatric MS patients and controls

(A) Frequency of EBV and HHV-6 viral detection in saliva specimens from MS and control pediatric cohorts. (B) Distribution of EBV and HHV-6 viral loads in saliva specimens from MS and control pediatric cohorts. Viral loads are expressed as viral copies per ml saliva. Only virus positive saliva samples are shown. Statistical significance is defined as $p<0.05$ C.I. 95% two-tailed based on chi-square and non-parametric t-tests. The asterisk indicates that this individual has HHV-6A chromosomal integration.

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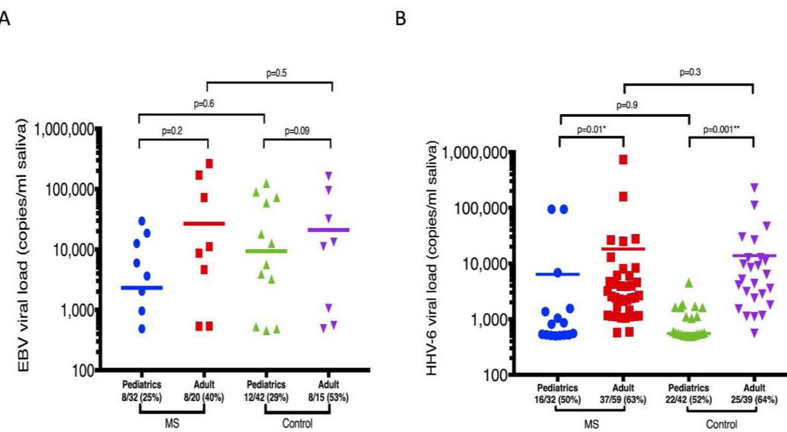

Figure 2. Pediatric saliva contains significantly lower levels of HHV-6, and equivalent levels of EBV, compared to adult saliva

(A) The average EBV viral load per ml saliva for the pediatric patients is 2.3×10^3 copies, compared to 2.7×10^4 copies for the adult patients. The average viral load per ml saliva for the pediatric controls is 9.3×10^3 copies/ml, compared to 2.1×10^4 copies for the adult controls. (B) The average HHV-6B viral load per ml saliva for the pediatric patients is 6×10^3 copies, compared to 1.8×10^4 copies for the adult patients. The average viral load per ml saliva for the pediatric controls is 5.5×10^2 copies/ml, compared to 1.3×10^4 copies for the adult controls. Statistical significance is defined as $p<0.05$ C.I. 95% two-tailed based on non-parametric t-test.

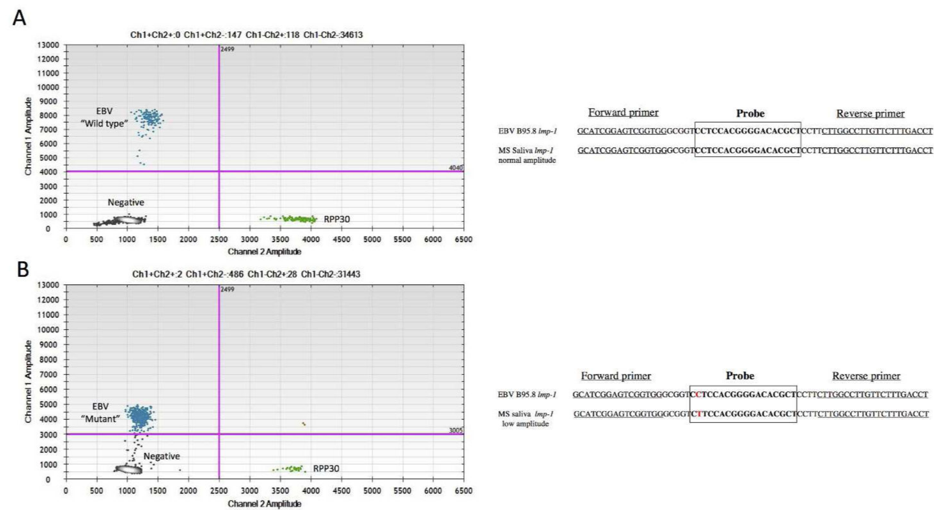

Figure 3. DdPCR detection of a low amplitude EBV positive population corresponds to a mutation in the target gene sequence

(A) EBV *lmp-1* positive droplets normally fluoresce around 8,000 (Channel 1 amplitude). The sequence of this amplicon from a patient with a 'wild type' EBV positive droplet population shows complete alignment with the sequence of an EBV transformed cell line, B95.8. (B) EBV *lmp-1* positive droplets with a lower fluorescence (around 4,500, Channel 1 amplitude). The sequence of this amplicon from a patient with a lower amplitude 'mutant' EBV positive droplet population reveals a single substitution mutation in the probe-binding region, compared with the sequence of B95.8. *RPP30* shown in channel 2 of (A) and (B) is a cellular housekeeping gene.

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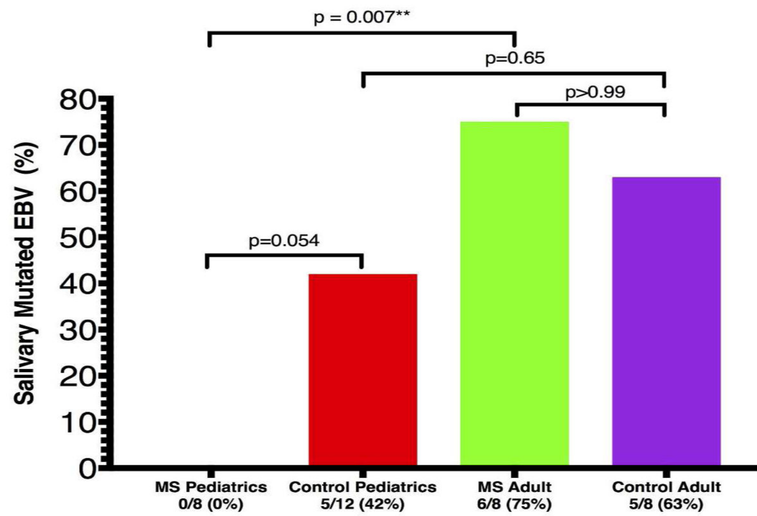

Figure 4. Frequency of low amplitude (mutated) EBV detected in the saliva of MS and control cohorts

Low amplitude EBV is detected at significantly lower frequencies in the saliva of both pediatric cohorts compared to adults. Statistical significance is defined as $p<0.05$ C.I. 95% two-tailed.

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Table 1

Demographic information for pediatric MS patients and age-matched controls

	Pediatric MS patients (n=32)	**Pediatric controls (n=42)**	
Age (year) ± SD	13.8±3.9	13.8±2.8	p=0.4
Sex	17 females 15 males	22 females 20 males	p=0.9
Disease-modifying therapy	Copaxone (n=9) Cytoxan (n=1) Interferon (n=7) Tysabri (n=1)	N/A	
Diagnosis	Confirmed MS	Juvenile arthritis (n=1) Type I diabetes (n=1)	
EBV serology	23/32 (72%) seropositive 1/32 (3%) undetermined	24/42 (57%) seropositive 2/42 (5%) undetermined	p=0.2 p>0.99