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EPSTEIN BARR VIRUS SHEDDING IN MULTIPLE SCLEROSIS: SIMILAR FREQUENCIES OF EBV IN SALIVA ACROSS SEPARATE PATIENT COHORTS

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ABSTRACT

Background:

Epstein Barr Virus (EBV) infection is closely associated with multiple sclerosis (MS), but the relationship between viral load and disease activity is unclear. This study tested the observed levels of salivary EBV in MS, as a first step in investigating this relationship.

Methods:

Real-time quantitative PCR (qPCR) was used to measure EBV DNA levels in saliva samples from three separate Multiple Sclerosis (MS) patient cohorts.

Results:

The qPCR assay was used to delineate EBV shedding, defined here as a reliably detectable level of extracellular EBV DNA in saliva. Frequency of EBV shedding was found to be similar across the groups, with 20-25% of subjects releasing virus on any given sampling date. Diurnal variation in EBV count was tested in one of the cohorts, in which 26% of subjects showed more than a 10-fold difference between the highest and lowest EBV levels on a single day. In the same cohort, elevated viral levels at one time point did not predict elevated viral levels at a subsequent time point.

Conclusions:

These results indicate that EBV lytic activity in a subject cannot be inferred from a single measure of EBV in saliva. Also, subjects do not appear to be behave constantly as "EBV shedders" or "nonshedders". The assay is useful in giving a clear indication of salivary gland EBV lytic activity across a patient cohort – for example, in testing anti-viral drugs in MS.

BACKGROUND

Epstein Barr Virus (EBV) infection is the virus most strongly associated with multiple sclerosis (MS) (Christensen, 2006; Pender & Burrows, 2014), with almost all MS patients (MSers) found to be seropositive for EBV (Bray, Bloomer, Salmon, Bagley, & Larsen, 1983; Wandinger et al., 2000).

Evidence for a pathogenic role of EBV in MS is supported by elevated EBV antibody titres in MS patient cohorts (Farrell et al., 2009; Latham et al., 2016). In one such study (Wandinger et al 2000), antibody response to EBV (IgG) early antigen (EA) was found in 73% of patients with disease exacerbations and in none of those with clinically stable disease. Early antigen response is a marker of acute viral infection, at which time extensive release of viral particles into saliva (viral shedding) occurs (Hadinoto, Shapiro, Sun, & Thorley-Lawson, 2009). Such particles consist of a lipid envelope containing virally-encoded glycoproteins, a protein capsid and viral DNA (Perera, Samaranayake, & Tsang, 2010). Arguably, increased antibody titres could arguably reflect immune dysregulation (Hunter & Hafler, 2000) and may not be linked temporally to viral reactivation. For this reason, measurement of EBV levels using qPCR may be preferable to antigen response assays as a measure of virus reactivation. One such study showed elevated salivary EBV levels in children with MS (Yea et al., 2013), and a recent investigation found detectable salivary EBV to be 20x more frequent in adults with relapsing MS compared with healthy controls, although this increase in shedding frequency was not found to be statistically significant (Giess et al., 2017). The temporal association between EBV release and MS disease activity measured by magnetic resonance imaging (MRI) is unclear. A recent investigation demonstrated a negative correlation between MRI activity and EBV titres 4-6 weeks before the MRI scan (Latham et al., 2016).

The purpose of the present study was to establish a reliable protocol for defining and quantifying salivary EBV, and then identify the normal patterns of salivary EBV levels in MS. Knowledge of expected levels of salivary EBV in MS would ensure that future studies have sufficient statistical power to identify the relationship, if any, between viral load and disease activity. A clear association between EBV shedding and disease activity would also justify a trial of antiviral agents targeting EBV in MS.

METHODS

1. Data sets were derived from three cohorts as defined below:

INSPIRE (Isentress Pilot Study in Relapsing MS). This comprised 20 patients with active relapsing MS. Samples were taken at monthly intervals over 7 months

ExIMS (Exercise Intervention for MS trial). This consisted of 120 patients with relapsing MS. Sampling was undertaken three times over one year. Four separate samples were taken at 3-hourly intervals on each sampling date.

MEAVIS (Measurement of EBV in Saliva). This consisted of 18 MSers who self-collected weekly samples over 3 months

Use of samples in all three cohorts approved by Ethics committees.

2. Analysis of Samples

All samples were analysed in the same laboratory at the Blizard Institute, Queen Mary University of London.

Approximately 1-2ml of saliva was collected at each sampling interval. DNA was isolated from these samples using standard protocols QiaSymphony (robot) SP Virus/Bacteria Mini-kit (Qiagen, Hilden, Germany) for the INSPIRE samples and Ambion PureLink 96 kit (Thermo Fisher Scientific, Waltham, Mass, USA) for the ExIMS and MEAVIS samples. No adjustment was made to DNA concentration.

The QPCR method was validated as part of the INSPIRE clinical trial, and an identical method applied to all three cohorts. EBV DNA was detected by a Taqman assay targeted to a non-repeated sequence of the EBV polymerase gene (Gallagher et al., 1999) and a pyruvate dehydrogenase (PDH) sequence (Bissett et al., 2011) was included as a reference gene in a multiplex PCR. The assay used a Qiagen QuantiTect Multiplex PCR Kit with a standard protocol (95°C 15 mins; (95°C 60 s, 60°C 60s) x 40 cycles, reaction volumes of 25µl, primer and probe concentrations 0.4µM and 0.1µM respectively). Patient samples were run in duplicate, and a standard curve was included on each PCR plate using a dilution series derived from quantitated EBV DNA (Agilent). The CT (cycle threshold) value was set at the midpoint of the exponential phase of the PCR and normalised across PCR runs to give uniformity across all PCR runs. The assay returned an absolute copy number of EBV per sample (copies per µl of saliva), calculated directly from the standard curve.

The PDH amplification was used to confirm the presence of genomic DNA but was not used to calculate the EBV copy number. A negative amplification of both EBV and PDH may indicate a failed PCR or a failed DNA extraction. Analysis of samples was repeated once if both EBV and PDH failed to amplify. Analysis was also repeated if one of the two EBV replicates failed to amplify, and samples were classed as missing data if this repetition was unsuccessful.

RESULTS

1. Reliability of Assay

An important goal of the study was to establish a reliable definition of EBV shedding. In earlier studies, the presence of EBV in saliva (at levels of as low as 1 copy per □l) has been taken to indicate viral shedding: this assumption was found to be unreliable in our study. Although low copy numbers were detectable with this assay, the

analysis was only found to be fully reproducible (with coefficient of variation <1.4) for EBV levels above 3 copies/µl (equivalent CT value >36).

As cellular remnants are present in saliva, very low titres of EBV DNA sample could be due to the presence of cellular DNA in the samples. For this reason, the presence of cellular (genomic) DNA was measured in the ExIMS cohort using the PDH primer/probe set. No correlation was found between PDH and EBV titres in this cohort (Spearman Rank Correlation, R_s= 0.0457, p=0.33), which suggests that EBV detected in these assays was mainly extracellular and due to active viral shedding.

For the purpose of this assay, shedding was defined as a salivary EBV concentration greater than 5.8 copies/µl: this is equivalent to one standard deviation above the median in the ExIMS dataset. This level is above the minimum reliable detection limit for this assay, and also minimises the effects of cellular remnants in saliva.

2. EBV Shedding in MS: comparison of 3 MS cohorts

Table 1 compares salivary EBV in our 3 populations to help predict the expected level of shedding in a typical MS population.

Population	Patients	Samples	EBV present		Shedding (>5.8 copies/µl)		% of subjects with EBV detected*	
	N	N	N	%	N	%	EBV detected %	Shedding %
INSPIRE	20	87	36	41.4	21	24.1	70.0	45.0
ExIMS	119	280**	107	35.2	64	22.9	68.6	37.3
MEAVIS	18	114	43	37.7	24	21.1	61.1	44.4

Table 1. Number of samples collected per cohort: (sample number, shedding, etc.)

(* subjects with any positive EBV samples during the study)

(** number of sampling dates, in which 4 samples were taken within a 24h period)

The results show that 20-25% of subjects were found to be releasing salivary EBV at >5.8 copies/µl (shedding EBV) on a given date.

3. Temporal patterns of salivary EBV levels in the EXIMs population.

In the ExIMS study, sampling occurred in all months of the year, so it was possible to test seasonal variation in EBV counts. Cold months were assigned lower scores (January = 1, December & February = 2, November & March = 3) and warmer months assigned higher scores (October & April = 4, September & May = 5, August & June = 6, July = 7). No correlation found between these scores and salivary EBV levels taken in these months (r^2 = 0.0038), strongly indicating no seasonal effect.

In the ExIMS population, sampling at four time points on a single day allowed diurnal variation to be measured. Although morning EBV counts were on average >60% higher than afternoon values, in 10% of samples there was more than a 30-fold difference between maximum and minimum EBV counts on a single day. Similarly,

26% of samples showed more than a 10-fold difference between maximum and minimum EBV counts.

4. Are some subjects either "shedders" or "non-shedders"?

This analysis investigated whether the distribution of viral load scores was non-random with respect to subject. As consecutive high scores 1 month apart could relate to a single shedding episode, this was exclusively tested in the ExIMS population in which sample dates were at least 3 months apart.

The null hypothesis was that there was no difference in subsequent shedding behaviour between subjects found to be shedding or not at baseline:

21 subjects were found to be shedding at baseline, of whom 8 were shedding 3 months later.

73 were not shedding at baseline, of which 16 were found to shedding 3 months later.

No significant difference was observed between these groups (Fisher's exact test, $p=0.16$), supporting the null hypothesis: repeat sampling showed that people with MS could not be divided into discrete groups of "shedders" and "non-shedders".

DISCUSSION

a. Methodology:

Previous studies have equated detectable EBV with shedding, but this study found the detection of low levels of salivary EBV to be unreliable. Additionally, as saliva typically contains a cellular fraction, any sample from an EBV positive subject will potentially contain trace levels of intracellular EBV. This study applied a threshold level for EBV shedding for the detection of extracellular EBV across separate cohorts.

b. EBV shedding across separate MS groups:

The percentage of samples (subjects/date) with EBV levels above the defined shedding threshold was found to be very similar across the three cohorts (20-25%). This figure provides an expected frequency of EBV shedding in MS samples, which should be useful in future studies.

 Across the three cohorts, between 37% and 45% of subjects were found to be shedding EBV at some point, which is similar to the result of a previous study in which 41% of subjects were found to be shedding (Hollsberg et al., 2005). An important caveat is that the percentage of subjects found to be shedding will

increase with increased sampling frequency and study duration; therefore this figure should not be used in predicting the necessary sample size in future studies.

c. Variation in EBV counts in individuals:

The present study found that salivary EBV levels in a single subject can vary markedly over short periods; 10% of patient sample sets showed a greater than 30-fold difference between the highest and lowest EBV counts on a given day. In one previous study, similar variation in copy number was observed across samples taken at 3-hourly intervals, although the extent of variation was obscured by reporting EBV levels as log copies per ml – leading to the erroneous (and cited) conclusion that virus shedding is relatively stable over short periods (Hadinoto et al., 2009; Huynh & Rong, 2012).

Data from the ExIMS study showed that for any given subject, EBV copy number at one time-point did not predict the EBV level at a subsequent time point. This echoes the results of a previous study that found the distinction between "high shedders" and "low shedders" was a function of variation within individuals over time, rather than variation between individuals (Hadinoto et al., 2009).

d. Implications for future studies

To date, it has proved difficult to establish a relationship between MS and EBV, given that around 95% of most healthy populations are positive compared to about 99% of MSers (Ascherio & Munger, 2007). The example of clinical poliomyelitis shows that a similarly ubiquitous infectious agent can be largely asymptomatic and yet cause a serious condition in some. Therefore, if EBV is found to have a pathogenic role in MS, it may be possible to prevent it by eliminating EBV infection completely or by blocking the pathway that leads from EBV infection to disease development.

Although relapses were reported by some of our subjects, the comparison of disease activity and EBV copy number was not a primary goal in any of the three studies described here. The logical follow-up to this study is to test whether EBV shedding is related to disease activity across an entire cohort, for example, by comparing salivary EBV levels with MRI activity. If salivary EBV levels are found to be linked to MS disease activity, it would then be important to test whether EBV antiviral drugs can be used to treat or even prevent the disease.

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