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A semiquantitative PCR method (SQ-PCR) to measure Epstein–Barr virus (EBV) load: its application in transplant patients

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Abstract

Background: High Epstein–Barr virus load has been related to an increased risk of Posttransplant Lymphoproliferative Disorders (PTLD) in transplant recipients. *Objectives:* Development of a method to quantitate EBV DNA levels in peripheral blood mononuclear cells (PBMC) and evaluate its usefulness in transplant patients. *Study design:* We designed a semiquantitative nested PCR based on a limiting dilution analysis to detect high viral loads in PBMC. This method was applied to 25 healthy carriers, and 85 solid organ transplant recipients as follows: (A) 53 asymptomatic patients; (B) 24 symptomatic patients; (C) eight patients with PTLD. *Results:* In healthy carriers the reciprocal of the limiting dilution (RLD) ranged between non-detected (ND) and 1, the median RLD was ND, which is equivalent to a viral load of <1 copy per 10^5 PBMC. In the transplant population the medians RLD (range) were: (A) asymptomatic group: ND (ND-64), median equivalent to a viral load of <1 copy per 10^5 PBMC; (B) symptomatic group: 4 (ND-256), median equivalent to a range of viral load of 4–64 copies per 10^5 PBMC. (C) PTLD group: 256 (16–16 384), median equivalent to a range of viral load of 256–4096 copies per 10^5 PBMC. Statistically significant differences were found between all groups: A + B vs. C ($P < 0.0001$); A vs. B ($P < 0.0001$); A vs. C ($P < 0.0001$), B vs. C ($P < 0.0001$). We also observed a good correlation between viral loads and clinical findings in four follow-up patients. Considering the RLD = 256 as a cutoff point to detect transplant patients with PTLD, resulted in sensitivity 75%, specificity 96.7%, positive predictive value 60%, negative predictive value 98.3%. *Conclusion:* This SQ-PCR method enables us to differentiate between transplant patients with and without PTLD; therefore, it could be applied as a marker for early detection of this pathology.

Keywords: Epstein–Barr virus; DNA quantitation; Viral load; PTLD; Transplant patients

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1. Introduction

Epstein–Barr virus (EBV) is a globally spread herpesvirus. It is the causal agent of infectious mononucleosis and is associated with an increasing number of benign and malignant diseases (Rickinson and Kieff, 1996). EBV enters the host through the oropharynx epithelium where it infects B-lymphocytes. These are immortalized and proliferate in lymphoid tissue where changes in viral genomic expression give rise to different latency programs (Faulkner et al., 2000). Infected lymphocytes as resting memory B cells pass into peripheral blood, which is the site of virus persistence (Miyashita et al., 1997; Babcock et al., 1998). Lymphoproliferation is controlled by neutralizing antibodies, NK cells, antibody dependent cell mediated cytotoxicity and, principally, specific cytotoxic lymphocytes (Khanna et al., 1995; Rickinson and Kieff, 1996). Thus, in the immunocompetent host the virus establishes a lifelong silent infection. In transplant patients cellular immune response must be iatrogenically diminished to prevent graft rejection; therefore, EBV can cause posttransplant lymphoproliferative disorders (PTLD). This is a complication of solid and bone marrow transplantation affecting graft and patient survival and involves a wide range of disease states (Nalesnik, 2001) with different clinical presentation and prognosis (Paya et al., 1999). In solid organ transplant patients the incidence varies between 1 and 20% depending on the organ transplanted, type and intensity of immunosuppression, age, and EBV status prior to transplant (Cockfield, 2001); reported mortality ranges between 50 and 80% (Paya et al., 1999).

A number of authors have reported high EBV loads in peripheral blood of transplant patients with PTLD compared to transplant patients without PTLD or healthy EBV carriers (Riddler et al., 1994; Kenagy et al., 1995; Rowe et al., 1997). Moreover, this elevated viral load precedes the development of PTLD (Kenagy et al., 1995; Green et al., 1996). Thus the monitoring of blood viral levels may detect transplant patients at risk of PTLD. This is an important issue because in many cases preemptive therapy may prevent the development of this pathology (Green et al., 1999; Rowe et al., 2001; Green et al., 2001). A decline in EBV levels following PTLD regression and disappearance of clinical symptoms have been reported, suggesting a good response to therapy (Kenagy et al., 1995; Green et al., 1999).

Different strategies have been applied to determine EBV load (Rowe et al., 2001; Stevens et al., 2001): semi-quantitative limiting dilution methods (SQ-PCR) (Allen et al., 2001; Meerbach et al., 2001); competitive methods (QC-PCR) (Rowe et al., 1997; Stevens et al., 1999; Baldanti et al., 2000); real time PCR (Kimura et al., 1999; Niesters et al., 2000; Jabs et al., 2001). QC-PCR and real time methods are more accurate, but SQ-PCR can also detect differences in viral load between transplant patients with and without PTLD (Rowe et al., 2001). SQ-PCR methods are easier to run than QC-PCR and less expensive than real time PCR; they are based on a qualitative end point (all or none) and on the premise that one or more target copies will give a positive signal (Sykes et al., 1992). Usually, a PCR or nested PCR from serial dilutions of a sample and a limiting dilution analysis of the agarose gel are performed.

We describe a SQ-PCR method to measure EBV DNA in peripheral blood and evaluate its usefulness in transplant patients.

2. Materials and methods

2.1. SQ-PCR development

2.1.1. DNA extraction

RAJI cells (ATCC CCL-86) containing an average of 50 copies of EBV genomes per cell (Adams, 1987; Kieff, 1996) were used as positive controls. RAJI cells were lysed in extraction buffer (Tris–ClH, pH 8, 10 mM; Tween-20, 0.45%; Igepal 40, 0.45%; proteinase K, 100 μg/ml) at 56 °C for 1 h; and heated at 95 °C 10 min to inactivate proteinase K. DNA was purified by a phenol–chloroform procedure (Harvard Medical School, 1992) and resuspended in buffer TE. DNA was quantified by spectrophotometry at 260 nm and stored at −80 °C in a concentration of 10^5 copies of EBV genomes per μl. PTP cells (from a human foreskin fibroblast cell line) were extracted,

purified, quantified as described for RAJI cells and stored at −80 °C.

2.1.2. PCR standardization

In healthy carriers the copy number of EBV genomes has been estimated between 0.01 and 0.1 copies per 10^5 peripheral blood lymphocytes (Wagner et al., 1992). This assay was developed for application in transplant patients with high viral loads, so we decided to work with DNA corresponding to 10^5 cells, which is equivalent to 366 ng of human DNA. To mimic blood sample conditions, controls were prepared by mixing different quantities of EBV genomes (10^6, 10^3 and 10) from RAJI stocks (10^5 copies/μl) with 366 ng of EBV negative DNA from PTP cells. Serial fourfold dilutions of each control (initially containing 10^6, 10^3 and 10 copies) were prepared and assayed as described below until limiting dilution was reached.

2.1.3. Semiquantitative PCR (SQ-PCR)

A nested PCR was designed to amplify a fragment of the BWRF1 gene of the EBV genome. The outer primer pair (nucleotide positions: 14 571–14 588 and 14 777–14 768) amplifies a 206 bp fragment of the B95-8 EBV strain (NC001345 GenBank); and the inner primer pairs (nucleotide positions: 14 613–14 631 and 14 736–14 718) amplify a 122 bp fragment (Saito et al., 1989). The PCR was performed in a 50 μl volume reaction containing 1 × PCR buffer, 1.5 mM Cl_2Mg, 100 μM dNTPs, 1 μM of each primer, 1 U of Taq DNA polymerase (Promega) and in the outer PCR: 366 ng of DNA and the serial fourfold dilutions; in the inner PCR: 10 μl of 1/100 dilution of the first round products. Cycling conditions: 3 min at 94 °C, and 15 cycles of 1 min at 94 °C, 30 s at 58 °C, 1 min at 72 °C; and 10 min at 72 °C (first round); 3 min at 94 °C, and 35 cycles of 1 min at 94 °C, 30 s at 55 °C, 1 min at 72 °C; and 10 min at 72 °C (second round). Each PCR reaction included as negative controls: water, DNA from PTP cells; recommended PCR procedures were followed to avoid contamination. The amplified products were run in a 3% agarose gel stained with ethidium bromide.

2.1.4. Sensitivity

Fourfold dilutions from various controls were examined.

2.1.5. Specificity

To evaluate cross-reaction, DNA from other herpesviruses (HSV, CMV, VZV, HHV-6, HHV-8) were tested.

2.1.6. Reproducibility and accuracy

These were checked by measuring the intra- and inter-assay variation of the limiting dilution of different control mixes

2.2. SQ-PCR clinical application

2.2.1. Study population

In order to evaluate the clinical application of the method used, four groups were analyzed:

I-25 immunocompetent healthy viral carriers. All samples were obtained from blood bank donors. They all tested positive for VCA-IgG antibodies against EBV and negative against all pathogens routinely tested in the blood bank control (25 samples).

II-85 solid organ transplant patients with evidence of EBV infection (presence of VCA-IgG antibodies or positive qualitative PCR on PBMC) as follows:

A) 53 asymptomatic solid organ transplant recipients (83 samples).
B) 24 symptomatic solid organ transplant recipients. Symptomatic patients were defined as those presenting fever, lymphadenopathies, tonsil or adenoid hypertrophy, and/or leukopenia/thrombocytopenia (37 samples).
C) 8 symptomatic solid organ transplant recipients with histologic diagnosis of PTLD (8 samples).

2.2.2. Samples

EDTA anticoagulated blood samples from each patient were collected and peripheral mononuclear cells (PBMC) were purified (Lymphoprep GIBCO) and stored at −20 °C until used. DNA from PBMC was extracted and processed as mentioned above. Each PCR reaction included, as positive

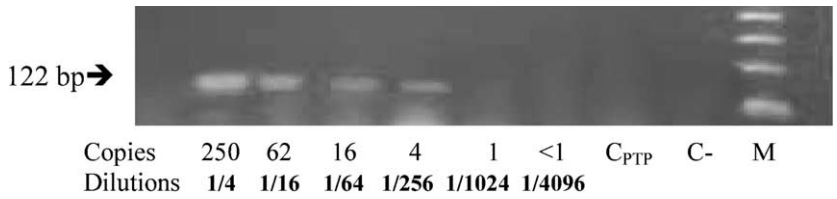

Fig. 1. SQ-PCR sensitivity. Serial dilutions of a control, initially containing 10^3 copies. Four copies of EBV genome could be detected in the limiting dilution. M: 100 bp DNA ladder.

control, fourfold dilutions of a control mix initially containing 10 copies until the limiting dilution was reached; these had to be within the range of sensitivity of the assay. In order to check for the presence of inhibitors, human β-globin gene was assayed in samples with non-detectable levels of viral load.

2.2.3. Statistical analyses

Data were compared by the non-parametric Mann–Whitney *U*-test for unpaired data. *P* values were calculated by the two-sided test. Analyses were done with the PEPI Program Finder version 4.0.

3. Results

3.1. SQ-PCR standardization

The median sensitivity of the assay was four copies (Fig. 1), as determined by measuring the limiting dilution from various controls, but the range was between 1 and 16 copies (Fig. 2). No cross-reaction was observed when DNA from HSV, CMV, VZV, HHV-6, and HHV-8 were assayed. The inter-assay variation was always ± 1 fourfold dilution (Fig. 2); similar results were obtained for the intra-assay variation, though the variation was smaller (data not shown). In all of the controls analyzed, the limiting dilution detected the same range of theoretically added copies of EBV genomes (1–16); the same sensitivity was obtained when measuring controls with low or high copy numbers.

Table 1 describes features of the study population and shows the EBV DNA levels detected by SQ-PCR. The end point can be converted into viral copies/10^5 PBMC by multiplying the sensitivity of the assay by the reciprocal of the limiting dilution (RLD). As the sensitivity of the method was a range of 1–16, we can assume equivalent ranges of viral load for each RLD value.

A significant difference was observed comparing RLD values of transplant patients without PTLD (asymptomatic plus symptomatic patients) and transplant patients with PTLD ($P < 0.0001$). Statistically significant differences were also found between asymptomatic and symptomatic recipients ($P < 0.0001$), asymptomatic and PTLD patients ($P < 0.0001$), symptomatic and PTLD patients ($P < 0.0001$).

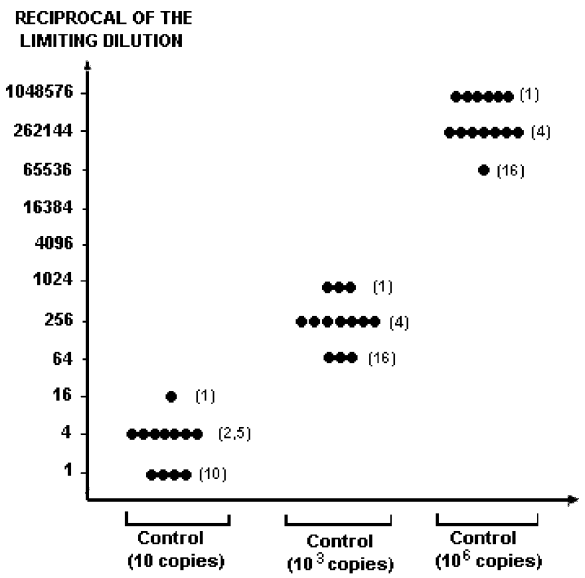

Fig. 2. Inter-assay variation of SQ-PCR. Reciprocal of the limiting dilution of different controls initially containing 10, 10^3 and 10^6 copies of EBV genomes. The sensitivity of the assay ranged between 1 and 16 copies.

Table 1
Features and viral load in the study population

	Healthy controls ($n=25$)	Asymptomatic SOT[a] ($n=53$)	Symptomatic SOT[a] ($n=24$)	PTLD patients ($n=8$)
Type of organ transplanted	–	Liver (17)	Liver (22)	Liver (5)
		Renal (36)	Renal (2)	Renal (3)
Mean age ± SD	34.1 ± 10.5 yrs	131.7 ± 85.3 mo	77.3 ± 48.3 mo	66.9 ± 40.9 mo
(range)	(19–50)	(6–252)	(8–204)	(12–144)
Minimum RLD value	ND(#)	ND(#)	ND(#)	16
Maximum RLD value	1	64	256	16384
Median RLD value	ND(#)	ND(#)	4	256

The mean age ± SD of the total transplant population was 110.2 ± 77.8 months.
[a] SOT: solid organ transplant recipients. ND(#): not detected.yrs: years; mo: months.

SQ-PCR performance to detect transplant patients with PTLD and the analysis of different cutoff points are shown in Table 2.

The dynamic of viral load in four patients is exhibited in Fig. 3. TA, a liver recipient developed increased viral load at the time that a lymphocytic hyperplasia was diagnosed on a tonsil biopsy. A transient reduction in immunosuppression was followed by a decrease in EBV load. One year later a monomorphic monoclonal lymphoma was diagnosed on an intra-abdominal mass biopsy (EBER+); simultaneously high EBV levels were detected. Her treatment was withdrawal of immunosuppression and infusion of anti-CD20 monoclonal antibodies; low viral loads were noticed. As there was evidence of tumor progression, chemotherapy was applied. Regression of tumor mass was observed. Low viral loads persisted. The residual mass was later surgically removed and no tumor cells were observed. At present, she is alive and well. GE, MP and JC are three liver transplant patients from whom various samples were obtained over periods of 1, 2 and 1 year, respectively. They presented no EBV related clinical episode, and their EBV DNA levels remained low.

4. Discussion

The level of viral load is the most widely used method to identify transplant patients at risk of PTLD and also to monitor their response to therapy. Different methodological strategies, standardization controls and types of samples have been used (Rowe et al., 2001; Stevens et al., 2001), which makes it difficult to compare these reports. However, all the studies agree that patients with PTLD have very high circulating levels of EBV. Therefore, it is essential for each assay to determine which level of viral load indicates increased probability of developing a lymphoproliferative disease. Our objective was to develop a semi quantitative method to quantify circulating viral copies for the early detection of transplant patients at risk of developing PTLD.

Table 2
Performance of the SQ-PCR test

Cutoff point (RLD)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Odds ratio
≥ 16	100	80	25	100	∞
≥ 64	87.5	91.7	41.1	99.1	77
≥ 256	75	96.7	60	98.3	87
≥ 1024	25	100	100	95.2	0

The prevalence of PTLD in the study population was 6.2%.

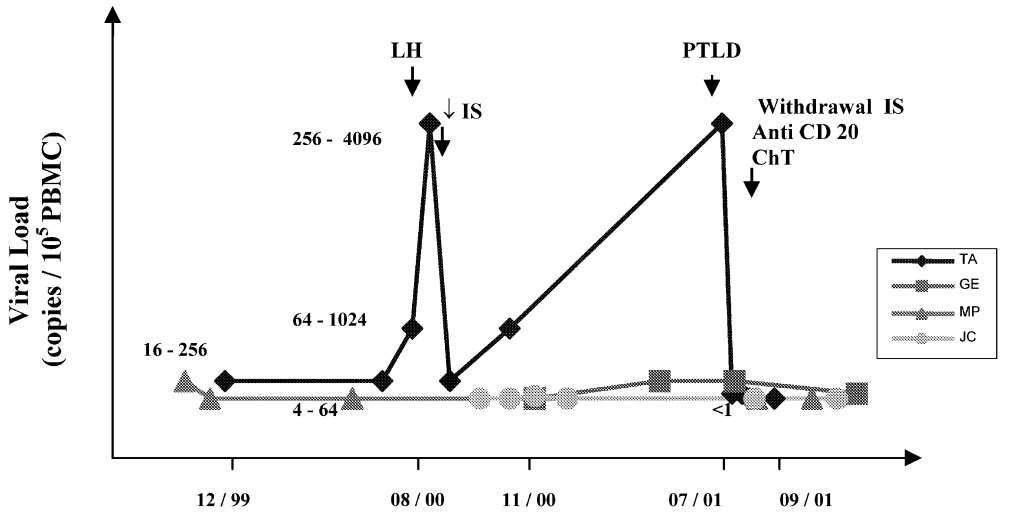

Fig. 3. Dynamics of viral load in four patients. LH, lymphocytic hyperplasia; IS, immunosupression; ChT, chemotherapy.

The group of healthy carriers included seropositive EBV adult individuals without clinical manifestation of disease or evidence of infection by the pathogens routinely controlled by the blood bank; therefore, this group may be a good example of latent infection by this virus (Miyashita et al., 1997). The viral load detected in this group varied between <1 copy per 10^5 PBMC and a range of (1–16) copies per 10^5 PBMC, which is similar to what other methodologies have detected: Rowe et al. (1997) detected <1–2 copies/10^5 PBMC; Khan et al. (1996) 1–50/10^6 B cells; Wagner et al. (1992) 1–50/10^6 B cells.

The transplant population we analyzed included patients who had received different solid organs (liver and kidney), treated in various medical centers with different protocols of immunosuppression, and the time lapse between the transplant and the extraction of the sample was variable (from 1 month to more than 10 years). Although these facts have imposed a limit on the inferences that can be made, we were able to demonstrate the usefulness of the method described for the detection of different circulating levels of viral DNA.

As others have previously reported (Riddler et al., 1994; Kenagy et al., 1995; Baldanti et al., 2000; Allen et al., 2001), our results showed a generally higher level of viral load in transplant patients than healthy carriers. This demonstrates the relative inability of these patients to control the EBV induced lymphoproliferation, as a consequence of the immunosuppression to which they are subjected. We also observed different levels of EBV DNA in the three groups of transplant individuals studied; the highest levels were found in the group of patients with PTLD. The differences in the levels of viral load observed between the asymptomatic and the symptomatic groups could be explained by a higher probability of activation of EBV in the patients in the latter group. In the symptomatic group, one of the patients with a high EBV DNA level: 256–4000/10^5 CMP, developed a PTLD 11 months later. Although no samples were studied during that lapse, these data could show that the increase in circulating viral copies precedes the development of PTLD, even earlier than previously described (Riddler et al., 1994; Kenagy et al., 1995; Green et al., 1999). It would be useful to determine the length of the period between the increase in viral load and the development of PTLD more precisely.

The frequency of infected cells in peripheral blood varies between 5 and 500 per 10^7 B cells, but is quite stable in each particular individual (Khan et al., 1996; Thorley Lawson, 1999); this indicates the importance of monitoring the viral load in each patient. In this study, the follow-up of the circulating levels of EBV DNA in four patients

(Fig. 3) correlated well with the clinical findings; and in the case of PTLD the viral load decreased with the treatment applied, although progression of the tumor has been observed after the initial withdrawal of immunosupression and administration of anti-CD20 monoclonal antibodies. As previously described, patients treated with these monoclonal antibodies have shown an almost immediate and dramatic decline in viral loads even in those cases whose PTLD progressed during therapy (Yang et al., 2000).

To determine the RLD level that could be considered at risk for PTLD with this assay, different cutoff points were analyzed. A cutoff point of 1000 was discarded because of its low sensitivity. Considering that the detection of patients at risk of PTLD would probably lead to a decrease of the immunosuppression applied, we preferred to use the RLD cutoff point with a better positive predictive value; thus, of the three remaining cutoff points analysed we chose a RLD of 256, which also showed an acceptable odds ratio (>50) for this assay (Jekel et al., 1996).

We consider it necessary to increase the number of viral load follow-ups in transplant recipients, and also to study its relation to the type of organ transplanted, the different immunosuppression protocols used and the evolution of each patient. Moreover, the use of complementary parameters such as the expression of marker genes of EBV latency or replication (Qu et al., 2000), or the measurement of the viral load in the B cell subclasses (Rose et al., 2001) or the levels of EBV DNA in other sites (Nadal et al., 2002) could help in the identification of patients at risk of PTLD.

We conclude that the method of viral load assay described is simple (a nested PCR with a limiting dilution analysis), relatively rapid (results in 48–72 h), and could be useful for the early detection of transplant patients at risk of PTLD.

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