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Duplex realtime PCR method for Epstein–Barr virus and human DNA quantification: its application for post-transplant lymphoproliferative disorders detection

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ABSTRACT

Introduction: The quantification of circulating Epstein–Barr virus (EBV) DNA is used to monitor transplant patients as an early marker of Post-Transplant Lymphoproliferative Disorders (PTLD). So far no standardized methodology exists for such determination.
Objective: Our purpose was to develop and validate a real-time PCR assay to quantify EBV DNA in clinical samples from transplant recipients.
Methods: A duplex real-time PCR method was developed to amplify DNA from EBV and from a human gene. The EBV load was determined in peripheral blood mononuclear cells (PBMC), plasma and oropharyngeal tissue from 64 non-transplanted patients with lymphoid-hypertrophy (Non-Tx), 47 transplant recipients without PTLD (Tx), 54 recipients with PTLD (Tx-PTLD), and 66 blood donors (BD). WinPEPI, version 11.14 software was used for statistical analysis.
Results: Analytical validation: the intra and inter-assays variation coefficients were less than 4.5% (EBV-reaction) and 3% (glyceraldehyde 3-phosphate dehydrogenase – GAPDH reaction). Linear ranges comprised 10^7–10 EBV genome equivalents (gEq) (EBV-reaction) and 500,000–32 human gEq (GAPDH-reaction). The detection limit was 2.9 EBV gEq (EBV-reaction). Both reactions showed specificity. *Application to clinical samples*: higher levels of EBV were found in oropharyngeal tissue from transplanted groups with and without PTLD, compared to Non-Tx ($p < 0.05$). The EBV load in PBMC from the groups of BD, Non-Tx, Tx and Tx-PTLD exhibited increasing levels ($p < 0.05$). In BD, PBMC and plasma, EBV loads were undetectable.
Conclusions: The performance of the assay was suitable for the required clinical application. The assay may be useful to monitor EBV infection in transplant patients, in particular in laboratories from low-income regions that cannot afford to use commercial assays.

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Introduction

The quantification of Epstein–Barr virus (EBV) peripheral DNA is used to monitor transplant patients as an early marker of Post-Transplant Lymphoproliferative Disorders (PTLD).[1–3] It has been proven that EBV load in peripheral blood samples from transplant patients with PTLD is higher than in transplant recipients without this disorder.[4–6] Along the last decade, different quantitative PCR assays (semi-quantitative, competitive, real-time) have been used for surveillance, diagnosis, monitoring response to treatment, and determination of the degree of immunosupression to be applied. So far, there is no reference strategy to determine EBV load, including for instance the calibrator, the EBV region to be amplified, or the best sample type for identifying PTLD risk.[7,8]

No international standards were available until 2012, when the World Health Organization introduced the first WHO International Standard for Epstein–Barr virus, intended to be used for nucleic acid amplification techniques.[9] Thus, the literature describes a variety of controls used to analytically validate EBV quantification assays, including cell lines,[10–12] plasmids with EBV-genome fragments inserted,[13,14] and commercially available controls containing viral particles.[15,16] As no international standard or consensus-accepted control have been developed,[17–19] each laboratory decided what calibrator to use to validate its own EBV quantification method. Different fragments of EBV genes were chosen for amplification in a variety of quantification assays, including repeated (BamHI-W region) or single (EBERs, EBNA-1, LMP-2, etc) viral genome regions[20,21] with different degrees of sensitivity or accuracy, as previously described.[22,23] Moreover, several blood sample types (peripheral blood mononuclear cells, plasma, and whole blood) were analyzed to identify PTLD. Most studies described and/or recommended using cell-associated blood samples over plasma/serum, but both specimen types appear to be informative and each laboratory determines its preference.[24,25] Also, the extraction methods, the amount of sample to be analysed, the report format and the characteristics of the study populations vary between published data.[7,20,21] Thus, all these factors have affected the comparison between methods.

Several years ago, our laboratory developed a semiquantitative PCR strategy to measure EBV load[26] and since then it has been used to monitor this viral infection in transplanted population from most of the institutions that perform organ transplantation in Argentina. The method is quite cumbersome and time-consuming; results demand at least 48 hours. Currently, real-time PCR quantification methods are widely applied to assess EBV load due to their advantages over conventional PCR assays.[3,7,21] Moreover, the simultaneous amplification of an internal control along with the target DNA is widely used to detect the presence of inhibitors; it also allows to quantify the amount of sample present in the reaction, which permits viral load normalization.[10,27]

Despite the current availability of commercial assays, many laboratories from low income regions are unable to afford them; thus, less-costly in-house methods may be the only option to monitor EBV load in transplant patients. Their development and validation could be extremely useful for the prevention of PTLD in these settings.

Therefore, the aim of the present study was to develop and analytically validate a duplex real-time PCR assay to quantify EBV and human DNA in different types of clinical samples, in order to determine the EBV load in transplant patients regarding the risk of PTLD.

Materials and methods

Patients and samples

Children treated in "Prof. Dr Juan P. Garrahan" Pediatric Hospital, Austral University Hospital, "Sor María Ludovica" Children's Hospital" and Favaloro Foundation, and blood donors of the "J. F. Muñiz" Infectious Diseases Hospital were included as follows:

(A) 64 non-transplanted patients with lymphoid hypertrophy in the oropharyngeal tissue.
(B) 101 solid organ transplant patients (75 liver, 24 kidney, 2 heart), 54 of them with histological diagnosis of PTLD (including categories 1, 2, 3 and 4), according to the World Health Organization: IARC, 2008.[28]
(C) 66 blood donors, with negative results for all infections screened in routine blood bank protocol (hepatitis C virus, hepatitis B virus, human T lymphotropic virus I/II, human immunodeficiency virus, syphilis, brucellosis, and Chagas' infection).

All patients were infected with EBV according to the presence of IgG antibodies against viral capsid antigen (VCA) and/or viral DNA in peripheral blood.

Patients in the transplant group were on an immunosuppressive regimen consisting of cyclosporine, tacrolimus or sirolimus, azathioprine or mycophenolate mofetil and steroids.

Peripheral blood and oropharyngeal lymphoid tissue samples were taken due to oropharyngeal lymphoid hypertrophy, following the treatment protocol for transplant and non-transplanted patients. An informed consent was obtained in all cases as per the Helsinki declaration and other national and international regulations.

Peripheral blood mononuclear cells (PBMC) and plasma were separated from 2.5 to 5 mL of EDTA-anticoagulated whole blood samples by centrifugation on a density gradient (Histopaque-1077, Sigma–Aldrich) and stored at −20 °C. Oropharyngeal lymphoid tissue samples obtained through surgical removal from patients with lymphoid hypertrophy were stored at −80 °C.

Controls and Calibrators

- *EBV-specific reaction (EBNA-1 reaction)*

A plasmid containing a deleted fragment of the EBNA-1 coding gene from the EBV genome was used as calibrator of the real-time PCR quantification method. It had been previously developed in our laboratory to be used as competitor in