

Detection of the Epstein-Barr virus by the polymerase chain reaction in immunosuppressed and immunocompromised patients

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Abstract. DNA extracted from the blood of immunosuppressed and immunocompromised individuals and from patients with infectious mononucleosis, leukaemias and lymphomas were studied using the Polymerase Chain Reaction (PCR) technique. The oligonucleotide primers used for the detection of the Epstein-Barr virus (EBV) amplify a 375bp sequence from the EcoRI B fragment of the viral genome. EBV specific sequences were amplified from the blood samples of 18 out of 65 patients, most of which were transplant patients (9 out of 31). The results confirmed the association of EBV with clinical disorders in immunodeficient and immunocompromised patients and the importance of PCR method in routine diagnosis.

Introduction

Epstein-Barr Virus (EBV) is a member of the human herpes virus family and the causative agent of infectious mononucleosis (1). EBV has a B-lymphocyte tropism and is strongly associated with post-transplanted lymphoproliferative disorders, Hodgkin's disease, nasopharyngeal carcinoma, Burkitt's lymphoma and lymphoproliferative disorders of primary and secondary immunodeficiency (2,3).

The virus, which has been detected worldwide, invades B-lymphocytes that have EBV receptors in common with receptors for the complement components. Selected epithelial cells of different organs bearing the same receptors may also become infected. Primary infection occurring in childhood is mostly asymptomatic but in later childhood and adolescence may result in lymphoproliferative diseases. In immunosuppressed transplant patients and in individuals with the acquired immunodeficiency virus, in whom there is a loss in T-lymphocyte mediated control of stimulated B

lymphocytes, malignant lymphomas may arise from activation of latently EBV-infected B lymphocytes (4).

The detection of EBV infections is limited by the lack of routine laboratory techniques and diagnosis of primary or reactivated EBV infection is often based on serological tests (1,5). Recently, *in vitro* amplification of specific nucleic acid sequences by the PCR technique has been applied for the identification of EBV DNA in immunosuppressed patients (6,7).

PCR provides a specific, rapid and sensitive means for the detection of viral genomes and may be used in routine diagnosis (3,5,6,8,9).

We have employed the PCR technique for the detection of EBV in blood samples from various groups such as kidney and heart transplanted patients, AIDS patients and patients with infectious mononucleosis, Hodgkin's disease, and different lymphomas.

Materials and methods

DNA extraction. DNA was extracted from leukocytes from the blood of 65 immunosuppressed patients. 3-5 ml of each blood sample were diluted with equal volume of 0.9% NaCl. The mixture was overlaid onto an equal volume of Lymphoprep (Nycomed AS). Samples were then centrifuged at 1,800 rpm for 35 min and the white cell layer was collected. Cells were washed twice with 0.9% NaCl. 400 µl of TES (10 mM Tris HCl, pH 8.0, 1 mM EDTA, 0.1% SDS) containing 100 µg/ml Proteinase K was added to the resulting pellet. After one hour of incubation at 60°C, fresh Proteinase K was added at concentration 100 µg/ml and incubation was continued for another hour. Samples were then extracted with equal volume of phenol, phenol/chloroform and chloroform. DNA was precipitated with the addition of 20 µl 5 M NaCl and 1 ml ethanol. The samples were stored at -20°C overnight. DNA was recovered by centrifugation at 13,000 rpm for 15 min at 4°C, washed with 70% ethanol and the pellet was resuspended in 50-100 µl distilled water.

Oligonucleotide primers and PCR. One set of primers was used for the amplification of a 375bp region of the EBV EcoRI B fragment of the virus genome (10,11) (Table I).

PCR was performed with 1-3 µg genomic DNA in 100 µl of the reaction containing buffer (Perkin-Elmer Cetus), 10X

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Table I. Primers used for the amplification of a 375bp fragment from the EBV genome.

Primers	Sequences	Location on EBV genome Nucleotide no.
EBV 1	5' GTGTGCGTCGTGCCGGGGCAGCCAC 3'	102669-102694
EBV 2	5' ACCTGGGAGGGCCATCGCAAGCTCC 3'	103019-103044

10 mM Tris HCl at pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 200 mM dNTPs, 40 pmoles of each primer and 2.5U of Taq polymerase (Advanced Biotechnologies). Before the addition of Taq polymerase, samples were heated at 98°C for 5 min. Amplification was performed in a Perkin Elmer Cetus Thermal Cycler with the following parameters: denaturation at 95°C for 1 min, annealing and extension at 72°C for 1 min, for 35 cycles. 20 µl aliquots of the amplification products were analysed on a 2% agarose gel and visualised under UV illumination after staining with ethidium bromide.

Restriction endonuclease cleavage. 10 µl aliquots of the PCR product were separately digested with 20U of HindIII, BstNI and PvuII (New England Biolabs), following the conditions recommended by the suppliers.

Results

Sixty-five blood specimens, including samples of infectious mononucleosis, Hodgkin's disease, AIDS, different leukemias and lymphomas and samples from heart and kidney transplant patients were examined. Positivity of the samples were judged by the presence of a 375bp following the electrophoretic analysis of the PCR products (Fig. 1).

EBV-specific sequences were amplified in the blood of 7 out of 21 kidney transplant patients, 2 out of 10 heart transplant patients and all individuals with infectious mononucleosis and angioblastic lymphathenopathy. EBV was not detected in 4 patients with Hodgkin's disease. Additionally, EBV was detected in 1 out of 6 leukemias and lymphomas, 3 out of 17 patients with high titres of IgG and IgM and 1 out of 4 AIDS patients. The results of EBV detection by PCR are summarised in Table III.

To ensure the specificity of the reaction, PCR products were separately digested with HindIII, BstNI and PvuII. The cleavage sites of the enzymes and the expected restriction fragments are shown in Table II. The digestion products were analysed on a 2% agarose gel and visualised under UV illumination after staining with ethidium bromide (Fig. 2). All EBV amplified sequences showed the expected restriction fragments.

Discussion

EBV infects B lymphocytes leading to their cellular proliferation that is controlled by a T lymphocyte response. Thereby, infection with EBV has been described as a viral process that generates a prominent immune response. A prominent T-lymphocyte proliferation occurring in response to EBV-carrying B lymphocytes results in infectious

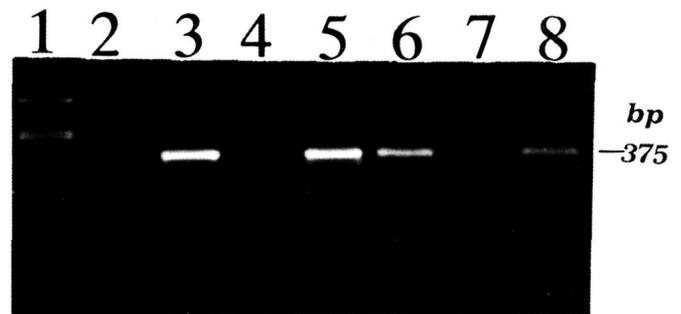


Figure 1. EBV amplification products analysed in a 2% agarose gel stained with ethidium bromide. The presence of a 375bp band indicates the presence of EBV DNA in the sample. Lane 1: pUC18/HaeIII molecular weight marker. Lane 2: negative control, lane 3: positive control, lanes 5, 6 and 8: positive samples, lanes 4 and 7: negative samples.

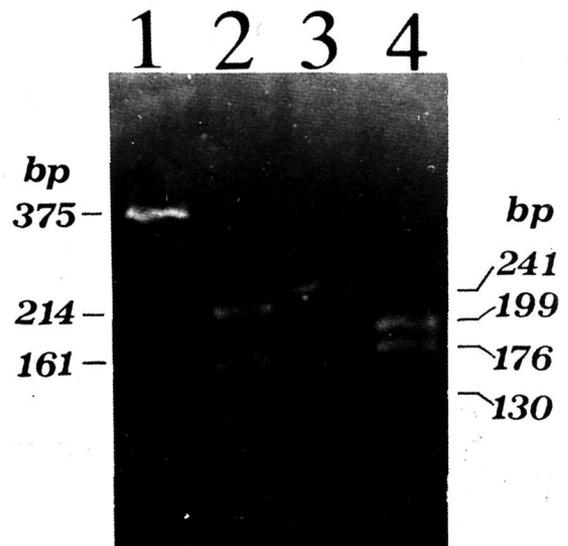


Figure 2. Amplification products of the digested EBV-specific sequence. Lane 1: EBV positive sample, lanes 2, 3 and 4: EBV band digested with HindIII, BstNI and PvuII respectively.

Table II. Restriction endonuclease enzymes and their sites of cleavage of EBV-specific sequence.

Restriction enzyme	Cleavage site	Restriction products bp
HindIII	A/AGCTT	214, 161
BstNI	CC/AGG	130, 241, 4
PvuII	CAG/CTG	199, 176

Table III. Detection of EBV by PCR.

Study cases	No. of patients	EBV positive
Kidney transplants	21	7
Heart transplants	10	2
Angioblastic lymphathenopathy	1	1
Leukemias and lymphomas	6	2
AIDS patients	4	1
High IgG, IgM titres	17	3
Hodgkin's disease	4	0
Infectious mononucleosis	2	2
Total	65	18

mononucleosis. Lymphoproliferative disorders following organ transplantation or AIDS represent an unchecked proliferation of EBV infected B-lymphocytes (7).

The diagnosis of EBV infections has generally been based on clinical features, a Monospot test or on serological evidence.

The PCR technique, because of its ability to detect reduced numbers of viral copies, is an important diagnostic tool in diseases caused by EBV to immunosuppressed and immunocompromised individuals. It is a rapid, specific and sensitive method and is independent of the humoral immune status of the patients. The absence of increasing levels of antibody to EBV in some patients may reflect impaired humoral immunity rather than lack of active infection.

In transplant recipients taking immunosuppressive agents post-transplant lymphoproliferative disorders have emerged as potentially life-threatening complications. Early recognition of these disorders is important because many patients would recover with modulation of their immunosuppressive therapy.

Finally, establishing the presence of EBV has prognostic importance because EBV-associated tumor progression can be reversible upon reduction or discontinuation of immunosuppression.

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