Detection of human cytomegalovirus and Epstein-Barr virus by the polymerase chain reaction in patients with ß-thalassaemia

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Abstract. Infections caused by Human Cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) are common in multiple transfused patients, such as patients with ß-thalassaemia. The ability of the Polymerase Chain Reaction (PCR) to amplify HCMV and EBV DNA from blood and other samples makes this technique a valuable diagnostic tool for the detection of both viruses in the early stages of the infection. PCR was used for the amplification of a 435 bp region of the immediate early-1 (IE-1) gene of HCMV and a 375 bp sequence from the EcoRI B fragment of EBV genome. Blood samples from 80 patients with ß-thalassaemia were examined. HCMV was found in 14 and EBV in 12 patients. The results obtained confirm the implications of HCMV and EBV in the diagnosis of viral infections in multiple transfused patients as well as the importance of PCR technique as a valuable diagnostic tool.

Introduction

HCMV and EBV are members of the herpes viruses family; HCMV is a ubiquitous viral pathogen provoking serious infections in specific populations such as immunosuppressed and immunocompromised patients, pregnant women, low birthweight neonates and multiple transfused patients (1). EBV is the causative agent of infectious mononucleosis and has been incriminated in the pathogenesis of Hodgkin's disease, Burkitt's lymphoma, nasopharyngeal carcinoma and lymphoproliferative disorders (2). Even though, primary infection occurring in childhood is mostly asymptomatic, in later childhood and adolescence it may result in lymphoproliferative diseases. Activation of latent EBV-infected B lymphocytes may also lead to the development of malignant lymphomas (3).

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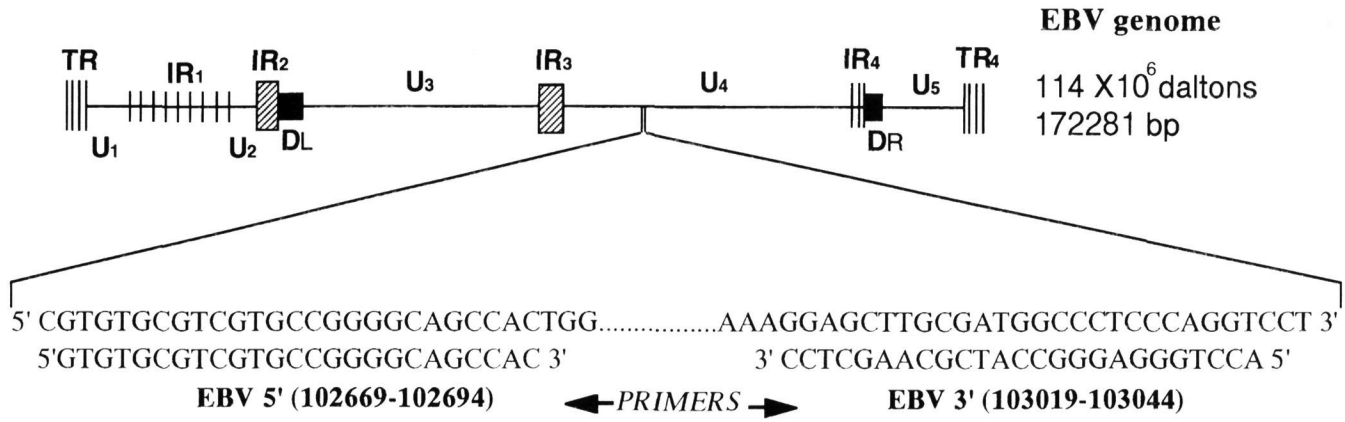
Current laboratory techniques used for the detection of HCMV and EBV are based on serological tests (e.g. ELISA, latex agglutination) while active infection can be determined using cell culture. However, serology as well as cell culture have disadvantages of major importance such as the high level of expertise required for the detection of antibodies, and the cost and time required for the cell culture (4,5). Recent advances in molecular biology have allowed *in vitro* amplification of specific nucleic acid sequences by the Polymerase Chain Reaction (PCR). The use of PCR for the detection of HCMV and EBV DNA have been previously reported (6,7). PCR provides a specific, rapid and sensitive means for the detection of viral genomes (8,9) as well as being a technique with other medical applications (10,11). We employed the PCR technique for the detection of HCMV and EBV DNA in peripheral blood leucocytes from 80 frequently transfused patients with ß-thalassaemia.

Materials and methods

Patients. Blood samples were collected from selected groups of patients with homozygous ß-thalassaemia who undergo frequent transfusion treatment in the Thalassaemia Unit of the 1st Department of Pediatrics of the University of Athens at St. Sophia's Children Hospital. 80 patients with homozygous ß-thalassaemia were divided into four groups, as follows: group I, consisted of 10 patients with ß-thalassaemia and AIDS, group II, consisted of 15 patients with ß-thalassaemia and increased transaminases, group III 49 splenectomized patients with ß-ALT level thalassaemia, and group IV 6 patients awaiting for bone marrow transplantation.

DNA extraction. Leucocytes were isolated from 3 ml blood using Lymphoprep (Nycomed AS) and lysed with the addition of 400 µl TES buffer containing 100 µg/ml Proteinase K. After incubation at 37°C O/N samples were extracted once with phenol and once with phenol/chloroform. DNA was precipitated with the addition of 20 µl 5 M NaCl and 1 ml ethanol. DNA was recovered by centrifugation at 13,000 rpm for 15 min at 4°C, washed once with 70% ethanol and resuspended in 100 µl of double-distilled water.



PCR Amplification

5' GTGTGCGTCGTGCCGGGGCAGCCACTGG.....AAAGGAGCTTGCGATGGCCCTCCCAGGT 3'

PCR product (375 bp)

Figure 1. Schematic diagram of EBV oligonucleotide primers and the EBV genome.

Control DNA. HCMV: DNA extracted from the CMV plasmid pRR 47 was used as a positive control. EBV: DNA extracted from peripheral blood leucocytes derived from a patient with infectious mononucleosis was used as positive control.

Oligonucleotide primers. Two pairs of primers were used. One specific for the amplification of a 435 bp long sequence from the IE-1 gene of HCMV. This set of oligonucleotide primers, termed MIE-4 and MIE-5, has been previously described (12). The second set of primers, termed EBV-1 and EBV-2, was specific for the amplification of a 375 bp sequence from the EcoRI B fragment of EBV (13). All the primer sequences used are shown in Table I. The oligonucleotide primers of EBV with the EBV genome are shown in Fig. 1. The HCMV primers with the HCMV IE-1 sequence have been previously described (14).

PCR amplification. HCMV: 500 ng DNA of each sample was amplified in a volume of 100 µl containing 200 µm of dNTPs, 50 pmoles of each primer, 75 mM Tris-HCl (pH 8.4), 200 mM $(NH_4)_2SO_4$, 0.01% (w/v) Tween 20, 2.5 mM $MgCl_2$ and 2.5 units Taq polymerase (Advanced Biotechnologies). Amplification was performed in a thermal cycler (Perkin Elmer Cetus) using the following conditions: denaturation at 94°C for 50 sec, annealing at 62°C for 30 sec and extension at 72°C for 50 sec, increasing the extension time by 1 sec per cycle. Samples were subjected to 40 cycles of amplification.

EBV: PCR was performed with 500 ng genomic DNA in 100 µl of the reaction containing, 10 mM Tris HCl at pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 mM dNTPs, 40 pmoles of each primer and 2.5U of Taq polymerase (Advanced Biotechnologies). Samples, prior to the addition of Taq polymerase, 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.

Table I. Primer sequences for HCMV and EBV PCR.

HCMV Immediate early-1 gene		
Sense primer:	MIE-4	5' CCA AGC GGC CTC TGA TAA CCA AGC C 3'
Antisense primer:	MIE-5	5' GGT CTC CTT CTC CTC CTA CCA CGA C 3'
Product=435 bp		
EBV EcoRI B fragment		
Sense primer:	EBV 1	5' GTG TGC GTC GTG CCG GGG CAG CCA C 3'
Antisense primer:	EBV 2	5' ACC TGG GAG GGC CAT CGC AAG CTC C 3'
Product=375 bp		

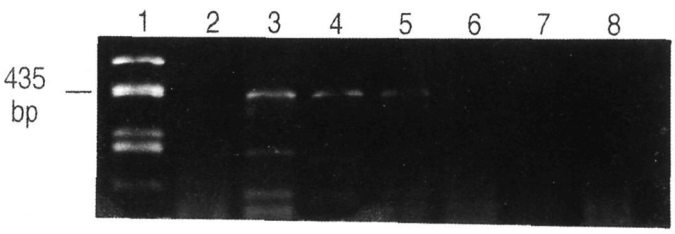


Figure 2. Detection of HCMV DNA by PCR. Amplification products were electrophoresed through a 2% agarose gel. Lane 1: pUC18/HaeIII molecular marker, lane 2: negative control, lane 3: positive control, lanes 4,5: positive samples, lanes 6,7,8: negative samples.

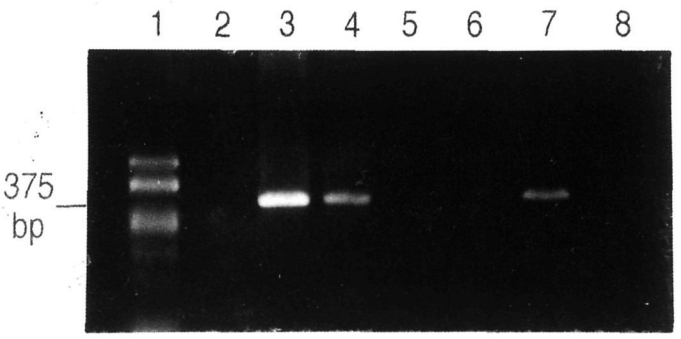


Figure 3. Detection of EBV DNA by PCR. Amplification products were electrophoresed through a 2% agarose gel. Lane 1: pUC18/HaeIII molecular marker, lane 2: negative control, lane 3: positive control, lanes 4,7: positive samples, lanes 5,6,8: negative samples.

Twenty µl of the amplification products were run on a 2% agarose gel and visualised under UV illumination after staining with ethidium bromide.

Results

HCMV DNA was detected in 14 (17.5%) while EBV DNA in 12 (15%) out of the 80 patients. The presence of HCMV and/or EBV was judged from the amplification of a 435 bp long sequence and of 375 bp long sequence respectively (Figs. 2 and 3). HCMV DNA was detected in 2 out of 10 patients in group I, 2 out of 15 patients in group II, 9 out of 49 patients in group III and 1 out of 6 patients in group IV. Similarly, EBV DNA was detected in 4 out of 10 patients in group I, 2 out of 15 patients in group II, 4 out of 49 patients in group III and 2 out of 6 patients in group IV. Our results are summarised in Table II. None of the 80 examined patients were found to be co-infected with HCMV and EBV. Differences in the prevalence of HCMV and EBV infection among the groups studied were not found.

Discussion

HCMV and EBV can be transmitted via transfussion of blood or blood products given and/or bone marrow transplantation. Both viruses may cause serious infections in immunosuppressed and immunocompromised individuals and severe complications in transplant or multiple transfused patients who are under immunossuppression (15,16). In these cases, the early recognition of the viruses in the blood is essential. Tissue culture is time consuming, expensive and does not detect latent viruses. Serological tests are not reliable since the absence of increased levels of antibody to HCMV or to EBV may reflect impaired humoral immunity rather than lack of active infection. As much of the seropositive blood comes from blood donors who may not currently have an HCMV and/or EBV infection but who have been previously exposed to the virus(es), it seems apparent that the ability to detect the virus(es) itself/themselves in populations of blood donors has significant importance especially for certain groups of patients (17). It is strongly supported by two recent investigations which have shown that human cytomegalovirus DNA can be removed from donor blood by filtration and the potential for HCMV disease in very low birth weight infants can be reduced by removal of white blood cells from red cell concentrates (18,19).

In this study, we examined 80 patients with homozygous ß-thalassaemia who are on frequent transfusion treatment, with packed red cell preparations, leucocytes poor. HCMV DNA was detected in 14 out of the 80 (17.5%) while EBV DNA was detected in 12 out of the 80 examined patients (15%). Our data show that there is an implication of both HCMV and EBV in multiple transfused patients. As both viruses are associated with latent infections the detection of HCMV or EBV DNA does not indicate active viral infection. Application of the Reverse Transcription Polymerase Chain Reaction (RT-PCR) is required for the detection of mRNA transcripts which provide evidence of an active viral infection. However, in the case of latent viral infection, the immunosuppression which may arise due to red cell transfusions, may lead to reactivation of endogenous viruses. As, HCMV and EBV infections are not hospital based infections, it is essential that the HCMV and EBV status of

Table II. Summary of results on the detection of HCMV and EBV by PCR.

Groups studied	Number of patients	CMV positive	EBV positive
I. Patients with ß-thalassaemia and AIDS	10	2	4
II. Patients with ß-thalassaemia and splenectomy	49	9	4
III. Patients with ß-thalassaemia and increased transaminases	15	2	2
IV. Patients with ß-thalassaemia undergoing marrow transplantation	6	1	2
Total	80	14	12

blood used for transfusion to such patients is determined and that the clinical outcome is monitored (20). It must be stressed, that none of the patients studied had clinical signs of active infections, despite the fact that in the group of patients with anti-HIV antibodies immune deficiency was present.

PCR has been proved to be a sensitive, accurate and specific method with two great advantages when applicable for the detection of viral genomes. It does not depend on the immune status of the patient that can be defective, either due to post-operative pharmaceutical immunosuppression or due to immune disorders and it is able to detect small amounts of viral copies in clinical specimens, including body fluids and biopsies, present either in latent or in replicative forms.

Thus, PCR is a powerful diagnostic tool and may have an important role in the preparation of HCMV and EBV negative blood and blood products. By the use of HCMV and EBV negative blood the morbidity and mortality rate in susceptible patients (immunosuppressed and immunocompromised) is expected to decline. The prevalence of infection with HCMV and EBV, in patients with ß-thalassaemia is low compared to infection with HBV and HCV. This may be due to the fact, that during the last fifteen years, patients have been transfused with leucocyte poor, packed red cell preparations.

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