

# Detection of HSV, CMV and EBV by the polymerase chain reaction technique in patients with inflammatory eye diseases

M. ERGAZAKI<sup>1,2</sup>, G. XINARIANOS<sup>1</sup>, A. GIANNOUDIS<sup>1</sup>, N. MARKOMICHELAKIS<sup>3</sup>,  
J. TSAMPARLAKIS<sup>4</sup> and D.A. SPANDIDOS<sup>1,2</sup>

<sup>1</sup>Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, Athens; <sup>2</sup>Laboratory of Clinical Virology, University of Crete, Medical School, Heraklion; <sup>3</sup>Ophthalmology Clinic, General Hospital of Athens;

<sup>4</sup>Ophthalmology Clinic, University Hospital, Heraklion, Greece

Received September 9, 1994; Accepted October 4, 1994

**Abstract.** Herpes simplex virus (HSV), cytomegalovirus (CMV) and Epstein-Barr virus (EBV) have been recognized as pathogenic agents of intraocular inflammatory conditions. The ability of the Polymerase Chain Reaction (PCR) technique to amplify HSV, CMV and EBV DNA from aqueous specimens makes this technique a valuable diagnostic tool for the detection of these viral pathogens in patients with ophthalmic lesions. We used PCR for the amplification of a 476 bp long sequence from the *pol 1* gene of HSV genome, a 435 bp region of the immediate early-1 (IE-1) gene of CMV and a 375 bp sequence from the EcoRI B fragment of EBV genome. We examined 22 aqueous humour specimens from patients with uveitis and retinitis, inflammatory eye diseases, diagnosed clinically. We found HSV in 4 (18.2%), CMV in 6 (27.3%) and EBV in 1 (4.5%) out of the 22 examined patients. None of the 22 examined samples was found to be infected with more than one of the examined viral pathogens. These data confirm the implications of the members of Herpesvirus family in inflammatory inner eye diseases and the importance of PCR technique as a diagnostic tool in clinical virology.

## Introduction

Intraocular inflammation caused by viral infection is one of the major known types of endogenous inflammatory eye diseases. Herpesviruses are common pathogens of retinitis in both immunosuppressed and immunocompromised patients (1). In particular, cytomegalovirus retinitis has been reported as the initial manifestation of the acquired immune deficiency syndrome (AIDS) (2,3). Cell culture of intraocular specimens testing for cytopathic effect (CPE) is the standard method for

making a specific virologic diagnosis of inflammatory intraocular disease. However, it appears to be particularly difficult when only a very small quantity of intraocular fluid is available (4). Moreover, cell culture is both expensive and time-consuming. Recently, molecular biology techniques have allowed *in vitro* amplification of specific nucleic acid sequences by PCR. The use of PCR for the detection of HSV, CMV and EBV DNA (5-7) and its specificity and sensitivity for the detection of viral genomes as well as other medical applications have been reported (8-10).

In this study, we employed PCR to detect HSV, CMV and EBV DNA in aqueous humour from 22 patients with clinically diagnosed uveitis or retinitis.

## Materials and methods

**DNA extraction.** NaOH was added to final concentration 0.1 N and samples were boiled for 5 min. 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 50 µl of double-distilled water.

**Control DNA.** The positive control used for HSV was DNA extracted from the aqueous humour from a patient with diagnosed herpetic uveitis, for CMV DNA extracted from the CMV plasmid pRR 47 and for EBV DNA extracted from peripheral blood leucocytes from a patient with infectious mononucleosis.

**Oligonucleotides and PCR amplification.** HSV: A set of primers, termed HSV-a and HSV-b were used which have been previously described (13) to amplify a 476 bp long sequence from the DNA polymerase gene of both HSV I and HSV II.

500 ng DNA of each sample were amplified in a volume of 100 µl containing 200 µM of dNTPs, 75 pmoles of each primer, 75 mM Tris-HCl (pH 8.4), 200 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% (w/v) Tween 20, 2.5 mM MgCl<sub>2</sub> and 2.5 units Taq polymerase (Advanced Biotechnologies). Amplification was performed in a thermal cycler (Perkin Elmer Cetus) using the

---

**Correspondence to:** Professor D.A. Spandidos, Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48 Vas. Constantinou Avenue, Athens 116 35, Greece

**Key words:** HSV, CMV, EBV, inflammatory eye diseases, PCR

Table I. Oligonucleotide primer sequences for the detection of HSV, CMV and EBV by the PCR technique.

Virus	Oligonucleotide primers	Position	Amplified region	Amplified PCR product (bp)
HSV	5' CAGTACGGCCCCGAGTTCGTGA 3'	5921-5942	pol	476
	5' GTAGATGGTGC GG GTGATGTT 3'	6367-6387		
CMV	5' CCAAGCGGCCTCTGATAACCAAGCC 3'	731-755	IE-1	435
	5' CAGCACCATCCTCCTCTTCCTCTGG 3'	1150-1165		
EBV	5' GTGTGCGTCGTGCCGGGGCAGCCAC 3'	102669-102694	EcoRI B	375
	5' ACCTGGGAGGGCCATCGCAAGCTCC 3'	103019-103044		

following conditions: denaturation at 94°C for 60 sec, annealing at 64°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.

CMV: A pair of primers, termed MIE-4 and MIE-5, which have been previously described (11) were used to amplify a 435 bp long sequence from the immediate early-1 gene of HCMV. The primers along with the HCMV IE-1 sequence have been described previously (12).

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<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% (w/v) Tween 20, 2.5 mM MgCl<sub>2</sub> and 2.5 units Taq polymerase. Amplification was performed in a thermal cycler 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: A set of primers specific for the amplification of a 375 bp sequence from the EcoRI B fragment of EBV, termed EBV-1 and EBV-2, were used (14,15). The primers along with the EBV genome have been described previously (16).

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<sub>2</sub>, 0.001% gelatin, 200 mM dNTPs, 40 pmoles of each primer and 2.5 U of Taq polymerase. Prior to 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.

The sequences of the oligonucleotide primers used, their location in the viral genomes and the amplified regions are shown in Table I.

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

*Endonuclease cleavage for the differentiation of HSV1 and HSV2.* Ten to twenty µl of the amplification products of the samples which showed the 476 bp band were further cleaved using 80 units of the restriction endonuclease *Ava*II (New England Biolabs). Samples were digested O/N at 37°C. The conditions followed for digestion were those recommended by the supplier. There is a single *Ava*II site in the amplified sequence of HSV2 that cleaves the 476 bp

product into 87 and 389 bp fragments. An additional *Ava*II site is present in the amplified fragment of HSV1, resulting in fragments of 87, 183 and 206 bp. This allows differentiation of HSV1 and HSV2 with a single restriction enzyme cleavage.

Digestion products were electrophoresed through a 2% agarose gel and photographed on a UV transilluminator after staining with ethidium bromide.

*Endonuclease cleavage for the reconfirmation of the specificity of EBV DNA amplification.* Thirty µl of the amplified PCR product showing the 375 bp band was digested with 100 U from the restriction endonuclease *Hind*III (New England Biolabs). Samples were digested O/N at 37°C. The conditions followed for digestion were those recommended by the supplier. There is a single *Hind*III site in the amplified sequence of EBV that cleaves the 375 bp product into 214 and 161 bp fragments.

Digestion products were electrophoresed through a 2% agarose gel and photographed on a UV transilluminator after staining with ethidium bromide.

Results

We examined 22 aqueous humour samples from patients with active, clinically diagnosed retinitis (N=7) or uveitis (N=15). Our results are summarised in Table II. HSV DNA was detected in 4 (18.2%) (Fig. 1), CMV DNA in 6 (27.3%) (Fig. 2) and EBV DNA in 1 (4.5%) (Fig. 3) out of the 22 examined samples. HSV DNA was detected in 1 and CMV DNA in 3 out of the 7 examined patients with retinitis. EBV DNA was not detected in any of the examined samples from patients with retinitis. HSV DNA was detected in 3, CMV DNA in 3 and EBV DNA in 1 out of the 15 examined patients with uveitis. None of the 22 examined patients was found to be co-infected with HSV, CMV or EBV. Samples which were found positive for HSV were followed by endonuclease cleavage using the restriction endonuclease *Ava*II for the differentiation between the two types of HSV. All the samples were found to be HSV 1 (Fig. 4). Ocular infection caused by EBV is not very common. Thus, the restriction endonuclease *Hind*III was used to reconfirm the specificity of the amplification of EBV DNA. There is a unique *Hind*III cleavage site in the amplified fragment of EBV whereas the amplified PCR product is digested in two fragments of 214bp and 161bp (Fig. 5).

Table II. Summary of results on the detection of HSV, CMV and EBV in patients with inflammatory eye diseases.

Study cases	No. of patients	HSV positive	CMV positive	EBV positive
Retinitis	7	1	3	0
Uveitis	15	3	3	1
Total	22	4 (18.2%)	6 (27.3%)	1 (4.5%)

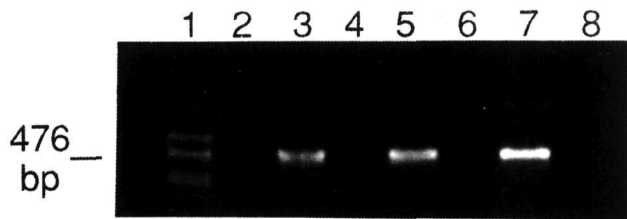


Figure 1. Detection of HSV DNA by PCR. Amplification products were electrophoresed through a 2% agarose gel. Lane 1: pUC18/HaeIII molecular marker, lane 2: negative control, lane 3: positive control, lanes 4, 7: positive samples, lanes 5, 6, 8: negative samples.

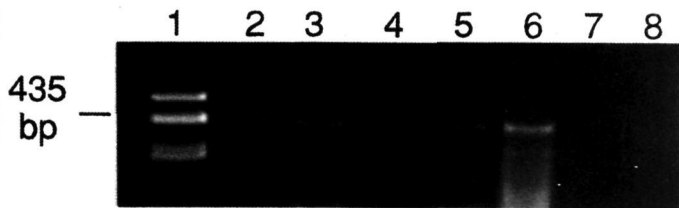


Figure 2. Detection of CMV DNA by PCR. Amplification products were electrophoresed through a 2% agarose gel. Lane 1: pUC18/HaeIII molecular marker, lane 2: negative control, lane 3: positive control, lanes 4, 8: positive samples, lanes 5, 6, 7: negative samples.

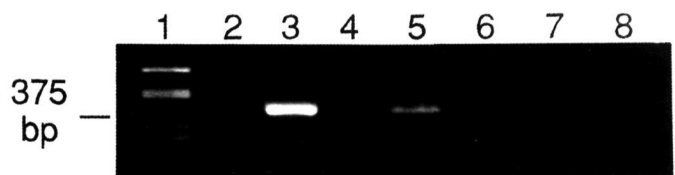


Figure 3. Detection of EBV DNA by PCR. Amplification products were electrophoresed through a 2% agarose gel. Lane 1: pUC18/HaeIII molecular marker, lane 2: negative control, lane 3: positive control, lanes 4, 6, 7, 8: negative samples, lane 5: positive sample.

## Discussion

Intraocular inflammation caused by viral infection represents one of the major known types of endogenous inflammatory eye diseases (17). Infections caused by herpesviruses are very common in immunosuppressed and immunocompromised patients. Moreover, ocular diseases in these specific populations are strongly associated with infections caused by

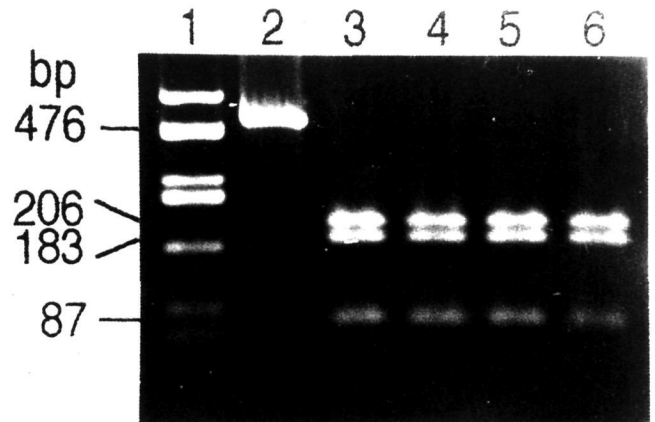


Figure 4. Endonuclease cleavage for the differentiation of HSV type. Digestion products were electrophoresed through a 2% agarose gel. Lane 1: pUC18/HaeIII molecular marker, lane 2: undigested PCR product, lane 3: HSV 1 positive control, lanes 4, 5, 6: HSV 1 samples.

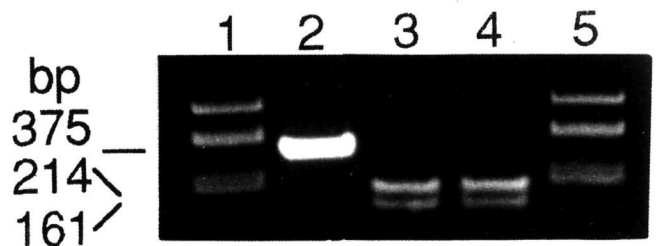


Figure 5. Endonuclease cleavage for the reconfirmation of the specificity of EBV DNA amplification. Digestion products were electrophoresed through a 2% agarose gel. Lane 1: pUC18/HaeIII molecular marker, lane 2: undigested PCR product, lane 3: EBV positive control, lane 4: EBV positive sample.

members of the herpesvirus family (18,19). However, establishment of the diagnosis of ocular disease due to viral infection is usually difficult. Although a clinical diagnosis of viral ocular infection is most frequently made, based on the typical appearance of chorioretinal lesions, in patients with atypical features, it may be difficult to differentiate, based on clinical findings alone, between viral infections due to herpes group viruses or other nonviral pathogens. As the recommended antiviral treatments differ for the various herpes group viruses, optimal management and treatment require a rapid as well as specific laboratory diagnosis for establishing appropriate antiviral therapy. Current laboratory techniques for the detection of herpesviruses in ophthalmic

lesions are based on serology and cell culture. However, both approaches have significant disadvantages such as low specificity and sensitivity. Also, cost and time consumed must be considered. PCR has been proved to be accurate and very sensitive when applied for the detection of viral DNA in almost every type of clinical sample (20-24).

In this study, we employed PCR to detect herpesvirus DNA in aqueous humour from patients with clinically diagnosed retinitis, or uveitis. None of the 22 examined patients was immunosuppressed or immunocompromised at the time of examination. Presence of HSV, CMV and EBV DNA was consequently judged from the amplification of a 476, 435 and 375 bp long sequences (Figs. 1-3).

Samples which were found to be HSV positive were further checked for the differentiation of HSV type. All HSV positive samples were found to be HSV type 1. This result was based on the 206, 183 and 87 bp fragments after the endonuclease cleavage of HSV PCR products (Fig. 4).

Our data suggest that HSV 1 and CMV may play an important role in ocular inflammatory diseases. Also, the use of the PCR technique for the direct detection of herpesvirus DNA in aqueous humour samples seems to be of major importance. PCR may be important in the laboratory for the detection of viral agents in the early stages of the inner eye infection offering the opportunity for prompt treatment, especially during the acute stage of the disease.

## References

1. Fox GM, Crouse CA, Chouang EL, Pflugfelder SC, Cleary TJ, Nelson SJ and Atherton SS: Detection of herpesvirus DNA in vitreous and aqueous specimens by the polymerase chain reaction. *Arch Ophthalmol* 109: 266-271, 1991.
2. Friedman AH, Orellana J, Freeman WR, Luntz MH, Starr MB, Tapper ML, Spigland I, Rotterdam H, Tejada RM, Brauhut S, Mildvan D and Mathur U: Cytomegalovirus retinitis: a manifestation of the acquired immune deficiency syndrome (AIDS). *Br J Ophthalmol* 67: 372-380, 1983.
3. Henderly DE, Freeman WR, Smith RE, Causey D and Rao NA: Cytomegalovirus retinitis as the initial manifestation of the acquired immune deficiency syndrome. *Am J Ophthalmol* 103: 316-320, 1987.
4. Usui M, Usui N, Goto H, Minoda H and Rai T: Polymerase chain reaction for diagnosis of herpetic intraocular inflammation. *Ocul Immunol Inflamm* 1: 105-112, 1993.
5. Shibata D, Martin WJ, Appleman MD, Causey DM, Leedom JM and Arnheim N: Detection of cytomegalovirus DNA in peripheral blood of patients infected with human immunodeficiency virus. *J Inf Dis* 158: 1185-1192, 1988.
6. Telenti A, Marshall WF, Smith TF: Detection of Epstein-Barr virus by polymerase chain reaction. *J Clin Microbiol* 28: 2187-2190, 1990.
7. Puchamer-Stöckl E, Popow-Kraupp T, Heinz FX, Mandl CW and Kunz C: Establishment of PCR for the early diagnosis of herpes simplex encephalitis. *J Med Virol* 32: 77-82, 1990.
8. Nikolaidou A, Liloglou T, Malliri A, Ergazaki M, Tiniakos G, Tiniakos D and Spandidos DA: Detection of hepatitis B virus DNA and mutations in *K-ras* and *p53* genes in human hepatocellular carcinomas. *Int J Oncol* 3: 593-596, 1993.
9. Eisenstein BI: The polymerase chain reaction: A new method of using molecular genetics for medical diagnosis. *N Engl J Med* 322: 178-183, 1990.
10. Wright PA, Wynford-Thomas D: The polymerase chain reaction: Miracle or mirage? A critical review of its uses and limitations in diagnosis and research. *J Pathol* 162: 99-117, 1990.
11. Demmler GJ, Buffone GJ, Schimbor CM and May RA: Detection of cytomegalovirus in urine from newborns by using polymerase chain reaction DNA amplification. *J Inf Dis* 158: 1177-1184, 1988.
12. Ergazaki M, Xinarianos G, Koffa M, Liloglou T and Spandidos DA: Detection of human cytomegalovirus by the polymerase chain reaction in immunosuppressed and immunocompromised patients. *Oncol Rep* 1: 805-808, 1994.
13. Rogers BB, Josephson SL and Mak SK: Detection of herpes simplex virus using the polymerase chain reaction followed by endonuclease cleavage. *Am J Pathol* 139: 1-6, 1991.
14. Rogers BB, Alpert LC, Hine EAS, Buffone GJ: Analysis of DNA in fresh and fixed tissue by the PCR. *Am J Pathol* 136: 541-548, 1990.
15. Liloglou T, Giannoudis A, Ergazaki M, Koffa M and Spandidos DA: Detection of Epstein Barr virus by the polymerase chain reaction in immunosuppressed and immunocompromised patients. *Oncol Rep* 1: 809-812, 1994.
16. Ergazaki M, Xinarianos G, Giannoudis A, Koffa M, Liloglou T, Kattamis Ch and Spandidos DA: Detection of human cytomegalovirus and Epstein-Barr virus by the polymerase chain reaction in patients with  $\beta$ -thalassaemia. *Oncol Rep* 1: 813-816, 1994.
17. Yoser SL, Forster DJ and Rao NA: Systemic viral infections and their retinal and choroidal manifestations. *Surv Ophthalmol* 37: 313-352, 1993.
18. Drew LW: Nonpulmonary manifestations of cytomegalovirus infection in immunocompromised patients. *Clin Microbiol Rev* 5: 204-210, 1992.
19. Masur H: Clinical implications of herpesvirus infections in patients with AIDS. *Am J Med* 92 (suppl 2A): 1-2, 1992.
20. Gulley ML and Raab-Traub N: Detection of Epstein-Barr virus in human tissues by molecular genetic techniques. *Arch Pathol Med* 117: 1115-1129, 1993.
21. Mao EJ and Smith CJ: Detection of Epstein-Barr virus (EBV) DNA by the polymerase chain reaction (PCR) in oral smears from healthy individuals and patients with squamous cell carcinoma. *J Oral Pathol Med* 22: 12-17, 1993.
22. Cathomas G, Morris P, Peckle K, Cunningham I and Emanuel D: Rapid diagnosis of cytomegalovirus pneumonia in marrow transplant recipients by bronchoalveolar lavage using the polymerase chain reaction, virus culture, and the direct immunostaining of alveolar cells. *Blood* 81: 1909-1914, 1993.
23. Rogers BB, Josephson SL, Mak SK and Sweeney PJ: Polymerase chain reaction amplification of herpes simplex virus DNA from clinical samples. *Obstet Gynecol* 79: 464-469, 1992.
24. Spandidos DA, Xinarianos G, Ergazaki M, Giannoudis A and Tsaparakis J: The presence of herpesviruses in pterygium. *Int J Oncol* 5: 749-752, 1994.