ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (2003) 21: 55-61

Detection and Differentiation of Human Herpesviruses 1-5 by Consensus Primer PCR and RFLP

Kamol Sakulwira[1], Pijitra Vanapongtipagorn[2], Apiradee Theamboonlers[2], Parvapan Bhattarakosol[3], Siriwan Wananukul[4], and Yong Poovorawan[2]

SUMMARY **Eight human viruses of the *Herpesviridae* family represent a significant public health problem world-wide. Detection and typing of five of the human herpesviruses (HSV-1, HSV-2, VZV, EBV, and CMV) was performed by applying a consensus primer polymerase chain reaction (PCR). The amplified PCR products from the five human herpesviruses were typed based on their restriction enzyme digestion polymorphism with *Hinf* I and *Alu* I. Fifteen clinically suspected specimens from herpesvirus-infected patients were also evaluated. A fragment of the DNA polymerase gene from each of the five human herpesviruses was successfully amplified by the set of consensus primers. Their amplicons obtained by PCR from the template DNAs were subjected to restriction endonuclease digestion and human herpesviruses 1-5 could be clearly differentiated and typed. This method can be used to detect and differentiate between the five human herpesviruses in clinical specimens. This study demonstrates the value of testing for five human herpesviruses by consensus PCR and restricted fragment length polymorphism (RFLP). These procedures are simple and straightforward techniques for the investigation of clinical specimens.**

Eight human viruses of the *Herpesviridae* family- herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpesviruses 6, 7 and 8 (HHV-6, 7 and 8) - are a significant public health problem world-wide. These viruses have been classified as members of the alpha-herpesvirus subgroup (HSV-1, HSV-2 and VZV), beta-herpesvirus subgroup (CMV, HHV-6 and HHV-7) and gamma-herpesvirus subgroup (EBV and HHV-8).[1,2] Whereas some of the agents have been known for decades and are well characterized, little is known about the pathogenic potentials of some of the more recently described members of this viral family.

Before the introduction of molecular techniques, laboratory diagnosis of viral infections relied on cell culture for virus isolation, detection of specific antibody or detection of viral antigen. The clinical signs produced by alpha-herpesviruses (HSV-1, HSV-2 and VZV) are fever with vesicular lesions. In normal hosts, it is easy to differentiate the clinical presentations of HSV and VZV. In immunocompromised hosts, however, skin lesions are not typical. Tzanck smear and skin biopsy can not differentiate between HSV and VZV.[3] Serological diagnosis is of value only to determine past exposure. VZV is a highly contagious virus that needs isolation and requires higher doses of antiviral therapy. CMV and EBV manifest as fever with maculopapular rashes. They have clinical importance in immunocompromised-hosts because all human herpesvi-

From the [1]Inter-Department of Medical Microbiology, Faculty of Graduate Studies, Chulalongkorn University, Bangkok 10330, Thailand, [2]Viral Hepatitis Research Unit, Department of Paediatrics, [3]Department of Microbiology, [4]Department of Paediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.
Correspondence: Yong Poovorawan

ruses retain a certain latency after their initial infection and their reactivation may cause a fatal outcome. Therefore, tests based on polymerase chain reaction (PCR) have assumed an important role in the laboratory detection of these agents.

In this study, we presented a consensus primer PCR assay for detection and restriction fragment length polymorphism (RFLP) for typing of the five human herpesviruses: HSV-1, HSV-2, VZV, EBV, and CMV.

MATERIALS AND METHODS

Positive control specimens

HSV-1 (KOS) and HSV-2 (Baylor 186) seeds, as well as CMV-DNA positive serum were provided by the Virology Unit, Department of Microbiology, Faculty of Medicine, Chulalongkorn University. CMV (AD169) cell culture was provided by the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University. VZV was taken from vesicles of children with chicken pox. The vesicles were ruptured and the intravascular fluid was dissolved in PBS. The EBV strain B95-8 with a known concentration (2×10^6 template molecules per 100 ng of human DNA) was provided by the Genetics Unit, Department of Anatomy, Faculty of Medicine, Chulalongkorn University. All specimens were kept at -70^0C until tested.

Negative control specimens

Asymptomatic healthy hepatitis B carrier sera were used in parallel with sterile distilled water as negative control.

Clinical specimens

Fifteen specimens were collected from highly suggestive or clinically diagnosed herpesvirus-infected patients. Eleven of those were taken from the vesicle fluids (chicken pox, 5; herpes zoster, 1; herpes stomatitis, 3; herpes vaginalis, 2) while four were taken from the sera of nasopharyngeal carcinoma patients with positive EBV-DNA test.[4]

Human herpesviral DNA extraction

One hundred microliters of specimens were extracted with proteinase-K/SDS in Tris buffer pH 8.0, followed by phenol/chloroform extraction and ethanol precipitation.[5] The pellet was dissolved in 20 µl of sterile distilled water and directly subjected to the polymerase chain reaction.

Human herpesviral DNA detection

DNA was amplified in an automated thermocycler (Perkin Elmer Cetus, Branchburg, NJ, USA) using nested primers on DNA polymerase gene as described elsewhere.[6] Briefly, PCR mixture (50 µl) using 5 µl of viral template DNA, 1.5 units of *Taq* polymerase in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ (QIAGEN, Hilden, Germany), 200 µM each of the four deoxyribonucleotide triphosphates (Pharmacia Biotech, NJ, USA) and 15 pmol of each primer. We used distilled water as negative control in parallel with sera of asymptomatic hepatitis B carriers. Primary PCR mixtures contained two upstream primers (DFA, 5'-GAYTTYGCNAGYYTNTAYCC-3'; and ILK, 5'-TCCTGGACAAG-CAGCARNYSGCNMTNAA-3') and one downstream primer (KG1, 5'-GTCTTGCTCACCAGNTCNA-CNCCYTT-3') in a multiplex format. The reactions were cycled 45 times with 30 seconds of denaturation at 94°C, 1 minute of annealing at 46°C and 1 minute of strand extension at 72°C. After cycling, the reaction mixtures were incubated for 7 minutes at 72°C and were then held at 4°C. Secondary PCRs were performed on 5 µl of the primary PCR mixture in a 50-µl volume with the upstream primer TGV (5'-TGTAACTCGGTGTAYGGNTTY-ACNGGNGT-3') and downstream primer IYG (5'-CACAGAGTCCG-TRTCNCCRTADAT-3') under the same conditions used for the primary reaction. The secondary PCR products were analysed on a 2% agarose gel made up in 0.5x TBE and containing 0.5 µg of ethidium bromide per ml. The assay amplified a short (215 to 315 bp) region of herpesviral DNA polymerase-coding sequences.[6]

PCR sensitivity

Ten-fold serial dilutions were prepared for the EBV strain B95-8 of a known concentration (50 ng or 10^6 copies/µl) and were used to perform sensitivity testing.

Herpesviral DNA sequencing

The herpesviruses 1-5-PCR products were purified for sequencing by the QIAquick Gel Extraction Kit (Qiagen Inc, Valencia, CA, USA) according to the manufacturer's specifications and subsequently subjected to 2% agarose gel electrophoresis in order to ascertain their purity. DNA was subjected to cycle sequencing by dye-labeled terminators which represented a rapid and convenient method for performing enzymatic extension reactions for subsequent DNA sequencing on the ABIPRISM^{TM}310

Genetic Analyser (Perkin Elmer Cetus, Branchburg, NJ, USA). This round of amplifications was performed according to the manufacturer's specifications using the forward primer TGV and reverse primer IYG to amplify the particular DNA strand of interest for further sequencing. Cycle sequencing consisted of 25 cycles at 96°C for 10 seconds (denaturation), 50°C for 5 seconds (annealing), and 60°C for 4 minutes (extension). The reaction was terminated by cooling the thermal ramp to 4°C. The extension products were subsequently purified from excess un-incorporated dye terminators by ethanol precipitation, according to the manufacturer's specifications (Perkin Elmer Cetus, ABIPRISM™310 Genetic Analyser). For all the subsequent steps we referred to the ABIPRISM™ 310 Genetic Analyser user's manual (Perkin Elmer Cetus, Branchburg, NJ, USA). Herpesviruses 1-5-DNA sequence analysis of the PCR products was sequenced by the inner sense primer, TGV and confirmed by the inner anti-sense primer IYG. The nucleotide sequences were compared to the DNA sequences of HSV-1, HSV-2, VZV, EBV and CMV from the GenBank accession numbers M10792, M16321, AB059830, V01555 and AF291828, respectively, using the BLAST program, NCBI.

Table 1 Polymorphism among different human Herpesviruses based on RFLP analysis of *Hinf* I and *Alu* I.

Human herpesvirus	PCR products (bp)	Fragment sizes (bp)	
		Hinf I	*Alu* I
HSV-1	231	185, 38, 8	144, 87
HSV-2	231	223, 8	144, 87
VZV	234	226, 8	234
EBV	231	197, 34	176, 55
CMV	303	196, 107	303

Fig. 1 Ethidium bromide stained nested PCR products of human herpesviruses with consensus primers. M: 100-bp marker; 1: HSV-1 (231 bp); 2: HSV-2 (231 bp); 3: VZV (234 bp); 4: EBV (231 bp); 5: CMV (303 bp) and N: negative control.

Human herpesvirus typing

Human herpesviral DNA PCR products were used for human herpesvirus typing by RFLP. We further applied the restriction endonuclease most suitable for specific genotyping by selecting from a table of enzyme names. Computer predictions of restriction fragment sizes from PCR products indicated that both *Hinf* I and *Alu* I should produce differentiable cleavage patterns of each of the five human herpesviruses. Accordingly, we selected *Hinf* I and *Alu* I for typing the five human herpesviruses. Twenty units of enzyme were added to 20 µl of secondary PCR product and subsequently incubated at 37°C for 4 hours. The cluster products were subjected to electrophoresis on a 3% nusieve agarose gel and their respective sizes compared to that of a suitable nucleotide size marker (100 bp DNA ladder, NEB, New England, USA). The expected sizes of the polymorphisms after RFLP were shown in Table 1.

RESULTS

A fragment of the DNA polymerase gene from all 5 human herpesviruses was successfully amplified by the set of consensus primers located within a highly conserved region of the gene. The presence of PCR products of the expected sizes (231, 231, 231, 234 and 303 bp corresponding to HSV-1, HSV-2, EBV, VZV and CMV, respectively) on ethidium bromide-

stained gel are shown in Fig. 1. Negative control and HBV positive sera were shown to be absent of any bands. The sensitivity of this nested PCR was found at a concentration of 40 copies of EBV DNA polymerase template per 100 ng of DNA.

Herpesviral positive control PCR products were subsequently sequenced for specificity of the test and submitted to the Genbank (accession numbers AY114170-AY114174). The fragment of strains tested were shown to have 96%, 98%, 99%, 99% and 96% nucleotide identity to HSV-1, HSV-2, VZV, EBV and CMV (Genbank accession number M10792, M16321, AB059830, VO1555 and AF291828), respectively.

The CMV amplified products could be accurately identified by their PCR- product sizes (Fig 1). The remaining four human herpesviruses led to PCR products of similar sizes and therefore needed further characterization. Species identification was done by restriction enzyme digestion. Amplicons obtained by PCR from the template DNAs described above were subjected to restriction endonuclease digestion with *Hinf* I and *Alu* I (Fig. 2). After digestion and electrophoresis, each viral DNA yielded a characteristic fragment pattern that

Fig. 2 The sequences and restriction sites of *Hinf* I (**G/ANTC**) and *Alu* I (**AG/CT**) of the amplified products of the herpesviruses 1-5.

```
AY114170        TGTAACTCGGTGTATGGCTTCACAGGAGTGCAGCACGGACTCCTGC-CGTGCCTGCACGT
AY114171        TGCAACTCGGTGTACGGGTTCACCGGGGTGCAGCACGGTCTTCTGC-CCTGCCTGCACGT
AY114172        TGTAATTCCGTGTACGGTTTTACTGGAGTTGCGCAGGGATTTCTGC-CATGTTTATACGT
AY114174        TGCAACGCTTTCTACGGTTTTACCGGCGTGGTCAACGGCATGATGC-CGTGTCTGCCCAT
AY114173        ------------------CTCTCTGAAAACCCCCGGCACTCGATAAACCTGCCT-CTCGT
                                  *  * *                  *   * **  *   * *

AY114170        TGCCGCGACGGTGACGACCATCGGCCGCGAGATGCTGCTCGCGACCCGCGAGTACGTCCA
AY114171        GGCCGCCACCGTGACGACCATCGGCCGCGATATGCTCCTCGCGACGCGCGCGTACGTGCA
AY114172        AGCGGCCACTGTCACTACAATTGGCCGTCAAATGTTATTAAGTACCAGAGATTATATTCA
AY114174        CGCCGCCAGCATCACGCGCATCGGTCGCGACATGCTAGAGCGCACGGCGCGGTTCATCAA
AY114173        CGCCGAGACGGTGACGCTGCAGGGCCGCACGATGTTGGAGCGGGCCAAGGCCTTCGTGGA
                 ** *  *   * **       ** **    *** *        *       *   *  *

AY114170        CGCGCGATGGGCGGCCTCGAACCAGCTCCTGGCCGATTTCCCGGAGCCGGCCGACA---T
AY114171        CGCGCGCTGGGCGGAGTTCGATCAGCTGCTGGCCGACTTTCCGGAGGCGGCCGGCA---T
AY114172        TAATAACTGGGCCGCATTTGAACGTTTTATTACAGCGTTTCCAGACATTGAAAGTAGCGT
AY114174        AGACAATTTTTCAGAGCCGTGTTTTTTGCACAATTTTTTTAATCAGGAAGACTATGTAGT
AY114173        GGCCCTGAGCCCCGCCAACCTGCAGGCCCTGGCC-CCTCCCCGGACGCCTG-GGCGCCCC
                          * *                        *      *

AY114170        GCGCGCCCCCGGGCCCTATTCCATGCGCATCATCTACGGGGAC-ACGGACTCCATA----
AY114171        GCGCGCCCCCGGTCCGTACTCCATGCGCATCATTTACGGAGAC-ACGGACTCCATT----
AY114172        TCTCTCCCAAAAAGCGTACGAGGTAAAGGTTATATATGGAGAT-ACGGATTCTGTG----
AY114174        GAGAACGCGGGAGGGGGATTCGGAGGAGAGCAGCACGTTACCGGAGGGGCTCGAAACATC
AY114173        TCAACCCCGAGGGCCAGCTTCGAGTCATCGTCTCGGCGATGGAGAGGCAGGGAAAGAGGC
                     * *                                      * *

AY114170        ------------------------------------------------------------
AY114171        ------------------------------------------------------------
AY114172        ------------------------------------------------------------
AY114174        GTCAGGGGGCTCGAACGAACGGCGGGTGGAGGCGCGGGTCATCTACGGGGACACGGACAG
AY114173        CGTTGGCCACCC------------------------------------------------

AY114170        ----
AY114171        ----
AY114172        ----
AY114174        CGTG
AY114173        ----
```

corresponded to that predicted by DNA sequence analysis as shown in Figs. 3 and 4.

The clinical suspected specimens taken from vesicle fluids of five chicken pox and one herpes zoster patients showed clearly positive RFLP patterns to VZV. Three herpes stomatitis patients were positive to HSV-1, and two herpes vaginalis patients were positive to HSV-2. In three sera of the four nasopharyngeal carcinoma patients with EBV viremia, EBV-DNA was detectable by using consensus primers and they also showed EBV patterns on RFLP.

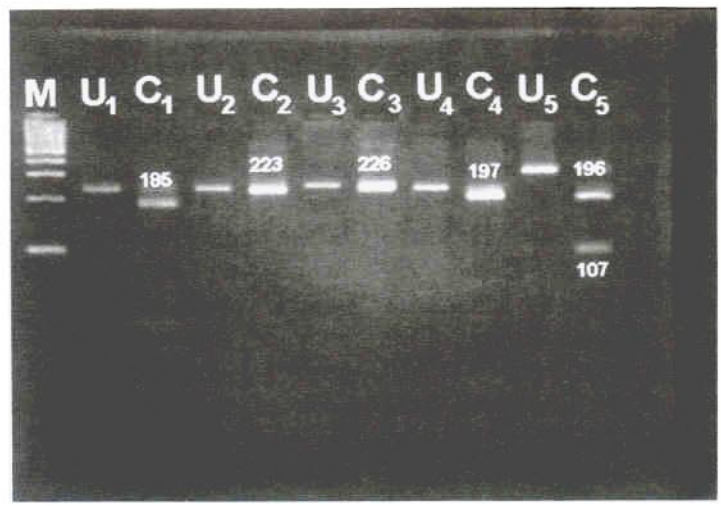

Fig. 3 RFLP pattern of human herpesviruses digested with endonuclease *Hinf* I. M: 100-bp marker; U_1-U_5: undigested PCR products (HSV-1, HSV-2, VZV, EBV and CMV, respectively); C_1-C_5: digested PCR products (HSV-1, HSV-2, VZV, EBV and CMV, respectively).

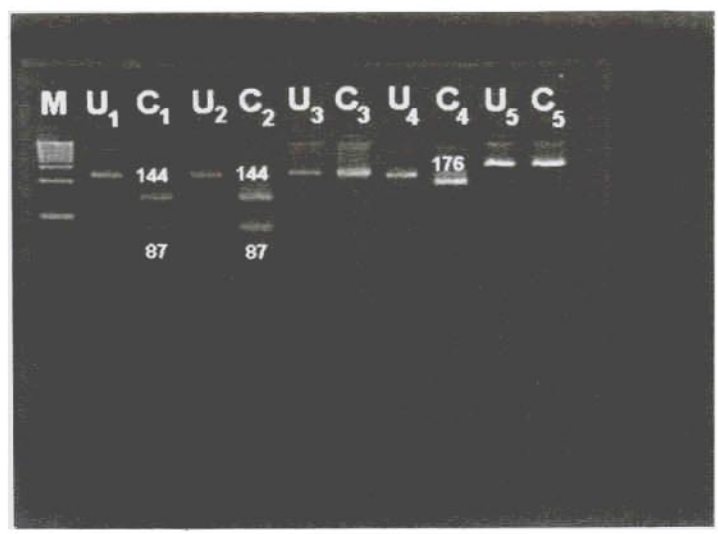

Fig. 4 RFLP pattern of human herpesviruses digested with endonuclease *Alu* I. M: 100-bp marker; U_1-U_5: undigested PCR products (HSV-1, HSV-2, VZV, EBV and CMV, respectively); C_1-C_5: digested PCR products (HSV-1, HSV-2, VZV, EBV and CMV, respectively).

DISCUSSION

Detection and differentiation of human herpesviruses have been a time- consuming and labour intensive processes. Differentiation is made even more difficult by the extensive genetic relatedness of these viruses. The Tzanck smear was detectable in 60% of HSV cases and 64-75% of VZV cases. Viral culture was positive in 83% and 26-44% of HSV and VZV cases, respectively.[7,8] However, the antigenic cross-reactivity of most viral polypeptides in particular has impeded the development of reliable and sensitive serodiagnostic tests. In an attempt to circumvent the antigenic cross-reactivity of these viruses, the development of diagnostic tests based on the detection of viral DNA has been explored. PCR gives rapid and early results relative to the onset of clinical symptoms. In our study, we used consensus primers to amplify five members of the *Herpesviridae* family. A simple digestion of the amplified products by two enzymes, *Hinf* I and *Alu* I, allowed us to identify accurately and rapidly the different types of the human herpesvirus.

A diagnostic assay was developed for the detection of only three human herpesviruses.[9] The herpesvirus consensus PCR and RFLP described here appear to be reliable for use with clinical samples. This is a system to detect five

different human herpesviruses in clinical specimens (vesicular fluids and sera) by a technique suitable for routine uses. This method helps to determine the etiologies of patients that came with vesicular and maculopapular rashes. There are clear advantages to this method: it requires less amount of sample material, reagents and time. Differentiation of HSV-1, HSV-2 and VZV helps clinicians with the diagnosis, treatment and containment of the disease. Differentiation of CMV and EBV will definitely help clinicians with their decision of antiviral therapy because CMV responses to ganciclovir but EBV does not.

Three of four clinical specimens from sera of nasopharyngeal carcinoma patients with detectable EBV-DNA were positive by using consensus primers. The results may relate to the amount of DNA in the sera. The relative sensitivity of consensus primer PCR in detecting different human herpesviral DNA polymerase genes was determined by assaying for known quantities of viral DNA polymerase fragments from human herpesviruses 1 to 6 in the presence of normal human DNA (from 10^5 to 1 template molecule per 100 ng of human DNA). The sensitivity of this technique was previously demonstrated at a concentration of 1, 10 and 100 copies of DNA polymerase template per 100 ng of DNA for HSV-1 and HSV-2, CMV and HHV-6, and VZV and EBV, respectively.[6] However, in this study, the sensitivity in detecting the EBV strain B95-8 was found to be 40 copies of DNA polymerase template per 100 ng of DNA. The addition of 5% dimethylsulfoxide (DMSO) lowered the threshold of detection of EBV DNA polymerase target fragments from 10^5 to 10^2 copies per 100 ng of DNA.[6,10]

The simplicity of the method that we have described here will have an impact on the management of human herpesvirus infections, especially in immunocompromized patients. Unfortunately, our study did not include the human herpesviruses 6, 7 and 8. Human herpesviruses 6 and 7 are closely related beta-herpesviruses that have been linked with acute febrile illness, roseola infantum, as well as invasion of the central nervous system.[2,11] Human herpesvirus 8 is associated with Kaposi's sarcoma in HIV infected patients.[12] Further studies are needed to evaluate the specificity and sensitivity of using consensus primer PCR and RFLP for diagnosis and typing of these human herpesviruses, as compared to using the gold standard technique.

In conclusion, we have described a method for detection and typing of human herpesviruses by using consensus primer PCR and RFLP which can amplify and identify five different human viruses of the *Herpesviridae* family. In this current state of development, these procedures are simple, streamlined and cost-effective. They are well-suited for routine clinical laboratory practice.

ACKNOWLEDGEMENTS

The authors would like to thank the Department of Microbiology and Department of Anatomy, Faculty of Medicine, Chulalongkorn University; Associate Professor Surang Tantivanich, Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, for providing the positive specimens; Division of Venereal Diseases, Department of Diseases Control, Ministry of Public Health for providing the clinical specimens, and the Thailand Research Fund, Senior Research Scholar, the Royal Jubilee Ph.D. Program, the Viral Hepatitis Research Unit and the Centre of Excellence Fund, Ratchadaphisek Somphot Endowment, Chulalongkorn University, for supporting the study. We also thank Ms. Pisanee Saikarin and Venerable Dr. Mettanando Bhikkhu of Wat Rajaorasaram, Bangkok, for reviewing the manuscript.

REFERENCES

1. Arao Y, Schmid DS, Pellett PE, Inoue N. Herpesviruses beyond HSV-1 and HSV-2. Clin Microbiol Newslett 1999; 21: 153-9.
2. Levy JA. Three new human herpesviruses (HHV-6, 7, 8). Lancet 1997; 349: 558-62.
3. Cohen JI, Brunell PA, Straus SE, Krause PR. Recent advances in varicella-zoster virus infection. Ann Intern Med 1999; 130: 922-32.
4. Mutirangura A, Pornthanakasem W, Theamboonlers A, *et al.* Epstein-Barr viral DNA in serum of patients with nasopharyngeal carcinoma. Clin Cancer Res 1998; 4: 665-9.
5. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A Laboratory Manual, Second edition, Part 3. Cold Spring Harbor Laboratory Press, Plainview, New York, 1989.
6. Van Devanter DR, Warrener P, Bennett L, *et al.* Detection and analysis of diverse herpesviral species by consensus primer PCR. J Clin Microbiol 1996; 34: 1666-71.
7. Nahass GT, Goldstein BA, Zhu WY, Serfling U, Penneys NS, Leonardi CL. Comparison of Tzanck smear, viral culture, and DNA diagnosis methods in detection of herpes simplex and varicella-zoster infection. JAMA 1992; 268: 2541-4.
8. Sadick NS, Swenson PD, Kaufman RL, Kaplan MH. Comparison of detection of varicella-zoster virus by the Tzanck smear, direct immunofluorescence with a monoclonal antibody, and virus isolation. J Am Acad Dermatol 1987; 17: 64-9.
9. Aono T, Murakami S, Yanagihara N, Yamanishi K. Detection of human alpha-herpesvirus DNA using consensus

primers and specific probes. Acta Otolaryngol Suppl 1994; 514: 132-4.
10. Winship PR. An improved method for directly sequencing PCR amplified material using dimethyl sulphoxide. Nucleic Acids Res 1989; 17: 1266.
11. Yoshikawa T, Ihira M, Suzuki K, *et al.* Invasion by human herpesvirus 6 and human herpesvirus 7 of the central nervous system in patients with neurological signs and symptoms. Arch Dis Child 2000; 83: 170-1.
12. Ascoli V, Lo Coco F, Torelli G, *et al.* Human herpesvirus 8 associated primary effusion lymphoma in HIV patients: a clinicopidermiologic variant resembling classic Kaposi's sarcoma. Haematologica 2002; 87: 339-43.