Detection and Typing of HSV-1, HSV-2, and VZV by a Multiplex Polymerase Chain Reaction

Panayotis Markoulatos,^{1*} Amalia Georgopoulou,¹ Constantin Kotsovassilis,²
Paraskevi Karabogia-Karaphillides,³ and Niki Spyrou¹

¹*Hellenic Pasteur Institute, Department of Virology, Athens, Greece*

²*Laboratory of Clinical Chemistry, General Hospital of Athens, Athens, Greece*

³*Laboratory of Virology, General Hospital of Athens, Athens, Greece*

The development of a multiplex polymerase chain reaction method for the rapid and accurate detection and typing of HSV-1, HSV-2, and VZV from clinical specimens is described. A sensitive multiplex polymerase chain reaction was achieved by optimization of parameters such as the primers, magnesium, and dNTPs concentrations. False-negative results that sometimes arise due to inhibitors of DNA amplification or failure of DNA extraction procedure used may be avoided by assaying each specimen with α -tubulin primers. Multiplex PCR amplified

viral sequences from all 55 specimens obtained from patients with clinical evidence of HSV or VZV infection indicated 100% sensitivity. From 55 patients who were investigated by multiplex PCR, HSV-1 was detected in 28, HSV-2 in 20, and VZV in 7 specimens. The reported results indicate that the present multiplex PCR assay has a potential application in clinical diagnosis when a rapid and accurate detection and typing of involved viruses HSV-1, HSV-2, or VZV is needed. *J. Clin. Lab. Anal.* 14:214–219, 2000. © 2000 Wiley-Liss, Inc.

Key words: polymerase chain reaction (PCR); multiplex; herpes simplex virus types 1 and 2; varicella zoster virus

INTRODUCTION

Herpes simplex (HSV) types 1 and 2 (HSV-1, HSV-2) and varicella zoster virus (VZV) are ubiquitous agents causing a wide range of acute central nervous system (CNS) diseases; they are important human pathogens and belong to the subfamily of alpha herpesviruses. The clinical diagnosis of herpes simplex, varicella, and zoster presents few difficulties once the eruption has developed. Virological confirmation is required in the case of zosteriform herpes simplex and in atypical VZV and HSV infections of immunosuppressed patients (1). Both primary and recurrent HSV or VZV infections may lead to CNS infection and disease (2).

Although some herpesviral diseases are associated unequivocally with only one virus, most of the herpesviral syndromes can be due to different herpesviruses. The severity of some of these diseases and their high incidence in immunocompromised patients has led to the development of specific antiviral treatment (3,4). Nevertheless, the high frequency of syndromes due to different viruses often makes it necessary to undertake time-consuming differential diagnosis (5,6). Analysis of viral DNA with restriction enzymes is a specific method and the restriction patterns of HSV-1 are readily distinguished from those of HSV-2. Typing of HSV isolates by restriction-enzyme-DNA analysis is time consum-

ing, involves the use of radioisotopes, and is too cumbersome for most clinical laboratories to be performed on a large scale (7,8).

In recent years, the polymerase chain reaction (PCR) assay has proven to be a powerful tool for detecting a broad range of infectious agents, which can be present in extremely small amounts in human tissues or in body fluids even from samples such as crust from lesions. Moreover, the PCR assay has been shown to be capable of detecting herpesvirus-specific DNA sequences in clinical samples that yielded negative results by the current virus isolation procedures (9,10). The specificity of the PCR process relies on the chosen primers, which hybridize to the target DNA sequences. A multitude of different primer pairs may be used for the detection of a given viral genome. Sensitivity and specificity may vary with different primer pairs and the genetic variability of naturally occurring strains of viruses is an important influencing factor. Many different primer pairs have been suggested for the diagnosis of HSV and VZV infections (1,2,8,11–15).

*Correspondence to: Panayotis Markoulatos, Hellenic Pasteur Institute, Department of Virology, 127 Vas. Sofias Avenue, 11521 Athens, Greece. E-mail: vresearch@hol.gr

Received 8 March 2000; Accepted 20 March 2000

Considerable time and effort can be saved by simultaneously amplifying multiple sequences in a single reaction, a process referred to as multiplex PCR (16–19). Multiplex PCR requires that primers lead to amplification of unique regions of DNA, both in individual pairs and in combinations of many primers, under a single set of reaction conditions. The development of an efficient multiplex PCR usually requires strategic planning and multiple attempts to optimize reaction conditions.

In this study, the development of a multiplex PCR assay for simultaneous diagnosis and typing of HSV-1, HSV-2, and VZV isolates is reported. This assay could avoid the need to test each clinical specimen separately for each primer pair.

MATERIALS AND METHODS

Specimen Collection: Inoculation, Cell Cultures, and Viruses

Clinical specimens were collected from 45 adult patients using sterile cotton swabs from oral, genital, and skin sites. The swabs were transferred into 3 ml of Hank's balanced salt solution containing 100 µg/ml streptomycin, 100 units/ml penicillin, and 1 µg/ml mycostatin and were frozen at -70°C . Specimens were inoculated into triplicate tubes (0.3 ml per tube) of MRC-5 cells (American Type Culture Collection, Manassas, VA), grown in MEM-D medium supplemented with 2% fetal calf serum, and examined daily until observation of typical cytopathic effect (CPE) could be made. When 70–80% CPE was observed, the tubes were frozen at -70°C until their use in DNA extraction procedure. From ten more patients crust material from older lesions was collected for direct typing by multiplex PCR. The sterile cotton swab was first moistened with sterile water, and then the crust material was suspended in 500 µl of sterile water and spun in a microfuge for 10 min. The supernatant was discarded and the pellet was resuspended with 20 µl of sterile water.

As positive controls, herpes simplex virus type 1 (HSV-1) strain F, herpes simplex virus type 2 (HSV-2) strain G, and varicella zoster virus (VZV) strain Ellen (American Type Culture Collection) were propagated in MRC-5 cells. Uninfected MRC-5 cells as well as human cytomegalovirus

(HCMV) strain AD-169 and Epstein-Barr virus (EBV) strain P-3 (American Type Culture Collection) were used as negative controls.

DNA Extraction

Uninfected MRC-5 cells or cells infected with one of the HSV-1, HSV-2, VZV, or CMV viruses, as well as with MRC-5 cells inoculated with clinical specimens were harvested by centrifugation at 550g for 5 min when 70–80% CPE was observed. EBV was obtained from NC-37 cells maintained in suspension; the cells were harvested as above. The supernatant was discarded and the cell pellet subjected to DNA extraction using Xtreme Genomic DNA purification kit II (Pierce, Rockford, IL).

The efficiency of the DNA extraction procedure used, and the presence of inhibitors of DNA amplification in each specimen was evaluated with α -tubulin primers in a separate PCR assay (19).

Primers

Primers with nearly identical optimum annealing temperatures should work under fairly similar conditions if they anneal with single-copy sequences. Primer concentration is a critical parameter for successful PCR (18). The PCR primers were synthesized with an Applied Biosystems oligonucleotide synthesizer and their sequences are reported on Table 1.

Primers H₁P₃₂/H₁M₃₂, H₂M₄₀/H₂P₄, and VP₂₂/VM₂₀ were chosen from the DNA sequence of HSV-1, HSV-2, and VZV complete genome, respectively.

Sequences were accessible from Gene Bank accession numbers X14112 for HSV-1, Z86099 for HSV-2, and X04370 for VZV.

Primers sequences were designed by Primer 3 (Whitehead Institute, Cambridge, MA) elaborated by Steve Rozen and Helen J. Skaletsky (1996, 1997), (code available at <http://www.genome.wi.mit.edu/genomesoftware/other/>).

DNA Amplification: Multiplex PCR

The amplification was performed with a Perkin-Elmer GeneAmp PCR system 9600. Forty amplification cycles of

TABLE 1. Amplification primers

Primers	Sequences (5'→3')	Location	Length of amplimers ^a
H ₁ P ₃₂	TGGGACACATGCCTTCTTGG	6621–6640	147
H ₁ M ₃₂	ACCCTTAGTCAGACTCTGTACTTACCC	6741–6768	
H ₂ M ₄₀	GTACAGACCTTCGGAGG	56320–56336	227
H ₂ P ₄	CGCTTCATCATGGGC	56533–56547	
VP ₂₂	CACACGATAATGCCTGATCGG	9796–9816	275
VM ₂₀	TGCTGATATTTCCACGGTACAGC	10049–10071	

^aExpressed in base pairs.

20 sec at 94°C, 20 sec at 60°C and 20 sec at 72°C were carried out in a 50- μ l final volume containing 5 μ l of 10 $\times$ reaction buffer (Minotech, Crete, Greece), 0.3mM from each of deoxynucleotide triphosphate (dNTP), 0.1 μ M from each of the 6 primers, 2 units of Taq Polymerase (Minotech, Crete, Greece), and 1.5mM MgCl₂. Five μ l of appropriate DNA sample (positive controls, HSV-1 strain F, HSV-2 strain G, VZV strain Ellen; negative controls uninfected MRC-5 cells, CMV strain AD 169, EBV strain P-3, and clinical samples) were added to the reaction mixture. After the last cycle, the samples were incubated for 15 min at 78°C to complete the extension of primers.

To optimize the buffer concentration of multiplex PCR, we performed a series of titrations of primer concentrations and then, the Mg⁺⁺ and dNTP levels were titrated (19).

Ten μ l of each amplified product were analyzed by agarose gel electrophoresis on 3% agarose (Gibco BRL, ultra pure agarose, electrophoresis grade) containing 1 μ g/ml of ethidium bromide in 1 $\times$ TBE buffer and visualized in a UV transilluminator FOTO/PHORESIS I, FOTODYNE (Hartland, WI).

All procedures were carried out under strict conditions that minimized the risk of DNA carry over. DNA from negative controls gave negative results for HSV-1, HSV-2, and VZV in each assay run, confirming the efficiency of these preventive measures.

Specificity and Sensitivity

The specificity of the PCR reaction was tested with 10 ng and 1 ng of genomic MRC-5 cell DNA infected with either HSV-1, HSV-2, or VZV in the presence of 1 μ g of uninfected MRC-5 cell DNA.

To evaluate the sensitivity of the PCR assay, serial dilutions (10 ng and 1 ng) of genomic MRC-5 cell DNA infected with either HSV-1, HSV-2, or VZV were amplified.

The specificity and sensitivity assays were performed for each primer pair and for the combined three primer pairs in the multiplex PCR.

RESULTS

Different concentrations of dNTPs and MgCl₂ were assayed in order to optimize the buffer system for the single and the multiplex PCR. The MgCl₂ and dNTP concentrations are dependent on each other, as well as on the concentration of primers. The primer concentrations were first titrated (0.1–0.5 μ M). The selected concentration for all 6 primers was 0.1 μ M as all the primers in the reaction anneal with equal efficiencies when the concentrations of MgCl₂ and dNTPs were 1.5 mM and 1.2 mM respectively.

Optimization of Single-Target PCRs

Using each primer's pair for HSV-1, HSV-2, and VZV in a series of single-target PCRs, the detection limit was 1 ng of

genomic MRC-5 cell DNA infected with either HSV-1, HSV-2, or VZV.

Increasing the number of cycles from 40 to 45 had no considerable effect on these sensitivities. With MgCl₂ concentrations of 1, 1.25, 1.5, and 2 mM and dNTP concentrations of 0.4, 0.8, and 1.2 mM optimal amplification (maximum band intensity and minimal background, nonspecific staining) was achieved at 1.5 mM MgCl₂ and 1.2 mM dNTPs.

Optimization of Multiplex PCR

As the MgCl₂ concentration is dependent on free dNTPs, MgCl₂ and dNTP were titrated simultaneously with magnesium concentrations of 1, 1.25, 1.5, and 2 mM and dNTPs concentrations of 0.4, 0.8, and 1.2 mM. Optimal amplification was performed with 1.5 mM MgCl₂ and 1.2 mM dNTPs, while lower levels resulted in a decrease in the efficiency of amplification. Reduction of the dNTP concentrations can lead to a considerable increase in the formation of primer artifacts as a consequence of the excess of free Mg⁺⁺ resulting from the lowered dNTPs concentration (18). Lower levels of dNTPs also led to inconsistent amplification results and frequently the reactions produced only a subset of the targeted amplicons (18).

Specificity and Sensitivity

At each stage in the optimization of the single primer pair, the two- and three-primer pair PCRs and serial 10-fold dilutions of HSV-1, HSV-2, and VZV were used to define the sensitivity and specificity achieved. The aim was to achieve the lowest possible detection limit for all three primer pairs in the multiplex DNA amplification.

The specificity of the multiplex PCR reaction was tested with 10 ng and 1 ng of genomic MRC-5 cell DNA infected with either HSV-1, HSV-2, or VZV in the presence of 1 μ g of uninfected MRC-5 cell DNA. When the three primer pairs were added to a single reaction tube in the presence of 1 μ g of negative control, bands of the predicted size were visualized on agarose gel by ethidium bromide staining (Fig. 1, lanes 1 and 2). In such conditions, negative control DNAs (uninfected MRC-5 cells or HCMV strain AD 169 and EBV strain P-3) were always negative (Fig. 1, lane 3).

Various amounts of genomic MRC-5 cell DNA infected with either HSV-1, HSV-2, or VZV were amplified. The sensitivity of the reaction was equivalent with each of the three primer pairs. On the electrophoresed agarose gel stained with ethidium bromide, HSV-1, HSV-2, and VZV were consistently detected from 10 ng of genomic MRC-5 cell DNA infected with the corresponding viral strain (Fig. 1, lanes 4, 6, and 8). Faint bands of the predicted size also were observed from 1 ng of genomic MRC-5 cell DNA (Fig. 1, lanes 5, 7, and 9).

When all the three sets of primers were combined in a single reaction tube, the sensitivity of the assay was equivalent to 10 ng of genomic MRC-5 cell DNA (Fig. 1, lane 10) whereas

Fig. 1. Specificity and sensitivity of the multiplex PCR to detect HSV-1, HSV-2 and VZV. Amplification was performed with 1.2 mM of dNTPs and 1.5 mM of $MgCl_2$. All three primer pairs were used at the concentration of 0.1 μ M. Amplification conditions were as follows: 40 cycles of 20 sec at 94°C, 20 sec at 60°C and 20 sec at 72°C. The amplified products were subjected to a 3% agarose gel electrophoresis containing 1 μ g/ml of ethidium bromide in TBE buffer. M, molecular-weight markers corresponding to a Hinf I digest of pBR 322 DNA (Minotech, Creta, Greece); Lanes 1 and 2, amplification performed on 10 ng and 1 ng, respectively, of genomic MRC-5 cell DNA infected with either HSV-1, HSV-2, or VZV in the presence of 1 μ g of uninfected MRC-5 cell DNA; lane 3, negative control, amplification performed on 1 μ g of MRC-5 cell DNA; lanes 4 and 5, 6 and 7, 8 and 9, amplifications with primer pairs H_1P_{32}/H_1M_{32} , H_2M_{40}/H_2P_4 , and VP_{22}/VM_{20} respectively; lanes 10 and 11, amplification with the three sets of primers; lanes 4, 6, 8, and 10, amplification performed on 10 ng of genomic MRC-5 cell DNA infected with either HSV-1, HSV-2, or VZV; lanes 5, 7, 9, and 11, amplification performed on 1 ng of genomic MRC-5 cell DNA infected with either HSV-1, HSV-2, or VZV. The predicted size of each amplicon is shown on the right.

amplification from 1 ng gave also visible but faint bands (Fig. 1, lane 11).

The ratios of target DNA in the multiplex PCR were adjusted in order to establish if a very low concentration of one target could be amplified in the presence of high concentrations of one or both of the other two targets. Initially, the limiting dilution of one target (10 ng) was amplified in the presence of high levels of the second (1 μ g) and low levels of the third (10 ng). Thus, HSV-1 at concentrations corresponding to its limits of detection (10 ng) was amplified in the presence of 1 μ g of HSV-2 and 10 ng of VZV or in the presence of 1 μ g of VZV and 10 ng of HSV-2 (Fig. 2, lanes 1 and 2). Using the same procedure for HSV-2 and VZV at concentrations corresponding to their limits of detection (10 ng), HSV-2 and VZV were amplified in the presence of high levels of the second (1 μ g) and low levels (10 ng) of the third target (Fig. 2, lanes 3–6).

However, when the DNA level of both of the other two

Fig. 2. Ratio of DNA targets in the multiplex PCR assay. Amplification conditions with the three sets of primers are as described in Figure 1. M, molecular-weight markers corresponding to a Hae III digest of Φ X 174 DNA (GIBCO BRL Life Technologies). Lanes 1 and 2, 10 ng of HSV-1 were amplified in the presence of 1 μ g of HSV-2 and 10 ng of VZV (lane 1), or in the presence of 1 μ g of VZV and 10 ng of HSV-2 (lane 2); lanes 3 and 4, 10 ng of HSV-2 were amplified in the presence of 1 μ g of HSV-1 and 10 ng of VZV (lane 3), or in the presence of 1 μ g of VZV and 10 ng of HSV-1 (lane 4); lanes 5 and 6, 10 ng of VZV were amplified in the presence of 1 μ g of HSV-1 and 10 ng of HSV-2 (lane 5), or in the presence of 1 μ g of HSV-2 and 10 ng of HSV-1 (lane 6); lane 7, 10 ng of HSV-1 were amplified in the presence of 1 μ g of VZV and HSV-2; lane 8, 10 ng of HSV-2 were amplified in the presence of 1 μ g of HSV-1 and VZV; lane 9, 10 ng of VZV were amplified in the presence of 1 μ g of HSV-1 and HSV-2. The predicted size of each amplicon is shown on the right.

targets was high (1 μ g) the sensitivity of the third target was identical to that seen previously for HSV-1, while it was lowered for HSV-2 and prevented for VZV (Fig. 2, lanes 7–9).

Detection and Typing of Clinical Isolates by Multiplex PCR

All the 55 clinical specimens tested after cell culture and DNA extraction or directly from crust-material were positive in PCR assay with α -tubulin primers (Fig. 3, lanes 1, 3, 5, and 7; 527-bp band) proving the efficiency of DNA extraction procedure. PCR with primer pairs H_1P_{32}/H_1M_{32} , H_2M_{40}/H_2P_4 and VP_{22}/VM_{20} amplified viral DNA sequences with all 55 specimens which were obtained from patients with clinical evidence of HSV or VZV infection. The lengths (bp) of the amplified products were 147 bp for HSV-1, 227 bp for HSV-2, and 275 bp for VZV as predicted by the DNA sequences of viruses. From 55 patients who were investigated by multiplex PCR, HSV-1 was detected in 28 specimens, HSV-2 in 20, and VZV in 7 specimens (Table 2).

All samples positive by cell culture also were positive by

Fig. 3. Detection and typing of clinical isolates by multiplex PCR. Amplification conditions were the same as are described in Figure 1. M, molecular-weight markers corresponding to a *Hinf* I digest of pBR 322 DNA (Minotech, Creta, Greece). Lanes 1, 3, 5, and 7, amplifications performed with α -tubulin primers on clinical isolates; lanes 2, 4, 6, 8, and 9, detection and typing of corresponding clinical isolates; lane 2, clinical isolate from skin typed as HSV-1 after cell culture; lane 4, genital isolate typed as HSV-2 after cell culture; lane 6, clinical isolate from skin typed as VZV after cell culture; lanes 8 and 9, clinical isolates from older skin lesions of two patients typed as HSV-1 by multiplex PCR directly from cotton swabs. The predicted size of each amplicon is shown on the right.

multiplex-PCR. In addition, the use of multiplex PCR allowed the detection of HSV-1 and VZV viruses in 10 more cases (18.18%) from crust material (6 HSV-1 and 4 VZV strains).

A representative gel showing the results obtained with clinical specimens is shown in Figure 3. No patient presented a simultaneous infection by more than one virus.

This assay allowed a rapid detection and typing of HSV-1, HSV-2, and VZV isolates. No isolate needed to be further typed by another method. Our data also were reinforced by the fact that 6 HSV-1 and 14 HSV-2 isolates that had been included in a previous study (8) and were analyzed by 4 different methods; indirect ELISA, capture ELISA, restriction enzymes DNA analysis, and PCR; were tested by the present multiplex PCR

TABLE 2. Results of testing 55 samples from oral, genital, and skin sites by multiplex PCR

	Multiplex PCR positive			Total
	HSV-1	HSV-2	VZV	
Cell culture positive <i>No. of samples: 45</i>	22	20	3	45
Cell culture negative <i>No. of samples: 10</i>	6	0	4	10
Total	28	20	7	55

assay. All these 20 isolates were detected and typed in perfect concordance with the previous reported results.

DISCUSSION

This article describes a rapid and accurate multiplex PCR method for detecting and typing HSV-1, HSV-2, and VZV. In a multiplex PCR assay there are several parameters that have to be considered which may affect primer-template affinities and competition between different primers. The melting temperature of primer-template complexes has a major influence on the efficiency of amplification. The importance of adjusting the primer sequence and concentration reflects the competition between simultaneous DNA amplifications occurring in the same tube.

A sensitive assay was achieved only by careful choice of primers and adjustment of $MgCl_2$, dNTP and primer concentrations. In each multiplex PCR, two units of Taq DNA polymerase were used providing adequate enzymatic activity when one DNA amplification is less efficient than another in a simultaneous reaction (18,19). Forty cycles of amplification were chosen to drive PCR into the linear phase of amplification when the amount of primers or enzyme became limiting. Different template concentrations should become compensated, since smaller amounts of template will be amplified exponentially during more cycles, before the plateau phase of the PCR is reached. Such a compensation step should reduce the possible influence of differences in the primary template concentrations on the final results.

Diagnosis of HSV and VZV must be accurate because antiviral therapy with acyclovir is effective, particularly if given early in the disease process (20). Differences between HSV types 1 and 2 are especially apparent in the viruses' susceptibility to antiviral drugs (3) and the site and rate of recurrence (4). This may be important for patient/partner counseling in that HSV-1 genital herpes recurs less frequently than HSV-2 genital herpes.

In this regard the present assay is a potentially rapid and sensitive means of providing clinical and epidemiological information.

Study of target ratios in our multiplex PCR assay revealed a lack of sensitivity with VZV target when the two others (HSV-1 and HSV-2) were in great excess (Fig. 2, lane 9). Failure to detect a low level of VZV DNA in cell culture containing an excess of HSV-1 and HSV-2 DNA would most likely result merely in failure to document a clinically irrelevant VZV infection in a patient with HSV disease. In MRC-5 cells inoculated with HSV (HSV-1 or HSV-2) and VZV, HSV would produce an early cytopathic effect destroying the cell cultures and thereby preventing detection of any VZV-induced cytopathic effect. In those circumstances, multiplex PCR directly from cotton swabs would be preferable to virus isolation in cell culture, because only the former would diagnose the VZV infection. In the present study this problem

was considered unimportant as a simultaneous triple infection by HSV-1, HSV-2, and VZV seems extremely unlikely to occur. This hypothesis is also reinforced by the fact that none of the ten cotton swabs examined directly by multiplex PCR revealed any dual or triple infection.

Primers of α -tubulin were used in a separate PCR assay to evaluate the efficiency of DNA extraction procedure used and the eventual presence of DNA amplification inhibitors. All the treated swabs as well as the inoculated cultures yielded α -tubulin DNA by PCR indicating successful extraction of amplifiable DNA. This observation suggested that failure of DNA extraction was an unlikely explanation for failure to detect viral DNA in a clinical specimen that could lead to unidentifiable false negative results for HSV-1, HSV-2, or VZV DNA and untreated atypical VZV and HSV infections.

Protocols that use consensus primers followed by restriction enzyme analysis of amplified products have been reported (10,14). Nevertheless, the assay described by Rozenberg and Lebon cannot detect VZV. Recently a duplex nested PCR assay has been reported (21). This assay cannot differentiate HSV-1 from HSV-2 and needs a two-round PCR assay for 30 and 35 cycles, which is expensive, time consuming, and also has the potential drawback of carryover contamination by the amplified products. The present multiplex PCR assay permits the detection and characterization of HSV-1, HSV-2, and VZV in a single reaction tube and in a single PCR round, avoiding the need to test clinical specimens with separate PCR assays, or the digestion of amplified products by restriction enzyme analysis.

The reported results showed that all clinical specimens positive by cell culture were also positive by multiplex PCR but, this assay detected a further 18.18% of samples indicating a 100% sensitivity. In all these cases clinical evidence gave a clear support to the positive PCR diagnosis (old lesions from vesicular rashes).

It is concluded that the multiplex PCR assay described in the present report has an extensive spectrum of potential application in clinical diagnosis when a rapid and accurate diagnosis and typing of involved viruses HSV-1, HSV-2, or VZV is needed.

REFERENCES

1. Penneys NS, Goldstein B, Nahass GT, Leonardi CL, Zhu WY. Herpes simplex virus DNA in occult lesions: demonstration by the polymerase chain reaction. *J Am Acad Dermatol* 1991;24:689–692.
2. Casas I, Tenorio A, Ory F, Lozano A, Echevarria JM. Detection of both herpes simplex and varicella-zoster viruses in cerebrospinal fluid from patients with encephalitis. *J Med Virol* 1996;50:82–92.
3. De Clercq E, Descamp J, Verhelst G, et al. Comparative efficacy of antiherpes drugs against different strains of herpes simplex virus. *J Infect Dis* 1980;141:663–574.
4. Reeves C, Corey L, Adams H, Vontver L, Holmes K. Risk of recurrence after first episodes of genital herpes: relation to HSV type and antibody response. *N Engl J Med* 1981;305:315–319.
5. WHO Meeting. Diagnosis of human herpesviruses: memorandum from a WHO meeting. *Bull WHO* 1991;69:277–283.
6. Nahass GT, Goldstein BA, Zhu WY, Serfling U, Penneys NS, Leonardi CL. Comparison of Tzanck smear, viral culture and DNA diagnostic methods in detection of herpes simplex and varicella-zoster infection. *JAMA* 1992;268:2541–2544.
7. Lonsdale DM. A rapid technique for distinguishing herpes simplex virus type 1 from type 2 by restriction enzyme technology. *Lancet* 1979;i:849–851.
8. Markoulatos P, Fountoucidou P, Marinakis G, et al. Clear detection and typing of herpes simplex virus types 1 and 2 by an indirect Elisa assay: comparison with three different combined methods—capture Elisa, restriction enzymes and polymerase chain reaction. *J Clin Lab Anal* 1997;11:146–153.
9. Nahass GT, Mandel MJ, Cook S, Fan W, Leonardi CL. Detection of herpes simplex and varicella zoster infection from cutaneous lesions in different clinical stages with the polymerase chain reaction. *J Am Acad Dermatol* 1995;32:730–733.
10. Rozenberg F, Lebon P. Amplification and characterization of herpes virus DNA in cerebrospinal fluid from patients with acute encephalitis. *J Clin Microbiol* 1991;29:2412–2417.
11. Matsumoto T, Yamada O, Itagaki A, Ishida S, Kamahara T, Kurimura T. Rapid DNA diagnosis of herpes simplex virus serotypes. *J Virol Methods* 1992;40:119–126.
12. Shimizu C, Shimizu H, Mitsuda T, Tsukuda M, Ichikawa S, Yokota S. One step determination of herpes simplex virus type I and II by polymerase chain reaction. *Molec Cellul Probes* 1994;8:193–198.
13. Lucotte G, Bathelier C, Lespiaux V, Bali C, Champenois T. Detection and genotyping of herpes simplex virus type 1 and 2 by polymerase chain reaction. *Molec Cellul Probes* 1996;9:287–290.
14. Baron JM, Rübben A, Grubendorf-Conen FI. Evaluation of a new general primer pair for rapid detection and differentiation of HSV-1, HSV-2 and VZV by polymerase chain reaction. *J Med Virol* 1996;49:279–282.
15. Hawrami K, Harper D, Breuer J. Typing of varicella-zoster virus by amplification of DNA polymorphisms. *J Virol Methods* 1997;57:169–174.
16. Vandeveld C, Verstraete M, Van Beers D. Fast multiplex polymerase chain reaction on boiled clinical samples for rapid viral diagnosis. *J Virol Methods* 1990;30:215–228.
17. Repp R, Rhiel S, Heermann KH, et al. Genotyping by multiplex polymerase chain reaction for detection of endemic hepatitis B virus transmission. *J Clin Microbiol* 1993;31:1095–1102.
18. Markoulatos P, Samara V, Siafakas N, Plakokefalos E, Spyrou N, Moncany M. Development of a quadriplex polymerase chain reaction for human cytomegalovirus. *J Clin Lab Anal* 1999;13:99–105.
19. Markoulatos P, Mangana-Vougiouka O, Koptopoulos G, Nomikou K, Papadopoulos O. Detection of sheep poxvirus in skin biopsy samples by a multiplex polymerase chain reaction. *J Virol Methods* 2000;84:161–167.
20. Whitley RJ. Herpes simplex viruses. In: Fields BN, Knipe DM, editors. *Virology*, 2nd ed., vol. 2. New York: Raven Press; 1990. pp.1843–1888.
21. Beards G, Graham C, Pillay D. Investigation of vesicular rashes from HSV and VZV by PCR. *J Med Virol* 1998;54:155–157.