

A Diagnostic Multiplex Polymerase Chain Reaction Assay for Ocular Herpetic Infections

Short Communication

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Summary

A multiplex PCR (m-PCR) technique was used to diagnose and differentiate ocular herpes virus infections including Varicella zoster virus (VZV) and herpes simplex virus (HSV) types 1 and 2. Conjunctival swab, corneal scraping, and ocular fluid samples were collected from 28 patients. Using a specific pair of primer, for thymidin kinase gene, both HSV-1 and HSV-2 DNAs were amplified. A set of primer flanking a 208bp of the DNA-polymerase gene was also used to amplify VZV DNA. The sensitivity of the m-PCR for detection of HSV and VZV in clinical samples was 80.9% and 95%, respectively. Parts of the specimens were cultured on Vero, Hep II, and MRC-5 cell lines. The sensitivity of the cell culture for isolation of HSV and VZV was 62.9% and 72.7%, respectively. Using statistical analysis of the results a significant difference ($P=0.001$) between virus isolation and m-PCR for detection of HSV and VZV in clinical specimen was noticed. Both HSV and VZV DNAs were detected in 1 out of 28 (3.5%) specimens exclusively by m-PCR. Results indicate that m-PCR is a more reliable method in rapid diagnosis of herpes viruses DNA in clinical samples than virus isolation.

Key words: multiplex polymerase chain reaction, ocular herpetic infections, VZV, HSV

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Introduction

Viral infections are the most common identifiable etiology of eye disease (Ganatra *et al* 2000). Varicella zoster virus (VZV) and herpes simplex virus (HSV) have both been implicated as causative agents in severe ocular diseases associated with poor visual prognosis (Ganatra *et al* 2000, Morgolis *et al* 1991). The diagnosis of intraocular herpes infections is usually based on clinical manifestations, reaction to antiviral treatment or by means of invasive methods. However, in a subset of patients atypical appearance can necessitate additional testing to support a clinical diagnosis. Rapid laboratory diagnosis of ocular herpes infection facilitates patient management and possible initiation of antiviral therapy. Serology, virus culture, and cytology have been used for identification of viral etiology of ocular herpes (Messmer *et al* 1998, de Boar *et al* 1996). The majority of the methods have been designed for detection of single etiological infection. Although virus isolation has traditionally been accepted as the gold standard test, virus culture from the eye has poor recovery. Recent years, the polymerase chain reaction (PCR) has been used in diagnosis of ocular herpes (Mc Cann *et al* 1995, Short *et al* 1997). The PCR can directly detect DNA of causative agents, with sensitivity and specificity often greater than that of culture. One impediment to routine use of PCR technique is the necessity of performing a separate PCR for each pathogen in the differential diagnosis. The serial detection of individual pathogen is time consuming and may be prohibitively expensive if a large panel of potential of pathogens is tested. Multiple-PCR (m-PCR) is a technique, which performed simultaneously for multiple pathogens in single reactions (Short *et al* 1997). Recently Cassinotti and Seigl (1998) have been used m-PCR for detection and differentiation of viruses in clinical specimen. Few studies have been designed for simultaneous diagnosis of HSV-1, HSV-2, and VZV in ocular specimen.

In this study we used m-PCR technique to diagnose and differentiate ocular herpes infections and compared the results with the virus isolation method.

Materials and Methods

Patients and specimen. Between December 2001 and June 2002, a total of 28 ocular specimens were collected from 19 males and 9 females aged 3-71 years at Shiraz University of Medical Sciences teaching hospitals. Patterns of diseases were characterized by experienced ophthalmologist. There were. Indication for viral study included keratitis (n=7), dendritic conjunctivitis (n=4) corneal ulcer (n=6) and unknown diagnosed (n=11). Conjunctival swab, corneal scraping, and ocular fluid samples were collected into viral transport medium. Approximately 200 μ l was removed for virus isolation and the remaining volume was stored at -70°C prior to processing for PCR assay.

Virus isolation. A volume of 200 μ l was inoculated to MRC5, Hep-II and Vero monolayer cells. Cultures were incubated at 37 C and inspected for cytopathic effect (CPE) in a light microscope (100–200 \times magnification) during a period of one week for HSV and two weeks for VZV. HSV isolates were typed by using monoplex HSV-1 and HSV-2 PCR. VZV CPE was also confirmed by VZV-PCR.

PCR assay. 100 μ l of specimen boiled for 15min and cooled for 5min then centrifuged at 14000g at room temperature for 5min. The supernatant was used for PCR assay (Behzad-Behbahani *et al* 1997). Primers for HSV-1, HSV-2, and VZV DNA (Klapper *et al* 1991, Echevarria *et al* 1994) were selected from published sequences and checked for correctness by computer-assisted analysis (BLAST searches on European Molecular Biology laboratory, EMBL, Gene Bank databases). Using a specific pairs of primer for thymidin kinase gene, 351bp of both HSV-1 and HSV-2 DNAs was amplified. Aset of primer flanking a 208bp of the DNA-polymerase gene was also used to amplify VZV DNA. Purified VZV and HSV DNAs were used to optimize the sensitivity of each monoplex and multiplex PCR. The individual monoplex and multiplex PCR cycling conditions and extension temperatures; MgCl_2 concentration, number of cycles and concentration of primers

for each virus were separately optimized. Negative control including DNase and RNase free water, uninfected Vero and Hep II cell lines and positive control including VZV (vaccine strain), HSV-1, and HSV-2 clinical isolates were also tested by the PCR. For each multiplex reaction 5µl of samples was add to 45µl PCR mixture contained 1.4XPCR buffer, 50 pmol of each VZV and HSV primers, 7% DMSO, 2 units *Taq*, 200mM dNTPs and 2mM MgCl₂. Following denaturation at 94°C for 5min the materials were subjected to 39 cycles of 94°C for 1min, 58°C for 1min, 75°C for 1min and finally 72°C for 5min. Subsequently 10µl of each amplified product was analyzed by conventional gel electrophoresis followed by ethidium bromide staining (10µl/ml). In according to standard procedure for preventing contamination each procedure for PCR processing was performed in physically separate rooms.

Determination of PCR sensitivity and specificity. The sensitivity of the PCR procedure was tested by serial log dilutions of the template DNAs. The specificity of the mPCR was confirmed by testing DNA extracted from herpes virus group other than HSV and VZV and from uninfected Vero and Hep-II cell lines.

Statistical analysis. Using SPSS software version 9.0, Chi-square, two-tail Fisher's exact test, and Spearman correlation methods were analyzed.

Results and Discussion

Detection of HSV and VZV DNA by multiplex PCR in ocular samples. The m-PCR results of examined ocular samples are shown in figure 1. VZV DNA was detected in 7 out of 28 samples (25%). Clinical diagnosis included keratitis (n=3), dendritic conjunctivitis (n=1), corneal ulcer (n=1) and unknown diagnosis (n=2). HSV DNA was found in 11 out of 28 sample (39.2%). Diagnosis feature of this group of patients was corneal ulcer (n=2), dendritic conjunctivitis (n=1), keratitis (n=3), and unknown diagnosis (n=3). However, 18 (64.3%) of the ocular samples were found positive for either HSV or VZV DNA by multiplex PCR (Table 1). Double infection

of both HSV and VZV was found in a patient, who was 60 years man with any underlying disease. Virus isolation was negative after 10 days.



Figure 1. Detection of HSV and VZV DNA by m-PCR in some ocular samples. Lanes 1,2: HSV-DNA positive, lane 3: VZV-DNA positive, lane 4: negative control, lane 5: negative sample, lane 6: HAS-DNA positive, lane 7: negative sample, lane 8: double infection of both HSV and VZV. lane 10: empty well and lane 11: 100bp DNA ladder

Table 1. Detection of HSV and VZV DNA in clinical specimens by m-PCR

Clinical status	No. patient	HSV	VZV	Total
Keratitis	7	3	3	6
Corneal ulcer	5	2	1	3
Dendritic conjunctivitis	5	3	1	4
Unknown diagnosis	11	3	2	5
Total	27	11	7	18

Sensitivity and specificity of the m-PCR. Ten fold serial dilution of each HSV (10^{12} particles/ml) and VZV (10^8 particles/ml) were used as the template for PCR. The limit sensitivity of VZV and HSV PCR was found to be 2×10^2 and 5×10^1 DNA molecules/PCR tube, respectively. The Specificity on the PCR procedure was examined by using DNA template isolated from adenovirus and cytomegalovirus. No amplification was detected from any source of DNA other than HSV-1, HSV-2, and VZV.

Virus isolation from clinical samples. Cell culture system was used for virus isolation from clinical specimens and checked for demonstration of CPE up to 10 days. CPEs were demonstrated in 11 (39%) of the specimens. Confirmation and differentiation of virus isolation test was performed using developed m-PCR. Seven of the positive culture were also positive by m-PCR i.e. HSV (n=5) and VZV (n=2). Three out of four negative m-PCR were confirmed adenovirus infection by clinical features of the patients and CPE characteristic in cell culture. However, fungal infection was determined in only one specimen. Using statistical analysis of the results a significant difference between virus isolation and m-PCR for detection of HSV and VZV in clinical specimen was noticed ($P=0.001$). In one case both HSV and VZV was diagnosed by PCR, which could not be diagnosed either clinically or by virus isolation.

The comparison of the different methods showed that the detection rate of VZV in clinical specimens could be increased more than three times by PCR compared to virus isolation (Espy *et al* 2000). Additionally, the isolation is dependent on the cell line used, the kind of lesion, the presence of viable virus, and the transportation time (Lakeman 1997).

Using statistical analysis of the results a significant difference ($P=0.001$) between virus isolation and m-PCR to detect HSV-1, HSV-2 and VZV in clinical specimen was noticed. Results show that m-PCR is a more reliable method in rapid diagnosis of herpes virus DNA in ocular samples than virus isolation. However, more studies for application of m-PCR in clinical diagnosis of viral infection are recommended.

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