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Multiplex Polymerase Chain Reaction for Detection of Herpes Simplex Virus Type 1, Type 2, Cytomegalovirus, and Varicella-Zoster Virus in Ocular Viral Infections

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Purpose: To detect simultaneously herpes simplex virus type 1 (HSV-1), type 2 (HSV-2), cytomegalovirus (CMV), and varicella-zoster virus (VZV) in ocular specimens suspected of indicating viral infection, and to compare the results of multiplex polymerase chain reaction (PCR) with those of uniplex PCR.

Methods: Forty specimens, collected from 33 patients with clinically suspected herpes virus ocular infection, were tested. DNA was extracted from the specimens and amplified by multiplex and uniplex PCR.

Results: Both multiplex PCR and uniplex PCR gave the same results. Nineteen (19/33, 57.6%) patients were PCR-positive, among whom HSV-1 was detected in 13 (13/19, 68.4%) patients, and VZV in 6 (6/19, 31.6%) patients.

Conclusion: These results demonstrated that multiplex PCR is as reliable as uniplex PCR, and is an accurate and a cost-saving method to identify several agents from a single specimen. **Jpn J Ophthalmol 2003;47:260–264**

Key Words: Cytomegalovirus, herpes simplex virus type 1, herpes simplex virus type 2, polymerase chain reaction, varicella-zoster virus.

Introduction

Herpetic ocular disease is a major cause of blindness throughout the world, and early diagnosis is necessary to initiate early therapy. Herpes simplex virus type 1 (HSV-1), type 2 (HSV-2), cytomegalovirus (CMV), and varicella-zoster virus (VZV) all belong to the same herpes family, the *Herpesviridae*, that can cause ocular diseases.[1] They are difficult to differentiate by clinical findings alone. An accurate and rapid identification of these viruses is important to avoid incorrect diagnosis and to initiate early treatment.

Polymerase chain reaction (PCR) has been used to detect viral nucleic acid, and the amplification of different sequences has been conventionally performed by separate PCR reactions for different types of viruses.[2–5] These reports indicated that PCR is useful as a rapid and sensitive technique. However, the individual amplifications are a time-consuming and costly technique. In addition, it may be impossible if the amount of the clinical specimen is limited.

More recently, a multiplex PCR for rapid and simultaneous diagnosis of viral disease has been reported.[6–8] We have tested whether a multiplex PCR technique can be performed on several ocular specimens to detect simultaneously HSV-1, HSV-2, CMV, and VZV. We have compared its sensitivity and reliability with uniplex PCR on the same specimens.

Materials and Methods

Clinical Specimens

Appropriate informed consent was obtained from each subject before the collection of specimens. Forty specimens, including 1 corneal button, 3 tear samples, 7 skin crusts from cases of cutaneous herpes, 20 conjunctival

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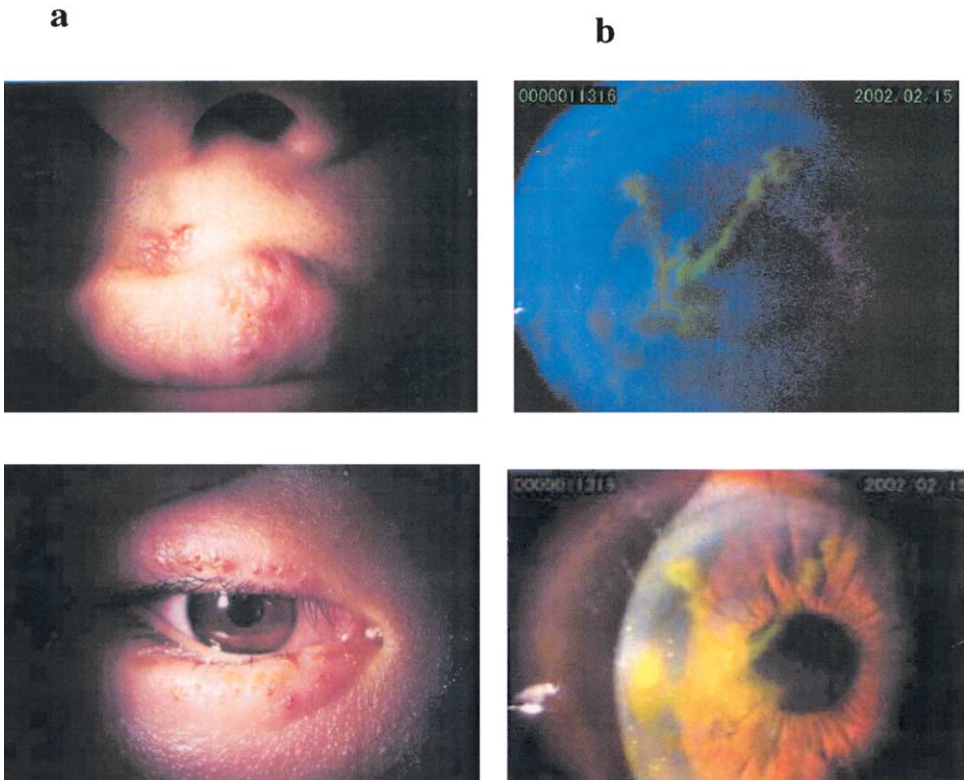

Figure 1. Slit-lamp photos of symptoms in 2 patients with herpes virus infection. (**a**) Patient no. 2 in Table 2. Top: Herpetic vesicular lesions on labia. Bottom: Skin lesions on eyelid. (**b**) Patient no. 13 in Table 2. Typical herpetic dendritic ulcer stained with fluorescein (top and bottom).

swabs, 6 skin swabs, 1 ulcer swab, 1 soft contact lens (SCL), and 1 pseudomembrane were collected from 33 patients (20 men and 13 women) with clinically suspected ocular viral infection. The mean age of the patients was 48 years with a range of 17–80 years. The symptoms of 2 patients are shown in Figure 1.

DNA Extraction

DNA was extracted from the corneal button, pseudomembrane, and skin crusts with the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA). The DNA from swabs, tears, SCL, and fluorescence filter paper (FFP) was extracted as follows. The specimens were placed into 50 μL of 10 mM Tris-HCL, 1 mM EDTA, pH 8.0 (TE) containing 2.5 μL of 0.1% Tween20 and incubated at 55ºC for 2 hours followed by boiling at 99ºC for 10 minutes. The DNA concentration was assessed using Gene Spec 1 (Naka Instrument, Tokyo). The extracted DNA was diluted 10 times and stored at 4ºC until used.

Primer Design

The primers were constructed according to the entire DNA sequence of the glycoprotein D (K02372) of HSV-1 and HSV-2,[9] of CMV (M14709),[10] and of VZV (X04370, M14891, M16612).[11] Each primer set (Table 1) consisted of a part of the primers described previously.[12]

PCR Preparation and Conditions

All of the PCR tests were carried out adhering to stringent precautions to avoid contamination as described.[13] The 25-μL multiplex PCR reaction mixture was composed of 2.5 uL of 10× PCR buffer, 5 μL Q-Solution,

Table 1. Properties of Oligonucleotide Primers

Target*	Sequence	Products†
HSV-1	F: GTTAGGGAGTTGTTCGGTCATAAGCT R: TCGGCCATCTTGAGCATCC	208 bp
HSV-2	F: GTCGGTGTGGTGTTCGGTCATAAGCT R: GGCTGAATCTGGTAAACACGCTTC	276 bp
CMV	F: CACGGCCGCCACCAAGGT R: AGTGGTTGGGCAGGATAAA	392 bp
VZV	F: ATCGCGGCTTGTTGTTTGTCTAAT R: GGGCGAAATGTAGGATATAAAGGA	355 bp

*HSV: herpes simplex virus, CMV: cytomegalovirus, VZV: varicella-zoster virus.

†bp: base pair.

Jpn J Ophthalmol
Vol 47: 260–264, 2003

and 0.625 U *Taq* DNA Polymerase. These three reagents were obtained from the Qiagen *Taq* DNA Polymerase Kit (QIAGEN, Hilden, Germany). In addition, 2.5 μL (10 mM) of deoxynucleotide triphosphates, 1 μL (12.5 pmol/μL) of each primer, and 5 μL appropriate template DNA or double distilled water, as a negative control, were added. Uniplex PCR was performed using each pair of primers individually and 0.6 U *Taq* DNA polymerase/reaction. In the present study, the optimal DNA amount for PCR was 5 μL of 10 ×-diluted DNA.

The PCR conditions for both multiplex and uniplex PCR were as follows: denatured at 94ºC for 7 minutes, 34 cycles each of 1 minute at 94ºC, 2 minutes at 56ºC, 1.5 minutes at 75ºC, and final extension at 75ºC for 7 minutes. The PCR products were analyzed by electrophoresis on 2% agarose gel prestained with ethidium bromide.

Control DNA

The Fukuda strain of HSV-1, the G strain of HSV-2, the AD 169 strain of human CMV, and the CaQu strain of VZV were used as positive control DNA. Ten conjunctival swabs, collected from 10 patients with nonviral diseases were used as negative control.

Results

Nineteen (24 specimens) of 33 patients were PCR-positive (19/33, 57.6%), and 14 patients (16 specimens) were PCR-negative (14/33, 42.4%) by both multiplex and uniplex PCR. The results of multiplex and uniplex PCR by gel electrophoresis are shown in Figure 2. The clinical diagnoses, analyzed specimens, and the type of virus detected by PCR on the 19 PCR-positive patients are shown in Table 2. Among them, HSV-1 was found in 13 (13/19, 68.4%) patients, and VZV was found in 6 (6/19, 31.6%) patients. HSV-2 and CMV DNA were not detected in this trial, and none of the 10 negative control patients showed infectious agents.

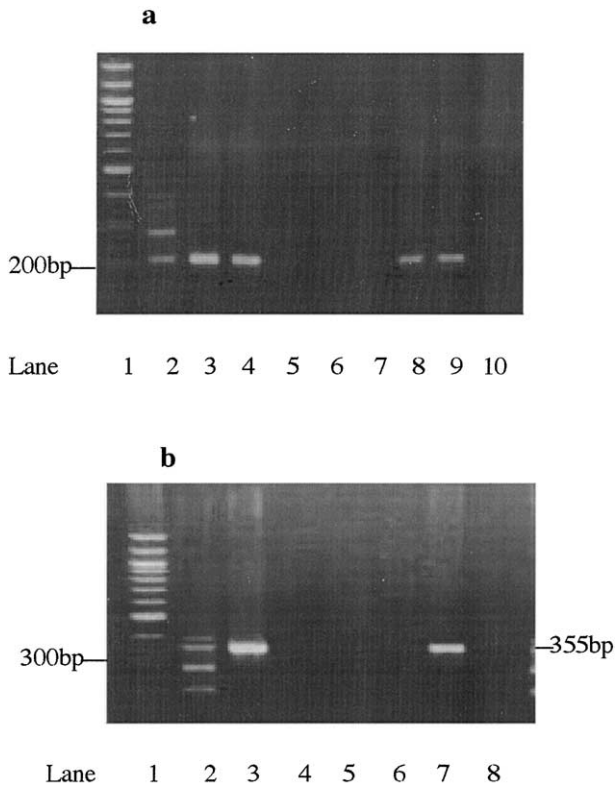

Figure 2. Electrophoreses of polymerase chain reaction (PCR) products obtained by multiplex and uniplex PCR. (**a**) Detection of herpes simplex virus type 1 (HSV-1) in patient nos. 1 and 3 in Table 2. Lane 1, 100 bp DNA Ladder; lane 2, positive control by multiplex PCR for HSV-1 (208 bp), HSV-2 (276 bp), cytomegalovirus (CMV; 392 bp), and varicella-zoster virus (VZV; 355 bp); lane 3, patient no. 1, HSV-1 positive by multiplex PCR; lanes 4–7, patient no. 1 by uniplex PCR for HSV-1, HSV-2, CMV, and VZV, respectively, where the patient was positive only to HSV-1, the same as in multiplex PCR testing; lanes 8 and 9, patient no. 3, HSV-1 positive by multiplex and uniplex PCR, respectively; lane 10, negative control. (**b**) Detection of VZV (355 bp) by multiplex PCR and uniplex PCR in patient no. 2 in Table 2: Lane 1, 100 bp DNA Ladder; lane 2, positive control of HSV-1 (208 bp), HSV-2 (276 bp), CMV (392 bp), and VZV (355 bp) by multiplex PCR; lane 3, patient no. 2, positive to VZV by multiplex PCR; lanes 4–7, uniplex PCR for HSV-1, HSV-2, CMV, and VZV, respectively, where the patient was positive only to VZV, the same as in multiplex PCR testing; lane 8, negative control.

Discussion

PCR has been demonstrated to be significantly more sensitive and reliable than other laboratory techniques for detecting viral nucleic acid.[14] Multiplex PCR, using different pairs of primers, was used in the hope that the different fragments could be amplified independently and simultaneously.[15–17]

For eye disease, two or three agents including adenovirus and HSV,[7] and adenovirus, HSV and *Chlamydia trachomatis*[8] have been identified by multiplex PCR from ocular specimens. In the present study, we have tried the multiplex PCR technique with four different pairs of primers to detect HSV-1, HSV-2, CMV, and VZV simultaneously, using several specimens from a corneal button, tear samples, skin crusts, conjunctival swabs, skin swabs, an SCL, and a pseudomembrane. The multiplex and uniplex PCR gave identical results, and all of the PCR-positive patients showed clinical improvement by antiviral treatment after PCR diagnosis.

Table 2. Clinical Diagnosis, Specimens, and Detected Virus DNA of Polymerase Chain Reaction (PCR) positive Patients

Patient No.	Clinical Diagnosis*	Specimen Type†	PCR Result‡
1	HK, HC	c swab	HSV-1
2	ZO	s swab and crust	VZV
3	HB, HC	c swab	HSV-1
4	HK, HC, HB	pseudomembrane	VZV
5	HB	crust	HSV-1
6	EKC	c swab	HSV-1
7	HC, HB	c swab and s swab	HSV-1
8	HC, HB	c swab	VZV
9	ZO	s swab and crust	VZV
10	HB	crust	HSV-1
11	HK, HC	c swab	HSV-1
12	HK, HB, HC	crust	HSV-1
13	LH	s swab	HSV-1
14	HB	s swab and crust	VZV
15	HB, HC	c swab	HSV-1
16	HB	s swab and crust	HSV-1
17	ZO	c swab	VZV
18	HB, HC	c swab	HSV-1
19	HK, HC	c swab	HSV-1

*EKC: epidemic keratoconjunctivitis, HB: herpetic blepharitis, HC: herpetic conjunctivitis, HK: herpetic keratitis, LH: labial herpes, ZO: zoster ophthalmicus.

†c swab: conjunctival swab, s swab: skin swab.

‡Results of multiplex PCR have been also confirmed by uniplex PCR.

Table 3. Clinical Diagnosis and Specimens of Polymerase Chain Reaction (PCR) negative Cases

Patient No.	Clinical Diagnosis*	Specimens†
1	DB	c swab
2	AC	c swab
3	RCE	tear
4	AC	c swab
5	VZV-K	corneal button
6	RCE	c swab and SCL
7	TK	tear
8	EKC	c swab
9	CP	tear and c swab
10	RCE	c swab
11	DK	c swab
12	EKC	c swab
13	DK	c swab
14	CU	u swab

*AC: allergic conjunctivitis, CP: corneal phlyctenule, CU: corneal ulcer, DB: drug-induced blepharitis, DK: drug-induced keratitis, EKC: epidemic keratoconjunctivitis, RCE: recurrent corneal erosion, TK: Thygeson's superficial punctate keratitis, VZV-K: zoster keratitis.

†SCL: soft contact lens, c swab: conjunctival swab, u swab: ulcer swab.

Fourteen patients were PCR-negative by both multiplex PCR and uniplex PCR (Table 3). Among them, three cases seemed to have drug-related symptoms (nos. 1, 11, and 13). In Case 1, nypradilol (Hypadil; Kowa, Tokyo) induced blepharitis. The blepharitis had caused some spots of erosion and crusts on the lid. Herpetic blepharitis was suspected at first. However, acyclovir ointment was not effective and even exacerbated the condition. The discontinuation of nypradilol and a steroid ointment resulted in rapid improvement. In Case 11, isopropyl unoprostone (Rescula; Fujisawa Pharmaceutical, Osaka) induced keratitis. After the discontinuation of unoprostone the patient's condition soon improved. Case 13 was latanoprost-induced linear superficial punctate keratopathy (SPK). We discontinued latanoprost (Xalatan; Pharmacia, Piscataway, NJ, USA) and started to use acyclovir ointment. The patient had used latanoprost, timolol, and dorzolamide at the same time. The use of timolol alone was continued, together with the acyclovir ointment. SPK disappeared after 5 days of acyclovir use. We thought the case was latanoprost-induced herpetic keratitis. However, neither multiplex PCR nor uniplex PCR exhibited any positive PCR results. Therefore, SPK seemed to be due to the cytotoxicity of latanoprost or the combined use of the antiglaucoma agents.

Patient no. 5 was a case with regrafted cornea. The patient had undergone penetrating keratoplasty 10 years previously because of corneal opacification due to zoster ophthalmicus; however, the graft opacified again after rejection. The corneal button in Case 5 was the result of regraft surgery. Therefore, PCR did not show the presence of VZV DNA.

The possibility of viral infection has been suspected as the cause for Thygeson superficial punctate keratitis in Case 7, and for the recurrent corneal erosion in Cases 3 and 10. We expected to detect the DNA of the herpes virus family in these cases for which the cause had not been identified. However, PCR showed no herpes virus DNA at all. Other PCR-negative cases did not demonstrate such inconsistency between clinical diagnosis and PCR results.

An amplification of a DNA fragment such as rhodopsin or G3PDH from human host genome DNA, which will be contained in DNA samples extracted from specimens, may be helpful to find some failure in our DNA isolation or in our purification from the small amount of specimens. As the next step, we are planning to use the multiplex PCR technique to support negative results.

Our results showed that the four pairs of primers for HSV-1, HSV-2, CMV, and VZV worked well together, and the accuracy of each primer in multiplex PCR was the same as that in uniplex PCR. These results demonstrated that careful optimization of PCR conditions can provide good PCR productivity in a multiplex reaction.

Jpn J Ophthalmol
Vol 47: 260–264, 2003

In conclusion, multiplex PCR is sensitive, reliable and cost-saving. This method enabled us to screen the four pathogens simultaneously, thus saving template DNA, and the results can be obtained within a few hours. Although the multiplex PCR will be more useful for simultaneous detection of several viral infections, further study should be done to confirm the sensitivity and specificity of the multiplex PCR technique.

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