
Original Article

Quick detection of herpes viruses from skin vesicles and exudates without nucleic acid extraction using multiplex PCR

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Summary

Distinguishing herpes virus infection from other skin diseases is sometimes difficult. This study aims to detect herpes virus DNA by multiplex real-time PCR without nucleic acid extraction in a short period of time. Specimens of cutaneous vesicles and swabs were obtained from 23 patients suspected of having herpes virus infection. These specimens were stored at -80°C after dissolving them in sterilized water. DNA extraction was not performed. Specific real-time PCR primers for herpes simplex virus (HSV) 1 and 2 and varicella-zoster virus (VZV) were designed. These primers were used to perform real-time PCR with the frozen solution as template. Results clearly revealed a type-specific dissociation curve. Agarose gel electrophoresis was also performed and produced a single band of the expected size. In addition to using multiplex PCR, other steps were used to reduce the time even further. Each experiment took only 2 h to complete; the type of Herpes virus was successfully detected by multiplex real-time PCR without nucleic acid extraction in a short period of time. In conclusion, omission of the nucleic acid extraction step prior to real-time PCR does not negatively affect downstream reactions. Using multiplex PCR may allow more rapid qualitative analysis of HSV1, 2 and VZV.

Keywords: Herpes virus, Real-time PCR, Multiplex PCR

1. Introduction

Occasionally, skin lesions may present problems in terms of their diagnosis. Distinguishing herpes simplex virus infection from herpes zoster infection or other skin diseases is sometimes difficult. Rapid and accurate detection and typing of herpes simplex virus type 1 (HSV-1), type-2 (HSV-2) or varicella-zoster virus (VZV) is crucial to clinical diagnosis and therapy as early as possible. In Japan, the tests most commonly used for the diagnosis of herpes virus infections of the skin are the Tzanck test and immunofluorescence of the serum. However, the Tzanck test does not distinguish between herpes simplex and varicella-zoster virus. The immunofluorescence method only has a sensitivity of 32% (1). Herpes viruses are DNA viruses, and therefore

no extra procedures are required to obtain DNA, providing a significant reduction in the cost and time involved in the experimental procedure. The possibility of contamination of the sample during the DNA extraction procedure is also eliminated. Several recent reports have indicated that extraction and purification of nucleic acid is not always necessary to perform real-time PCR (2,3). To the extent known, there are no reports in the field of dermatology that have discussed omission of DNA extraction, but there are reports in other fields (4,5). The liquid from cutaneous vesicles and exudates from patients with a possible diagnosis of HSV or VZV infection was examined. Uniplex PCR was initially used; multiplex PCR was later performed on the same specimens using the same primers.

2. Materials and Methods

2.1. Samples

In the period from December 2006 to September 2007, clinical specimens ($n = 25$) suspected for herpes viruses

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Table 1. Patient profiles, clinical appearance, clinical diagnosis, and results of uniplex PCR / multiplex PCR. Both types of PCR analysis yielded the same results, and the dissociation temperatures of each target differed (84.25°C for HSV1, 88.75°C for HSV2, and 79.75°C for VZV)

Specimens No.	Age	Gender	Lesions	Appearance	Clinical diagnosis	Specimen	Dissociation T (°C)	Uniplex PCR result	Multiplex PCR result
1	16	M	Rt. Humerus	Vesicle	Zoster	Skin	79.75	VZV	VZV
2	78	F	Rt. Humerus	Herpes	Zoster	Skin	79.75	VZV	VZV
3	0	F	Genital	Ulceration	Herpes simplex	Swab	84.25	HSV1	HSV1
4	74	M	Lt. forehead	Herpes	Zoster	Skin	79.75	VZV	VZV
5	70	F	Upper lip	Herpes	Herpes simplex	Skin	84.25	HSV1	HSV1
6	71	M	Lip	Herpes	Herpes simplex	Swab	—	—	—
7	71	M	Lip	Herpes	Herpes simplex	Skin	—	—	—
8	84	F	Rt dorsal trunk	Herpes	Zoster	Skin	79.75	VZV	VZV
9	13	M	Rt dorsal trunk	Herpes	Zoster	Skin	79.75	VZV	VZV
10	58	F	Finger	Abcess	Whitlow	Swab	—	—	—
11	80	F	Rt femoral	Herpes	Zoster	Skin	79.75	VZV	VZV
12	54	F	Rt. forearm	Herpes	Zoster	Skin	79.75	VZV	VZV
13	69	F	Lower lip	Herpes	Herpes simplex	Skin	84.25	HSV1	HSV1
14	74	F	Rt trunk	Herpes	Zoster	Skin	79.75	VZV	VZV
15	72	F	Buttocks	Ulceration	Herpes simplex	Swab	88.75	HSV2	HSV2
16	66	F	Upper lips	Herpes	Herpes simplex	Skin	84.25	HSV1	HSV1
17	72	M	Lower lip	Erosion	HSV or Drug eruption	Swab	—	—	—
18	55	F	Genital	Ulceration	Genital ulcer	Swab	88.75	HSV2	HSV2
19	56	M	Lt. femoral	Herpes	Zoster	Skin	79.75	VZV	VZV
20	68	M	Genital	Ulceration	Genital ulcer	Swab	88.75	HSV2	HSV2
21	34	F	Genital	Ulceration	Ulcer	Swab	—	—	—
22	72	M	buttocks	Ulceration	Ulcer	Swab	—	—	—
23	27	F	Rt. Lower lip; Lt. feroarm; Lt. forefinger; Lt. chest	Herpes	Zoster (multiple lesion)	Skin	79.75	VZV	VZV
24	62	F	Lt. femoral	Ulceration	Varicella	Swab	79.75	VZV	VZV
25	62	F	Rt. Humerus	Herpes	Varicella	Skin	79.75	VZV	VZV

Table 2. Oligonucleotides used for uniplex and multiplex PCR

Primer		Product (bp)	Sequence (5'-3')
Herpes simplex virus type 1	Forward	90	TGGCGATCCAGATTCCAAAG TAACCGCTGGCTGGAAACC
	Reverse		
Herpes simplex virus type 2	Forward	119	CACGATTGTAGGTGCGGATAGG TCCAACAAGACGCTATCCCG
	Reverse		
Herpes zoster virus	Forward	89	CATTACCGCACCCAAAGTGAA CTAACGCTTCCACCTCGGGT
	Reverse		

infection were collected from outpatients and inpatients at Kumamoto University Hospital (Table 1). Specimens were collected after rupturing the skin vesicles and swabbing erosions or ulcers from various anatomical sites. Vesicle skin samples were dipped into 100 µL of sterilized water and swabs were washed in 400 µL sterilized distilled water and discarded. The solution alone was used as a template, and specimens were stored at -80°C.

2.2. Primer and settings

The reaction mixture contained 0.5 µL of specimen, 11 µL of deionized water, 0.5 µL each of the forward and reverse primer, and 12.5 µL of Ex Taq DNA polymerase (SYBR® *Premix Ex Taq*™, TaKaRa, Shiga, Japan). The primer sequences used are shown in Table 2. All reactions were performed in a Thermal Cycler Dice® Real Time System (TaKaRa). The thermal cycler was pre-heated to 95°C for 10 sec followed by 40 cycles of annealing at 95°C, extension for 30 sec at 60°C, and denaturation for 5 sec at 95°C. Dissociation was performed at the end of this reaction.

2.3. Agarose gel electrophoresis

PCR amplification was examined with agarose gel electrophoresis. Five-µL aliquots of the amplification products from each primer were run on 2% Agarose gel (Ultra PURE Agarose, Life Technologies, Gaithersburg, USA).

2.4. Uniplex PCR

Initially, all specimens were examined for HSV1, HSV2 and VZV infection by uniplex real-time PCR. Five or six specimens were examined at a time, with each PCR run lasting 2.5 h. For all 25 specimens, PCR was performed 6 times.

2.5. Multiplex PCR

A master mix containing forward and reverse primers for all viruses, deionized water, and polymerase was made. Aliquots of 0.5 µL were taken from all 25 specimens, 22 µL of master mix was added, and multiplex PCR was performed with a Thermal Cycler.

3. Results

Table 1 shows the results of real-time PCR for 25 specimens obtained from 23 patients. Figure 1 shows a typical clinical profile of HSV 1, HSV 2, and VZV patients, respectively. Figure 2 shows the respective amplification plots and dissociation curves for patients no. 15, 16, and 19 using uniplex PCR. Nineteen of the 25 specimens (76%) were positive for one of the three target viruses; HSV 1 ($n = 4$), HSV 2 ($n = 3$), VZV ($n = 12$), respectively. Their dissociation curves had an exact

peak temperature specific to each virus DNA: 84.25°C for HSV1, 87.75°C for HSV2, and 79.75°C for VZV. The experiments took about 2.5 h to complete using uniplex PCR, and experiments were performed 6 times to obtain results for all 25 specimens.

All of the 25 specimens were retrospectively analyzed at a time using multiplex PCR. Nineteen of the 25 specimens (76%) were positive for one of the three target viruses (HSV 1 ($n = 4$), HSV 2 ($n = 3$), and VZV ($n = 12$)). Figure 3 shows the respective amplification plots and dissociation curves for patients no. 15, 16, and 19



Figure 1. Clinical presentation of patients. (a) Patient no.16. A bullous lesion was present on her upper lip (HSV1). (b) Patient no.15 (HSV2). (c) Patient no.19. A bullous lesion was present in his left femoral area (VZV).

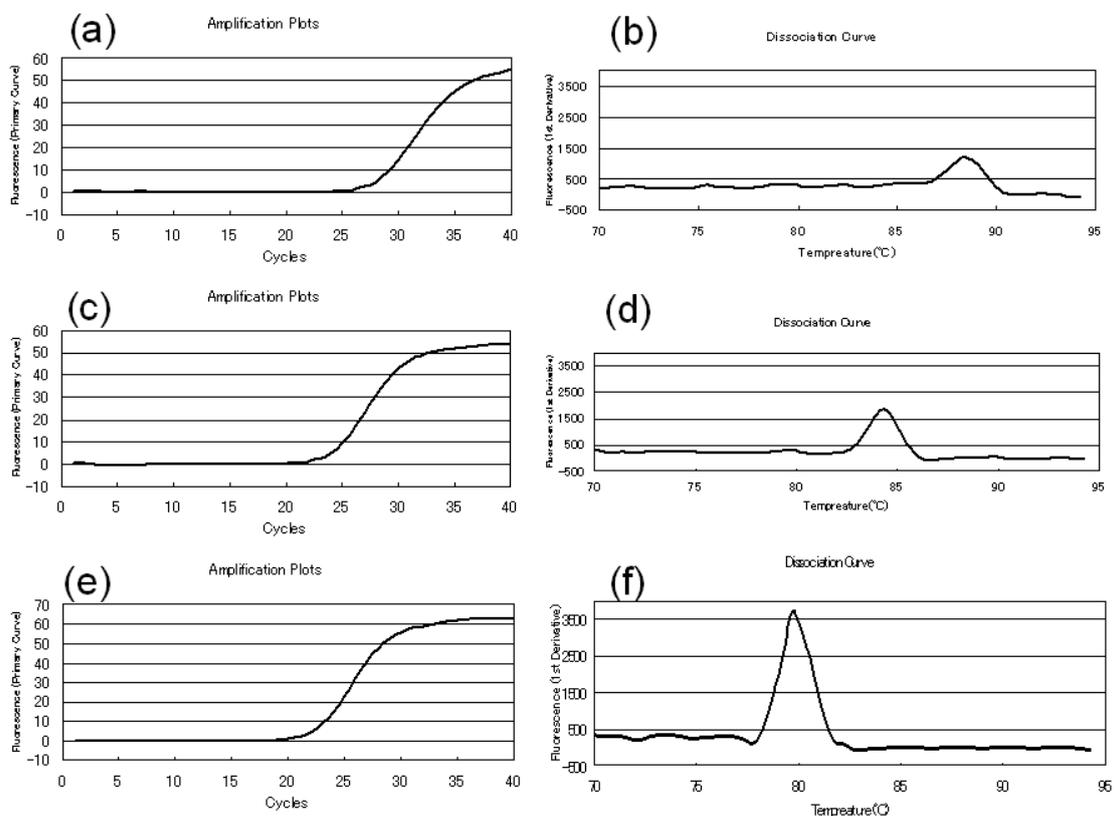


Figure 2. Amplification plots and dissociation curves for three patients by uniplex PCR. (a, b) Patient no.15 sample with HSV2-specific primers. Peak temperature of 88.75°C on the dissociation curve. (c, d) Patient no.16 sample with HSV1-specific primers. Peak temperature of 84.25°C. (e, f) Patient no.19 sample with VZV-specific primer. Peak temperature of 79.75°C.

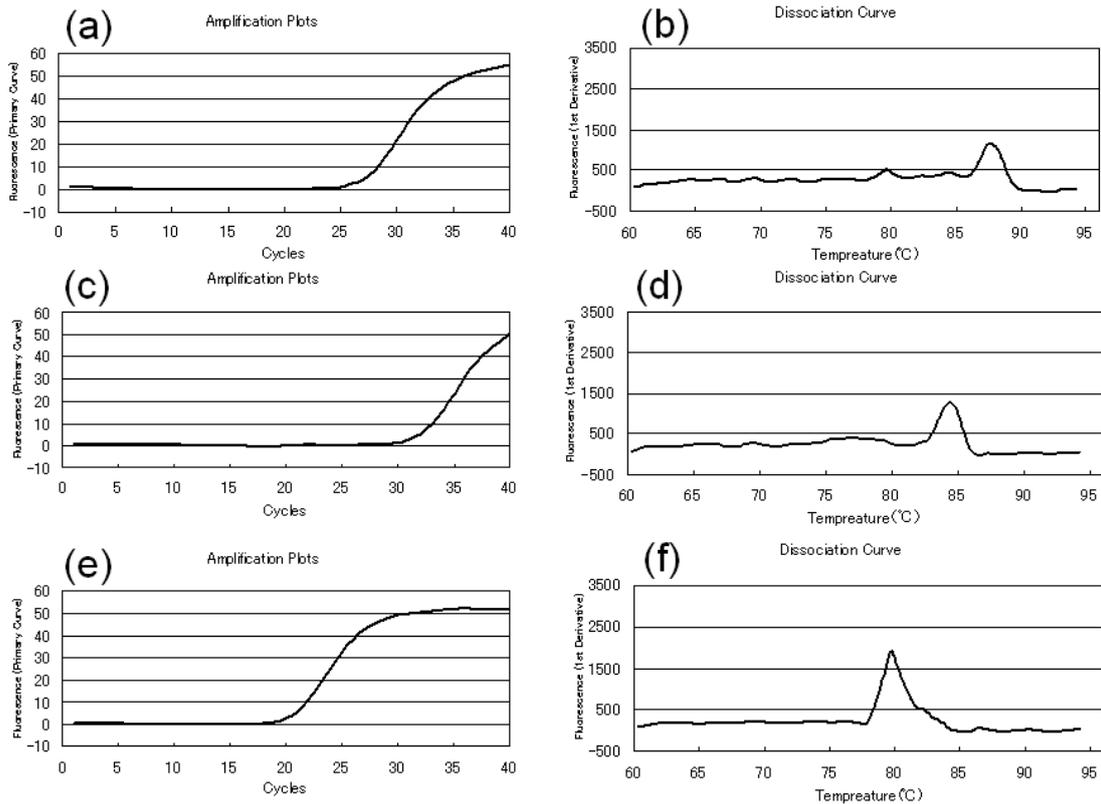


Figure 3. Amplification plots and dissociation curves for three patients by multiplex PCR. (a, b) Patient no.15 (HSV2) sample with master mix. Peak temperature of 88.75°C on the dissociation curve. (c, d) Patient no.16 (HSV1) sample. Peak temperature of 84.25°C. (e, f) Patient no.19 (VZV) sample. Peak temperature of 79.75°C.

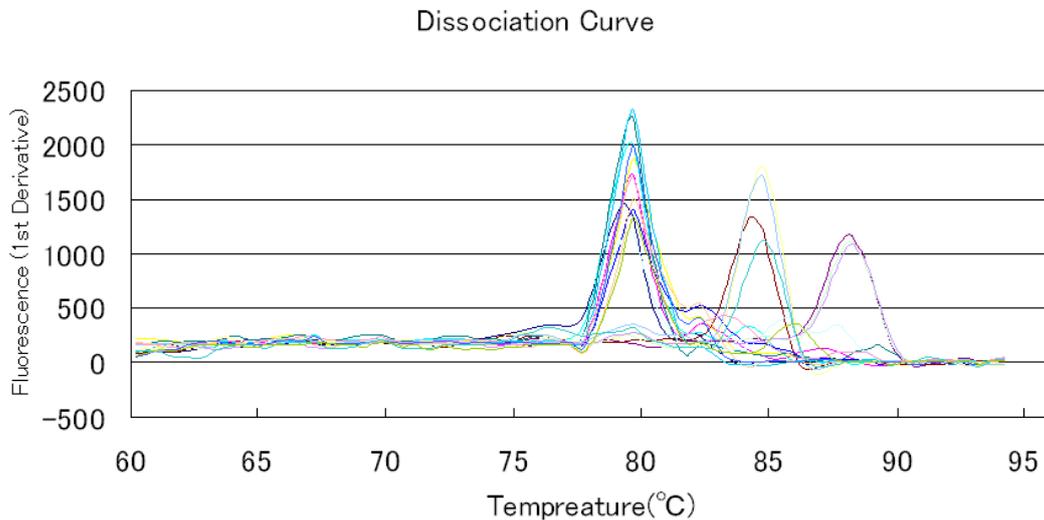


Figure 4. Dissociation curves of all positive specimens ($n = 19$). Each curve had only one peak temperature, and these temperatures were exactly the same as those obtained with uniplex PCR.

using multiplex PCR. Multiplex PCR results for all of the positive specimens were exactly the same as those obtained with uniplex PCR in terms of the dissociation curve and temperature (Table 1, Figure 4). Analyzing all 25 specimens took only 2 h. Multiplex PCR reduced the experimental time 30 min although the number of specimens increased.

Frozen solutions of the sample were able to withstand multiple freeze-thaw cycles; this was

confirmed by results indicating the same dissociation curves, even for specimens that were stored for up to 11 months.

Agarose gel electrophoresis was performed to check the bands of PCR amplification. Five microliters of the amplification products were run in 2% agarose gel, and the results are shown in Figure 5. The difference in band sizes was reflected by the migration distance, which also confirmed the dissociation curve results.

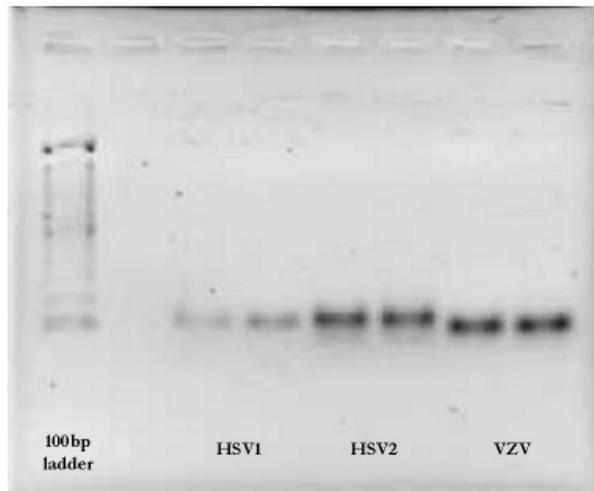


Figure 5. Agarose gel electrophoresis. As shown in Table 2, the longest product in 3 was the HSV2 primer product of 119 bp and the shortest was the VZV primer product of 89 bp. Electrophoresis results coincided with the length of each product.

4. Discussion

Nucleic acid extraction is expensive, time-consuming, and may result in specimen contamination prior to analysis. To counter such problems, this study has described a reliable and speedy way of confirming the diagnosis of herpes viruses without nucleic acid extraction.

Real-time PCR is a speedy and sensitive method for the detection and genotyping of infectious diseases such as herpes viruses. Earlier methods include isolation of the virus in cell culture and detection of the virus followed by immunofluorescence microscopy, but this method was laborious. In the current study, clinical specimens of skin vesicles and swabs from patients suspected of being infected by herpes viruses (HSV type 1/2, VZV) were analyzed by real-time PCR without nucleic acid extraction or purification. Mark *et al.* investigated PCR performance with or without nucleic acid isolation from specimens (2). They found only one peak of the dissociation curve, which means that omission of DNA extraction did not negatively affect data analysis. Omission of the DNA extraction step provides a significant reduction in time and cost.

Specific primers for each virus amplified each specific DNA arrangement and were distinguishable by dissociation temperature. A stock cocktail containing the primers of both (HSV1/2, VZV), polymerase, and de-ionized water was prepared to identify ways to further reduce the experimental time, cost (of tubes), and possibility of contamination. As shown in Figure 4, the same dissociation curve for each positive specimen was obtained by using multiplex PCR. Recently, the use of multiplex PCR for the diagnosis of herpes has become more and more common (4-8). Several reports compared uniplex and multiplex PCR (4,6). Multiplex PCR was found to be more rapid, specific,

and sensitive than uniplex PCR. Multiplex PCR assay also offers increased sensitivity, typing, and improved turnaround time compared to traditional viral culture and immunofluorescence techniques (8).

5. Conclusions

The current results indicate that omission of the nucleic acid extraction step prior to real-time PCR does not negatively affect downstream reactions. Multiplex PCR is a rapid, sensitive, time-saving, and cost-effective assay when many specimens are being examined. A sample/water solution was stored in a -80°C freezer for several months without deterioration in quality, even after several freeze-thaw cycles.

On the basis of these results, work to devise methods to further reduce experimental time will continue.

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