

## Rapid Diagnosis of Herpes Simplex Virus Infection by a Loop-Mediated Isothermal Amplification Method

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**Primers for herpes simplex virus type 1 (HSV-1)-specific loop-mediated isothermal amplification (LAMP) method amplified HSV-1 DNA, while HSV-2-specific primers amplified only HSV-2 DNA; no LAMP products were produced by reactions performed with other viral DNAs. The sensitivities of the HSV-1- and HSV-2-specific LAMP methods, determined by agarose gel electrophoresis, reached 500 and 1,000 copies/tube, respectively. The turbidity assay, however, determined the sensitivity of the HSV-1- and HSV-2-specific LAMP methods to be 1,000 and 10,000 copies/tube, respectively. After initial validation studies, 18 swab samples (in sterilized water) collected from patients with either gingivostomatitis or vesicular skin eruptions were examined. HSV-1 LAMP products were detected by agarose gel electrophoresis in the 10 samples that also demonstrated viral DNA detection by real-time PCR. Nine of these 10 samples exhibited HSV-1 LAMP products by turbidity assay. Furthermore, both the agarose gel electrophoresis and the turbidity assay directly detected HSV-1 LAMP products in 9 of the 10 swab samples collected in sterilized water. Next, we examined the reliability of HSV type-specific LAMP for the detection of viral DNA in clinical specimens (culture medium) collected from genital lesions. HSV-2 was isolated from all of the samples and visualized by either agarose gel electrophoresis or turbidity assay.**

Viral isolation and serological assays are standard methods of herpes simplex virus (HSV) diagnosis. Both viral isolation and serological testing, however, require substantial time to obtain accurate final results. More rapid detection has been achieved by modification of cell culture techniques by centrifugation of inocula on cell monolayers and the use of immunofluorescence techniques (6). Recent studies have suggested that detection of HSV DNA by PCR increases the sensitivity of viral infection detection compared to antigenic detection or cell culture methods (3, 4, 11, 13, 14). While quantitative analysis of viral DNA by real-time PCR may become a valuable tool for bedside monitoring of HSV infection and progression (1, 2, 7, 10, 17, 21, 22), it has not yet become a common procedure in hospital laboratories due to the requirement of specific expensive equipment (a thermal cycler).

Recently, Notomi et al. (18) reported a novel nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP), which is used to amplify DNA under isothermal conditions with high specificity, efficiency, and speed. The most significant advantage of LAMP is the ability to amplify specific sequences of DNA between 63 and 65°C without

thermocycling. Thus, the technique requires only simple and cost-effective equipment amenable to use in hospital laboratories. The LAMP method also exhibits both high specificity and high amplification efficiency. As the LAMP method uses four primers which recognize six distinct target DNA sequences, the specificity is extremely high. This method also exhibits extremely high amplification efficiency, due in part to its isothermal nature; as there is no time lost due to changes in temperature and the reaction can be conducted at the optimal temperature for enzyme function, the inhibition reactions that often occur at later stages of typical PCR amplifications are less likely to occur. Thus, this method could potentially be a valuable tool for the rapid diagnosis of infectious diseases (5, 8, 9, 12, 19, 23) in both commercial and hospital laboratories. In this study, we sought to establish a LAMP-based HSV type-specific DNA amplification method and examine its reliability for the detection of HSV DNA from clinical specimens.

HSV-1 (KOS) DNA and HSV-2 (186) DNA were used as positive controls to determine the appropriate conditions for HSV type-specific LAMP and to establish the baseline sensitivity and specificity levels. HSV-1 (KOS), HSV-2 (186), varicella-zoster virus (VZV) (Oka), human cytomegalovirus (HCMV) (AD-169), human herpesvirus type 6B (HHV-6B) (Z29), and HHV-7 (RK) DNA were used to determine the specificity of HSV type-specific LAMP. Plasmids containing

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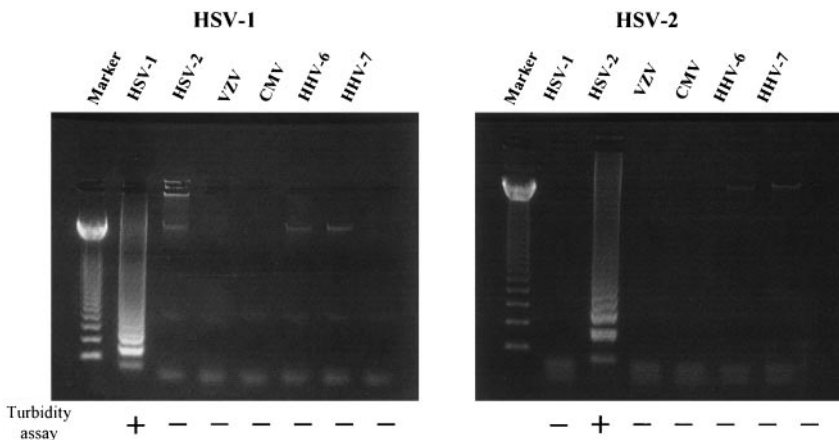


FIG. 1. DNA extracted from HSV-1 (KOS)-, HSV-2 (186)-, VZV (Oka)-, HCMV (AD-169)-, HHV-6 B (Z29)-, and HHV-7 (RK)-infected cells was amplified by using HSV-1- and HSV-2-specific LAMP to determine method specificity. The detection of LAMP products was assessed by agarose gel electrophoresis and turbidity assay using an LA-200. Marker, 123-bp DNA ladder marker.

defined 0.1 as the cutoff value for discrimination between positive and negative samples.

Real-time PCR quantitated the amount of either HSV-1 or HSV-2 DNA in each sample. The genes encoding HSV-1 and HSV-2 glycoprotein G were selected for HSV type-specific real-time PCR. The sequences of the primers and probes used for these experiments were described previously by Pevenstein et al. (20).

To develop an effective assay for rapid measurement of HSV DNA content, we first evaluated the specificity of our HSV type-specific primers. HSV type-specific LAMP was performed on DNA extracted from HSV-1 (KOS)-, HSV-2 (186)-, VZV (Oka)-, HCMV (AD-169)-, HHV-6B (Z29)-, and HHV-7 (RK)-infected cells. As the LAMP products contained several inverted-repeat structures, positive samples exhibit multiple bands of different sizes upon agarose gel electrophoresis. HSV-1-specific primers amplified only HSV-1 DNA and HSV-2-specific primers amplified only HSV-2 DNA (Fig. 1), and no LAMP products were detected in reactions performed with DNA from other viral infections. We also tested the specificity of the primers by using a turbidity assay. The use of HSV-1-

specific primers elevated sample turbidity only in HSV-1 DNA-containing samples. Similar specificity was observed for HSV-2-specific primers (Fig. 1).

We also determined the sensitivity of this method. Serial dilutions of either pGEMHS1 or pGEMHS2 plasmid containing the target sequences determined the detection limits of HSV type-specific LAMP. The sensitivities of the HSV-1- and HSV-2-specific LAMP determined by agarose gel electrophoresis were 500 and 1,000 copies/tube, respectively (Fig. 2). Detection by the turbidity assay, however, produced sensitivity levels of 1,000 and 10,000 copies/tube for HSV-1- and HSV-2-specific LAMP, respectively.

After these initial validation studies, we determined the reliability of this HSV type-specific LAMP as a method of viral DNA detection from clinical specimens. Eighteen swab samples (sample numbers 1 to 18) collected from patients with either gingivostomatitis or vesicular skin eruptions were examined (Table 2). Neither HSV-1 nor HSV-2 LAMP products were detected in samples (sample numbers 1 to 8) from which no HSV DNA could be detected by real-time PCR. In contrast, HSV-1 LAMP products were detected by agarose gel electro-

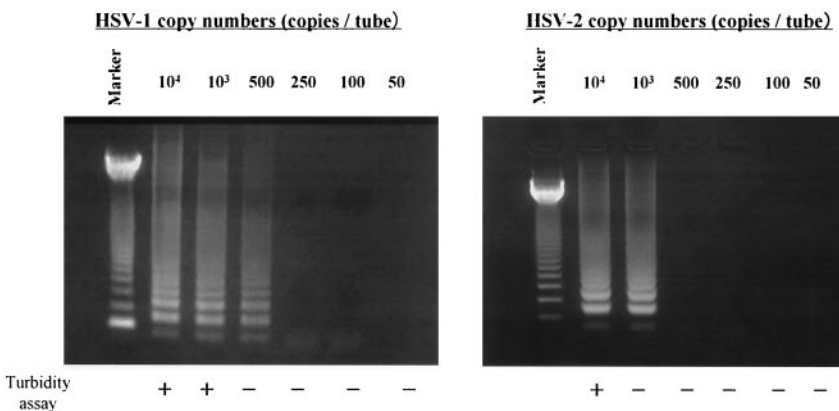


FIG. 2. To determine the respective sensitivities of each assay, serial dilutions of pGEMHS1 and pGEMHS2 plasmid DNAs were amplified by the HSV-1- and HSV-2-specific LAMP, respectively. The detection of LAMP products was assessed by agarose gel electrophoresis and turbidity assay using an LA-200. Marker, 123-bp DNA ladder marker.

TABLE 2. Comparison between HSV type-specific real-time PCR and HSV type-specific LAMP for detection of HSV DNA in swab samples collected from patients with gingivostomatitis or vesicular skin eruptions (samples 1 to 18) and genital HSV infection (samples 19 to 23)

Sample no.	HSV-1 <sup>a</sup>						HSV-2 <sup>a</sup>					
	Real-time PCR (copies/tube)	LAMP (DNA extraction) result		LAMP (direct <sup>b</sup> ) result		Real-time PCR (copies/tube)	LAMP (DNA extraction) result		LAMP (direct <sup>b</sup> ) result			
		Agarose gel electrophoresis	Turbidity assay	Agarose gel electrophoresis	Turbidity assay		Agarose gel electrophoresis	Turbidity assay	Agarose gel electrophoresis	Turbidity assay		
1	0	-	-	-	-	0	-	-	ND	ND		
2	0	-	-	-	-	0	-	-	ND	ND		
3	0	-	-	-	-	0	-	-	ND	ND		
4	0	-	-	-	-	0	-	-	ND	ND		
5	0	-	-	-	-	0	-	-	ND	ND		
6	0	-	-	-	-	0	-	-	ND	ND		
7	0	-	-	-	-	0	-	-	ND	ND		
8	0	-	-	-	-	0	-	-	ND	ND		
9	6,350	+	+	+	+	0	-	-	ND	ND		
10	9,200	+	-	-	-	0	-	-	ND	ND		
11	29,450	+	+	+	+	0	-	-	ND	ND		
12	129,650	+	+	+	+	0	-	-	ND	ND		
13	194,050	+	+	+	+	0	-	-	ND	ND		
14	283,000	+	+	+	+	0	-	-	ND	ND		
15	669,950	+	+	+	+	0	-	-	ND	ND		
16	2,629,400	+	+	+	+	0	-	-	ND	ND		
17	3,769,650	+	+	+	+	0	-	-	ND	ND		
18	86,136,700	+	+	+	+	0	-	-	ND	ND		
19	0	-	-	ND	ND	641	+	+	-	-		
20	0	-	-	ND	ND	22,430	+	+	-	-		
21	0	-	-	ND	ND	89,929	+	+	-	-		
22	0	-	-	ND	ND	413,117	+	+	-	-		
23	0	-	-	ND	ND	443,963	+	+	-	-		

<sup>a</sup> +, positive; -, negative.

<sup>b</sup> Swab samples were directly (without DNA extraction) used for LAMP reaction. ND, not done.

phoresis in the 10 HSV-1-positive samples, correlating perfectly with the results of real-time PCR. When the turbidity assay was used, HSV-1 LAMP products were detected in all but 1 (sample number 10) of these 10 positive samples. No HSV-2 DNA could be detected in these samples by either real-time PCR or LAMP. As rapidity and simplicity of the method are critical for commercial and hospital laboratory use, we investigated the requirement for DNA extraction in HSV type-specific LAMP. Either agarose gel electrophoresis or turbidity assay directly detected HSV-1 LAMP product in all 10 swab samples (sample numbers 9 to 18) (sterilized water), with the exception of sample number 10, regardless of the presence or absence of DNA extraction (Table 2). As genital herpes is another important clinical manifestation of herpes infection, we next examined the reliability of HSV type-specific LAMP for the detection of viral DNA in clinical specimens (culture medium) collected from genital lesions (sample numbers 19 to 23) (Table 2). High copy numbers of HSV-2 DNA (ranging between 641 and 443,963 copies/tube) were detected in these samples by HSV-2 type-specific real-time PCR. Both agarose gel electrophoresis and turbidity assay detected HSV-2 LAMP products in all of the samples. To determine the necessity of DNA extraction by this method, we again tried to detect HSV-2 LAMP products in the samples with or without (culture medium) DNA extraction. In contrast, while HSV-2 LAMP products were detected in samples after DNA extraction, no HSV-2 LAMP products were detected in the samples without DNA extraction (Table 2). To determine if the culture medium contained an inhibitor of LAMP, we attempted to detect HSV

LAMP products from both sterilized water and culture medium containing plasmid DNA which contained the target sequences. Although both HSV-1 and HSV-2 LAMP products could be detected in sterilized water containing the target sequences, no LAMP products were detected in culture medium containing these DNAs (data not shown).

HSV-1- and HSV-2-specific LAMP specifically amplified HSV-1 and HSV-2 DNA, respectively, exhibiting no cross-reactivity with other human herpesviruses, including another member of the subfamily *Alphaherpesvirinae*, VZV (Fig. 1). This specificity was confirmed by agarose gel electrophoresis and turbidity assay. Although the capability to distinguish between HSV-1 and HSV-2 infection is not crucial for correct administration of antiviral drugs, this discrimination is important from an epidemiological or public health standpoint. As a consequence of the experiment used for determination of the assay sensitivity, it was suggested that the turbidity assay is a less sensitive detection method than agarose gel electrophoresis, as previously suggested (23). However, the turbidity assay is more appropriate for bedside monitoring due to its ease and rapidity. Additionally, turbidity measurement of LAMP products allows a reduction in operation time and reduces contamination risks because of the absence of agarose gel electrophoresis.

We also evaluated the reliability of HSV type-specific LAMP in the detection of viral DNA from different clinical specimens. Although HSV-1 LAMP products were detected by agarose gel electrophoresis in 10 of the 18 swab samples (sterilized water) collected from patients with vesicular skin lesions

and gingivostomatitis suspected as HSV infection, no HSV-2 LAMP products were detected in these samples. All five swab samples (culture medium) collected from the lesions of patients with genital herpes contained HSV-2 LAMP products. These results corresponded well with those from real-time PCR analysis, suggesting that HSV type-specific LAMP is a reliable method for the detection of viral DNA in clinical samples. Although an HSV-1 LAMP product could not be detected in one HSV-1-positive sample by turbidity assay, this inconsistency is probably due to low copy numbers. As the majority of clinical samples (e.g., skin eruptions, oral ulcers, and genital lesions) contain large quantities of viral DNA, the sensitivity of type-specific LAMP by turbidity assay is likely sufficient for the evaluation of most clinical samples. Moreover, all amplification steps are completed within 30 min with an LA-200, and it is a cheaper piece of equipment than that required for real-time PCR, which are major advantages for hospital laboratory use.

Interestingly, when the swabs were collected in sterilized water, HSV LAMP products could be detected directly from the samples without DNA extraction. In contrast, LAMP products could not be detected directly from the culture medium containing viral DNA, regardless of the HSV strain. DNA target sequences, however, became detectable in samples after DNA extraction, suggesting that culture medium contains inhibitors of the LAMP reaction. As the DNA extraction step requires approximately 30 min, omission of DNA extraction could save both time and labor for preparing the samples for LAMP, a major advantage for rapid diagnosis in hospital laboratories. To our knowledge, this is the first report to demonstrate direct amplification of viral DNA from sterilized water containing viral nucleic acids without DNA extraction. We emphasize that the swab should be placed into sterilized water for direct amplification of viral DNA by LAMP. Direct amplification from swab samples in combination with assessment by turbidity assay would accomplish the entire amplification within 30 min. This system would therefore allow large increases in throughput, which is highly relevant for clinical laboratory use. Furthermore, as HSV DNA could be directly detected from swab samples without DNA extraction, the lesions of HSV infection may contain a large quantity of naked viral DNA as well as complete virions. Thus, direct detection of viral DNA from swab samples may be possible by additional DNA amplification methods such as real-time PCR. Further investigation will be necessary to confirm this hypothesis in the future.

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