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Loop-mediated isothermal amplification assay for the diagnosis of retinitis caused by herpes simplex virus-1

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Abstract

A loop-mediated isothermal amplification (LAMP) assay was developed for the detection of herpes simplex virus 1 (HSV-1). The specificity of the assay was tested using DNA extracted from HSV-1-infected rabbit corneal epithelium cultures, HSV-2 grown on Vero cell line, cytomegalovirus (CMV) (AD-169), varicella zoster virus (VZV) (Oka-vaccine), adenovirus, *Aspergillus flavus* and *Staphylococcus aureus*. The specificity of LAMP was confirmed by bidirectional sequencing of the amplicons. The sensitivity of the LAMP assay was tested using different concentrations of HSV-1 DNA. To evaluate the application of the LAMP assay in clinical diagnosis, we tested vitreous samples from 20 patients with suspected viral retinitis using LAMP and real-time PCR for HSV-1. The LAMP primers amplified only HSV-1 DNA; no LAMP products were detected with the DNAs of HSV-2, CMV, VZV, adenovirus *A. flavus* and *S. aureus*. The sequences of the positive HSV-1 LAMP products perfectly (99–100%) matched the HSV-1 sequences deposited in the GenBank database. LAMP is as sensitive as real-time PCR, with the lowest detection limit being 10 copies/μL of HSV-1 DNA. Of the 20 patients with suspected viral retinitis, four tested positive for HSV-1 using real- time PCR and LAMP. A 100% concordance was observed across the two methods. The LAMP assay is a rapid, highly specific and sensitive method for the diagnosis of retinitis caused by HSV-1.

Keywords: HSV-1, LAMP, real-time PCR, retinitis, sensitivity, specificity
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Introduction

Viral retinitis is an important vision-threatening infectious disease of the retina that can occur in both immunocompetent and immunocompromised individuals [1–4]. The most common aetiological agents of viral retinitis are herpes simplex virus (HSV), varicella zoster virus (VZV) and cytomegalovirus (CMV) [4]. Rapid detection of the specific causative agent helps in the timely institution of specific antiviral therapy because this differs for each group of herpes viruses [5]. Conventional diagnostic methods for viral retinitis include the detection of viral antigen and virus isolation from intraocular specimens [4].These tests have been shown to have low sensitivity in the detection of viruses and are not currently recommended for the diagnosis of viral retinitis [4]. PCR comprises a sensitive and specific technique that has been successfully used to detect viral DNA in ocular samples from patients presenting with viral retinitis [6]. However, PCR requires high-cost precision instruments (a thermal cycler) for amplification, which is a major obstacle to its widespread use.

Loop-mediated isothermal amplification (LAMP) was recently developed and evaluated for the detection of viruses [7–9]. The main advantage of LAMP is its ability to amplify specific sequences of DNA under isothermal conditions in the temperature range 63–65°C, thereby obviating the need for a thermal cycler [7]. The LAMP method depends on autocycling strand displacement DNA synthesis performed by *Bst* DNA polymerase and the isothermal reaction relies on recognition of the target by six independent primers, thereby making the assay highly specific [7]. In the present study, we describe the development and evaluation of LAMP, a simple, rapid and cost-effective method for the detection of HSV-1 in patients with viral retinitis.

Materials and Methods

Clinical samples, positive controls and DNA extraction

The study (Ref. No. LEC 08013) was approved by the Institutional Review Board of L. V. Prasad Eye Institute, Hyderabad, India. Vitreous samples were collected from 20 patients with clinically suspected viral retinitis between January 2009 and May 2009. HSV-1 DNA extracted from HSV-1 grown on rabbit corneal epithelial cells was used as a positive control. The viral DNA was extracted from clinical samples and from the HSV-1-infected cell line using the Qiagen DNA minikit (Qiagen, Hilden, Germany). The viral DNA was finally eluted in 75 μL of elution buffer and stored at −20°C. All the patient samples were tested with both the LAMP assay and with a real-time PCR assay.

LAMP reaction

The primers were selected based on criteria described by Notomi *et al.* [7]. The primers were designed using Primer Explorer V4 software (http://primerexplorer.jp/lamp4.0.0/index.html). A set of six primers was used, comprising two outer, two inner and two loop primers that recognize the distinct regions on the target site (UL-1 to UL-2 gene regions of the HSV-1 virus). The LAMP primer sequences are shown in Table 1. The two outer primers are described as forward outer primer (F3) and backward outer primer (B3), whereas the inner primers were described as forward inner primer (FIP) and backward inner primer (BIP). In addition, two loop primers (Loop F and Loop B) were used.

The LAMP reaction was performed using in-house prepared reaction mixtures and each assay was conducted with 25 μL of the reaction mixture consisting of 0.6 μL of (5 μM) each of the outer primers, 0.6 μL (40 μM) each of the inner primers, 0.6 μL of (20 μM) each of the loop primers, 2.5 μL of 10× *Bst* DNA polymerase reaction buffer (New England Biolabs, Beverly, MA, USA), 1.0 μL of 50 mM $MgSO_4$, 5.0 μL of 5 M Betaine (Sigma-Aldrich, St Louis, MO, USA), 2.5 μL of dNTPs (1.4 mM), 1.0 μL of 8 U/μL Bst DNA polymerase (New England Biolabs), 5.0 μL of target DNA and 4.4 μL of autoclaved milli-Q water (Millipore, Billerica, MA, USA). The reaction mixture was incubated in a water bath at 65°C for 1 h and heated at 80°C for 2 min to terminate the reaction. Positive and negative controls were included in each run. The amplified product was detected by adding 1 μL of (× 1000) SYBR green dye to each reaction tube and incubated for 15 min in the dark at room temperature. The yellowish–green colour indicated a positive reaction and the reddish orange (i.e. the colour of the unbound dye) indicated a negative reaction. In addition, the LAMP products were detected with agarose gel (2%) electrophoresis with UV light transillumination.

TABLE 1. Sequences of the primers used in the loop-mediated isothermal amplification assays

Primer	Sequence
HSV1 F3	5′-CAGCCACACACCTGTGAA-3′
HSV1 B3	5′-TCCGTCGAGGCATCGTTAG-3′
HSV1 FIP (F1-F2)	5′-CCAGACGTTCCGTTGGTAGGTCTTTTACTTTGACTGTTCGCGCACC-3′
HSV1BIP (B1-B2)	5′-CCATCATCGCCACGTCGGACTTTTTCGGCGTCTGCTTTTTGTG-3′
HSV1 LPF	5′-AAATCCTGTCGCCCTACACAGCGG-3′
HSV1 LPB	5′-CACCCCGCGACGGGACGCCG-3′

Sensitivity and specificity of the LAMP assay

The specificity of the LAMP assay was evaluated using HSV-2 grown on a Vero cell line, CMV DNA (AD-169), VZV DNA (Oka-Vaccine), adenovirus DNA, DNA extracted from *Aspergillus flavus* isolated from clinical samples and *Staphylococcus aureus* (ATCC 25923). Specificity was further tested by bidirectional sequencing of the HSV-1-positive LAMP product using the Big Dye terminator cycle sequencing kit (forward primer: 5′-CAGCCACACACCTGTGAA-3′; reverse primer: 5′-TCCGTCGAGGCATCGTTAG-3′, UL-1 to UL-2 gene regions) on an ABI 3130 XI automated sequencer in accordance with the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The sequences were analysed and identified using the MEGABLAST search program (http://www.ncbi.nlm.nih.gov) from the GenBank database. The analytical sensitivity of the LAMP assay was tested using 10, 100, 1000 and 10000 copies/μL of HSV-1 DNA.

Real-time PCR

Real-time PCR was used to confirm the presence of HSV-1 DNA in the clinical samples. This was performed using the artus HSV-1/2 TM Real-Time PCR kit (Qiagen) on ABI PRISM 7900 (Applied Biosystems) in accordance with the manufacturers' instructions.

Results

The LAMP primers amplified only HSV-1 DNA. No LAMP products were detected with the DNAs of HSV-2, CMV, VZV, adenovirus, *A. flavus* and *S. aureus* (Fig. 1). The LAMP reaction tubes were examined with the naked eye to detect a positive reaction upon the addition of SYBR green dye. Upon addition of SYBR green dye to the LAMP reaction tubes, the colour changed to yellowish green in a positive reaction (Fig. 2, tube 1) and remained reddish–orange (i.e.

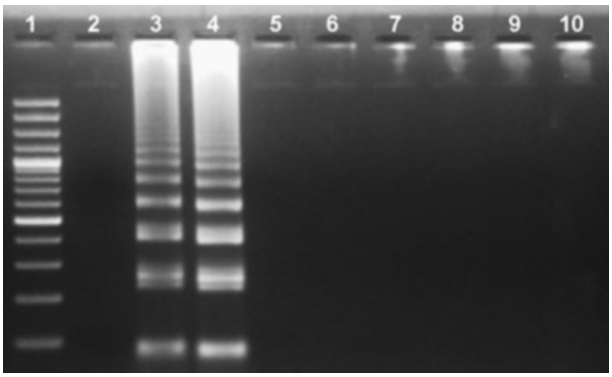

FIG. 1. Electrophoretic analysis of loop-mediated isothermal amplification (LAMP) products. Lane 1, 100 bp DNA ladder. Lane 2, negative control; lanes 3 and 4, herpes simplex virus (HSV)-1 LAMP products; lanes 5, 6, 7, 8, 9 and 10, no LAMP product with other organisms (HSV-2, cytomegalovirus, varicella zoster virus, adenovirus, *Aspergillus flavus* and *Staphylococcus aureus*).

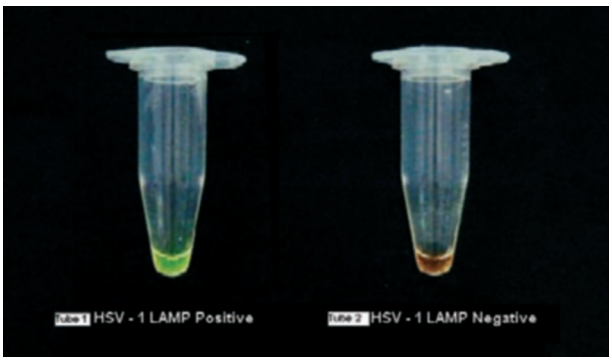

FIG. 2 Visual detection of loop-mediated isothermal amplification products. The tube with a positive reaction (tube 1) shows a yellowish–green colour change, which can be distinguished from the reddish–orange colour of a negative reaction (tube 2).

the colour of the unbound dye) in the negative reactions (Fig. 2, tube 2). All the samples that were judged positive by visual detection of the colour change were also positive by gel electrophoresis (and vice versa). The sequences of the HSV-1 LAMP products were perfectly (99–100%) matched with the HSV-1 sequences (FJ593289, X14112, D00373, AB252711, U53683, DQ889502, U53684, AY120884) deposited in the GenBank database. The sequence similarity (100%) of the LAMP products with one of the sequences deposited in GenBank is shown in Fig. 3. The HSV-1 LAMP was positive at all the different concentrations of DNA (10, 100, 1000, 10000 copies/μL of HSV-1 DNA) used to test sensitivity. LAMP is as sensitive as real-time PCR, with its lowest detection limit being 10 copies/μL of HSV-1 DNA.

Vitreous specimens from 20 patients with clinically suspected viral retinitis were screened for HSV-1 virus by

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CTGTGTAGGGCGACAGGATTTGGGACCTACCAACGGAACGTCTGGACGGACCCCGGTTCT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CTGTGTAGGGCGACAGGATTTGGGACCTACCAACGGAACGTCTGGACGGACCCCGGTTCT
GCCGCCGGACGATGAAGCGGGCCTGCAGCCGAAGCCCCTCACCACGCCGCCGCCCATCAT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GCCGCCGGACGATGAAGCGGGCCTGCAGCCGAAGCCCCTCACCACGCCGCCGCCCATCAT
CGCCACGTCGGACCCCACCCCGCGACGGGACGCCGCCACAAAAAGCAGACGCCGACGACC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CGCCACGTCGGACCCCACCCCGCGACGGGACGCCGCCACAAAAAGCAGACGCCGACGACC
CCACTCCCGGCGCCTCTAACGATGCCTCGACGGAAACCCGTCCGGGTTCGGGGGGGCGAAC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CCACTCCCGGCGCCTCTAACGATGCCTCGACGGAAACCCGTCCGGGTTCGGGGGGGCGAAC
CGGCCGC(247)
|||||||
CGGCCGC(247)
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FIG. 3. Sequence alignment of the herpes simplex virus 1 (HSV-1) positive loop-mediated isothermal amplification product (Red) with deposited sequence of HSV-1 (Black) in the GenBank (D00373).

real-time PCR and LAMP. The LAMP assay detected four positive samples and 16 negative samples from patients with retinitis. The real-time PCR also detected four positive samples and 16 negative samples. A concordance of 100% was observed between the two techniques (Table 2).The copy numbers of HSV-1 DNA detected by real-time PCR and the LAMP assay among the four positive samples were: sample 1 ($1.2 \times 10^2/\mu$L), sample 2 ($4.0 \times 10/\ \mu$L), sample 3 ($2.0 \times 10/\mu$L) and sample 4 ($1.5 \times 10^2/\mu$L).

Discussion

The diagnosis of viral retinitis is generally based on clinical examination. However, overlapping funduscopic findings may make a clinical diagnosis of CMV, VZV or HSV retinitis difficult, particularly early in the course of the disease [6]. PCR assays are used for the diagnosis of viral agents in intraocular fluid samples in cases of retinitis where the diagnosis cannot be established by clinical examination [6]. PCR requires precision equipment such as a thermal cycler for the amplification of DNA and an electrophoretic apparatus and gel documentation system for the detection of the amplified product. In the present study, we developed and evaluated a LAMP assay, a novel nucleic acid amplification method for the detection of herpes simplex virus 1 in vitreous samples. The LAMP assay is a simple diagnostic tool in which the

TABLE 2. Detection of herpes simplex virus 1 in vitreous samples by real-time PCR and loop-mediated isothermal amplification (LAMP) assays

	LAMP	
Real-time PCR	**Positive**	**Negative**
Positive	4	0
Negative	0	16
Total	4	16

reaction takes place in a single tube that contains buffer as well as target DNA, *Bst* DNA polymerase and primers. The tube containing this reaction mixture is incubated at 65°C in a regular laboratory water bath or heat block that maintains a constant temperature. There is no need for a thermal cycler because the entire amplification cycle can be achieved at a constant temperature. Moreover, there is no need for electrophoresis apparatus and a gel documentation system for the detection of amplified product in the LAMP assay. The final amplified product in this assay is detected by unaided visual examination by adding SYBR green dye to the reaction tube. A positive reaction is indicated if the product turns yellowish–green in colour in the reaction tubes and remains reddish–orange (i.e. the colour of the unbound dye) in the negative reaction tubes. The time required for the amplification and the detection of product is approximately 75 min in the LAMP assay compared to 4 h for PCR. Additionally, unlike a regular PCR, LAMP is less prone to inhibitors that can otherwise hamper the amplification process.

LAMP is highly specific because it uses four primers (excluding the loop primers) to detect six distinct regions on the target DNA [7]. In the present study, the assay specifically amplified only HSV-1 and no cross-reactivity was observed with other viruses, bacteria or fungi, and the amplified product on sequencing was similar to the HSV-1 sequences deposited in GenBank. The LAMP assay is highly sensitive; we found that the lower detection limit of the assay was 10 copies of DNA/μL. This indicates that the LAMP assay has almost the same sensitivity as real-time PCR.

Of 20 patients with viral retinitis, four were positive for HSV-1 DNA by both LAMP and real-time PCR and the remaining 16 patients were negative for HSV-1 DNA by both LAMP and real-time PCR assays. A 100% concordance was observed between the two techniques. We cannot rule out infection with other viral agents such as HSV-2, VZV, CMV or Epstein–Barr viruses in the remaining 16 patients who were negative for HSV-1, and the vitreous samples of the 16 patients were not tested for other viral agents. These initial findings are extremely promising and suggest the need to evaluate this test further in different laboratories using a larger cohort of patients.

The LAMP assay was initially developed by Notomi *et al.* [7] and was recently applied to the diagnosis of the severe acute respiratory syndrome, West Nile fever, Dengue, Japanese encephalitis and Chikungunya and BK virus infections [8–12]. In the present study, we evaluated LAMP for the detection of HSV-1 in patients with retinitis and found a good correlation between real-time PCR and LAMP in the detection of the HSV-1 virus. To conclude, HSV-1 LAMP assay comprises a highly sensitive, specific, rapid, cost-effective and reliable diagnostic tool for the detection of HSV-1 in patients with retinitis.

Transparency Declaration

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