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

A. K. Reddy1, P. K. Balne1, R. K. Reddy2, A. Mathai2 and I. Kaur3
1) Jhaveri Microbiology Centre, Hyderabad Eye Research Foundation, 2) Smt. Kanuri Santhamma Retina Vitreous Centre and 3) Kallam Anji Reddy Molecular Genetics Laboratory, Prof. Brien Holden Eye Research Centre, Hyderabad Eye Research Foundation, LV Prasad Eye Institute, Hyderabad, India

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

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 5 M Betaine (Sigma-Aldrich, St Louis, MO, USA), 1000 copies/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.

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’-TCCGTGAGCCATCGTTAG-3’, UL-1 to UL-2 gene regions) on an ABI 3130 XL 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.
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 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 × 10^2/µL), sample 2 (4.0 × 10^3/µL), sample 3 (2.0 × 10^4/µL) and sample 4 (1.5 × 10^5/µL).

**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**

| 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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**References**

1. Lau CH, Missotten T, Salzmann J, Lightman SL. Acute retinal necrosis. Features, management, and outcomes. *Ophthalmology* 2007; 114: 756–762.
2. Hillenkamp J, Nolle B, Bruns C, Rautenburg P, Fickenscher H, Roider J. Acute retinal necrosis: clinical features, early vitrectomy, and outcomes. *Ophthalmology* 2009; 116: 1971–1976.
3. Wiegand TW, Young LH. Cytomegalovirus retinitis. *Int Ophthalmol Clin* 2006; 46: 91–110.
4. Cordero-Coma M, Anzaar F, Yilmaz T, Foster CS. Herpetic retinitis. *Herpes* 2007; 14: 4–10.
5. Madhavan HN, Priya K, Biswas J. Current perspectives of herpes viral retinitis and choroiditis. *Indian J Pathol Microbiol* 2004; 47: 453–468.
6. Knox CM, Chandler D, Short GA, Margolis TP. Polymerase chain reaction-based assays of vitreous samples for the diagnosis of viral retinitis. *Ophthalmol 1998*; 105: 37–45.
7. Notomi T, Okayama H, Masubuchi H et al. Loop-mediated isothermal amplification of DNA. *Nucleic Acid Res* 2000; 28: e63.
8. Poon LLM, Wong BWY, Chan KH et al. Evaluation of real time reverse-transcriptase PCR and real time loop mediated amplification assays for severe acute respiratory syndrome coronavirus detection. *J Clin Microbiol* 2005; 43: 3457–3459.
9. Parida MM, Guillermio P, Inoue S, Hasebe F, Morita K. Real time reverse transcription loop mediated isothermal amplification assay for rapid detection of West Nile virus. *J Clin Microbiol* 2004; 42: 257–263.
10. Parida MM, Horioke K, Ishida H et al. Rapid detection and differentiation of Dengue virus by real time reverse transcription loop mediated isothermal amplification assay. *J Clin Microbiol* 2005; 43: 2895–2903.
11. Parida MM, Santhosh SR, Dash PK et al. Rapid and real-time detection of Chikungunya virus by reverse transcription loop-mediated isothermal amplification assay. *J Clin Microbiol* 2007; 45: 351–357.
12. Bista BR, Ishwad C, Wadowsky RM et al. Development of a loop-mediated isothermal amplification assay for rapid detection of BK virus. *J Clin Microbiol* 2007; 45: 1581–1587.

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