Rapid detection of active human cytomegalovirus infection in pregnancy using loop-mediated isothermal amplification

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Abstract. Understanding the association between congenital human cytomegalovirus (HCMV) infection and active maternal HCMV infection during pregnancy is important for maternal and neonatal healthcare. In the present study, a loop-mediated isothermal amplification (LAMP) method was established for the detection of CMV DNA from whole blood or amniotic fluid samples, using reverse transcription-quantitative polymerase chain reaction. The results of the present study demonstrated that the CMV LAMP assay detection was specific for CMV DNA, whereas it did not detect viral DNA from herpes simplex type 1 (HSV-1), HSV-2, varicella zoster virus, HSV-6 or HSV-7. Sensitivity determination using serially-diluted CMV glycoprotein B-containing plasmids, demonstrated that >10 copies per tube were detectable using the CMV LAMP method. Furthermore, the detection results, using the LAMP method for 336 whole blood samples, demonstrated that at a threshold of 10^1-10^4 copies per tube, the sensitivity of this method was 86.96-100%, the specificity was 97.24-100%, the positive predictive value was 76.92-100% and the negative predictive value was 99.05-100%. The results for 11 amniotic fluid samples from pregnant women with whole blood CMV-positive and 15 control amniotic fluid samples, indicated that the CMV LAMP assay was sensitive and specific for CMV detection. In conclusion, in the present study, a CMV LAMP method was developed, which was shown to be sensitive, specific and efficient in the detection of HCMV infection. Furthermore, CMV LAMP is capable of detecting active CMV infection in pregnant women. Therefore, the current study provides novel insights into diagnostic approaches for active CMV infection in pregnant women.

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Introduction

Congenital infection with human cytomegalovirus (HCMV), which belongs to the herpesviridae group, and may be termed human herpes virus-5, is the most common intrauterine infection (1,2). Studies have demonstrated an association between active CMV infection of the mother and *in utero* HCMV transmission. The risk of congenital HCMV infection is higher in infants when the mother acquires an initial CMV infection during pregnancy, compared with that in infants when the mother acquires the infection prior to conception (3-7). Therefore, maternal antibodies against HCMV provide protection against congenital infection (8,9). Congenital HCMV infection poses a high risk of causing congenital disorders. Congenital HCMV infection (15-20%) leads to long-term disability including sensorineural hearing loss, visual impairment, mental retardation and cognitive defects. Furthermore, 4% of CMV-infected infants do not survive (3-5,8,10).

Understanding the association between congenital infection and active maternal HCMV infection during pregnancy is important for maternal and neonatal healthcare. Therefore, the identification of active HCMV infection during pregnancy is required. However, >95% pregnant females with primary CMV infection are asymptomatic and, therefore, clinical diagnosis is challenging (11,12). Seroconversion may be used to detect HCMV antibodies during pregnancy. However, it is rarely effective, due to the lack of antibody screening prior to conception, which would enable the identification of seronegativity. Routine viral culturing may be sufficiently sensitive for the identification of CMV. However, this method is labor-intensive and subjective, and it may take >14 days for the virus to be propagated and identified (13). Due to the limitations of culture-based methods, targeting the viral genome via quantitative polymerase chain reaction (qPCR) has become an important laboratory tool for the diagnosis and treatment of CMV infection. Previous studies have evaluated qPCR for the detection and quantification of CMV in plasma samples (14,15).

A novel nucleic acid amplification method, loop-mediated isothermal amplification (LAMP), has been reported to detect CMV viral genomic DNA (16). This method has been used

for the rapid diagnosis of a number of infectious diseases, including herpes viruses (17-19), Epstein-Barr virus (20), hepatitis B Virus (21) and CMV (22). LAMP is capable of amplifying specific sequences of DNA under homoeothermic conditions and requires relatively simple and cost-effective equipment, making it amenable for use in hospital laboratories.

In the present study, a simple LAMP assay was established for the detection of CMV in peripheral blood samples from pregnant women. This detection method exhibits the potential for use in point-of-care settings for CMV infection screening and follow-up during pregnancy.

Materials and methods

Clinical specimens and DNA extraction. Whole blood samples from 336 pregnant women, who were registered at Yantai Yuhuangding Hospital of Qingdao University (Yantai, China), were used in the present study. Amniotic fluid samples (11) were obtained from pregnant women exhibiting CMV-positivity, which was identified using reverse transcription qPCR (RT-qPCR) and LAMP assays. Informed consent was obtained from the patients, and the study was permitted by the Human Research Ethics Committee of Yantai Yuhuangding Hospital of Qingdao University. Samples were initially detected using RT-qPCR and then evaluated using a LAMP assay. Total DNA from whole blood samples or amniotic fluid samples was extracted using a QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Total extracted DNA was quantified by measurements at 260 nm optical density (OD) using a spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Wilmington, DE, USA). Extracted DNA samples were stored at -20˚C prior to use. Viral DNA isolation was performed from stock viruses of herpes simplex virus type 1 (HSV-1), HSV-2, varicella zoster virus (VZV), HSV-6, HSV-7 and CMV (Sinobio, Beijing, China) using the QIAamp DNA Mini kit.

Primer design for LAMP. The primers for LAMP amplification of the CMV glycoprotein B (gB) gene were designed based on CMV sequence data obtained from Genbank (accession number: M60931). Oligonucleotide primers that were used in the present study were designed using Primer Explorer V4 software (Eiken Chemical Co. Ltd., Tokyo, Japan). Designed primer sequences were subjected to BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to exclude the possibility of cross-reactivity with HSV-1, HSV-2, VZV, HSV-6 and HSV-7. CMV specific primers consisted of two outer (F3 and B3) and two inner primers: Forward inner primer (FIP) and backward inner primer (BIP). Inner primers that recognized both forward and reverse strands of the target DNA were connected by a 'TTTT' linker. And additional loop primers [forward loop primer (LF) and backward loop primer (LB)] were used to promote both the amplification efficiency and acceleration of the reaction. Details of the sequence and location of each nucleotide primer in the target DNA sequences are provided in Fig. 1.

Optimization of LAMP conditions. In order to determine the sensitivity of the CMV LAMP method, part of the gB gene containing the target DNA sequence was amplified using the following primers: Forward TGCCCGACGTCACGGTGGTC and reverse: ACCGACTTCAGGGTACTGG, which was cloned into pGEM-T-Easy plasmid (Promega Corporation, Madison, WI, USA). Optimization of LAMP conditions for CMV and sensitivity determination was determined by amplifying 10^0-10^7 copies of CMV gB-containing plasmids. The specificity of the LAMP assay was determined using HSV-1, HSV-2, VZV, HSV-6 and HSV-7 DNA samples as negative controls.

Amplifications were optimized using different conditions: Using 20, 25, 30 or 35 μl reaction volumes, including 2, 5 or 10 μl DNA template, 1 or 2 μM inner primers (FIP and BIP), 0.1, 0.3 or 0.5 μM outer primers (F3 and B3) and 0.5 or 1 μM loop primers (LF and LB), 0.5,1 or 2 μl Bst DNA polymerase (Large Fragment; New England Biolabs, Inc., Ipswich, MA, USA), 2 x reaction mix (0.5 of the total volume), and supplemented distilled and deionized water (ddH_2O). Reaction temperatures were screened at 59, 62 or 65˚C and at the following reaction times: 5, 10, 15, 20, 25, 30 and 35 min. A LAMP turbidimeter TERAMECS (LA200; Teramecs, Co. Ltd., Kyoto, Japan) was used to incubate the mixtures and to measure the turbidity following the LAMP reaction. The turbidity cut-off value was set at >0.1 mean ± 3 standard deviation of the turbidity, from the turbidity values of three negative samples. The LAMP products were also subjected to 1.5% agarose gel electrophoresis in order to validate the experiments. Gels were visualized under an ultraviolet light following ethidium bromide staining.

CMV-specific RT-qPCR assay of whole-blood and amniotic fluid samples. Primers for the CMV RT-qPCR assay were designed according to previously reported sequences (23) and were synthesized by Sangon Biotech (Shanghai, China). Primers were dissolved in ddH_2O, to 100 μM and stored at -20˚C. The RT-qPCR assay was performed using a One-Step PrimeScript RT-PCR kit (Takara Bio, Inc., Otsu, Japan), using LightCycle 2.0 (Roche Diagnostics GmbH, Mannheim, Germany).

Evaluation of LAMP with clinical specimens. In order to evaluate the LAMP assay in whole blood or amniotic fluid specimens from pregnant women, 336 whole blood samples were tested for CMV using RT-qPCR. Whole blood samples (336) and 11 amniotic fluid samples from RT-qPCR-confirmed CMV-positive pregnant women were then subjected to a LAMP assay using the optimized conditions (25 μl reaction volume, including 3 μL DNA template, 1 μM inner primers, 0.5 μM outer primers and 0.5 μM loop primers, 1 μl Bst DNA polymerase and ddH_2O. The reaction was performed at 62˚C for 35 min). Sensitivity, specificity, positive predictive value and negative predictive value from the LAMP assays were then calculated using standard formulas and the results of RT-qPCR were used as standards.

Results

Optimized conditions for LAMP assays. In order to optimize the conditions for CMV LAMP detection, LAMP was conducted under different conditions, including different Mg^{2+} concentrations, different concentrations of loop primers, and different temperatures and durations. The results suggested that LAMP

A

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AGTGATAATG ACTACGGCTA TGGCCACGAG GAT GATGGTG
F3
AACGCTCCGA AGGGGGTTTTTGAGGAAGGTG GCAACGCCTTCGA
F2 LPF
CCACGGA GGCCACCGCG CCACCCACGG CCCCAATGGCT ACGCCAACGG
F1
CCTTTCCCGCGGcgcccag gccgctcatg aggtCG TCCA GACCCTTGAG GTAGGGC
B1 LPB
ggt agcgggtcga ctacctt GTCCTCCACgtac tttacccgct gctt GTACGA GTTGAATTCG
B2 B3
CGCATGATCT CTTCGAGGTC AAAAACGTTG CTGGAACGCA GCTCTTTCTG

B

Primers	Sequence
F3	5' ggctatggccacgaggat 3'
B3	5' aagcagcgggtaaagtac 3'
FIB	5' agccattggggccgtgggtg TTTT acgctccgaaggggtttttg 3'
BIP	5' cgcccaggccgctcatgaggt TTTT aaggtagtcgacccgctacc 3'
LF	5' gaaggtggcaacgccttcg 3'
LB	5' gccctacctcaagggtctgga 3'

Figure 1. Locations and target sequences of the CMV gB gene and the primers for CMV LAMP. (A) Target sequences in the CMV gB gene. (B) Primer sequences for the CMV LAMP. F3, labeled sequence in the target sequence; B3, reverse complementary sequence in the target sequence; FIB, forward internal primer, reverse complementary sequence of F1 + TTTT + labeled F2 sequence; BIP, backwarrd internal primer, labeled B1 sequence + TTTT + reverse complementary sequence of B2; LF, forward loop primer, labeled LPF sequence in the target sequence; LB, backward loop primer, reverse complementary sequence of labeled LPB sequence; CMV, cytomegalovirus; LAMP, loop-mediated isothermal amplification; glycoprotein B, gB.

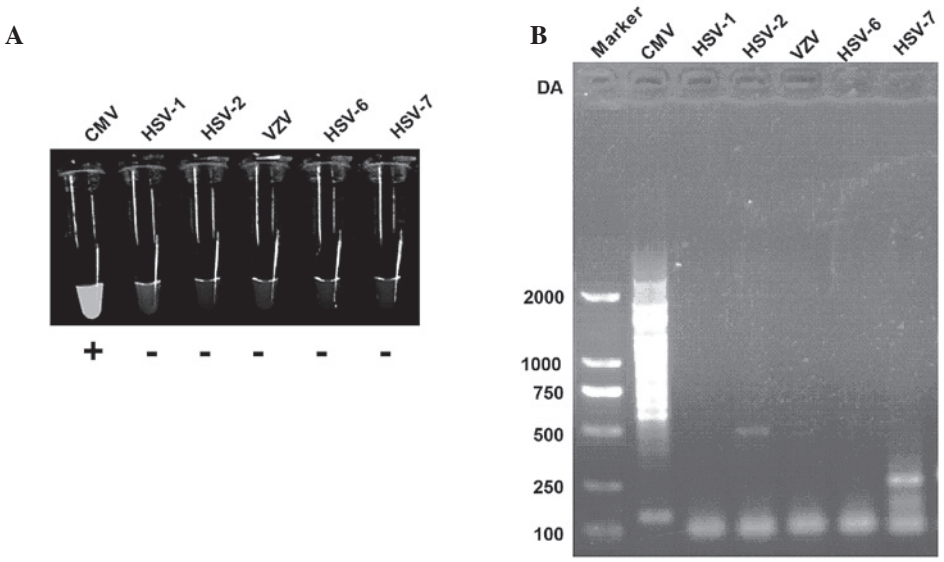

Figure 2. LAMP assay specificity to CMV, targeted to the CMV gB gene. (A) Visual inspection of LAMP assay for CMV, HSV-1, HSV-2, VZV, HSV-6 and HSV-7. (B) Electrophoretic analysis of LAMP product from samples of CMV, HSV-1, HSV-2, VZV, HSV-6 and HSV-7. LAMP, loop-mediated isothermal amplification; CMV, cytomegalovirus; HSV, herpes simplex virus; VSV, varicella zoster virus.

conditions were optimized at a 25-μl reaction volume. Final reaction mixtures consisted of 1.6 μM inner primers (FIP and BIP), 0.2 μM outer primers (F3 and B3), 0.8 μM loop primers (LF and LR), 10 mM $MgSO_4$, 1 μl Bst DNA polymerase and 5 μl DNA template. Amplifications were performed at 64˚C for 30 min and reactions were terminated at 85˚C for 5 min.

Specificity of LAMP using turbidity assays and gel electrophoresis. In the present study, the capability of the CMV LAMP assay for the discrimination of CMV from other members of herpesviridae, such as HSV-1, HSV-2, VZV, HSV-6 and HSV-7 was assessed. The results of the present study suggested that CMV exhibits bright turbidity following

Table I. Performance of the LAMP assay for active CMV infection in whole blood samples.

		LAMP (+/-)					
Copies/tube	Total number	RT-qPCR+	RT-qPCR-	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
$>10^1$	336	20/3	0/313	86.96	100	100	99.05
$>10^2$	336	21/2	1/312	91.30	99.68	94.45	99.36
$>10^3$	336	13/0	2/318	100	98.45	86.67	100
$>10^4$	336	10/0	3/317	100	97.24	76.92	100

PPV, positive predictive value; NPV, negative predictive value; LAMP, loop-mediated isothermal amplification; CMV, cytomegalovirus; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Table II. Diagnostic performance of LAMP assay for active CMV infection in amniotic fluid samples.

Copies per tube	CMV+ number	Positive	CMV- number	Negative
$>10^1$	11	10	15	15
$>10^2$	11	11	15	15
$>10^3$	11	11	15	15
$>10^4$	11	11	15	14

Positive total per tube = 11 and negative total per tube = 15; LAMP, loop-mediated isothermal amplification; CMV, cytomegalovirus.

a LAMP assay, whereas other members of herpesviridae were not detected using LAMP (Fig. 2A). LAMP products were then analyzed using gel electrophoresis and the results suggested that CMV was successfully amplified, while other viral DNA was not amplified (Fig. 2B). Therefore, primer sets developed in the present study exhibit specificities for the target CMV sequences.

Sensitivity of LAMP using turbidity measurement and RT-qPCR. LAMP assay sensitivity was analyzed using the serially-diluted CMV gB-containing plasmid. Serial dilutions of recombinant pGEM-T Easy plasmid ranging from 10^7-10^0 copies per tube were used in order to determine the detection limits of CMV LAMP. Results demonstrated that the sensitivity of the CMV LAMP assay was 10 copies per tube, according to a real-time turbidimeter at 650 nm OD (Fig. 3A and B). Reactions were repeated three times. Serially-diluted plasmids were examined, ranging from 10^6-10^{-1} copies per tube, using RT-qPCR (Fig. 3C). The threshold of RT-qPCR was 10^{-1} copies per tube, which was 10 times more sensitive than that of the CMV LAMP assay.

CMV LAMP assay of whole blood specimens from pregnant women (Tables I and II). In order to further evaluate the performance of the CMV LAMP assay for CMV infection detection in pregnant women, CMV LAMP assays were conducted in 336 whole blood samples and 11 amniotic fluid samples from pregnant women. Samples were tested using RT-qPCR. Positive samples (10, 13, 20 or 21), with a threshold of 10 copies, were

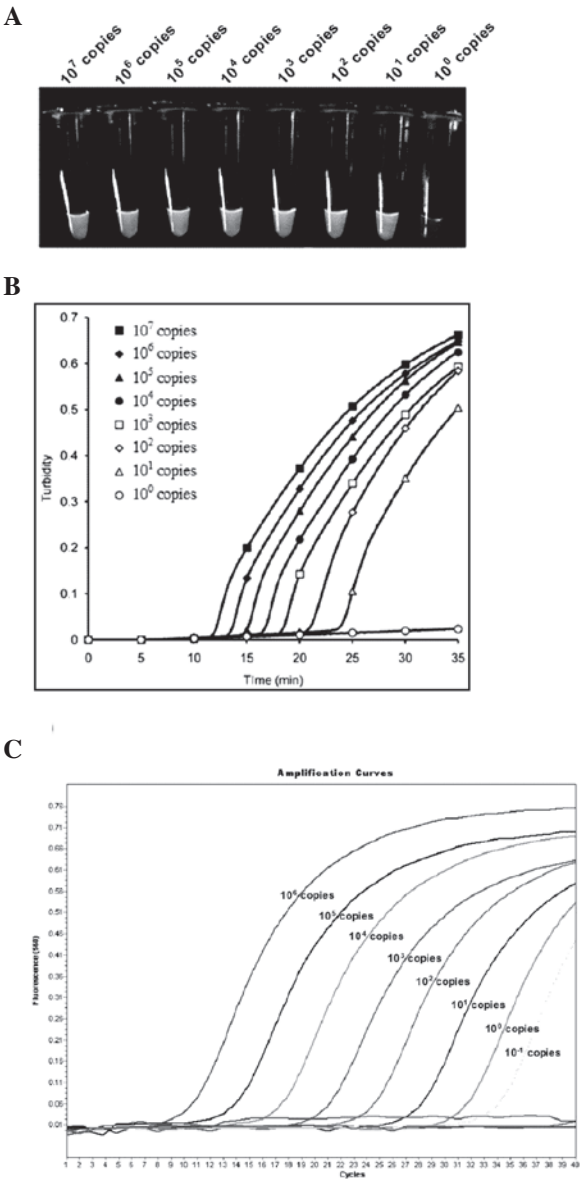

Figure 3. Sensitivity of the CMV LAMP assay. (A) Visual inspection of LAMP assay results for serially diluted (10^0-10^7 copies per tube) CMV gB-containing plasmids. (B) Turbidity results of the LAMP products for the serially diluted CMV gB-containing plasmids. (C) Reverse transcription quantitative-polymerase chain reaction results for the serially diluted (10^{-1}-10^6 copies per tube) CMV gB-containing plasmids. CMV, cytomegalovirus; gB, glycoprotein B; LAMP, loop-mediated isothermal amplification.

confirmed using RT-qPCR. RT-qPCR-positive samples, 10 and 13, were confirmed as positive according to the CMV LAMP, with a threshold of 10^3 or 10^4 copies per tube, respectively and 100% sensitivity. However, samples 2 and 3, which were negative according to RT-qPCR, were shown to be positive according to the CMV LAMP assay, with a specificity of 98.45 and 97.24%, PPV values of 86.67 and 76.92%, respectively; NPV for both thresholds were 100% (Table I). The following values were observed at 10^1 and 10^2 copies per tube compared with those at 10^3 and 10^4: Sensitivity decreased to 86.96 and 91.30%, specificity increased to 100 and 99.68%, PPV increased to 100 and 94.45%, and NPV decreased to 99.05 or 99.36%, at 10^1 and 10^2 copies per tube respectively. In order to reconfirm the sensitivity and specificity of the CMV LAMP assay, 11 amniotic fluid samples were examined from pregnant women with whole blood CMV-positive and 15 control amniotic fluid samples were examined, from pregnant women without active CMV infection. The results indicated that 11 samples were positive, with a threshold of 10^2, 10^3 or 10^4, while the 15 control samples were negative, with a threshold of 10^1, 10^2 or 10^3 (Table II). Overall, the CMV LAMP method performed well in the detection of CMV infection.

Discussion

LAMP reaction requires DNA polymerase with strand displacement activity and >4 specifically designed primers. During the first step, a stem-loop DNA structure is constructed, in which the sequences of both DNA ends are derived from the inner primers. Subsequently, one inner primer hybridizes to the loop on the LAMP cycle product and initiates strand displacement DNA synthesis, yielding the original stem-loop and new stem-loop DNA with a stem that is twice as long. The final products are termed stem-loop DNAs, and have several inverted repeats of the target DNA and cauliflower-like structures with multiple loops, amplifying $<10^9$ copies of the target. LAMP is a rapid and simple technique for the amplification of specific DNA sequences that has advantages over PCR (24,25). The most significant advantage of LAMP is its ability to amplify specific sequences of DNA at a constant temperature (63-65˚C), without thermocycling. In addition, <45 min are required in order to amplify the target sequences. Given these advantages, LAMP may be adopted for widespread use in hospital laboratories.

In the present study a CMV LAMP assay was established for the detection of CMV DNA in pregnant women with CMV infection, using RT-qPCR in order to confirm active CMV infection. Following optimization of the PCR protocol, the components for CMV LAMP included a 25-μl reaction volume with 1.6 μM inner primers, 0.2 μM outer primers, 0.8 μM loop primers, 10 mM $MgSO_4$, 1 μl Bst DNA polymerase and 5 μl DNA template. The amplification was conducted at 64˚C for 30 min. This PCR method was specific for the amplification of CMV DNA and it did not amplify HSV-1, HSV-2, VZV, HSV-6 or HSV-7, which belong to the same herpesviridae family. Sensitivity determination using the serially-diluted CMV gB-containing plasmids demonstrated that >10 copies per tube were detectable using the CMV LAMP method, which had a 10 fold lower sensitivity level compared with that of RT-qPCR.

Furthermore, the CMV LAMP assay performed well in the detection of CMV infection. The detection results for 336 whole blood samples demonstrated that, at a threshold of 10^1-10^4 copies per tube, the sensitivity of the LAMP assay for the detection of CMV infection was 86.96-100%, specificity was 97.24-100%, PPV was 76.92-100% and NPV was 99.05-100%. The LAMP assay was sensitive and specific for the detection of CMV in 11 amniotic fluid samples from CMV-positive pregnant women and in 15 control amniotic fluid samples. Overall, the CMV LAMP method performed well in the detection of CMV infection.

In conclusion, a CMV LAMP method was developed, which was highly sensitive, specific, simple and timesaving. Furthermore, it performed well in the detection of active CMV infection in pregnant women. Therefore, the present study provides novel insights into the detection of active CMV infection in pregnant women.

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