Community Acquired Infection

ORIGINAL ARTICLE

Detection of hepatitis A virus RNA with an improved loop-mediated isothermal amplification assay

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ABSTRACT

Background: The hepatitis A virus (HAV) is one of the pathogens responsible for acute viral hepatitis and human foodborne diseases. Simple and rapid techniques to detect this virus are urgently needed. **Methods:** In this study, an improved loop-mediated isothermal amplification (LAMP) assay based on the addition of an acceleration primer was developed for HAV nucleotide acid detection. **Results:** Precision and reproducibility analysis revealed high stability and reliability, with a coefficient of variance (CV) ranging from 0.51% to 0.93%. A comparison between the improved and conventional LAMP assays revealed that, in addition to its rapidity and simplicity of use, the former was more sensitive, with a detection limit of 5 TCID50/mL. The novel detection method displayed 100% consistency with the TaqMan Real-Time RT-PCR assay when applied to seven clinical specimens collected from patients with confirmed acute HAV infection. **Conclusion:** This technique is widely applicable as a simple diagnostic tool in the clinical field and for the surveillance and investigation of infectious diseases in developing countries where HAV is endemic.

Key words: diagnosis, hepatitis A virus, methodology, reproducibility, sensitivity

INTRODUCTION

The hepatitis A virus (HAV) is a small, nonenveloped, single-stranded RNA virus that belongs to the Hepatovirus genus within the Picornaviridae family.[1] The 7.5 kb genome is composed of 5' and 3' noncoding regions; a P1 region that encodes the structural proteins VP1, VP2, VP3, and putative VP4; and P2 and P3 regions that encode nonstructural proteins associated with replication.[2] To date, HAV strains worldwide have been classified into six genotypes (I–VI), with genotypes I–III being of human origin and genotypes IV–VI being of simian origin. In addition, each human genotype (I–III) possesses two subgenotypes (A and B).[3] HAV infection is the leading cause of acute viral hepatitis worldwide,[4] particularly in developing countries with poor sanitation. For example, the 1988 HAV epidemic in Shanghai, China—caused by the ingestion of contaminated raw clams—resulted in an infection rate of 4083 per 100,000 individuals, leading to an estimated 292,301 cases of HAV infection and 32 deaths.[5]

The anti-HAV immunoglobulin M (IgM) test is the preferred method of diagnosing acute hepatitis A owing to its high sensitivity and specificity when applied to specimens from patients with typical symptoms. However, IgM tests performed on individuals without hepatitis symptoms yield a high rate of false positives, resulting in unnecessary public health costs and further investigations. Therefore, the development of a widely applicable technique to effectively detect HAV remains an urgent medical requirement. A novel nucleic acid

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detection technique that is rapid, simple, and sensitive — known as loop-mediated isothermal amplification (LAMP) — was recently developed.[6,7] This amplification procedure relies on autocycling strand-displacement DNA synthesis performed with *Bst* DNA polymerase. The principal advantage of this method is that the reaction can be conducted under isothermal conditions (ranging from 60 ℃ to 65 ℃); therefore, denaturation of the DNA template is not necessary. Furthermore, LAMP is a highly specific detection method since at least six primer region sequences are required for recognition. The reaction leads to the generation of a large amount of amplified product in positive samples, resulting in easier detection, for instance, based on visual judgment of turbidity or real-time monitoring of the turbidity of the reaction mixture. LAMP assays have been widely and successfully used to detect numerous pathogenic viruses, such as monkeypox virus,[8,9] SARS-CoV-2,[10,11] Zika virus,[12,13] African swine fever virus,[14] and hepatitis C virus,[15] as well as some plant pathogens.[16]

In this study, we developed a modified LAMP assay to facilitate the rapid and economical detection of HAV RNA. Compared to conventional LAMP, the improved method incorporates an additional accelerating primer (AP). The effectiveness of the novel assay was further evaluated using clinical acute hepatitis A samples.

MATERIALS AND METHODS

Ethical issues

All aspects of the study were performed in accordance with the national ethics regulations and approved by the ethics committees of China's CDC (No. 202311).

Virus stock and clinical specimens from patients with acute HAV infection

The HAV H_2 attenuated vaccine strain was purchased from Zhejiang Pukang Biotechnology Co., Ltd. Viral stock suspensions were quantified as 3.6×10^6 $TCID_{50}$/mL. A panel of seven blood serum samples collected from patients with confirmed acute HAV infection was used to evaluate the improved LAMP assay. HAV cases were confirmed on the basis of a positive result in the enzyme-linked immunosorbent assay (Kehua Bio-engineering, Shanghai, China) for IgM antibodies against HAV. Based on phylogenetic analysis of nucleotide sequences in the VP1/2A junction region, among the HAV-infected samples, six HAV isolates clustered in subgenotype IA, and only one belonged to subgenotype IB. Both the HAV vaccine strain and serum samples were stored at – 20 ℃ for later use.

RNA preparation

Genomic viral RNA was extracted from 140.0 μL of viral stock using a QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted from the QIAamp spin column in a final volume of 50.0 μL with RNase-free water and stored at – 80 ℃ for later use.

Primer selection

The HAV H_2 attenuated vaccine strain (genotype IB) was used as a reference for generating the primer sets. All oligonucleotides, including two outer (F3 and B3) and two inner (Forward Inner Primer [FIP] and Forward Inner Primer [BIP]) primers, were designed according to the guidelines provided by PrimerExplorer V4 online software. In this set, FIP consisted of F1c complementary to the F1 sequence and F2, while BIP included the B1 sequence and B2c complementary to B2. The F3 and B3 sequences are located outside the F2 and B2 regions. To increase the rapidity of the conventional LAMP assay, an additional AP, located between the F1 and B1 positions, was incorporated into the primer set. The sequences and positions of the oligonucleotide primers used for the amplification of the target sequences are presented in Table 1.

RT-LAMP assays

The RT-LAMP reaction was performed with a Loopamp RNA amplification kit (Eiken Chemical Co. Ltd., Tokyo, Japan) in accordance with the manufacturer's protocol. Briefly, the 25.0 μL total reaction mixture contained 20 pmol of FIP inner primer, 40 pmol of BIP inner primer, 5 pmol each of the F3 and B3 outer primers, 12.5 μL of a 2× reaction mixture, 1.0 μL of enzyme mixture, and 5.0 μL of extracted RNA. Total reaction mixtures were incubated at 62.5 ℃ for 60 min in a Loopamp Realtime Turbidimeter LA-320C (Teramecs, Kyoto, Japan) and heated to 85 ℃ for 2 min to terminate the reaction. Real-time monitoring was performed every 6 s through spectrophotometric analysis by recording the optical density (OD) at 650 nm. A negative control containing water was analyzed using RT-LAMP for 60 min to define the turbidity signal value for discriminating positives from negatives in the assay. None of the negative controls displayed a turbidity signal greater than 0.1 within a reaction time of 50 min. Therefore, samples showing a change in turbidity of over 0.1 within 50 min were considered positive. The reaction mixture and conditions for the improved LAMP assay were similar to those described for conventional LAMP, except that 20 pmol AP was added to the mixture. A negative control was included for each LAMP run. Serial dilutions of the standard were used as templates to evaluate the sensitivity of the LAMP assay.

TaqMan real-time RT-PCR assays

To quantify viral RNA in HAV-positive clinical serum samples, TaqMan Real-Time RT-PCR assays were

Table 1: Specific LAMP primers designed for HAV detection used in this study

Primer name	Sequence (5' to 3')	Genome position
F3	GCATGGAGCTGTAGGAGTCT	293–312
B3	CACTCAATGCATCCACTGGA	520–539
FIP	ACCCGTAGCCTACCCCTTGTGG-TGTTTGGAACGTCACCTTG	385–406/329–347
BIP	TTGGATAGGGTAACAGCGGCG-CTCCGGCGTTGAATG	444–464/493–507
LF	TGAAAGCCAAGTTAACACTG	348–367
LB	GATATTGGTGAGTTGTTAAGAC	465–486
AP	TTAGGCTAATACTTCTATGA	415–434

F3 and B3 are two outer primers; FIP and BIP are two inner primers; LF and LB are two loop primers; AP, accelerating primer; LAMP, loop-mediated isothermal amplification; HAV, hepatitis A virus.

carried out in a 96-well format using the One Step PrimeScript™ RT-PCR Kit (Takara, Dalian, China). Briefly, 5.0 μL of extracted RNA was transferred into a capillary containing 15.0 μL of master mix. The probe and primer sequences were as follows. *F* (forward primer): 5'-GGT AGG CTA CGG GTG AAA C-3'; *R* (reverse primer): 5'-CCT CCG GCG TTG AAT GGT TT-3'; *P* (probe): 5'-FAM-ACA GCG GCG GAT ATT GGT GAG TTG TTA AGA T-BHQ-3'. RT-PCR was performed under optimized conditions and consisted of three steps. Specifically, reverse transcription was performed at 42 ℃ for 30 min, followed by denaturation at 95 ℃ for 10 s. Amplification was achieved *via* TaqMan Real-Time RT-PCR assay for 40 cycles of 95 ℃ for 8 s and 60 ℃ for 34 s. All reactions were performed on an ABI 7500 Real-Time RT-PCR system (Applied Biosystems).

For TaqMan real-time RT-PCR quantification of HAV, a fragment of HAV genome RNA was used as the standard and prepared by transcription from the linearized plasmid template *in vitro*. Briefly, 5 μg of plasmid was digested with the *Xba*I restriction enzyme to generate a linearized template. The RiboMAX™ Large Scale RNA Production System (Promega, Madison, WI) was used for full-length transcription of HAV RNA, followed by treatment with RQ1 DNase (1U/μg cDNA) for removal of the DNA template and purification using an RNeasy mini kit. The concentration of prepared RNA was calculated using Nanodrop, and the number of RNA copies in the solution was subsequently estimated.

RESULTS

Precision and reproducibility analysis of the improved LAMP assay

The threshold time (Tt) value, defined as the reaction time necessary to achieve a positive signal above the baseline, was applied to evaluate the precision of the improved LAMP assay. Virus dilutions were tested at six different concentrations. Each concentration was analyzed five times, and standard deviations and coefficients of variation (CVs) were calculated to assess assay precision. The CV is the ratio between the inverse of the mean and the standard deviation (CV = SD/M).

Since the CV only ranged from 0.51% to 0.93%, our data suggest that the improved LAMP assay is stable. The results are presented in Table 2.

Comparative evaluation of improved and conventional LAMP assays

The improved and conventional LAMP assays were compared by evaluating their sensitivity and threshold times using RNA extracted from 10^{-1} to 10^{-8} diluted HAV strain stock suspension. Both assays were simultaneously performed in quadruplicate on the same dilutions of the extracted RNA suspension. The detection limit of the conventional LAMP assay was 10^{-6} dilution, corresponding to 10 $TCID_{50}$/mL, while the improved LAMP assay displayed a slightly better performance, with a detection limit of 5 $TCID_{50}$/mL. The results are shown in Table 3.

Evaluation of clinical samples using the improved LAMP assay

The applicability of the improved LAMP assay for clinical diagnosis of HAV was validated in blood serum specimens collected from patients with confirmed acute infection using the HAV IgM test. We calculated RNA copies in each serum sample using the HAV TaqMan Real-Time RT-PCR assay and performed the improved LAMP assay in parallel. As shown in Table 4, data obtained with the improved LAMP assay were 100% consistent with TaqMan Real-Time RT-PCR findings in the seven samples examined, with a significant correlation in viral RNA detection between the two methods.

DISCUSSION

Besides LAMP, widely used isothermal techniques include rolling circle amplification (RCA) and helicase-

Table 2: Precision and reproducibility analysis of the improved LAMP assay

Virus dilution	Threshold time range (s)	Mean	*SD*	CV (%)
10^{-1}	942–954	949.2	5.02	0.53
10^{-2}	1098–1116	1,106.4	6.84	0.62
10^{-3}	1314–1338	1,329.6	6.84	0.51
10^{-4}	1536–1572	1,551.6	14.45	0.93
10^{-5}	1770–1812	1,789.2	15.53	0.87
10^{-6}	2076–2118	2,095.2	15.53	0.74

LAMP, loop-mediated isothermal amplification; CV, Coefficient of variation.

Table 3: Comparison of the sensitivities of the improved and conventional LAMP assays

Virus dilution	Improved LAMP assay		Conventional LAMP assay	
	No. of positive samples / no. of tested samples	Positive rate (%)	No. of positive samples / no. of tested samples	Positive rate (%)
10^{-1}	4/4	100	4/4	100
10^{-2}	4/4	100	4/4	100
10^{-3}	4/4	100	4/4	100
10^{-4}	4/4	100	4/4	100
10^{-5}	4/4	100	4/4	100
10^{-6}	4/4	100	4/4	100
10^{-7}	2/4	50	0/4	0
10^{-8}	0/4	0	0/4	0

LAMP, loop-mediated isothermal amplification.

Table 4: Evaluation of clinical samples using the improved LAMP and HAV TaqMan Real-Time RT-PCR assays

Patient	Improved LAMP assay	Real-time RT-PCR assay	
	Threshold time (s)	RNA copies/5 μL	Ct values
1	1104	247,000	26.82
2	1116	219,000	27.00
3	1110	197,000	27.15
4	1200	71,100	28.62
5	1506	3820	32.84
6	1542	3250	33.07
7	1794	518	35.72

LAMP, loop-mediated isothermal amplification; HAV, hepatitis A virus;

dependent amplification (HDA). RCA is often used for amplification of circular DNA templates. It can be performed with random primers, resulting in a branched DNA product, or with specific primers to increase specificity so that only the intended DNA sequences are amplified. HDA is an isothermal nucleic acid amplification technique that utilizes helicase enzymes to separate the DNA strands in a target sequence. This separation allows the primers to bind and initiate the amplification process. HDA is particularly useful for the rapid and sensitive detection of specific DNA or RNA sequences in various applications, including molecular diagnostics, research, and field-based testing. It offers advantages such as simplified reaction conditions, high specificity, and robust amplification performance at a constant temperature.

In this study, we developed an improved sensitive, rapid, and accelerated one-step RT-LAMP assay for the detection of HAV isolates and clinical samples. Based on an alignment of HAV sequences published in GenBank, a highly conserved region of HAV 5'-NCR was selected as the target for designing RT-LAMP primers. We devised an AP similar to the loop primer in conventional LAMP to provide an additional starting site for DNA synthesis during amplification, with a view to improving

the efficiency of RT-LAMP amplification. As expected, our results consistently suggest that the additional primer reduces the total reaction time compared with conventional RT-LAMP. Furthermore, the strategy using the additional primer is superior due to not only its proven ability to accelerate the LAMP reaction but also its convenience of primer design. For example, it is difficult to select FIP/BIP in general, since both ends of the secondary structures play a key role during amplification cycling in the LAMP-based assay. Accordingly, different FIP/BIP primer sets should be designed for reaction optimization. The addition of an AP may enhance the rapidity and sensitivity of the reaction, thereby avoiding the arduous screening of primers.

In addition to the major advantages afforded by rapidity and minimum requirements for laboratory equipment, the improved LAMP assay offers increased sensitivity and reproducibility. Precision and reproducibility tests were performed five times for each dilution of the HAV stock strain, and the accuracy of the improved amplification system was evaluated. Compared with earlier studies,[17–19] our detection method demonstrated a higher degree of reproducibility, with CVs ranging from 0.51% to 0.93%. Additionally, the analytical sensitivities of the improved and conventional LAMP assays were compared. The improved LAMP assay had a slightly better detection limit than the conventional assay and consistently achieved a lower limit of detection of 5 $TCID_{50}$/mL.

We observed complete agreement between improved LAMP and quantitative TaqMan Real-Time RT-PCR data when applied to the detection of clinical samples with a broad range of viral loads. Since the samples comprised the HAV genotypes IA and IB that are prevalent in China, the improved LAMP assay is expected to be effective for the majority of HAV-infected individuals in this region.

Despite the abovementioned advantages of the method, this study has some limitations. For example, only seven clinical specimens were tested for the validation of the improved HAV LAMP method. Therefore, we are collecting hepatitis A clinical samples to conduct a large-scale validation of the method.

In conclusion, rapidity and flexibility are the major advantages of the improved LAMP assay. This is an improvement on the present capacity of HAV LAMP detection, facilitating time saving and cost effectiveness. Furthermore, amplified products obtained using this method can be detected with the naked eye or real-time monitoring of turbidity, thus eliminating the need for gel electrophoresis. In conclusion, our improved assay presents a promising means of simple, cheap, and rapid diagnosis of HAV infection. Its potential applications are in infectious disease surveillance and investigations in developing countries where HAV is endemic as well as in rapid clinical diagnosis.

DECLARATIONS

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Author contributions
Su QD: Conceptualization, Methodology, Software. Qiu F: Data curation, Original draft preparation. Wang F: Visualization, Investigation. Zhang S: Reviewing and Editing. Xu K: Supervision. All authors have read and approve the final manuscript.

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Ethical approval
The study was approved by the ethics committees of China's CDC (No. 202311).

Informed consent
Not applicable.

Conflict of interest statement
The authors declare that they have no competing interests.

Data availability statement
No additional data

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