

Detection of hepatitis C virus by an improved loop-mediated isothermal amplification assay

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Abstract An improved, sensitive, specific, and rapid one-step reverse transcription loop-mediated isothermal amplification (LAMP) assay targeting the 5' untranslated region (UTR) was developed to detect hepatitis C virus (HCV) infection. Based on an accelerating primer (AP), the present assay, named AP-LAMP, has the advantages of rapidity and sensitivity over the routine LAMP method. The possible AP-based amplification pathway during the reaction was revealed by restriction enzyme digestion and electrophoresis. The detection limit of the AP-LAMP assay was approximately 84 IU/ml, and no cross-detection was observed. The assay was evaluated further with 126 clinical specimens, and the results indicated the suitability and simplicity of the test as a rapid diagnostic tool for detection of HCV RNA.

Introduction

The hepatitis C virus (HCV) pandemic has become a major public health concern, with such increasing prevalence that

nearly 200 million individuals are infected worldwide [1]. The majority of acute HCV infections present an asymptomatic course. Many infected individuals are therefore not seen in a medical setting [28]. Nearly 85% of infected people develop persistent infection and are at risk of long-term complications, ranging from mild liver damage to severe chronic hepatitis that can develop into cirrhosis, end-stage liver disease, or hepatocellular carcinomas [3]. Therefore, a rapid and accurate diagnosis of HCV is important for the prevention of viral transmission and management of disease progression. Screening of antibodies against HCV, however, is not a reliable method of diagnosing acute HCV infection, since the appearance of antibodies against HCV can be delayed in up to 30% of patients at the onset of symptoms [23]. Moreover, the window period can be even longer in immunocompromised patients, who need to be screened routinely for HCV viremia [21].

Nucleic-acid-based detection techniques are currently the most reliable methods for detecting HCV infection. A variety of molecular diagnostic assays, such as reverse transcriptase PCR [12], nucleic-acid-sequence-based amplification [13], transcription-mediated amplification [29], branched-chain DNA assay [26], and in-house real-time PCR [8], have been developed for the detection of HCV RNA. These assays, whether qualitative or quantitative, are relatively time-consuming, labor-intensive, and dependent on specialized equipment. In resource-limited or point-of-care settings, the cost and technology requirement limit their universal application. Since most HCV-infected individuals are asymptomatic, there are clear advantages to targeted screening for HCV in those who are at high risk. Earlier detection of infection results in earlier treatment and thus earlier recovery [25]. For this reason, there is still a great need for a tool to simplify the detection of HCV

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RNA with acceptable sensitivity and specificity, short turnaround time, and cost-effectiveness.

Loop-mediated isothermal amplification (LAMP) is a novel rapid, accurate, and economical nucleic acid test [19]. The method is characterized by employing a DNA polymerase with strand-displacement activity, along with two inner primers (FIP, BIP) and two outer primers (F3, B3) to form auto-cycling intermediates. Loop primers (LF, LB), first described by Nagamine *et al.*, could accelerate and enhance the sensitivity of the LAMP assay [21]. One-step LAMP assays have been successfully applied to the rapid detection of a number of RNA viruses, such as influenza virus [16], mumps virus [31], West Nile virus [24], severe acute respiratory syndrome corona virus [15], and HIV-1 [7]. LAMP assays have also been developed to detect hepatitis viruses, such as hepatitis B virus [5], hepatitis A virus, and hepatitis E virus [15]. At present, however, no HCV detection assay using this method has been reported. The attributes of the HCV target may interfere in complex ways with the LAMP method. For instance, although the viral 5' untranslated region (5'-UTR) is thought to be the most conserved portion of the HCV genome and is targeted by almost all of the commercial and in-house tests [9], one of the most important issues may be the existence of complex secondary structure across all of this region (Fig. 1B). Also, the 5'-UTR is generally considered to be variable enough to distinguish all of the major types and many subtypes of HCV [14].

In the study described here, we have developed a modified LAMP method for rapid and economical

detection of HCV RNA. In contrast to the routine LAMP (Pre-LAMP) method, we designed an accelerating primer (AP) and tested the performance of the AP-based LAMP assay (AP-LAMP). The new assay was further evaluated using clinical samples.

Materials and methods

Clinical specimens and standard

Twenty-five blood samples collected from patients with confirmed chronic HCV infection and 51 specimens obtained from patients suspected of having viral hepatitis who were admitted to Hangzhou 2th and Infectious Hospital were used for evaluation in this study. Confirmed cases of HCV infection were verified by a positive result in an enzyme-linked immunosorbent assay (Kehua Bio-engineering, Shanghai, China) for antibodies against HCV or a quantitative real-time PCR for HCV RNA. A panel of 50 samples collected from healthy blood donors was also included as negative controls. In addition, five each of anti-HIV-antibody-, anti-HAV-antibody- and HBV-DNA-positive samples obtained from the corresponding patients were also tested. Informed consent was obtained from all patients, and the study was approved by the local ethical committee, as per the Declaration of Helsinki (1995). Five milliliters of blood was collected from each subject in a tube containing 200 μ l of 0.5% EDTA. Plasma was immediately separated after centrifugation at 1500 rpm for

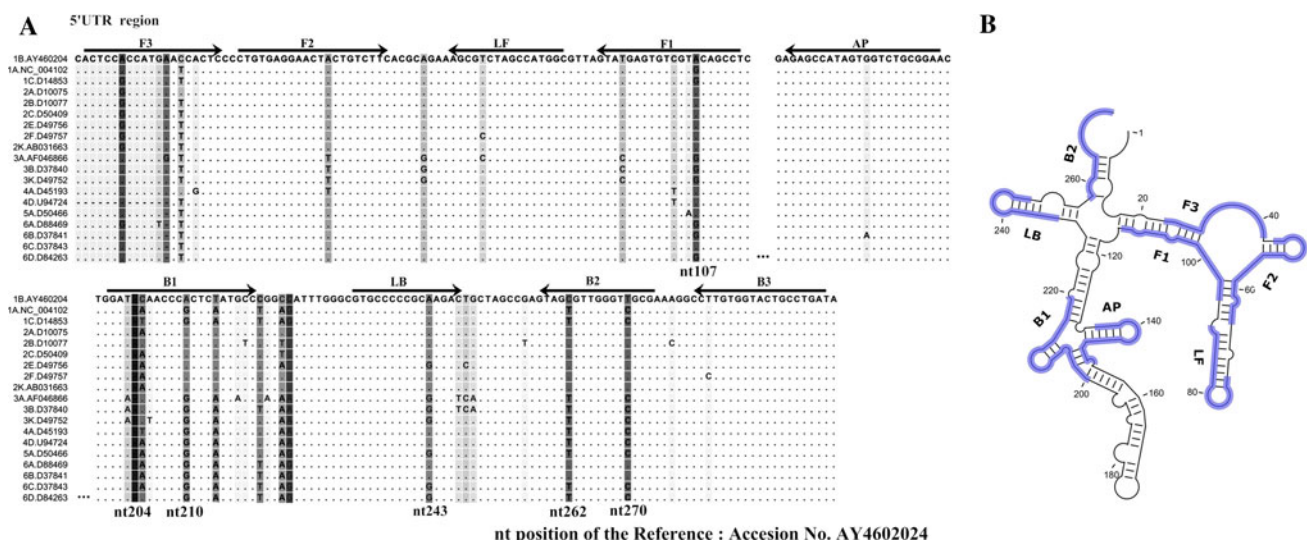


Fig. 1 LAMP primer set for HCV 5' UTR. **A** Sequence alignment of the target region for LAMP primers. This alignment shows a representative sequence for each of the six major genotypes and subtypes. Sequence heterogeneity of HCV isolates is highlighted in grey. Single nucleotide differences at positions 107 (A/G), 204 (C/A), 210 (A/G), 262(C/T) and 270(T/C) were accounted for by the use of

degenerate bases at those positions. Arrows indicate the selected primers. **B** Primary and secondary structure of the 5' UTR of the HCV genome used in this study. The structure is based on the model predicted using MFOLD. Binding sites of primers used by the LAMP assay are shown in bold

10 min at 4°C and stored in multiple aliquots at -80°C until further use.

The in-house HCV RNA standard was obtained by extracting RNA from a HCV-RNA-positive specimen with an HCV titer of 10⁶ IU/ml. This in-house standard was anti-HCV antibody positive and calibrated in triplicate in parallel with the national HCV RNA reference material (GBW09151, 2.26 × 10³ IU/ml -4.22 × 10⁷ IU/ml, genotype 1) using several commercial real-time PCR tests (Kehua Bio-engineering, Shanghai, China; DaAn Biotech, Guangzhou, China). The GBW09151 panel was calibrated using the WHO HCV International Standard (NIBSC 96/790) [29]. HCV genotyping was performed using sequencing of the NS5b region of the HCV genome as described previously [20]. Sequence analysis indicated that the HCV genotype of the in-house standard was 1b. Serial dilutions of the standard sample for experimental analysis were prepared in normal human plasma and stored at -80°C until testing.

RNA preparation

Viral RNA was extracted from 140 µl of plasma using a TIANamp Virus RNA Kit (Tiangen Biotech, Beijing, China) as per manufacturer’s instructions. This extraction protocol used a fast spin-column procedure. RNA was eluted in 60 µl of RNase-free water. The whole extraction procedure was done within an hour.

Primer

To design an assay that can detect most of the genotypes of prevalent HCV strains, 338 individual sequences of the 5’UTR region of HCV strains from the HCV database (<http://hcv.lanl.gov/>) were retrieved. Through alignment analysis, the conserved fragments of the 5’UTR were used to design the primer set. The HCV genotype 1b sequence (GenBank accession number AY460204), chosen as a representative strain, was used as a reference for generating the set of primers. All of the primers, including two outer (F3 and B3), two inner (FIP and BIP) and two loop primers (LF and LB), were designed according to the guideline

provided by PrimerExplorer V4 (<http://loopamp.eiken.co.jp/>). In this set, FIP consisted of F1c, complementary to the F1 sequence, and F2 sequence, and BIP consisted of the B1 sequence and B2c, complementary to the B2 sequence. F3 and B3 were located outside F2 and B2, while the loop primers recognized the region between F2 and F1, or B2 and B1. To strengthen the power of LAMP, an additional accelerating primer (AP), located between F1 and B1, was added to the primer set. A schematic representation of the locations of the primers along with a representative alignment of the main HCV strains is shown in Fig. 1. The details of the oligonucleotide primers used for the amplification are given in Table 1. All primers were synthesized by Invitrogen (Invitrogen, Shanghai, China).

HCV LAMP assay

The routine one-step LAMP (Pre-LAMP) reactions were carried out in a final volume of 20 µl containing 40 pmol each of the FIP and BIP inner primers, 20 pmol each of the LF and LB loop primers, 5 pmol each of the F3 and B3 outer primers, 1× ThermoPol buffer (New England Biolabs, Beverly, MA), 2 mM MgCl₂, 1 M betaine (Sigma Aldrich, USA), 1.4 mM each deoxynucleotide triphosphate, 8 U of Bst DNA polymerase (New England Biolabs, Beverly, MA), 0.125 U of AMV reverse transcriptase (TaKaRa, Dalian, China), and 6 µl of template. The mixture was incubated at 60°C for 60 min and then heated to 85°C for 2 min to terminate the reaction. For the AP-LAMP assay, the reaction mixture and the conditions were the same as those described for Pre-LAMP, except that 20 pmol of AP was added to the reaction mixture. A negative control was included for each LAMP run. Serial dilutions of the standard were used as templates for the LAMP assay to evaluate its sensitivity. The specificity of the assay was tested with samples from HAV-, HBV-, and HIV-infected patients and healthy donors.

Analysis of the LAMP product

After amplification, the amplified DNA products were analyzed by electrophoresis in a 1.5% agarose gel stained

Table 1 The specific LAMP primers designed for HCV detection used in this study

Primer name	Sequence (5’ to 3’)	Genome position ^a
F3	ACTCCACCATGAATCACTC	24-42
B3	ATCAGGCAGTACCACAAGG	279-297
FIP	AGGCTGYACGACACTCATAC-CTGTGAGGAACTACTGTCTTC	94-113/45-65
BIP	GGATMAACCCRCTCAATGCC-TCGCRACCCAACRCTAC	200-219/258-274
LF	GCCATGGCTAGACGCT	74-89
LB	GTGCCCCCGCRAGAC	233-247
AP	TTCCGCAGACCACTATGGCTCT	134-155

^a In AY460204

with ethidium bromide and visualized using a Bio-Rad Transilluminator. The restriction enzymes *NheI* and *SmaI* (New England Biolabs, Beverly, MA) were used to digest amplified products to confirm amplification specificity. Digested products were analyzed by gel electrophoresis on a 1.5% agarose gel.

For naked-eye visualization, one microlitre of $1000 \times$ diluted SYBR Green I (Invitrogen, Carlsbad, CA) was added to the reaction tube after amplification, and the reaction was observed visually. For a positive reaction, a change in the color of the reaction solution from orange to fluorescent green could be recognized.

For real-time monitoring of the LAMP reaction, the reaction was performed on an ABI Prism 7900HT Sequence Detection System, with $1 \times$ SYBR-Green I (Invitrogen, Carlsbad, CA) added to the reaction mixtures to provide the fluorescent signal. The run was set up as follows: 60 cycles of 1 min at 60°C (1 cycle corresponding to 1 min of reaction), with fluorescence reading at the end of each of these cycles.

Real-time PCR

A commercial HCV RNA real-time PCR detection kit (Kehua Bio-engineering, Shanghai, China) was used according to the manufacturer's instructions. As the template, $12.5 \mu\text{l}$ of RNA extract was used in a $25\text{-}\mu\text{l}$ reaction. The clinical sensitivity of this quantitation kit was 500 IU/ml [18].

Results

HCV-specific LAMP development

To design a LAMP primer set covering the main genotypes of HCV isolates, a multiple sequence alignment, including genotypes 1-6, was generated by retrieving and aligning the sequences stored in the HCV databases (<http://www.hcv.lanl.gov>). The primers were designed to maintain maximum conservation for annealing to the target regions. Mismatches at the 5' or 3' ends of FIP/BIP were substituted by degenerate bases (Table 1). A database search using BLAST from NCBI showed that all of the primers were specific for the HCV genome.

The routine LAMP format (Pre-LAMP) for the HCV-specific assay was performed by using RNA templates extracted from standard samples. The amplified DNA products were subjected to electrophoresis on 1.5% agarose gels and visualized under UV light after ethidium bromide staining. As a result, a typical LAMP laddering pattern was observed, indicating the different replication intermediates of the stem-loop amplification process, while no bands

were obtained from the no-template control. Electrophoresis-based monitoring of the Pre-LAMP product showed a sensitivity of 500 IU/ml (Fig. 2A). To test the specificity of the test, the amplified product was digested with the enzyme *NheI*, resulting in the detection of strong bands (Fig. 3B, lanes 2). Furthermore, the possibility of cross-reactivity with other viruses known to cause similar clinical signs was also investigated. No amplification of any viral RNA (or DNA) extracted from HBV-, HAV-, or HIV-positive samples was detected (Fig. 3E).

To improve the efficiency of detection, an accelerating primer (AP) was designed to form an additional synthesis-starting site. Different from the loop primer, which binds the single strand in the F1-F2 or B1-B2 region in the classical LAMP [21, 22], the AP designed here is complementary to one of the double-stranded regions between F1 and B1 (Fig. 5A). Adding AP to the Pre-LAMP reaction, the assay, named AP-LAMP, was carried out to evaluate its performance. Serial dilutions of the templates were amplified by the AP-LAMP assay. As shown in Fig. 4, the one-step AP-LAMP assay had a detection limit of a 50 IU/ml of RNA template. Specificity tests, including restriction enzyme analysis and the use of negative samples, were also conducted, and these showed no cross-reaction.

Since visualization of amplification products from LAMP reactions without special equipment would make the assay widely applicable and available, SYBR Green I was added to the reaction mixtures, resulting in a color change from orange to green. The amplified products yielded a green color in positive AP-LAMP reactions, demonstrating that the sensitivity of this assay is equal to that of electrophoresis (Fig. 4B).

Comparative evaluation of AP-LAMP assay

Given that the sensitivity of the AP-LAMP assay for detecting HCV is higher than that of the pre-LAMP method, the pathway of AP-based amplification was investigated. A dumbbell-like intermediate is the initial auto-cycling product for the subsequent amplification steps in the LAMP assay. Apart from the classical LAMP pathway that is followed when using FIP and BIP, as described elsewhere [22], the AP pathway involves the synthesis via AP priming to promote elongation followed by FIP self-priming. The characteristic feature of the products of the AP pathway is that the end products are partly derived from the concatenation of AP-FIP fragments (Fig. 5). More importantly, if the AP pathway is followed in the assay, it is logical to speculate that the amplification would still occur without the outer primer (named AP-B3 LAMP in this study), which is strictly required in the classical LAMP [22]. Three LAMP formats are shown in

Fig. 2 Detection of HCV RNA by electrophoresis analysis of the Pre-LAMP and AP-B3 LAMP end products. Lane M, DL2000 DNA ladder (TaKaRa, Dailian, China). The extracted RNA template was prepared from the standard plasma and was subjected to analysis by the Pre-LAMP and AP-B3 LAMP methods. **a** Pre-LAMP assay using serial dilutions from 5×10^5 IU/ml to 50 IU/ml. **b** AP-B3 LAMP assay using template concentrations from 5×10^5 IU to 50 IU/ml. **c** Low-concentration standards ranging from 500 to 50 IU/ml, detected by the Pre-LAMP assay. **d** The same standards ranging from 500 to 50 IU/ml, detected by the AP-B3 LAMP reaction. The dashed box indicates the band pattern with the most significant difference between the two assays

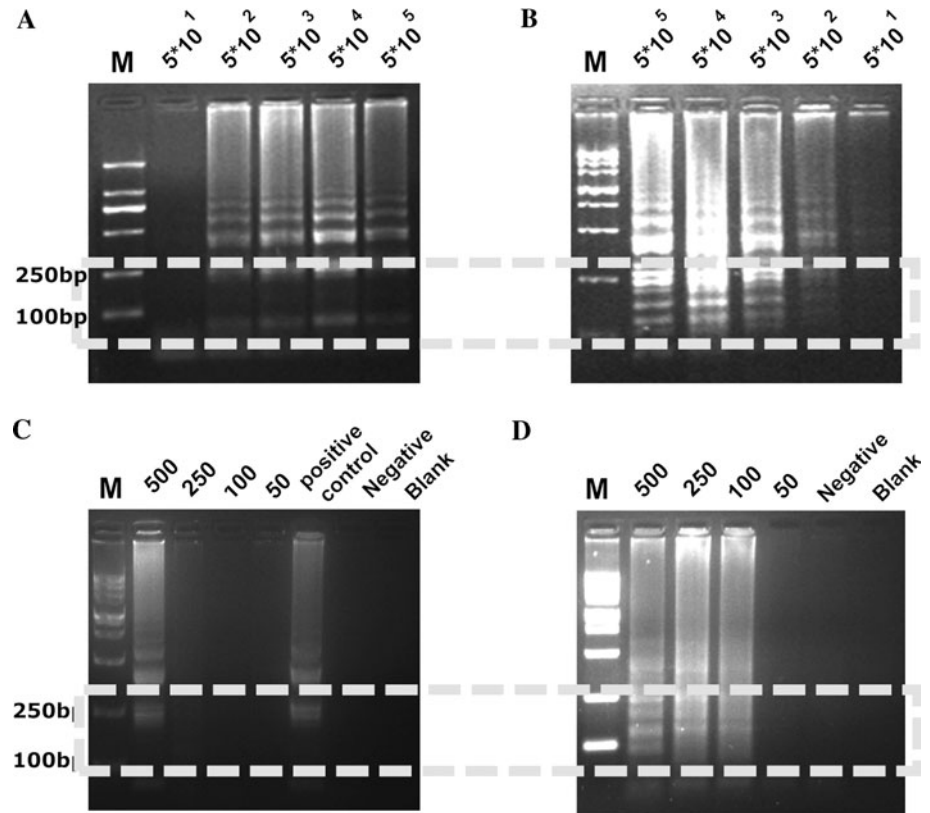


Fig. 5A. To test this hypothesis, we compared the Pre-LAMP, AP-LAMP, and AP-B3 LAMP assays using the same templates.

A panel of serial log dilutions of HCV RNA of known concentration was tested using the AP-LAMP, Pre-LAMP, and AP-B3 LAMP assays on the real-time 7900HT platform. The results for each dilution tested in batches of three replicates in two separate runs are given in Table 2. For the AP-LAMP assay, the average threshold time (Tt) required to detect a positive signal ranged from 17.99 ± 0.62 min (Mean \pm SD) when 5×10^5 IU RNA was present to 31.70 ± 0.43 min when 5×10^2 IU RNA was present, compared to 25.98 ± 0.58 - 42.71 ± 1.60 min in the Pre-LAMP assay and 16.00 ± 0.80 - 31.99 ± 1.06 min in the AP-B3 LAMP assay. The Tt value was defined as the reaction time necessary to achieve a positive signal above the baseline [10]. These results demonstrated that the AP-LAMP assay was faster by 9-12 min than the Pre-LAMP reaction and was much faster than real-time PCR.

Furthermore, using the assays to analyze low-concentration standards ranging from 500 to 10 IU, no amplification was obtained when the template concentration was less than 250 IU/ml in the Pre-LAMP assay. The real-time AP-LAMP assay consistently detected HCV RNA at levels of 5×10^1 IU/ml, while only three of six AP-B3 LAMP assays detected HCV RNA. By probit regression analysis, the AP-LAMP method detected 84 IU/ml with >95%

probability of a positive result, as compared to 617 IU/ml and 143 IU/ml for the pre-LAMP and AP-B3 LAMP assay, respectively. Therefore, the sensitivity of the AP-LAMP assay for detecting HCV is about two times higher than that of the AP-B3 LAMP assay and nine times higher than that of the pre-LAMP assay.

Characteristics of the AP pathway

When comparing the electrophoresis bands of the products of the AP-LAMP, AP-B3 LAMP and Pre-LAMP assays, the first two showed a similar pattern. The main difference between the AP-LAMP and Pre-LAMP assays is that there were ladder-like bands between 100 and 250 bp. The bands in this region (in rectangles with a broken line, Fig. 2) are likely to correspond to the self-primed amplification product bounded by the 5' ends of the AP and FIP stem-loop structure (~193 bp in size), which match the expected size of the AP-pathway product.

Moreover, the result of the AP-B3 LAMP assay provided evidence that the AP pathway is used during amplification. Several reports have indicated that the LAMP assay strictly requires the strand displacement function of the outer primers [22]. No LAMP amplification occurs when FIP, BIP, F3 or B3 is absent [2]. By using the AP in the assay, this study using real-time monitoring or agarose gel electrophoresis confirmed that the outer primer

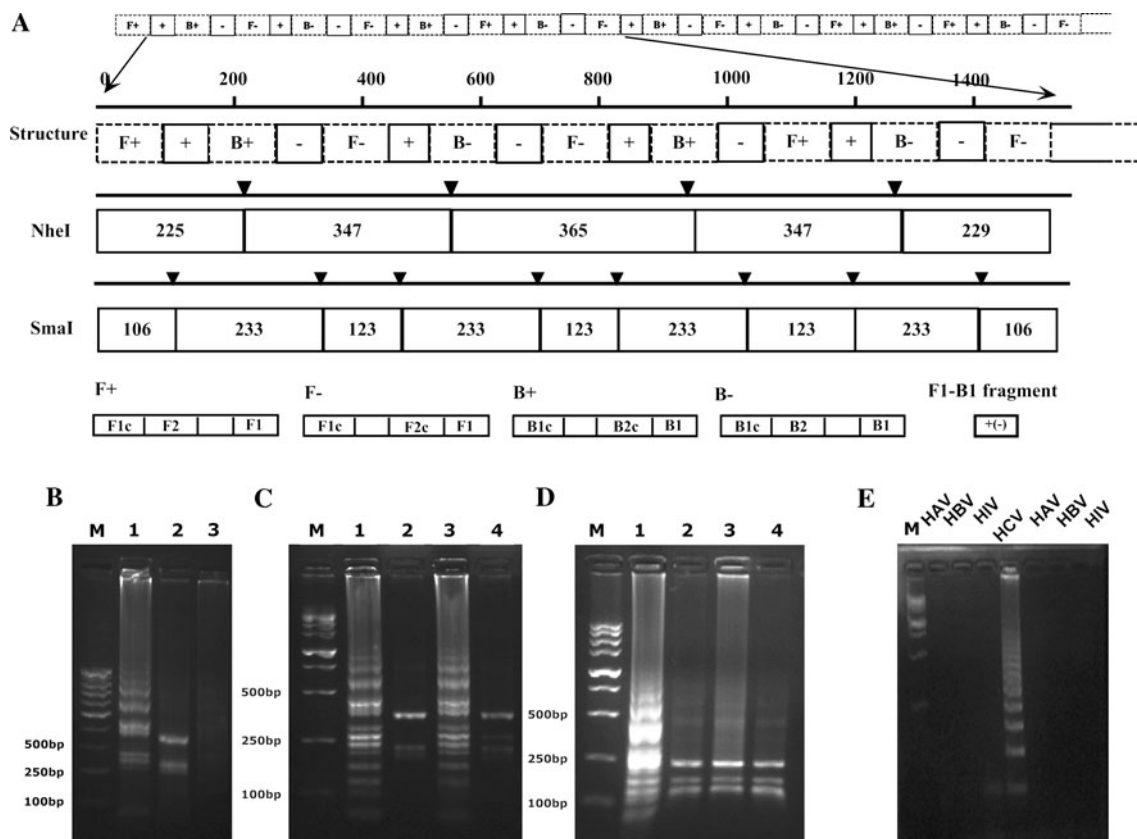


Fig. 3 Confirmation of the specificity and structure of the amplified product. **a** Schematic representation of the structure of the LAMP products. The concatenated structures in a linearized DNA form are shown in the boxes. +, the target sequence flanked by B1 and F1; -, the complementary sequence of +. Arrows indicate restriction sites. The sizes of the restriction fragments are shown in the boxes. HCV LAMP products would be fragmented into 225-, 229-, 347- and 365-bp products after *NheI* digestion, and 106-, 123- and 233-bp products after *SmaI* digestion. **b** M, DL5000 marker (Takara); lane 1, positive Pre-LAMP product; lane 2, Pre-LAMP product digested with

NheI; lane 3, negative control. **c** M, DL5000 (Takara); lane 1, positive AP-LAMP product; lane 2, AP-LAMP product digested with *NheI*; lane 3, AP-B3 LAMP product; lane 4, AP-B3 LAMP product digested with *NheI*. **d** M, DL5000 (Takara); lane 1, positive control; lane 2, AP-LAMP products digested with *SmaI*; lane 3, Pre-LAMP products digested with *SmaI*; lane 4, AP-B3 LAMP products digested with *SmaI*. **e** M, DL2000 (Takara); Specificity tests of the LAMP assay for HCV RNA. HAV-, HBV-, and HIV-positive clinical samples prepared in the current study were tested, and representative results are shown

is not required. The AP-B3 LAMP assay has higher sensitivity than the Pre-LAMP assay, but it is less sensitive than AP-LAMP. This assay type also showed lower stability than AP-LAMP, especially when the samples were at low concentration. In addition, taking into account the similar band patterns between the AP-LAMP and AP-B3 LAMP assay, these results indicate that AP-priming-based amplification is inferior to the classical pathway in the LAMP process.

The amplified products were digested with two restriction endonucleases to confirm the specificity and structure of the amplified products from three different LAMP assays. The restriction enzyme *SmaI* recognizes the sequence between F1 and B1, while *NheI* cuts between B1 and B2. If the products were amplified specifically and formed the structures shown in Fig. 3A, *NheI* digestion would yield fragments of 225, 229, 347, and 365 bp, and *SmaI* digestion would yield fragments of 106, 123 and

233 bp. Since the amplified product is a concatenation of DNA fragments of different sizes, the amplification kinetics probably affect the actual end products of the LAMP reaction. Due to cutting in the region between B1 and B2, *NheI*-digestion bands were observed at approximately 250–454 (225 + 229) bp in the Pre-LAMP product, in contrast to 220–400 bp in the AP-LAMP and AP-B3 LAMP products. While cutting the region between F1 and B1, the sizes of the fragments generated by *SmaI* digestion were approximately 120 and 230 bp (Fig. 3D), which is in good agreement with the predicted sizes.

Evaluation of AP-LAMP for clinical specimens

The feasibility of the AP-LAMP assay for detecting HCV in clinical material was assessed by using both positive and negative plasma specimens. The real-time PCR assays were performed simultaneously, and the results of both

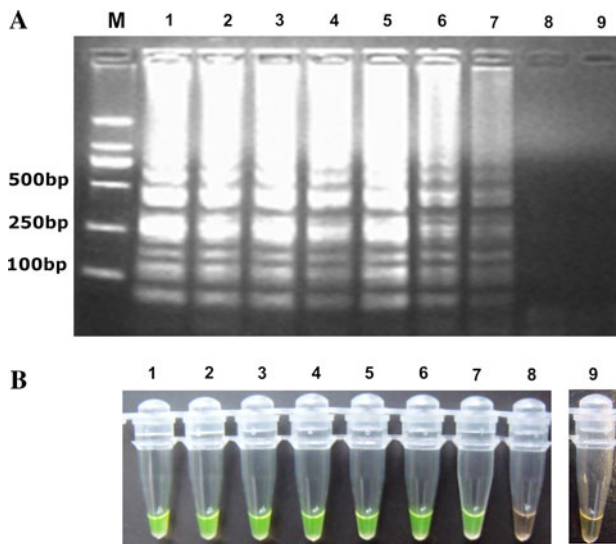


Fig. 4 Sensitivity of the AP-LAMP assay. **a** Agarose gel electrophoretic analysis of AP-LAMP products from RNA extracts of the templates ranging from 5×10^5 IU to 10 IU/ml; lane M, DL2000 DNA marker. Lanes 1 to 8, 5×10^5 IU to 10 IU/ml; lane 9, negative control. **b** Sensitivity of AP-LAMP for HCV as determined by a change in the color of SYBR Green I. A positive reaction is indicated by a color change from orange to green

methods were compared (Table 3). Of the 50 samples collected from healthy volunteers, all tested negative in both AP-LAMP and real-time PCR. A total of 25 samples were obtained from chronic HCV patients (genotype 1b, $n = 19$; genotype 2a, $n = 6$; all anti-HCV positive), with the viral load ranging from 1.35×10^3 to 3.12×10^6 IU/ml. None of the samples were missed by the AP-LAMP assay. Of 51 acute-phase samples collected from patients suspected of having viral hepatitis, three were anti-HCV positive, and these were also detected by AP-LAMP. Of the remaining 48 anti-HCV-negative cases, only two were positive for HCV RNA by AP-LAMP. These two patients later seroconverted after 1 and 3 months, respectively (Table 3). The AP-LAMP gave a total of 5 (9.8%) positive results, and the same result was obtained by real-time PCR. In total, the AP-LAMP method demonstrated 100% agreement with real-time PCR when used for analysis of clinical samples. These preliminary results suggest that the AP-LAMP assay described here can be applied for the diagnosis of HCV infection in a clinical setting.

Discussion

Among the nucleic acid amplification tests available to date, LAMP method has many characteristics that make it suitable for the rapid, sensitive, and simple detection of pathogens [19]. The adaptation of the LAMP technology for HCV detection in point-of-care or resource-limited

setting has many potential advantages. For examples, the reaction occurs under isothermal conditions and thus does not require special equipment. The powerful amplification efficiency of the LAMP assay makes it extremely rapid, and it exhibits high analytical sensitivity. Furthermore, the end product can be observed immediately by visual observation, through turbidity or dye staining [27]. Up to now, however, no HCV nucleic acid test has been available outside of the laboratory setting, possibly due to time, cost and technology limitations.

The use of multiple primer combinations is one of the key features of the LAMP method, but this can affect the primer selection for a given template, such as the HCV 5'UTR, which has a complex ordered secondary structure and genotypic variation sites simultaneously. We devised an accelerating primer to improve the efficiency of amplification. Just like the loop primer in LAMP, the AP provides an additional starting site for DNA synthesis (Fig. 5) during the amplification, thereby reducing the overall reaction time and increasing the sensitivity. It should be mentioned, however, that in the LAMP reaction, the loop region is always in a single-stranded state during the process. In contrast, the AP is located in the double-stranded region and is complementary to one of its strands. When comparing the performance of the AP-LAMP and Pre-LAMP assays, higher amplification efficiency was found in the former. A possible explanation for this is that apart from the classical amplification process in LAMP assay, there is an AP-based amplification pathway in the AP-LAMP method. This notion is supported by the following evidence: First, by comparing the band patterns of AP-LAMP and Pre-LAMP, the products of the AP pathway (AP-FIP) were observed in the former. Next, the AP-B3 LAMP assay still amplified the target in the absence of outer primer B3. In the classical LAMP format, the outer primer is important for strand displacement to form an auto-cycling intermediate product. No amplification occurs without this primer [2]. Finally, by the digesting the end products with restriction enzymes that recognize different sites in the target, the most favorable structure of the amplified products was found by length polymorphism analysis, as shown in Fig. 3.

It is well known that primer design in LAMP is more complex and difficult than that in PCR [2]. Since LAMP reaction efficiency and sensitivity strongly depend on primer selection, a more flexible way for LAMP primer design could promote the use of this method. Summing up the above points, the AP strategy could be applied in LAMP design to meet special demands under certain conditions. For instance, because both ends of the FIP/BIP secondary structure play a key role in amplification cycling in the LAMP-based assay, using an AP could reduce the problem of selecting FIP/BIP. Adding AP to the

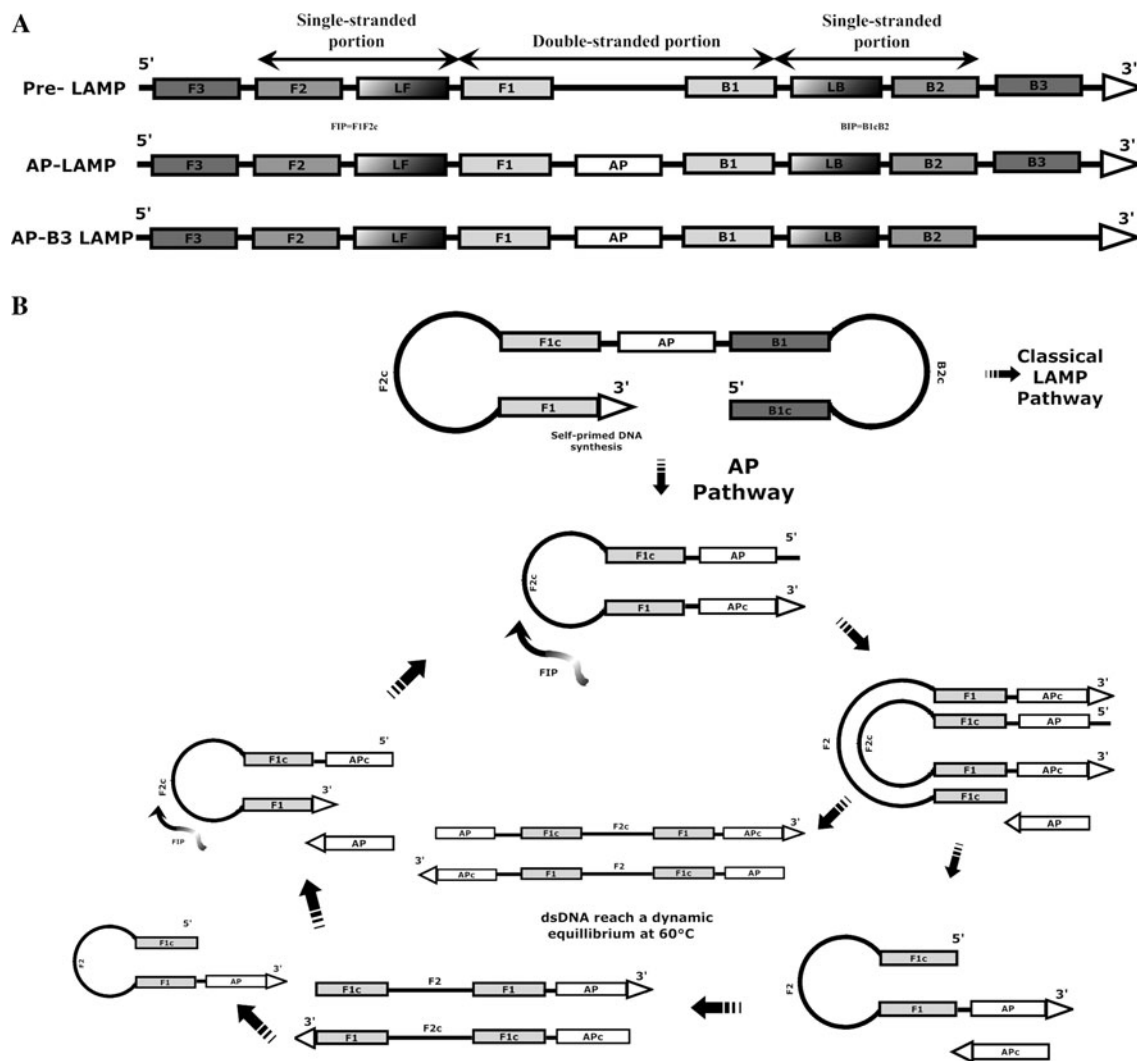


Fig. 5 Principle of AP in LAMP amplification. **a** The three LAMP formats in this study. The primers commonly used in the LAMP assay (Pre-LAMP) include inner primers FIP and BIP, outer primers F3 and B3, and loop primers LF and LB. The loop primer anneals the partially single-stranded portion. The AP devised in this study is the additional accelerating primer located between the F1 and B1

fragments. The AP-B3 LAMP assay does not use the outer primer B3. **b** The cyclic amplification step for the AP pathway is illustrated: The dsDNA reaches a dynamic equilibrium at 60°C, and thus the AP can bind the partly free 3' end of the template to initiate strand extension. Complementation of the hairpin structure (F1-F1c) induces self-primed DNA synthesis

Table 2 Comparative evaluation of the different HCV LAMP assays

HCV RNA (IU/ml)	Pre-LAMP			AP-LAMP			AP-B3 LAMP		
	No. positive/ tested	Positive ^a (%)	Tt	No. positive/ tested	Positive ^a (%)	Tt	No. positive/ tested	Positive ^a (%)	Tt
500000	6/6	100	25.98 ± 0.58	6/6	100	17.99 ± 0.62	6/6	100	16.00 ± 0.80
50000	6/6	100	29.42 ± 0.92	6/6	100	23.10 ± 0.77	6/6	100	22.32 ± 1.02
5000	6/6	100	35.55 ± 0.88	6/6	100	27.87 ± 0.16	6/6	100	27.88 ± 0.65
500	6/6	90.9	42.71 ± 1.60	6/6	100	31.70 ± 0.43	6/6	100	31.99 ± 1.06
250	2/6	45.4	45.51 ± 1.03	6/6	99.9	34.31 ± 0.71	6/6	99.4	34.94 ± 0.95
100	0/6	2.1	–	6/6	98.0	36.02 ± 0.57	5/6	85.2	38.59 ± 1.05
50	0/6	0.0	–	6/6	89.3	38.19 ± 0.73	3/6	51.2	41.88 ± 0.96
10	0/6	0.0	–	1/6	26.1	40.12	0/6	17.0	–

^a Probability determined by probit regression analysis

Table 3 Clinical samples evaluated using AP-LAMP

Group	Anti-HCV	AP-LAMP	Real-time PCR	Number of cases	Viral load (mean \pm SD) l g IU/ml
Confirmed	+	+	+	25	5.24 \pm 1.14
Suspected	+	+	+	3	7.13 \pm 0.57
	-	+	+	2	4.50 \pm 1.54
Healthy	-	-	-	50	

pre-optimized LAMP assay could avoid the need to design different primer sets for optimization.

The performance of AP-LAMP was investigated in this study. Running the AP-LAMP assay in a real-time PCR machine consistently achieved a lower limit of detection of 84 IU/ml by probit analysis. This sensitivity is comparable to a series of in-house tests published recently [4–6, 16, 30]. Although this method is essentially qualitative at the outset, the sensitivity limits represent good performance, since the HCV plasma viremia in acute infections is generally higher than 10^4 copies/ml [11]. As expected, a specificity test using seronegative samples and other non-targeted virus samples demonstrated 100% exclusivity of the assay.

By applying the AP-LAMP assay to clinical specimens, there was 100% agreement between AP-LAMP and the real-time PCR test. The AP-LAMP method consistently detected HCV-infected samples with a broad range of viral loads. Since the samples comprised the HCV genotypes 1b, 2a, which are prevalent in China [17], this AP-LAMP assay is expected to work for the majority of HCV-infected individuals in the local region. Because of the high mutation rate of the HCV 5'UTR, it is not easy to generate a single LAMP primer set to detect every viral strain of an individual subtype. Degenerate design is most the common way to address the issue of genotype inclusivity, but this may lower the diagnostic sensitivity due to a hybridization effect. For this reason, the AP strategy developed here would potentially be applicable in a situation like this. Future evaluation of detection efficiency using an extensive collection of HCV genotypes and larger samples would be desired to validate the performance of the assay.

In addition to the high sensitivity and specificity of the AP-LAMP assay, its other major advantages are its rapidity and the flexibility of its detection method. The AP-LAMP assay itself could be carried out in less than 45 min. Only 1.5 h (including the extraction step) was needed to perform the LAMP assay, compared to 2.5–3 h for the real-time PCR assay. Amplification in the LAMP assay can be detected with the naked eye in the form of visual fluorescence, e.g., the original orange color of the dye changes to green under natural light in the case of a positive amplification reaction [25], thus eliminating the need for gel electrophoresis or real-time monitoring. Our results, as

well as the results of previous studies using a fluorescent reagent to detect the LAMP product visually [24, 35], showed a similar detection efficiency when compared to real-time PCR or electrophoresis. Therefore, this HCV-specific LAMP assay may be applicable under clinical or field conditions.

In conclusion, as demonstrated using HCV, we have developed a LAMP test using a novel principle based on an accelerating primer and have provided an alternative way to design a LAMP assay for a complex target. This study presents a sensitive and specific LAMP method for screening for or confirming infection with HCV in a simple, rapid, and cost-effective manner.

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Conflict of interest The authors declare that they have no conflict of interest.

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