

*Full Length Research Paper*

# Rapid and simple detection of Hepatitis C virus by reverse transcriptase -loop- mediated isothermal amplification method

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Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV infection has now become a serious health problem because at least 170 million people worldwide are currently infected with HCV. Molecular diagnostics are revolutionizing the clinical practice of infectious disease. Loop-mediated isothermal amplification (LAMP) is a novel technique for nucleic acid amplification. This simple and rapid technique relies on strand-displacing DNA synthesis performed using the Bst DNA polymerase large fragment under isothermal conditions in the temperature range of 60 - 65°C, thereby obviating the need for a thermal cycler. The method has also been rendered applicable to RNA genomes by combining it with reverse transcription reactions (RT-LAMP). RT-LAMP is characterized by the use of 6 different primers. For 110 quantities sera, Nested RT-PCR and RT-LAMP were done. At the end of the LAMP reaction, SYBR Green was used for identifying negative and positive results. The PCR sensitivity up to 80 particles was observed and the LAMP sensitivity test was verified up to 8 particles. The data presented in this study suggested that the RT-LAMP assay is more sensitive than RT-PCR by picking up 5 additional cases that were negative by RT-PCR. These findings demonstrate that detection of HCV using RT-LAMP was more sensitive than the Nested RT-PCR. The RT-LAMP assay developed in this study is simple, rapid, and cost effective as well as highly sensitive and specific.

**Key words:** Hepatitis C virus, RT-LAMP, nested RT-PCR.

## INTRODUCTION

Hepatitis C virus (HCV) is an enveloped – positive standard RNA virus that belongs to the Hepacivirus genus of Flaviviridae family (Choo et al., 1989; Gondeau et al., 2009; Houghton et al., 1991; Lindenbach, 2001; Murphy et al., 1995; Rodriguez et al., 2009). High world wild prevalence of HCV infection (3%) remains significant

health problem all over the world providing major cause of chronic hepatitis that may progress to liver cirrhosis, and hepatocellular carcinoma (Alter, 2007; Chen et al., 2006; Levrero, 2006; Sidorkiewicz et al., 2009; Wasley, 2000). With the advancement of technologies, increasingly diverse methods are available which make it possible to detect and analyze any virus, including those, which can not be cultured. Three categories of virological assay can theoretically be used for the diagnosis and management of HCV infection: (I) serological assay, detecting antibodies specially directed to HCV antigens;

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(II) assays detecting and quantifying HCV antigens; (III) molecular biology - based assays detecting or quantifying HCV genomes and analyzing their sequence (Majid et al., 2002; Mondelli et al., 1999; Scott et al., 2007; Q et al., 1991). Serological assays can be performed on any kind of blood samples. Nevertheless, old samples or samples conserved at high temperatures may sometimes yield false positive or false negative results. In addition, these assays are time-consuming and tedious, more than a day for completion (Pawlotsky, 1999).

During the past decades, various forms of PCR (polymerase chain reaction) such as Nested PCR and multiplex PCR have been developed to address the need for rapid identification of viruses to serotype level with more accuracy (Ratcliff et al., 2007). Detection of viremia by reverse transcriptase polymerase chain reaction (RT-PCR) is being used extensively in the laboratory for the diagnosis and management of hepatitis C virus (HCV) infection (Radhakrishnan et al., 1999).

In addition to traditional RT-PCR, more rapid and sensitive real time PCR – based assay, such as Taq man RT-PCR and nucleic acid sequence - based amplification (NASBA) and branched - DNA methods, have been reported and are currently under extensive evaluation with blood samples (Chan et al., 1999; Daniela et al., 2008; Irshad et al., 2007; Sarrazin, 2002; Vernet, 2004).

However, all of these nucleic acid amplification methods have the intrinsic disadvantage of requiring either a high - precision instrument for amplification or an elaborate complicated method for detection of amplified products (Compton, 1991; Heid et al., 1996; Higuchi et al., 1993; Parida et al., 2004; Parida, 2008).

Recently, a novel nucleic acid amplification method termed loop-mediated isothermal amplification (LAMP) was developed by Notomi (Notomi et al., 2000). This method relies on auto cycling strand displacement DNA synthesis performed by using the *Bst* DNA polymerase large fragment (Uemura et al., 2008; Mori et al., 2001; Nagamine et al., 2002).

The characteristics of LAMP are rapidity under isothermal conditions, low reaction temperature (63 to 65°C), and high specificity for the target sequence. This is attributable to recognition of the target sequence at six independent sites in the initial stage and at four independent sites during the last stages. It requires only four primers, a DNA polymerase, and a regular laboratory water bath or heat block for reaction, making it simple to perform (Pham et al., 2005). Two inner primers and two outer primers define the target region, and an additional set of primers, termed loop primers, can be added to increase the sensitivity of the assay. The final products of the LAMP reaction are DNA molecules with a cauliflower-like structure of multiple loops consisting of repeats of the target sequence. The products can be analyzed by real-time monitoring of the turbidity resulting from the production of magnesium pyrophosphate precipitate during the DNA amplification reaction, by using an

intercalating fluorescent dye and visualization by natural light or with the aid of UV irradiation or by agarose gel electrophoresis (Le Roux, 2009; Mori et al., 2001, 2004).

The amplification of RNA template was accomplished through RT-LAMP assay by employing reverse transcriptase (RTase) for reverse transcription step in addition to the *Bst* DNA polymerase. RT-LAMP method can synthesize cDNA from template RNA and apply LAMP technology to amplify and detect them. RT-LAMP technique has been applied successfully for the detection of some RNA viruses (Chen et al., 2008; Hong et al., 2004; Parida et al., 2004; Pham et al., 2005; Poon et al., 2005).

## MATERIALS AND METHODS

### Serum samples

110 sera were studied. Serum HCV RNA load was determined with the quantitative Cobas Amplicor HCV Monitor Test (Roche Diagnostic Systems, Pleasanton, CA). HCV-positive serum samples were obtained from Kivan Laboratory.

### RNA extraction

HCV RNA was extracted from 100 µl of serum by RNX™ (-Plus) method (Cinagen), according to the manufacturer's instructions. The RNA was resuspended in 50 µl of diethyl pirocarbonate (DEPC)-treated water.

### cDNA synthesis and first round of nested PCR

5 µl of extracted RNA was applied to the optimized Nested PCR with primers ( SF1:5'- GTG-CAG-CCT-CCA-GGA-CCC-CC-3', SR 1: 5'-GGG-CAC-TCG-CAA-GCA-CCC-TAT-3') located in the highly conserved 5'non coding region (5'NCR). First PCR was performed in a 20 µl total reaction volume with 1.5 mM MgCl<sub>2</sub>, 1 X PCR reaction buffer, 0.8 mM dNTPs, 1.5 unit of Taq DNA polymerase enzyme (Bioflux), and 7 µl of the isolated RNA, 1 X RT reaction buffer, 0.4 µM/l of each forward and reverse primers, 200 U of Moloney Murine Leukemia Virus (MMLV) (Fermentas), 20 U of Rnasi (Fermentas). Thermal cycler was programmed as follows: 42°C for 30min, 94°C for 2 min, 60°C for 40 s, 72°C for 40 s, followed by 20 cycles of 93°C for 30 s, 60°C for 40 s and 72°C for 40 s.

### Second round of Nested PCR

2 µl of first PCR product was subjected to second round of nested PCR using nested PCR mixture containing, 1 X PCR reaction buffer, 0.2 mM dNTPs, 2 unit of Taq DNA polymerase enzyme (Bioflux) and 0.6 µM/l of second round primers. For comparison with the sensitivity of the RT-LAMP method, second PCR was performed by using the two outer primers, F3 and B3, used for the LAMP amplification as forward and reverse primers, respectively, in accordance with the standard protocol (Saiki, 1989).

Two-step PCR reaction profile was followed by 40 cycles of 93°C for 40 s, 58°C for 40 s and 72°C for 40 s. Amplified DNA (208 bp for HCV) was detected by staining with ethidium bromide (CinnaGen) after separation on a 2% agarose gel electrophoresis. All positive

**Table 1.** Details of Nested RT-PCR and RT-LAMP primers designed for 5'NCR gene sequence of HCV.

Primer name *	Genome position	Sequence
SF1	29 - 48	5'-TGCAGCCTCCAGGACCCCC-3'
SR1	269 -287	5'-TGCACGGTCTACGAGACCT-3'
F3	78 - 95	5'-CGGGAGAGCCATAGTGCT-3'
B3	267 - 285	5'-GTGCCAGATGCTCTGGAGG-3'
LB	210 - 218	5'-TGTTGGGTGCGCAAAGGCC-3'
LF	125 - 144	5'-AAAGGACCCGGTCGTCCTGG-3'
FIP	146 - 167	5'-GGCATTGAGCGGGTTGATCCAA
	104 - 122	CCGGTGAGTACACCGGAAT-3'
BIP	188 - 207	5'-CGCGAGACTGCTAGCCGAGT
	231 - 250	ACCCTATCAGGCAGTACCAC-3'

\* The primers of F3, B3, BL, FL, FIP and BIP were for RT-LAMP and primers of SF1 and SR1 were also applied in first round of Nested RT-PCR and cDNA synthesis.

and negative controls were tested in parallel with test samples throughout the entire procedures, starting with RNA extraction.

#### cDNA synthesis

A 259-bp fragment of 5'NCR cDNA was amplified with PCR using primers of SF1 and SR1. Synthesis of cDNA was undertaken by reverse transcription from 5 µl of RNA extracted at 42°C for 60 min and posteriorly 70°C for 10 min, in 20 µl solution containing 1 X RT reaction buffer, 0.4 µM/l of the primers, 200 U of Moloney Murine Leukaemia Virus (MMLV) (Fermentas), 20 U of Rnasin (Fermentas) and 0.2 mM dNTPs.

#### RT-LAMP primers designing

Designing of a highly sensitive and specific primer set is crucial for performing LAMP amplification. Oligonucleotide primers used for the LAMP assay of HCV were designed by using the 5'NCR gene sequences (GenBank accession no. EU256103). The name, location, and sequence of each primer are shown in Table 1. As LAMP primers, a set of six oligonucleotides comprising two outer (F3 and B3), two inner (FIP and BIP) and two loop primers (FL and BL) recognizing eight distinct regions on the target sequence were designed by using LAMP primer design support software (Primer Explorer version 4.0; Eiken Genome; <http://primerexplorer.jp/>).

#### RT-LAMP reaction

LAMP was performed in a total 25 µl reaction mixture containing 1.6 mM each of FIP and BIP primer, 0.2 mM each of outer primers (F3 and B3 primers), 0.8 mM each of loop primers F and B, 1.4 mM dNTPs, 8 U of Bst DNA polymerase large fragment (New England Biolabs) using the supplied buffer (Thermopol buffer containing 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 9 mM MgSO<sub>4</sub>, 0.1% Triton X-100), 0.8 M betaine and 5 µl cDNA. The reaction mixes without template were included as the negative controls. The sequences of primers are shown in Table 1. The RT-LAMP reactions were carried out at 65°C for 1 h.

#### Detection of LAMP products

The result can be visualised directly by the naked eye up on the

addition of an intercalating dye or by gel-electrophoresis: Five microliters of the RT-LAMP products were electrophoresed on a 2% molecular-grade agarose gel prepared in 0.5 X Trisborate-EDTA buffer stained with 0.5 µg/ml ethidium bromide. The amplification products were visualized using a transilluminator with UV light at 302 nm. The amplification by RT-LAMP showed a ladder-like pattern, whereas the RT-PCR showed a 208-bp amplicon (Figure 1). We could add SYBR Green I to the LAMP reaction products, for a positive reaction, orange changes to yellow fluorescence that can be detected with the naked eye or to green fluorescence under UV irradiation, while a negative reaction remains the orange (Figure 3A).

#### Cloning of PCR product as a positive control

After purification, the PCR product was cloned in pTZ57R vector by using T/A cloning kit (Fermentas).

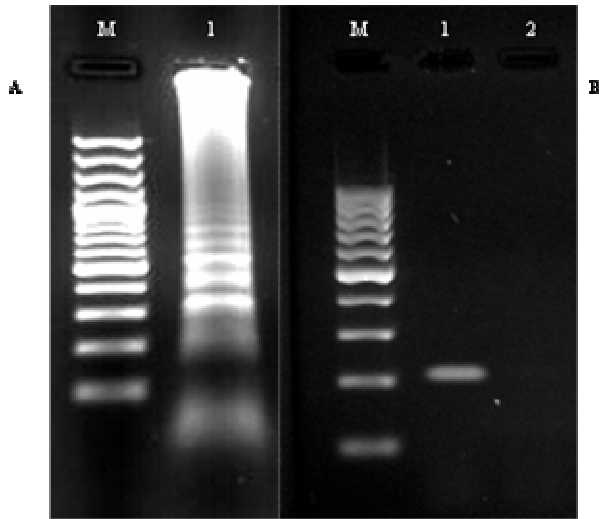
## RESULTS

### Sensitivity

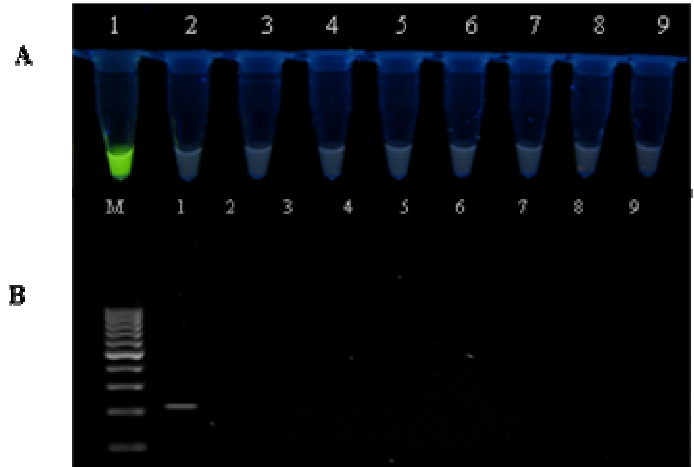
When the sensitivity of the RT-LAMP was compared with nested RT-PCR by testing serial dilutions of RNA prepared from infective serum containing 6 x 10<sup>4</sup> copies/ml of HCV RNA (16 x 10<sup>3</sup>, 16 x 10<sup>2</sup>, 160, 80, 8, 4 copies), the detection limits for the RT-LAMP assay and Nested RT-PCR were found to be 8 and 80 copies /ml of HCV RNA, respectively (Figure 2). Thus, the comparative sensitivity of RT-LAMP and RT-PCR indicated that the RT-LAMP was 10-fold more sensitive than RT-PCR.

### Specificity

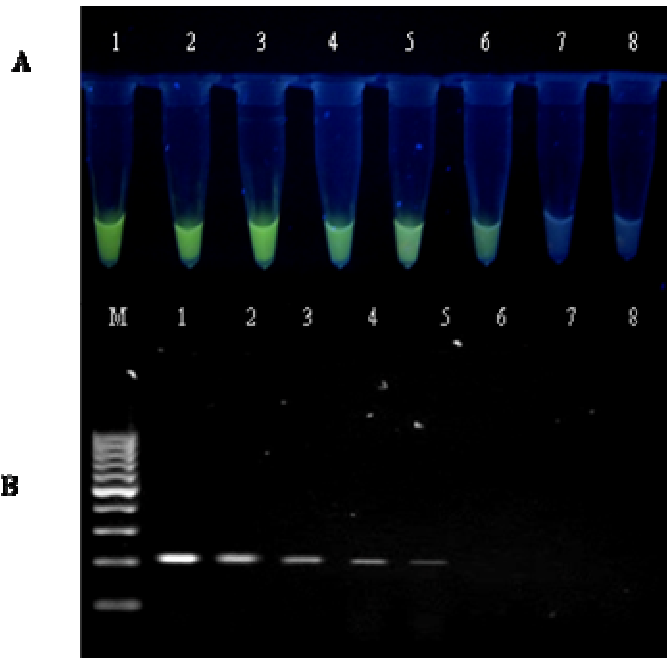
Specificity of primers was tested by LAMP and Nested RT-PCR with DNAs extracted from beings including *Hepatitis B virus*, *Toxoplasma gondii*, *Mice*, *Saccharomyces cerevisiae*, *Escherichia coli*, *Mycobacterium tuberculosis* and human. These DNA samples were not detected by both of LAMP and nested



**Figure 1. A.** Agarose gel electrophoresis of the RT-LAMP product of the 5'NCR Gene of Hepatitis C virus. Lanes: M, 100-bp DNA ladder (Fermentas);1, Ladder-like pattern product of RT-LAMP. **B.** Shows Nested RT-PCR roduct. Lanes: M, 100-bp DNA ladder (Fermentas); 1, Amplicon product of Nested RT-PCR, 2, Negative control.



**Figure 3.** Comparative Specificities of RT-LAMP and Nested RT-PCR. **(A)** DNAs extracted from 7 beings were amplified by the HCV RT-LAMP assay. From left to right: Tube 1, Positive control; 2, *Hepatitis B virus*; 3,*Toxoplasma gondii* ;4, *Mice* ; 5, *Saccharomyces cerevisiae*; 6, *Escherichia coli*; 7, *mycobacterium tuberculosis*; 8, *human*; 9, Negative control. **(B)** DNAs extracted from 7 beings were amplified by the HCV Nested RT-PCR assay. From left to right: Lane M, 100-bp DNA ladder(Fermentas) ; Lane 1, Positive control; 2, *Hepatitis B virus*; 3, *Toxoplasma gondii*; 4, *Mice*, 5, *Saccharomyces cerevisiae*;; 6, *Escherichia coli*; 7, *Mycobacterium tuberculosis*; 8, *human*.; 9, Negative control.



**Figure 2.** Comparative sensitivities of RT-LAMP and Nested RT-PCR. **(A)** Sensitivity of HCV RT-LAMP assay as monitored by agarose gel electrophoresis analysis. Tubes: 1, Positive control; 2-7 ,different RNA copy number of 'NCR gene of Hepatitis C virus ( $16 \times 10^3$ ,  $16 \times 10^2$  , 160, 80, 8 , 4 copies/tube spectively); 8, Negative control. **(B)** Sensitivity of HCV Nested RT-PCR as observed by agarose gel electrophoresis revealing a 208-bp amplicon specific for HCV.Lanes: M,100-bp DNA ladder (Fermentas); Lane1, Positive control , 2-7, different RNA copy number of 5'NCR gene of Hepatitis C virus ( $16 \times 10^3$  ,  $16 \times 10^2$  ,160, 80, 8 , 4 copies/tube respectively); 8, Negative.

PCR methods (Figure 3). Summing up, this assay was highly specific and showed a sensitivity of 8 copies *Hepatitis C virus* for a 60 min reaction in electrophoretic analysis. Considering this together with its ease of operation, rapid reaction and inexpensive system, the LAMP assay is more appropriate than the Nested RT-PCR assay at the genetic point of care in the hospital laboratory.

**DISCUSSION**

Nucleic acid amplification is one of the most valuable tools in virtually life science field, including application-oriented fields such as clinical medicine in which diagnosis of infectious diseases, genetic disorders and genetic traits are particularly benefited by this new technique. Although several amplification methods have been developed, PCR is the most widely used because of its apparent high simplicity and reliability (Yang et a l., 2004).

Within the last twenty years, HCV has emerged as a common cause of liver disease with an estimate of 170 million people infected annually on a worldwide basis (Anonymous, 1997; D.L, 2000; WHO, 1999). HCV infection is characterized by viral persistence and chronic liver disease in approximately 80% of cases (Antonelli et al., 2008; El Serag, 2001).

The complications of chronic hepatitis C include cirrhosis in 20% of cases and hepatocellular carcinoma

(Chen et al., 2006; Wasley et al., 2000), which has an incidence of up to 4 to 5% per year in patients with HCV-related cirrhosis (Perz et al., 2006<sub>a</sub>, 2006<sub>b</sub>). HCV-related end stage liver disease is now the principal indication for liver transplantation in industrialized countries (Gondeau et al., 2009; Pawlotsky, 2004). HCV infection is associated with very low level of circulating virus, hence requiring a genome amplification assay such as RT-PCR (Lin et al., 2002; Vernet, 2004).

Monitoring the viremia through the detection of viral RNA by use of qualitative RT-PCR and various quantitative methods has become the most frequently used and sensitive technique (El-Awady et al., 1999). This laboratory test is of particular use in some situation, that is useful in patients who fail to develop an antibody response, or whom presence of antibodies do not serve to differentiate between current infection and past infection; for deciding on dose and duration of antiviral therapy; and for documentation of perinatal transmission of HCV by demonstration of HCV RNA in blood of newborn (Thaler et al., 1991; Tsubota et al., 1994). Despite the obtainable magnitude of amplification, these methods are time consuming (e.g., RNA extraction, RT, and real-time PCR can be completed in 3 to 4 h) and more complicated and need a high-precision thermal cycler, and contamination of samples in laboratories may lead to false-positive results. On the contrary, the RT-LAMP assay reported here is advantageous due to its simple operation, rapid reaction, and easy detection. The RT-LAMP assay is a simple diagnosis tool in which the reaction is carried out at 65°C for 1 h in a regular laboratory water bath or heat block that provides a constant temperature of 65°C. The amplification can be performed in a shorter time than amplification by RT-PCR because there is no time loss due to thermal cycling (Chen et al., 2008). The LAMP method relies on auto-cycling strand displacement DNA synthesis that is performed by a DNA polymerase with high strand displacement activity and a set of two specially designed inner and two outer primers. In the initial steps of the reaction, all four primers are used, but later during the cycling reaction only the inner primers are used for strand displacement DNA synthesis.

The inner primers (FIP, BIP), respectively, each contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA, one for priming in the first stage and the other for self-priming in later stages (Notomi et al., 2000). Therefore, the specificity of the reaction is extremely high because it uses six primers (FIP, BIP, F3, B3, FL and BL) recognizing eight distinct regions on the target DNA. LAMP technology facilitates the detection of DNA or RNA of pathogenic organisms and, as such, is the basis for a broad range of clinical diagnostic tests for various infectious agents, including viruses and bacteria (Parida, 2008).

RT-LAMP assays for rapid detection of some of the recently emerged human viral pathogens viz. West Nile,

SARS, dengue, Japanese encephalitis, Norwalk, chikungunya, H5N1 highly pathogenic avian influenza (HPAI) viruses have been developed and evaluated (Hong et al., 2004; Imai et al., 2006; Parida et al., 2004, 2005, 2006, 2007; Toriniwa et al., 2006).

On comparison to conventional RT-PCR, RT-LAMP assay demonstrated RT-LAMP which has 10 to 100 fold more sensitivity than RT-PCR in all these cases, with all Nested-PCR-positive clinical samples being positive by LAMP. All specimens from uninfected HCV serums were negative by both methods. The data presented in this study suggested that the RT-LAMP assay is more sensitive than RT-PCR by picking up 5 additional cases that were negative by RT-PCR. It is pertinent to note that all 5 of these samples were collected from confirmed cases of HCV.

## Conclusion

In summary, we describe a LAMP-based method for the detection of HCV. This method is rapid, sensitive, and specific. Hence, LAMP can be a simple and valuable tool for the rapid diagnosis of infectious diseases by using very basic facilities and should be easily applicable in the clinical laboratories of developing countries.

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