

Original Article

Rapid Low-cost Detection of Hepatitis C Virus RNA in HCV-infected Patients by Real-time RT-PCR using SYBR Green I

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Abstract

Background: We intend to design and validate a low-cost assay for the detection of hepatitis C virus (HCV) RNA using rapid-cycle RT-PCR. The procedure is performed in a closed system with little risk of contamination allowing PCR and product identification to be performed within one or two hours.

Methods: A SYBR Green-based real-time RT-PCR for rapid detection of HCV. Amplicon synthesis was monitored continuously by SYBR Green I, which binds to double stranded DNA during PCR. The PCR products were identified by melting curve analysis. Standard sera with known concentrations of HCV RNA and 150 clinical samples were used to validate our assay.

Results: The minimum detection level of our assay was less than 50 IU/mL. The results on 100 plasma samples were comparable with commercial assays.

Conclusions: This method is useful for rapid qualitative detection of HCV infection and particularly suitable for routine diagnostic applications.

Keywords: hepatitis C virus, rapid detection, real-time RT-PCR, SYBR Green I

Introduction

The hepatitis C virus (HCV) is a single-stranded RNA virus of the *Flaviviridae* family. This virus is one of the most important causes of chronic liver disease, cirrhosis, and hepatocellular carcinoma.¹ Worldwide, it is estimated that over 170 million people are infected with HCV.² The prevalence of HCV infection in Iran's general population is estimated to be less than 1% and the major route of infection appears to be intravenous drug abuse and needle sharing.³

HCV is usually detected by serologic screening for antiviral antibodies using enzyme linked immunosorbent assays (ELISA), and then confirmed with recombinant strip immunoblot assay (RIBA) or PCR.⁴ Blood transfusion organizations routinely check donated blood for HCV using these tests, but the risk of contracting this virus through transfusion still exists. Assays for antiviral antibodies, including ELISA, and RIBA, are unable to detect HCV infection early in its course, leading to false negative results. Furthermore, they cannot differentiate between active and resolved infections.

Nucleic acid amplification tests (NAT) allow the detection of HCV much earlier in the course of infection, often as early as 11 days.^{5,6} These tests can also differentiate between active and past infection. In addition, NAT exhibits a very high sensitivity and

specificity, serving as an independent method for confirming HCV infection.⁶ Thus, it is not surprising that NAT is widely used as a clinical and research tool.^{5,7} Unfortunately NAT is more expensive than ELISA, thus its routine use as a screening tool for blood products or in clinical practice is limited.

Several methods are currently in use for the detection of HCV nucleic acid. These include branched DNA signal amplification,^{8,9} nucleic acid sequence-based amplification,^{6,10} strand displacement amplification,¹¹ ligase chain reaction,¹² transcription-mediated amplification,¹³ and PCR.^{14,15} The first-generation assays are technically demanding, have significant hands-on time, lengthy incubation periods, large sample volume requirements, high cost, are inconsistent in detecting genetic variants, and have a relatively high rate of false positivity and cross contamination.^{6,8,16} These characteristics make them unsuitable for high-throughput screening.

In recent years, PCR has been combined with fluorescence dye technologies in order to detect products during amplification in real-time. This combination allows convenient detection of PCR products and obviates the need for extensive assessment procedures.¹⁷ Another important advance in PCR technology has been the introduction of fluorescence rapid-cycle PCR systems.^{18,19}

Conventional PCR uses gel electrophoresis to identify the amplification product that takes several hours. Contamination of specimens by extraneous nucleic acid may occur from one specimen to another (cross-contamination) which is often the result of pipetting errors or splashes. Of much greater concern is the contamination of the specimen processing area by amplified nucleic acid (amplicons) generated from a previous PCR assay (amplicon carryover). This might lead to false positive results in subsequent PCR assays over a number of days.

We describe the design, optimization, and validation of a one-step real-time RT-PCR technique using SYBR Green I as a fluorochrome. The assay is simple, reliable, and rapid. We combine reverse transcription, amplification of HCV RNA and the detection

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Accepted for publication: 15 March 2011

of amplicons in the same closed glass capillary tube. This closed system minimizes the risk of carryover or contamination.

Materials and Methods

Clinical specimens

Plasma samples were obtained from 100 chronic HCV-infected patients who received combination therapy in different steps (before, 12, 24, and 48 weeks after therapy) and 50 healthy blood donors. HCV-infected samples were obtained from patients referred to a university hospital in Tehran. All patients had chronic HCV infection confirmed by RT-PCR (Cobas Amplicor HCV Monitor Test) carried out in an independent clinical virology lab. The HCV genotype was determined by PCR-RFLP. Genotype 1a was seen in 35 cases, and the rest were of the following genotypes: genotype 1b (7 cases), genotype 2a (1 case), genotype 3a (56 cases), and genotype 4 (1 case). HIV and HBV co-infections were ruled out in all cases.

Whole blood samples were collected into EDTA-containing tubes. The plasma was separated and stored at -80°C for later use.

Viral RNA extraction

To extract and purify HCV RNA, 140 μL of the plasma samples were processed with the QIAamp Viral RNA Mini Kit according to the manufacturer's instructions with slight modification for elution volume (Qiagen, Hilden, Germany). Purified RNA was eluted in 40 μL elution buffer and stored at -80°C . Reference plasma dilutions were similarly treated, in parallel.

Detecting polymerase inhibitors

The LightCycler RNA Control Kit was used for the detection of any Taq DNA polymerase inhibitor that might be present in the RNA preparations of clinical samples.

This kit provides a control RNA template and primers for amplification and specific detection of the human cytokine gene. To identify even weak inhibition events, 300 pg of cytokine RNA diluted in MS2 carrier RNA was amplified.

HCV reference standards

HCV reference preparations calibrated against the WHO Standard 96/798 were obtained from NIBSC (Hertfordshire, United Kingdom). Each vial was reconstituted with 0.5 mL of distilled water to yield 25,000 IU/mL of HCV genotype 1a. According to the data sheet, this concentration is equivalent to 100,000 RNA copies/mL.²⁰ End point dilutions were made to obtain concentrations of 1000, 500, 200, 100, 50, 25, and 10 IU/mL.

Qualitative HCV RNA detection by HCV RT-PCR kit

RNA was purified using the QIAamp viral RNA purification protocol (Qiagen, Hilden, Germany). Then, RT-PCR was performed with the STRP™ Hepatitis C Virus Detection Kit according to the manufacturer's instructions (Cinnagen, Tehran, Iran). Our experience shows that this qualitative assay detects HCV RNA with a lower detection limit of 100 IU/mL (experiments with standard reference dilutions).

RNA quantification by commercial HCV RT-PCR kit

HCV RNA was extracted from plasma samples and quantified by the Real-time TaqMan amplification method according to the manufacturer's instructions. The amount of HCV RNA was ex-

pressed as international unit per milliliter (IU/mL). The lower detection limit is 50 IU/mL according to the manufacturer.

Oligonucleotide primers

HCV 5' NCR-specific primers were used to avoid major mismatches due to HCV genome variability. The oligonucleotide sequences were designed using Gene Runner software. The sequence of sense and antisense primers were: 5'-AGC GTC TAG CCA TGG CGT-3' (HCV TF; 74 – 91) and 5'-GCA CGG TCT ACG AGA CCT-3' (HCV TR; 321-338), respectively. The length of the PCR product was 264 bp.

SYBR Green I-based real time qualitative RT-PCR optimization

For optimization of the assay we used the NIBSC standard panel, clinical samples with known viral loads and diluted samples.

To obtain maximum sensitivity, various concentrations of MgCl_2 and primers were tested. To determine the optimal MgCl_2 concentration for amplification of the template, a magnesium titration series (1 – 5 mM), including samples with varying concentrations, was performed.

Optimal primer concentration was achieved by using samples with different primer concentrations (0.1 – 0.5 μM). To minimize primer dimer formation, the amplification kit recruits Taqstart antibody which prevents primer extension at sub-stringent temperatures (Hotstart). The number of cycles was optimized at 45 so that 50 IU/mL of HCV RNA could be consistently identified.

HCV genotype reactivity

To confirm the ability of our procedure in detecting all genotypes, we tested a reference standard panel with genotypes 1 to 6 (NIBSC, UK) and plasma samples from patients with prevalent genotypes (predominantly: 1a, 1b and 3a; rarely: 2 and 4) in Iran.

Sensitivity and specificity

In order to determine the analytical sensitivity of our assay, RT-PCR experiments were performed on serial dilutions of NIBSC standard RNA, each in ten replicates. To evaluate the specificity, a panel of known plasma samples positive for HIV, HBV, HGV, HSV, and CMV were extracted and tested.

Real-time qualitative RT-PCR assay

SYBR Green I real-time RT-PCR was carried out in a 20 μL reaction volume that contained 5 μL of $5\times$ buffer 1 μL of 10 mM dNTP, 1 μL BSA, 3.5 μL of 25 Mm MgCl_2 , 0.5 μL of 40U/ μL RNase inhibitor (Qiagen, Hilden, Germany), 0.5 μL 1/1000 SYBR Green I dye (Roche, Germany), 0.2 μM of each HCV oligonucleotide primer, 2 μL one step RT mix (Qiagen, Hilden, Germany) and 3 μL of RNA obtained from plasma samples or from scalar dilutions of reference standard RNA. The process was monitored after each elongation step by SYBR Green I dye bound to the amplified 264 bp fragment products. Following the final amplification step, melting curve analysis was performed to identify the PCR product.

The RT-PCR protocol consisted of an RT step at 50°C for 30 min and enzyme activation step at 95°C for 15 min, followed by 45 cycles of denaturation (95°C for 2 seconds), annealing (59°C for 5 seconds), and extension (72°C for 15 seconds). The temperature transition rate was 20°C per second for each step.

Single fluorescence detection was performed in each cycle at 72°C to reveal the positive samples. Melting curve acquisitions were done immediately after the final amplification step by heating

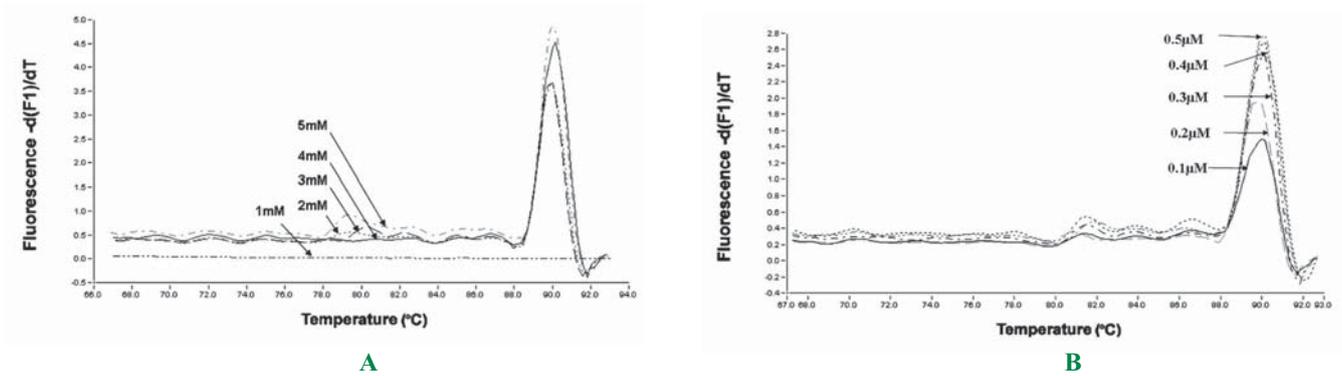


Figure 1. Optimization of the real-time RT-PCR assay. **A)** Amplification curves at various $MgCl_2$ concentrations. The optimal concentration is 4 and 5 mM $MgCl_2$. **B)** Amplification curves at various concentrations of primer. At 0.2 μM good amplification is observed without significant primer dimers.

at 95°C for 2 seconds, cooling to 65°C for 40 seconds, and heating slowly at 0.2°C per second to 95°C with continuous fluorescence recording. Melting curves were recorded by plotting fluorescence signal intensity versus temperature. Amplification, data acquisition and analysis were performed on the LightCycler system using LightCycler 3.5 software (Roche Diagnostics, Mannheim, Germany). All PCR products were run on 2.5% agarose gel electrophoresis.

Product specificity detection

Amplicon melting temperatures (T_m) were determined by calculating the derivatives of the curve using LightCycler software. The results were visualized by plotting the negative derivatives against temperature.

Ethics

The study protocol was approved by the Institutional Review Board and Ethics Committee of the Digestive Disease Research Center of Tehran University of Medical Sciences. All patients signed informed consent forms.

Results

In the first set of experiments, an HCV-positive plasma sample with known titer (10^6 IU/mL, by COBAS Amplicor HCV Monitor Test v2.0, Roche Diagnostics, Mannheim, Germany) was purified and used to optimize the RT-PCR assay on LightCycler. The amplification quality improved with increasing $MgCl_2$ concentrations. Since no significant difference was observed between 4 mM and 5 mM, an $MgCl_2$ concentration of 4 mM was used throughout the study (Figure 1A). Primer concentration was optimized by testing samples with different primer concentrations (0.1 – 0.5 μM). The concentration of 0.2 μM was selected since it produced better amplification without primer dimer formation (Figure 1B).

The dynamic range of our assay covered four orders of magnitude from 10^2 to 10^6 IU/mL. HCV-specific fluorescent peaks were detected in the positive plasma samples and standard panel dilutions to 50 IU/mL, but in none of the negative controls (Figure 2). The detection limit was consistently observed to be 50 IU/mL.

SYBR Green I fluorochrome binds all amplicons without distinguishing between HCV and non-HCV products. Thus, the amplification product was characterized by melting curve analysis. The HCV amplicon can be easily distinguished by its specific T_m value. Under our experimental conditions, analysis of the melting curve

profile of the PCR products indicated that the products peaked at about 90.0°C (Figure 3). The T_m for the plasma samples was slightly shifted to greater than 90.0°C. This phenomenon was due to sequence variability between positive samples.

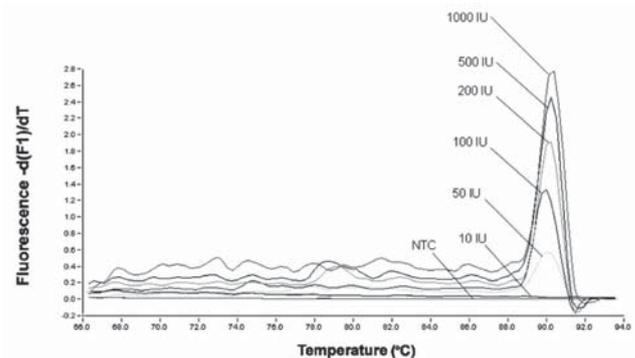


Figure 2. Analytical sensitivity of the real-time RT-PCR test showing a minimum detection limit of 50 IU/mL.

Agarose gel electrophoresis results confirmed the size of the amplicon. A 264 bp band specific for HCV was detected in the reference samples and all HCV-positive sera (analyzed in duplicate) but in none of the negative controls (Figure 4).

None of the plasma with known HIV, HBV, HGV, HSV, and CMV tested positive with our assay. The 100 HCV-positive plasma samples included all genotypes common in Iran (i.e., 1a, 1b, 2, 3a, and 4). The viral loads ranged from 211 to 39,600,000 IU/mL. All samples were tested by a qualitative HCV RT-PCR kit (Artus HCV RT-PCR Kit) and our assay. All samples that tested positive with the Artus RT-PCR Kit also tested positive with our assay. Therefore, the sensitivity and specificity of our assay was 100%, which was comparable to reference and commercial methods.

The standard panel included genotypes 1 through 6, which were all detected by our technique.

Discussion

The viral load in patients with chronic HCV infection is sometimes considerably lower than 5000 IU/mL,²¹ especially in those receiving treatment.¹⁸ Therefore, the Committee of Proprietary Medical Products (CPMP) of the European Union has released recommendations that require testing at higher sensitivities and the introduction of a run control with an HCV RNA concentration of 100 IU/mL.²²

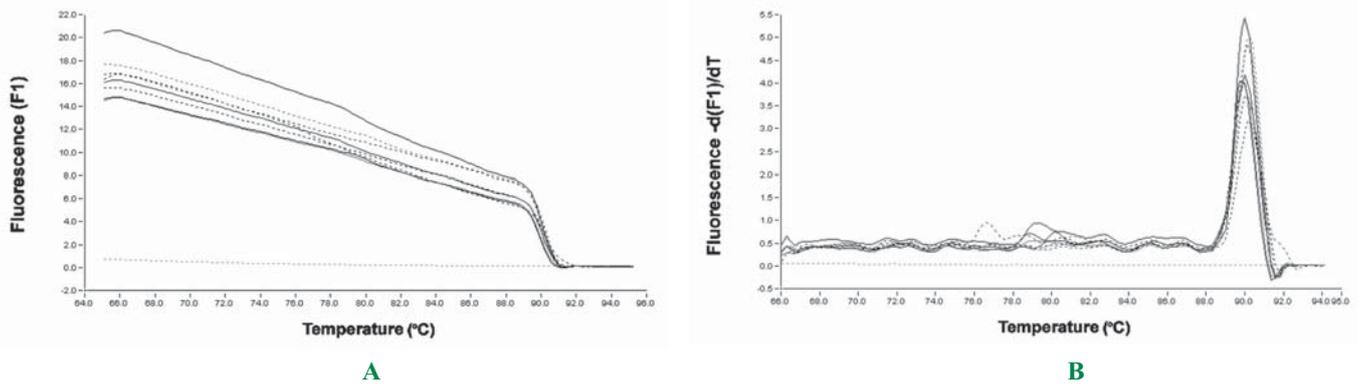


Figure 3. Calculation of amplicon T_m on patient samples (dots) and HCV reference standards (solid lines). **A)** Melting curves obtained after real-time RT-PCR. **B)** Plot of the negative derivative of the melting curves vs. temperature: peaks indicate the T_m of samples.

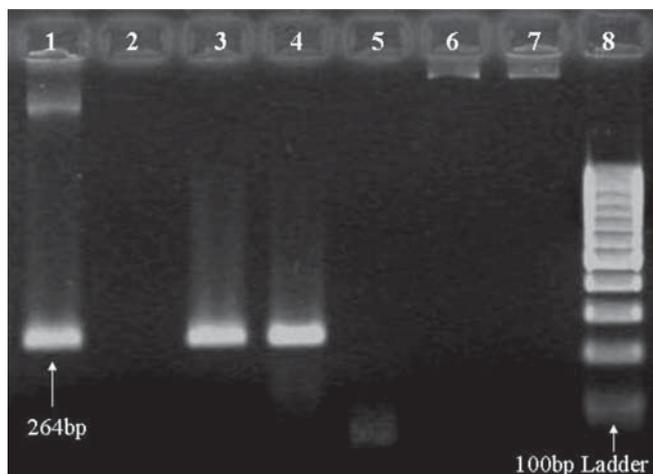


Figure 4. Agarose gel electrophoresis analysis of patients samples (lanes 1, 3, and 4), a healthy control (lane 2) amplified by SYBR Green I real-time RT-PCR assay, No template control (Lane 5), cDNA directly applied (lanes 6 and 7). Lane 2 represents molecular marker (100bp).

Conventional PCR techniques have been relatively cumbersome, difficult to interpret and prone to contamination.²³ During recent years, real-time PCR has gradually demonstrated its potential utility in the field of clinical virology and specifically, in the detection of HCV RNA.^{17,23} The advantages of this new technology over conventional PCR include higher sensitivity, higher specificity, possibility of accurate quantification, and most importantly, reduction of the likelihood of contamination.²⁴

Our assay identifies HCV genome by melting curve analysis of the product of amplification using SYBR Green I. SYBR Green I is a fluorescent molecule that has many advantages over other real-time PCR detection formats such as labeled probes. SYBR Green I is inexpensive and technically simple to use, whereas labeled probes are expensive and add complexity to both the oligonucleotide design and the parameters of the classical amplification reaction.²⁵ PCR artifacts may be noted, especially at amplification cycles beyond the 30th and the selected target sequence may undergo mutations. Such mutations may render the target sequence undetectable by specific probes (i.e., TaqMan).²⁶ Whereas in SYBR Green-based methods such artifacts are easily recognized during melting curve analysis.²⁷

During optimization of our assay, we focused on the compatibil-

ity of oligonucleotide pairs during amplification and on minimizing potential non-specific interactions such as primer dimers. In particular, the determination of primer and $MgCl_2$ concentrations were pivotal factors in enhancing sensitivity and specificity.

The SYBR Green I real-time RT-PCR assay depends on melting curve analysis to specifically identify the target. The T_m of the HCV melting curve was detected at 90.0°C with slight variations due to sequence variability between positive patients.

Although this assay is a qualitative technique, analysis of known reference plasma dilutions can give an approximate indication of its sensitivity. The analytical sensitivity of the assay was determined at 50 IU/ml for HCV RNA. This analytical sensitivity is below that required by European guidelines for blood safety.⁸

Viral loads between 10^5 and 10^6 IU/mL have been reported during the window period.^{26,28} In addition, the ramp up doubling time in the window period was calculated as 0.1 day for HCV, suggesting that our assay's sensitivity will be sufficient to detect HCV infection in this phase.²⁶ One of the advantages of this assay is its low cost. Commercial qualitative HCV RT-PCR per sample costs in different private laboratories in Iran range from \$40 to \$80, while our developed assay takes \$14.2 to obtain the same results, therefore it is \$25.8 less than the minimum available cost and at least four times less expensive than the average cost of this procedure in the routine Iranian market.

Another advantage is the short time required for performing this assay. The average time between starting the procedure to the end of the procedure is 20 min for RNA extraction and 50 min for amplification. The total time for this procedure will be on average 70 min, while routine procedures in the market take around 180 min (minimum 120 min and maximum 240 min).

In conclusion, besides a good level of sensitivity and specificity, the ease of handling, relatively low cost, low contamination rate and rapid analysis makes our assay potentially suitable for both routine clinical use and large-scale screening of donated blood. Given the better than required sensitivity, pooling blood samples may further reduce the cost of this assay in settings such as blood transfusion organizations.

Acknowledgments

The authors would like to thank Day General Hospital Laboratory for technical assistance. This study was supported by a grant from the Digestive Disease Research Center of Tehran University of Medical Sciences.

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