Development of a low-cost real-time reverse transcriptase-polymerase chain reaction technique for the detection and quantification of hepatitis C viral load

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Abstract

Background: It is necessary to develop a highly specific and sensitive assay to quantify the exact amount of hepatitis C virus (HCV) RNA in blood of patients with hepatitis C. For this reason, a real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay for quantification of HCV RNA in human plasma was developed.

Methods: A pair of primers as well as hybridization probes were selected. A real-time RT-PCR was set up and optimized. To establish the sensitivity of the assay, a serial dilution of HCV standards and reference sera, including the six major HCV genotypes, was used. The performance of the assay was evaluated with 191 known HCV-RNA positive and 100 negative samples.

Results: The real-time assay had a sensitivity of 50 IU/mL, with a dynamic range of detection between 105 and 109 IU/mL. The coefficients of variation of threshold cycle values in intra- and inter-day-runs were <1.77% and 3.40%, respectively. Measurement of HCV-RNA positive samples yielded reproducible data with 100% specificity.

Conclusions: The high sensitivity, simplicity, reproducibility, wide dynamic range, and low cost of this real-time HCV RNA quantification makes this method especially suitable for monitoring viral load during therapy and tailoring of treatment schedules accordingly.


Keywords: hepatitis C virus; hybridization probe; real-time reverse transcriptase-PCR; viral load.

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Introduction

Hepatitis C virus (HCV) is a positive-strand RNA virus from the Flaviviridae family. It is the major cause of transfusion-associated and community-acquired non-A, non-B hepatitis (1, 2). It is estimated that HCV affects 200 million people worldwide. Acute HCV infection is generally asymptomatic, but it progresses to chronic disease in about 85% of cases. Persistent HCV infection is associated primarily with chronic liver disease, which progresses to cirrhosis and hepatocellular carcinoma over time (3). It is known that many viral factors, such as HCV genotype, degree of viral diversity in one patient, and viral load correlate with disease activity, extent of liver damage, and response to antiviral therapy (4, 5). Among these parameters, viral load is routinely used for guiding treatment, especially in patients infected with genotype 1 (6). During antiviral therapy, HCV can circulate in blood with a low copy number and its genome is very heterogeneous, even though the virus titer can show a wide range from being undetectable to more than 107 copies/mL. For this reason, detection and quantification of HCV RNA in plasma requires highly specific and sensitive assays (7–9).

Different commercial assays have been used by many investigators to quantify the viral load in HCV infected patients during treatment. But many of these assays have limited dynamic range, and for exact quantification of samples, dilution and retesting is necessary (10–12). In competitive and non-competitive quantitative polymerase chain reaction (PCR) methods, the level of amplified products is measured at the end of the amplification process. Since PCR amplification has an exponential nature, a very small change in amplification efficiency can lead to dramatic differences in the amount of final product (13–15). In addition, these quantitative assays produce results that have considerable differences (16–18). To avoid these problems, quantitative PCR assays are necessary for measurement of the product during the logarithmic phase and before the plateau (19).

In the recent years, real-time reverse transcriptase-PCR (RT-PCR) analysis has been employed successfully both for basic research and clinical applications (20–22). Several groups of investigators have reported the use of real-time RT-PCR assay for detection and quantification of HCV RNA (23). In real-time RT-PCR technique, kinetic quantification is measured during the log-phase of PCR (24).

Recently, commercial HCV RNA viral load assays are widely used for early diagnosis and monitoring of HCV infection. These assays are frequently expensive, often over $150, and sophisticated.
In the present study, we describe a specific, sensitive, reproducible, and low cost real-time RT-PCR assay developed inhouse using specific primers and hybridization probes located in a highly conserved 5'-non-coding region (5'NCR) of HCV genome that quantifies HCV RNA in plasma samples.

Materials and methods

Material used for analytical evaluation

In order to validate the assay, a number of plasma samples with known viral loads were used. These samples, which belonged to patients with different viral loads (from low to high titer), had been quantified with the Cobas Amplicor HCV Monitor Test, version 2.0 (Roche Diagnostics, Mannheim, Germany).

HCV reference standards

HCV RNA reference preparations calibrated against the WHO Standard 96/798 were obtained from the National Institute for Biological Standards and Control (NIBSC) (Hertfordshire, UK). Prior to the RNA purification procedure, each vial of HCV stock was reconstituted with 0.5 mL of distilled water, then diluted in HCV-negative plasma and mixed to achieve several HCV dilutions (25).

Clinical specimens

Plasma samples were collected from 191 patients with chronic hepatitis C who were not under treatment, and 100 HCV seronegative blood donors. Samples were immediately stored in five aliquots in RNAse/DNAse/pyrogen free tubes at –70°C for further use.

All patients tested positive for HCV RNA and negative for human immunodeficiency virus. Other causes of hepatitis were excluded by appropriate serological testing and liver histology. The HCV genotype was determined by PCR-restriction fragment length polymorphism (RFLP) (26). The genotype was further confirmed by direct sequencing in 20 patients. There were 78 genotype 1, and 113 genotype 2 patients with different viral loads (from low to high titer), had been quantified with the previously published sequences of HCV (Gene Bank accession no. D10934).

RNA extraction

For the preparation of total RNA from human plasma, we used the QIAamp Viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions except for a slight modification for elution volume.

Optimization of the RT-PCR

The conditions for the RT-PCR were optimized for the annealing temperature, TaqDNA polymerase, primer, MgCl₂ and dNTPs concentrations. Both RT and LightCycler master mixes were assembled with a minimal pipetting volume of 2 μL, to minimize pipetting errors and to improve homogeneity between all reaction capillaries.

Reverse transcription

First strand cDNA synthesis was performed in a total volume of 20 μL using the Expand RT kit (Roche Molecular Biochemicals, Mannheim, Germany) containing 50 U reverse transcriptase, 20 U RNase inhibitor, 4 μL 5× reaction buffer, 2 μL DTT (10 mM), 2 μL dNTP Mix (1 mM), 2 μL specific primer (anti-sense) KY78s (50 μM) and 1 μg total RNA, as recommended by the manufacturer.

Serial dilutions of NIBSC Standard Panel were reverse transcribed and 1/20 of each reaction was used for generation of a standard curve (Figure 1). Following cDNA synthesis for 60 min at 42°C and inactivation of the enzyme at 95°C for 5 min, PCR was performed in the LightCycler. Known HCV-negative human serum was used for preparing the dilutions.

Real-time PCR assay

In order to prepare a standard curve of HCV RNA, a 10-fold serial dilution of HCV standard panel (10⁻¹⁰–10⁻⁶ IU/mL) was transcribed into cDNA, which was then amplified by the real-time PCR. The LightCycler system (Roche Diagnostics, Mannheim, Germany) was used for real-time analysis. For product analysis, a pair of FRET hybridization probes was selected to hybridize to the product between the primers. The upstream probe was labeled with fluorescein at its 5’ terminus to serve as donor; the downstream probe was labeled with LightCycler Red 640 at its 3’ terminus to serve as acceptor for FRET. For each PCR reaction, 1 μL of cDNA template was added to 19 μL of PCR Master Mixture comprised of 2.8 mM MgCl₂, 22 μM of each of KY80s and KY78s primers (20 pmol/μL stock solutions), 0.2 μM each of the FRET hybridization probes HCVFL and HCVLC (20 pmol/μL stock solution) (TIB MOLBIOL, Berlin, Germany) and 2 μL LC FS HP mix (LightCycler Fast Start DNA Hybridization Probes, Roche Diag-

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>D10934</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KY80s</td>
<td>AGCGTCTAGCCCATGGCAGTT</td>
<td>S</td>
<td>74–91</td>
</tr>
<tr>
<td>KY78s</td>
<td>CAAGACCACCTATCAGGCCAGT</td>
<td>A</td>
<td>288–308</td>
</tr>
<tr>
<td>HCVFL</td>
<td>GCGAGCCCTCAGGACCCCCC X</td>
<td>A</td>
<td>107–125</td>
</tr>
<tr>
<td>HCVLC</td>
<td>LC640-CCGGGAGAGCCATAGTGGTCTG P</td>
<td>A</td>
<td>128–150</td>
</tr>
</tbody>
</table>
Figure 1 Analysis of real-time RT-PCR sensitivity and linearity with HCV standard RNA. HCV standards RNA (10^3–10^6 IU/mL) were amplified by real-time RT-PCR.

(A) Amplification plot of 10-fold serial dilutions of HCV standard RNA. (B) The standard curve of 10-fold serial dilutions of HCV standard RNA showed a correlation coefficient (R^2) of 0.997. The curve was generated based on data shown in Table 2. The threshold cycle (Ct) values are the mean of 10 runs.

Quantification of the HCV genome in samples was performed using an external standard curve. From the fluorescence intensities recorded during each cycle of PCR, the baseline fluorescence was determined using baseline adjustment set to arithmetic mode (LightCycler Software version 3.5). Next, the threshold cycle (Ct) i.e., the first cycle with fluorescence intensities exceeding the baseline, was determined by applying the "second derivative maximum" calculation using two points. The noise band which cuts off the baseline fluorescence was then adjusted to 0.02 with the fluorescence channels set to F2/F1.

Having set the noise band, the crossing points used as measurement for quantification were automatically calculated. A standard curve was then generated using the NIBSC standard diluted to 1,000,000, 100,000, 10,000, and 1000. In addition, one negative control was assayed in parallel with each batch of samples tested.

Analysis of raw data

The quantitative analysis of the resulting LightCycler fluorescence raw data was performed using LightCycler Software version 3.5. The background readings were subtracted from the raw data and analyzed using the second derivative maximum method. The threshold value was adjusted to minimize the error of the calibration curve.

Gel electrophoresis of PCR product

For verification of exact length, LightCycler PCR products were separated by gel electrophoresis. Amplified HCV LightCycler PCR products were removed from the glass capillaries by reverse centrifugation into 1.5 mL reaction tubes. Samples were diluted in agarose gel loading buffer and loaded onto 2% agarose gels in 1× TBE buffer. Gel analysis was performed with Bio Doc analyzer (Biometra, Goettingen, Germany).

Specificity

Specificity of the assay was ensured by the selection of the primers and probes. The primers and probes were checked for possible homologies in Gene Bank's published sequences by sequence comparison analysis (Blast).
Table 2  Real-time RT-PCR analytical sensitivity evaluated by dilution analysis of plasma standards.

<table>
<thead>
<tr>
<th>HCV, IU/mL</th>
<th>Replicates (detected/replicates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,000,000</td>
<td>10/10</td>
</tr>
<tr>
<td>100,000</td>
<td>10/10</td>
</tr>
<tr>
<td>10,000</td>
<td>10/10</td>
</tr>
<tr>
<td>1000</td>
<td>10/10</td>
</tr>
<tr>
<td>500</td>
<td>10/10</td>
</tr>
<tr>
<td>100</td>
<td>10/10</td>
</tr>
<tr>
<td>50</td>
<td>10/10</td>
</tr>
<tr>
<td>25</td>
<td>10/10</td>
</tr>
</tbody>
</table>

In addition, the specificity was validated with 100 different HCV-negative plasma samples. Potential cross-reactivity of the assay was studied using available controls including human immunodeficiency virus 1, hepatitis B virus, hepatitis G virus, herpes simplex virus type 1 and 2 and human cytomegalovirus. In each case, samples from two different patients were used and each sample was tested in duplicate. In addition to patient samples, in cases with human immunodeficiency virus, hepatitis B virus, and herpes simplex virus, standards from NIBSC and VQC were also tested.

Commercial real-time RT-PCR

RNA was purified from patient samples using the QIAamp viral RNA purification protocol (Qiagen, Hilden, Germany). Quantification of HCV RNA was performed using the Artus HCV RT-PCR kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. This assay exhibits a lower detection limit of 50 IU/mL, according to the manufacturer.

Ethics

Informed consent was obtained from all patients. 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.

Results

Specificity

None of the 100 HCV-negative plasma samples and controls, including human immunodeficiency virus 1, hepatitis B virus, hepatitis G virus, herpes simplex virus type 1 and 2 and human cytomegalovirus, produced any signal. No cross-reactivity was observed with any of these samples.

Analytical sensitivity

In order to determine the assay’s lower limit of detection, PCR experiments were performed using serial 10-fold dilutions of reference standards obtained from NIBSC (genotypes 1, 2 and 5). Experiments were performed in duplicate with the amplification mixture containing two primers and two hybridization probe oligonucleotides. With the developed assay, 50 IU/mL of HCV could be detected in 10 of 10 replicates (100%).

The limit of detection was defined as the lowest concentration that yields positive results in 95% of the replicates. We determined the detection limit to be 50 IU/mL (Table 2).

Quantitative analysis of HCV in 10-fold serial dilution of standard RNA ($10^3$–$10^6$ IU/mL) was performed over 3 consecutive days (inter-assay) with quadruplicate measurements of each dilution in one run (intra-assay) using real-time RT-PCR. Tables 3 and 4 demonstrate the mean coefficient of variation (CV) of Ct values and the input IU (International Unit) of HCV RNA between different runs (Table 3), and within one run (Table 4).

Linearity and reliability of real-time PCR

Amplification of HCV standard RNA at different concentrations showed that the linear range extended over four orders of magnitude (Figure 1). The correlation coefficient ($R^2$) was 0.997 with a slope of $-3.5$. Amplification efficiency, calculated as $10^{\log_{10}(-1)} \times 100$, was 93%.

Reproducibility of the assay

To confirm the reproducibility of real-time RT-PCR in running clinical specimens, five HCV-RNA positive samples with different viral loads were run on 4 days with five different master mixes. Table 5 shows that the CV of these runs was $<1.68%$.

HCV genotype reactivity

In order to determine the utility of real-time RT-PCR for both research and clinical studies on HCV, its ability to identify various HCV genotypes was tested. Total RNA was extracted from 191 clinical cases (genotypes 1 and 3) and from ref-

Table 3  Inter-assay reproducibility of HCV real-time RT-PCR.

<table>
<thead>
<tr>
<th>HCV RNA concentration, IU/mL</th>
<th>Threshold cycle, Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^3$</td>
</tr>
<tr>
<td>Run 1</td>
<td>37.71</td>
</tr>
<tr>
<td>Run 2</td>
<td>35.84</td>
</tr>
<tr>
<td>Run 3</td>
<td>38.26</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>37.27±1.26</td>
</tr>
<tr>
<td>CV, %</td>
<td>3.40</td>
</tr>
</tbody>
</table>

CV, coefficient of variation; SD, standard deviation.
Table 4  Intra-assay accuracy of HCV real-time RT-PCR.

<table>
<thead>
<tr>
<th>HCV RNA concentration, IU/mL</th>
<th>Threshold cycle, Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^3$</td>
</tr>
<tr>
<td>Repetition 1</td>
<td>36.49</td>
</tr>
<tr>
<td>Repetition 2</td>
<td>37.67</td>
</tr>
<tr>
<td>Repetition 3</td>
<td>36.17</td>
</tr>
<tr>
<td>Repetition 4</td>
<td>36.63</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>36.74 ± 0.64</td>
</tr>
<tr>
<td>CV, %</td>
<td>1.77</td>
</tr>
</tbody>
</table>

CV, coefficient of variation; SD, standard deviation.

Table 5  Reproducibility of HCV real-time RT-PCR.

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1</td>
<td>38.30</td>
<td>35.75</td>
<td>31.23</td>
<td>27.72</td>
</tr>
<tr>
<td>Run 2</td>
<td>38.06</td>
<td>35.56</td>
<td>32.05</td>
<td>27.92</td>
</tr>
<tr>
<td>Run 3</td>
<td>38.77</td>
<td>36.16</td>
<td>31.49</td>
<td>27.88</td>
</tr>
<tr>
<td>Run 4</td>
<td>38.53</td>
<td>35.10</td>
<td>32.41</td>
<td>27.87</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>38.41 ± 0.30</td>
<td>35.64 ± 0.43</td>
<td>31.79 ± 0.53</td>
<td>27.84 ± 0.08</td>
</tr>
<tr>
<td>CV, %</td>
<td>0.79</td>
<td>1.23</td>
<td>1.68</td>
<td>0.31</td>
</tr>
</tbody>
</table>

CV, coefficient of variation; SD, standard deviation.

Table 6  Identification of HCV genotypes by real-time RT-PCR.

<table>
<thead>
<tr>
<th>HCV genotype</th>
<th>HCV real-time RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Qualification</td>
</tr>
<tr>
<td>1</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>Positive</td>
</tr>
<tr>
<td>4</td>
<td>Positive</td>
</tr>
<tr>
<td>5</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>Positive</td>
</tr>
</tbody>
</table>

It should be noted that HCV in Iran is almost exclusively genotypes 1 and 3; other genotypes are very rare (genotypes 2 and 4), or are never reported (genotypes 5 and 6). Thus, the clinical samples tested in the present study were exclusively genotypes 1 and 3. Other genotypes were tested using standards obtained from NIBSC.

Comparison of the in-house real-time RT-PCR with a commercial real-time RT-PCR

In order to evaluate the practical application of our real-time RT-PCR assay, we examined HCV RNA in clinical samples and compared the results with a commercial real-time RT-PCR kit (Arthus HCV RT-PCR, Qiagen, Hilden, Germany). A total of 191 clinical samples were tested in parallel using both tests. A typical result of the real-time RT-PCR assay is shown in Figure 2.

All 191 samples that tested positive with the commercial real-time RT-PCR assay also tested positive with the in-house real-time RT-PCR. All 100 samples that tested negative with the commercial kit also tested negative with the in-house real-time RT-PCR. Results obtained with the commercial kit and our in-house real-time RT-PCR assay were compared using linear regression and showed excellent correlation ($R^2 = 0.991$, Figure 3).

Agarose gel electrophoresis was used to confirm the exact length of the amplicon. A 235 bp band, specific for HCV RNA, was detected in HCV reference standards and all HCV-positive samples (Figure 4).
Discussion

We developed an in-house real-time RT-PCR assay for quantification of HCV viral load in plasma samples of patients with hepatitis C. We showed that our assay has good reproducibility and accuracy with a wide dynamic range for detection and quantification of HCV RNA.

It is believed that the amount of HCV RNA in the blood represents the steady state of viral replication and clearance. Therefore, it is important to develop a highly specific and sensitive assay for precise quantification of HCV RNA in the blood of HCV-infected patients. This is especially useful in tailoring antiviral therapy. Previous studies have shown that in patients with chronic HCV infection, the response to treatment is correlated with plasma HCV RNA concentrations (27, 28). Many investigators suggest that viral load is correlated with the degree of liver damage, but these results are controversial. The controversial results from different studies may be caused by lack of reliable methods for HCV RNA quantification (29, 30). Thus, there is an urgent need to develop a quantitative assay that is specific, sensitive, accurate, and reproducible for monitoring the progress of hepatitis C, response to antiviral therapy, and enable comparisons in clinical studies.

A sensitive assay should be able to detect low viral loads, even when patients are receiving effective antiviral therapy (31). Hence, the lower detection limit for diagnostic assays must be 10–50 IU/mL. Our in-house real-time RT-PCR assay has a sensitivity of 50 IU/mL when tested against end point dilutions of NIBSC standard RNA.

The linearity of our real-time RT-PCR assay was established using NIBSC standard RNAs with known viral concentrations. The variability of the inter- and intra-assay precision was not statistically significant using both HCV standard RNA (Tables 3 and 4) and clinical specimens (Table 5). This criterion is a basic requirement for a good quantitative assay. The %CV for both inter- and intra-assay precision was very low, <1.77% for intra-assay and 3.40% for inter-assay.

When applied to clinical specimens (seropositive and negative plasma), HCV real-time RT-PCR had a sensitivity and specificity of 100% and was able to amplify HCV RNA in the reference plasma with six major genotypes (Table 6). These results indicate that the assay is reliable and should

Figure 3 Comparison of a commercial real-time RT-PCR kit with our in-house real-time assay.

Figure 4 Agarose gel electrophoresis analysis of patients (lanes 1, 2, and 3), healthy donor (lane 4) plasma and HCV reference standards (lanes 6, 7, 8, and 9) amplified by real-time RT-PCR assay. Lane 5 represents a 100 bp molecular marker.
be able to find broad applications in the field of HCV research.

Sequence specific real-time PCR systems for quantification is primarily a probe-based analysis. HybProbe has been used for the construction of probes that are critical for real-time detection of nucleic acids hybridization events using the LightCycler system (32). Recent studies have employed HybProbe successfully for a variety of real-time PCR applications (21, 33).

The HybProbe detection format relies on the use of two oligonucleotide probes that hybridize next to each other to a sequence located between the amplification primers. The probes are designed to hybridize during the annealing step to the same strand in a head-to-tail arrangement at a distance of 1–5 nucleotides in order to bring the two dyes into close proximity to one another (hence the name “kissing” probes) (19, 34).

Our data demonstrates that the HCV real-time RT-PCR using HybProbe and primers that target the sequences in the highly conserved 5′NCR of HCV genome are highly specific, sensitive and reproducible, with a wide dynamic range for detection of HCV RNA (10^3–10^9 IU/mL of plasma). The regression coefficient of the standard curve was, on average, >0.997. Thus, there is no need to dilute or concentrate samples. In addition, the HCV real-time RT-PCR assay is much less cumbersome, and the costs associated with this system as a quantitative assay are the same, or less than, conventional RT-PCR assays. Because, there is no need for electrophoresis of the PCR-amplified products after real-time RT-PCR, this assay eliminates post-PCR processing, and also avoids variation and contamination caused by manipulation of post-PCR samples. The test is low cost. It can be performed in the Digestive Disease Research Center for US $22.6 per sample, including reagents for extraction (US $6), “master mix” (US $9), primer and probes (US $0.6), consumables (US $5 for microtubes, capillaries and filter tips), and other material (US $2). This assay costs 5- to 10-fold less than commercial HCV RNA quantitative tests (as charged in Iran). There are other advantages with the use of this assay. The primer and probes can be ordered once a year, given the long shelf-life of these freeze-dried reagents. This is an advantage over some other commercial assays, such as bDNA assays where reagents need to be ordered several times a year and require dry ice.

It should be noted that none of our HCV samples were co-infected with HBV or HIV. However, we see no reason why our test should not perform adequately in patients with co-infections, although we would recommend caution.

In summary, we developed a low-cost, HybProbe-based, real-time RT-PCR assay. We used this assay to successfully measure HCV RNA levels in both experimental and clinical specimens quantitatively. The data, in conjunction with reports by other investigators, demonstrate that this method is particularly useful for quantification of HCV RNA levels in plasma and serum. This sensitive, accurate, and reproducible assay may be an essential tool for diagnostic purposes in the field of HCV infection, and can be applied for assessment of response to antiviral therapy.

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Conflict of interest statement

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