Quantitation of hepatitis C virus using an in-house real-time reverse transcriptase polymerase chain reaction in plasma samples

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Abstract

Even with the most advanced 3rd-generation assays, the serologic window period of hepatitis C virus (HCV) is approximately 74 days. HCV RNA detection would reduce the risk of transmission during this period. Furthermore, quantitation of HCV RNA is necessary for proper planning of treatment, monitoring disease progression, and assessing response to antiviral therapy. We have standardized an in-house HCV real-time reverse transcriptase polymerase chain reaction (RT-PCR) for screening and accurate quantitation and detection of HCV RNA in plasma samples. The in-house real-time assay was compared with a commercial assay using 100 chronically infected individuals and 70 blood donors who are negative for hepatitis B surface antigen, HCV antibody, and HIV antibody. The lower limit of detection of this in-house HCV real-time RT-PCR as assessed against the World Health Organization (WHO) standard was 50 IU/mL. Interassay and intraassay coefficient of variation ranged from 1.3% to 6.4% and 0.0% to 2.3% respectively. Virus loads as estimated with this in-house HCV real-time assay correlated with the commercial artus HCV RG RT-PCR assay (\(r = 0.59, P < 0.0001\)). This assay could be used in screening and monitoring individuals on therapy, showing no genotype-dependent differences in detection.

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Keywords: Hepatitis C virus RNA; In-house real-time RT-PCR; Quantitation

1. Introduction

Approximately 1.85% of the Indian population is infected with hepatitis C virus (HCV), suggesting a national burden of about 19 million carriers (WHO, 1997). Screening of HCV is done mainly by detection of HCV antibody (HCV-Ab). However, even with the most advanced 3rd-generation assays, the HCV-Ab preseroconversion window period is approximately 74 days (Barrera et al., 1995). Furthermore, HCV-Ab assays cannot be used in patients and individuals with suppressed or impaired immunity (Radhakrishnan et al., 2000; Tashkandy et al., 2007). Screening of HCV using nucleic acid amplification techniques (NAT) such as polymerase chain reaction (PCR) will reduce the risk of transmission of HCV and aid in the early detection of HCV infections. Quantitation of HCV is necessary for the proper planning of treatment, monitoring disease progression, and assessing response to antiviral therapy. Many commercial assays are available albeit expensive. NAT for HCV can be done by reverse transcriptase PCR (RT-PCR) (Tashkandy et al., 2007), nucleic acid sequence-based amplification (Guichon et al., 2004), transcription-mediated amplification (Rigopoulou et al., 2005), branched-chain DNA assay (Sarrazin et al., 2006), or real-time RT-PCR (Castelain et al., 2004). The most widely used NAT method is the RT-PCR (Roth et al., 1999). Lately, real-time PCR has replaced the conventional RT-PCR for quantitative detection of most viral pathogens. In this study, we standardized a sensitive in-house HCV real-time RT-PCR for the accurate detection of HCV in plasma samples and compared it with a commercial real-time artus HCV RG RT-PCR assay, the diagnostic assay in use in our laboratory.
2. Materials and methods

2.1. Samples

This study was conducted on 100 patients who were referred from the Departments of Gastroenterology, Nephrology, and Hematology for HCV RNA detection. All patients were recruited after a verbal consent in addition to a general consent that was obtained in our hospital for all investigations as part of routine patient management in our hospital. In addition to these 100 patient samples, 10 each of HIV-1 RNA and hepatitis B virus (HBV) DNA-positive samples were tested. Samples with simulated dual infection HCV RNA and HIV RNA or HBV DNA (n = 5) and triple infection HCV RNA, HIV RNA, and HBV DNA (n = 5) were also tested. The dual and triple infection specimens were prepared using 10 samples each of HIV, HBV, and HCV monoinfection, with viral loads ranging from 10^4 to 10^6 IU/mL for HIV and HCV and 10^5 to 10^8 IU/mL for HBV. Two hundred microliters of each sample was taken and mixed to simulate different combinations of dual or triple infection. For samples simulating dual infection, 200 μL of negative plasma was added to make up the volume to 600 μL of sample. Two hundred microliters of mono/dual/triple infection samples were used for each extraction.

Seventy consecutive blood donor samples collected from individuals who are negative for HIV-1/2 antibody, hepatitis B surface antigen, and HCV antibody (as screened in the blood bank) were used for the standardization of this HCV real-time PCR assay.

Ten milliliters of blood was collected in tubes containing 200 μL of 0.5% EDTA and plasma separated after centrifugation at 1500 rpm for 10 min at 4 °C and stored in multiple aliquots at −60 °C until time of testing. The identity of all the samples including the blood donor samples was coded, aliquoted, and stored at −60 °C for testing within a week. The person performing the assay was blinded to the coded panel. Fresh frozen aliquot of plasma samples were used for testing.

2.2. In-house HCV standard

The in-house standard for HCV real-time RT-PCR was an HCV RNA-positive blood bag with a viral load of 10^6 IU/mL (courtesy of Scudder Memorial Blood Bank, Christian Medical College, Vellore, India). This in-house standard was tested in triplicate in parallel with the WHO 2nd HCV international standard 96/798. This WHO standard was tested in duplicate in log dilutions up to 5 IU/mL to generate the standard curve. Tenfold dilutions of this standard were made down to 10 IU/mL using normal human plasma and tested in all the runs to generate standard curve. Dilutions of this in-house standard were tested at log dilutions from 10^4 to 1 IU/mL in batches of 4 replicates on 6 separate runs, with a total of 24 replicates for each dilution to determine the positive cutoff point or lower limit of detection (Official Control Authority Batch Release of Blood Products [OCABR], 2001).

Interassay and intrasay variation for this in-house real-time RT-PCR assay was calculated using a panel of 3 samples with varying viral loads (10^6, 10^5, and 10^3 IU/mL) tested in triplicate in 3 different assays on different days.

2.3. HCV genotyping

This in-house real-time RT-PCR was further evaluated for its capacity to detect the various genotypes prevalent among individuals seen in this tertiary care center using samples that were collected from those who came to the Department of Clinical Virology with a request for HCV genotyping. HCV genotyping was performed using sequencing of the NS5b region of the HCV genome (Harris and Teo, 2001).

2.4. Nucleic acid extraction

This assay was performed using brome mosaic virus (BMV) (Promega, Madison, WI) as the internal control (IC) to rule out false negatives. Plasma viral RNA was extracted by using QIAamp MinElute Virus spin kit (QIAGEN, Hilden, Germany). This extraction protocol was used in this assay with a view to develop this assay as a multiplex screening assay in a blood bank. Five microliters of 5 × 10^-6 μg/μL of BMV RNA was added to 1 mL of lysis buffer (AL) with 22.4 μg of carrier RNA. RNA was extracted using 200 μL plasma sample as per kit protocol. Elution was done using 60 μL of elution buffer (AVE) provided by the manufacturer.

2.5. Amplification

Probes for the detection of HCV RNA amplifying the 5′ non-coding region (NCR) of the HCV genome and BMV RNA were labeled with 6-carboxyfluorescein (FAM) and 6-carboxy-4′,5′-dichloro-2′, 7′-dimethoxyfluorescein (JOE), respectively, as the reporter dye at the 5′ end and with 6-carboxy-tetramethyl-rhodamine (TAMRA) as the quencher.

### Table 1
Primer names used for in-house HCV real-time RT-PCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequences</th>
<th>Nucleotide position</th>
<th>Product size (base pair)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV TAQ 1</td>
<td>GTC TAG CCA TGG CGT TAG TA</td>
<td>77–96</td>
<td>90</td>
</tr>
<tr>
<td>HCV TAQ 2</td>
<td>GTA CTC ACC GGT TCC GC</td>
<td>166–150</td>
<td></td>
</tr>
<tr>
<td>HCV TAQ PR</td>
<td>FAM-CCC TCC CGG GAG AGC CAT AGT G-TAMRA</td>
<td>124–145</td>
<td></td>
</tr>
<tr>
<td>BMV TAQ 1</td>
<td>GTT CAC CGA TAG ACC GCT G</td>
<td>364–382</td>
<td>71</td>
</tr>
<tr>
<td>BMV TAQ 2</td>
<td>AAG AGC CCG GAA TGTCAA GA</td>
<td>415–434</td>
<td></td>
</tr>
<tr>
<td>BMV TAQ PR</td>
<td>JOE-CCT CAA GCT GAA ATG GCA CGG ATG-TAMRA</td>
<td>386–409</td>
<td></td>
</tr>
</tbody>
</table>
dye at the 3′ end. The primer and probe sequence used in this study were subjected to an HCV BLAST search, which indicated that the primer and probe had a high probability of amplifying all the 6 genotypes of HCV. An HCV primalign search indicated that this primer and probe sequence showed identity with 97% of strains in the HCV database with a maximum of up to 1 nucleotide difference (http://hcv.lanl.gov). Of the 161 strains used by the HCV database to compare with our primer and probe sequences, 2 strains (1.2%) of genotype 1b did not have homology. Amplification was performed using 10 μL of extract in a 25-μL volume containing 12.5 μL of 2× QuantiTect Probe RT-PCR buffer, 0.25 μL of RT enzyme mix (QIAGEN), 20 pmol of HCV primers (HCV TAQ 1, HCV TAQ 2) and BMV primers (BMV TAQ 1, BMV TAQ 2), and 10 pmol of HCV probe (HCV TAQ PR) and BMV probe (BMV TAQ PR) (Metabion, Martinsried, Germany). The primer and probe sequences are provided in Table 1. Primer and probe sequences for this in-house HCV real-time RT-PCR was kindly provided by Prof Richard Tedder (University College London, London, UK). The reaction mixture was amplified using the following thermal cycling conditions: 50 °C for 30 min for the RT step followed by 95 °C for 15 min and amplification for 45 cycles at 95 °C for 15 s and 60 °C for 60 s. Amplification and detection of HCV RNA and the IC were done using Rotor-Gene™ 3000 (Corbett Research, New South Wales, Australia). Standard curves were generated using the built-in software (Rotor-Gene version 6.0) in the Rotor-Gene™ 3000. Based on the fluorescent intensities of the sample, the HCV RNA viral load was derived from the standard curve. The IC serves as a control to rule out PCR inhibitors during the extraction and the amplification step. Samples found negative for HCV RNA with no amplification of the IC would be repeated. A sample result was accepted only when IC was amplified.

2.6. artus HCV RG RT-PCR assay

HCV RNA isolation was done using QIAamp viral RNA mini kit, which was supplied along with the artus HCV RG RT-PCR assay for extraction of nucleic acid from plasma samples. Elution was done using 50 μL of AVE buffer (provided by the manufacturer). This assay amplified a 240-bp region in the 5′NCR of the HCV genome, which was detected by the cycling A.FAM channel. This assay also uses an IC which was detected by the cycling A.JOE channel. The IC was added to the PCR mix. All the 4 standards provided by the manufacturer were preextracted. Amplification and detection of HCV RNA was done using Rotor-Gene 3000 as per manufacturer’s instructions. A sample result was accepted only when IC was amplified.

3. Results

3.1. Calibration of in-house standard against 2nd WHO international standard for HCV RNA 96/798

The viral load of this sample as assessed against the 2nd WHO international standard for HCV RNA 96/798 was 10^6 IU/mL. All the samples showed amplification using the IC BMV RNA. The results of the in-house standard tested in batches of 4 replicates in 6 separate runs (24 replicates at each dilution) are given in Table 2. The amplification plots obtained using the in-house standard and the standard curve are shown in Figs. 1 and 2.
3.2. Sensitivity (lower limit of detection)

Log dilutions of the 2nd WHO international standard for HCV RNA 96/798 were done to assess the sensitivity (analytic sensitivity) of this in-house HCV real-time RT-PCR. The sensitivity of this in-house HCV real-time RT-PCR as assessed by testing in duplicate the 2nd WHO international standard for HCV RNA 96/798 (supplied by the National Institute for Biological Standards and Control, UK) in log dilutions up to 5 IU/mL was 50 IU/mL. The analytic sensitivity of this assay calculated based on the probit analysis is 39 IU/mL using the HCV RNA-positive blood bag with a viral load of $10^6$ IU/mL.

3.3. Specificity

Seventy blood donor samples that were negative for HCV antibody were negative by this in-house assay. Ten samples each that were positive for HIV and HBV were also negative in this HCV real-time assay. Five samples with simulated dual infection (HCV with HIV or HBV) and triple infection (HCV, HIV, and HBV) were also correctly identified.

3.4. Reproducibility

The intraassay coefficient of variation (CV) of this in-house HCV real-time RT-PCR ranged from 0.0% to 2.3% (Table 3). The interassay CV of this in-house HCV real-time RT-PCR ranged from 1.3% to 6.4% (Table 4). When the actual copy numbers were used for calculation of CV, the interassay and intraassay CV ranged from 1% to 40% and 14% to 91%, respectively.

| Table 3: Intraassay variation of HCV viral RNA load in the in-house real-time HCV RT-PCR assay |
|---|---|---|---|---|---|---|
| Sample ID | Assay no. | Mean log (IU/mL) | SD | CV % |
| 06/H-1081 | 06/H-1085 | 06/H-1083 | 06/H-1081 | 06/H-1085 | 06/H-1083 |
| 1 | 2 | 3 | 1 | 2 | 3 |
| 4.6 | 4.6 | 4.7 | 4.6 | 0.058 | 1.3 |
| 4.6 | 4.6 | 4.7 | 4.6 | 0.058 | 1.3 |
| 6.0 | 6.2 | 5.9 | 6.0 | 0.153 | 2.6 |
| 7.5 | 7.1 | 6.6 | 7.1 | 0.451 | 6.4 |

HCV viral RNA load values are expressed in log units (IU/mL).

All values are shown in log units (IU/mL).

3.5. Correlation between artus HCV RG RT-PCR real-time assay

The in-house HCV real-time RT-PCR viral load compared well with the commercial artus HCV RG RT-PCR assay viral loads ($r = 0.59$, $P < 0.0001$, 95% CI for $r$, 0.45–0.71) (Fig. 3). The Bland–Altman plot showing the differences of the 2 assays is shown in Fig. 4. When different genotypes were compared, genotype 4 showed good correlation ($r = 0.8$), genotypes 3 and 1 showed moderate correlation ($r = 0.59$ and 0.69, respectively), but genotype 6 showed very poor correlation ($r = -0.29$) (not depicted in Fig.).

3.6. Detecting HCV genotypes prevalent in this region

Hundred plasma samples genotyped using the NS5b sequencing method were tested using this in-house HCV real-time RT-PCR. This in-house HCV real-time RT-PCR detected all the genotyped samples tested in this study. The

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**Table 4:** Interassay variation of HCV viral RNA load in the in-house real-time HCV RT-PCR

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Assay no.</th>
<th>Mean log (IU/mL)</th>
<th>SD</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>06/H-1081</td>
<td>06/H-1085</td>
<td>06/H-1083</td>
<td>06/H-1081</td>
<td>06/H-1085</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>4.6</td>
<td>4.6</td>
<td>4.7</td>
<td>4.6</td>
<td>0.058</td>
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<tr>
<td>4.6</td>
<td>4.6</td>
<td>4.7</td>
<td>4.6</td>
<td>0.058</td>
</tr>
<tr>
<td>6.0</td>
<td>6.2</td>
<td>5.9</td>
<td>6.0</td>
<td>0.153</td>
</tr>
<tr>
<td>7.5</td>
<td>7.1</td>
<td>6.6</td>
<td>7.1</td>
<td>0.451</td>
</tr>
</tbody>
</table>

HCV viral RNA load values are expressed in log units (IU/mL).

**Fig. 3:** Scatter plot comparing the HCV viral RNA load between artus HCV RG PCR assay with in-house HCV real-time RT-PCR assay ($n = 100$).

**Fig. 4:** Bland-Altman plot depicting the correlation of artus HCV RG RT-PCR assay viral load with in-house HCV real-time RT-PCR assay ($n = 100$). The graph displays a scatter diagram of the differences plotted against the averages of the 2 measurements. Horizontal lines are drawn at the mean difference and at the mean difference plus and minus 1.96 times the SD of the differences.
different HCV genotypes tested in this study were genotype 1 (n = 15), genotype 3 (n = 71), genotype 4 (n = 7), and genotype 6 (n = 7). The viral load of the genotyped samples ranged from 182 to 2.4 × 10⁸ IU/mL. Using Kruskal–Wallis test (SPSS 11.0; SPSS Inc., Chicago, IL), the in-house real-time RT-PCR did not demonstrate a significant difference in virus loads across these genotypes (P = 0.358).

4. Discussion

We describe an in-house real-time RT-PCR that quantitates the viral load in plasma samples of HCV-infected individuals with acceptable accuracy. This assay has good analytic sensitivity with a good dynamic range for detection of HCV RNA.

Diagnostic laboratories use different commercial assays for HCV RNA quantitation. These commercial assays are reliable, but many of these have limited dynamic range, so clinical samples will have to be diluted and retested to get the exact viral load. This in-house real-time RT-PCR exhibits a wide dynamic range of approximately 1 × 10⁶ IU/mL, and hence, samples do not have to be diluted and retested. However, newer real-time quantitative assays have wide dynamic range, and samples need not be diluted and retested.

HCV viral load estimation is an accurate marker for active HCV replication and is, hence, used to monitor patients while on therapy (Kim et al., 2005; Neumann et al., 2000). This should, in all probability, detect most of the HCV RNA-positive samples during therapy, where efficacious drugs are expected to lower the viral load. Low HCV viral RNA load may not be detected by assays that are not sensitive. Hence, it is suggested that diagnostic assays must have a lower detection limit of 10 to 50 IU/mL (Glynn et al., 2005; NIH, 2002). The in-house HCV RT-PCR has a sensitivity of 50 IU/mL when tested against the 2nd WHO international standard for HCV RNA 96/798. To avoid dual standards (difference in viral load between laboratories), the Food and Drug Administration (FDA) and the Paul Ehrlich Institute have introduced clear guidelines for testing samples. This in-house real-time RT-PCR meets the criteria set by the FDA, which requires a 95% detection limit of 100 copies/mL of HCV NAT for testing. According to the German guidelines for labile blood components, the sensitivity should be at least 5000 IU/mL per individual donation for HCV RNA detection. After standardization, this assay detected 100 IU/mL of standard 100% of the time when the standards were tested 24 times in 6 runs as recommended by OCABR (2001) for validation of NAT for detection of HCV RNA. Given the sensitivity of this assay, it could be used on blood banks adopting the pool testing approach.

This in-house assay exhibited very low interassay and intraassay variation, which is a basic requirement of a good quantitation assay. The intraassay and interassay CV of 2.3% and 6.4%, respectively, of this in-house real-time RT-PCR compares well with the other available protocols. Some commercial assays do not include an IC during the lysis step. This might be detrimental because the efficiency of nucleic acid purification and amplification for each sample will vary depending on the presence of PCR inhibitors present in the sample (Al-Soud and Radstrom, 2001). This assay uses BMV RNA as an IC from the nucleic acid extraction step to control the efficiency of the extraction and the amplification steps. Because this present assay uses BMV RNA and not plasmids, it further accounts for the efficiency of the extraction protocol. BMV RNA is commercially available and is used in many other assays as an IC for other RNA viruses (Grant et al., 2003).

Accurate detection of HCV RNA is essential for management of HCV-infected individuals (McHutchison et al., 1998; Poynard et al., 1998). To accurately compare HCV RNA viral load estimates between different laboratories, it is essential to use a common standard for assay calibration (Saldanha, 2001; Saldanha et al., 2000). We have used the 2nd WHO international standard for HCV RNA 96/798 to calibrate an internal standard, and the test results are, hence, reported in international units per milliliter. Compared with commercial assays that use preextracted plasmids as standards, this assay used plasma samples as standards that are validated against the WHO standard. These standards are extracted in each run along with the samples. When HCV viral load from this in-house real-time RT-PCR assay was compared with a commercial real-time HCV RT-PCR assay, it showed an overall moderate correlation (r = 0.59, P < 0.0001) with HCV genotype 6 showing the worst correlation. This probably could be because of the small number of HCV genotype 6 samples tested or could be due to the higher genetic heterogeneity of this genotype.

It is essential that the primer and probe used in an assay efficiently detect all the genotypes of HCV (Bukh et al., 1992). The in-house assay utilizes primer and probe from the 5′NCR of the HCV, which is the most highly conserved region of HCV. Matching of the 1st 2 or 3 nucleotides at the 3′ end of the primer is one of the most critical factors for annealing. HCV primalign search indicated that this primer and probe sequence used in this study showed good identity with 97% of strains in the HCV database (with a maximum of 1 nucleotide difference for some strains) (http://hcv.lanl.gov). The remaining 3% of the strains used from the HCV database had more than 1 nucleotide difference compared with our HCV primer and probe sequences. Of the 161 strains used from the HCV database for comparison, 2 strains (1.2%) of 1b did not have homology with the primer and probe sequence used in our study. Compared with other published sequences, our primers and probe sequence showed complete homology to a higher percentage of sequences from different regions in the HCV database. Our primers and probe sequences were also able to detect all the clinical samples included in this study comprising HCV genotypes 1, 3, 4, and 6, the prevalent genotypes in this region.

In this study, 2 different methods of extraction were used for the in-house real-time assay and the artus commercial
assay. The QIAamp MinElute Virus spin kit used for extraction of HCV RNA in the in-house real-time RT-PCR assay was used to improve the sensitivity of the assay and with a view to use this extraction method for both RNA and DNA extraction. A small difference in efficiency may exist between both extraction methods used. Also, a difference in elution volume of 10 μL between both extraction methods needs to be borne in mind.

This assay, which uses the manual nucleic acid extraction protocol and the real-time TaqMan chemistry, can be adapted for high-throughput screening of HCV in blood banks. The total assay duration is 5.5 to 6 h, including the extraction protocol. This has only a single round of amplification with no detection step avoiding cross-contamination, which is inherent to all conventional nested PCR where sensitivity matches real-time PCR. This assay is considerably inexpensive compared with the commercial quantitative HCV real-time PCR assays available. A rough estimate of the cost of this in-house assay amounts to about 50% of the commercial real-time assay. This in-house real-time assay with high sensitivity (50 IU/mL) is a reliable and more economical assay and can be used for screening and monitoring of HCV-infected individuals on treatment in diagnostic laboratories. Further detailed prospective evaluation of this in-house assay will be useful.

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