Quantitation assay of hepatitis C virus RNA using real-time PCR technique

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Abstract

Hepatitis C virus is a major cause of acute and chronic infections leading to fibrosis, cirrhosis, and hepatocellular carcinoma. A real-time PCR analysis has been employed successfully for both basic research and clinical applications. The ability to monitor the real-time progress of the amplification greatly improves the PCR-based quantitation of Hepatitis C virus RNA. The study was determine the viral load of hepatitis C virus RNA by real-time PCR technique and the influence of the gender on HCV viral load. The presence of HCV antibodies were assessed in clinically suspected HCV infection patients using commercial HCV Ab ELISA test kit, then all positive test were tested for detection and quantitative of HCV RNA by real-time PCR at Public Health Laboratory in Erbil, Iraq. Among 445 patients of HCV sero positive with ELISA screening test, RNA HCV was detected in 195(43.82%) patients by quantitative real-time PCR. Based on the distribution of viral load, 38.97% of patients had viral loads of $10^6$ IU/ml, 0.51% had viral loads between $10^8$ and $10^9$ IU/mL, and 6.67% had $10^3$IU/ml. In conclusion, the highest rate of HCV was detected among sero positive males.

Keywords: HCV; antibody; real-time PCR; ELISA; quantitative assay

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Introduction

Hepatitis C virus (HCV) is a single stranded RNA virus belonging to the Flavirviridae family, it has been identified as a major pathogen of post transfusion and community transmitted hepatitis [1, 2]. It is estimated that 170 million people worldwide are infected with HCV [3, 4].

The infection of HCV results in a higher rate of chronic infection compared to HBV infection with approximately 80% of infected subjects showing viral persistence and chronic liver diseases[5, 6], thus an accurate diagnosis is essential to identify the presence of HCV RNA in apparently symptom free carriers[7, 8]. Patients with HCV infection develop chronic liver disease, in this group, about 30% progress to cirrhosis, and 1-2% develops hepatocellular carcinoma per year [9, 10].

The main mode of HCV transmission is blood transfusion. HCV may also contaminate through tissue transplantation, drug usage via injection, sexual intercourse with infected individuals, and used contaminated tools [4, 9].

ELISA test has been introduced as serological assay for early detection of lately developed responses of HCV antibody with many limitations [1, 11]. One of the limitation is the lack of a highly sensitive and specific assay to measure viral loads in plasma or serum of which HCV circulates in the blood have a low copy number and its genome is extremely heterogeneous [12, 13]. These limitations include a requirement for confirmation by other assay to reduced specificity in low risk populations, reduced sensitivity in cases of early infection, immunosuppressant patients, and the inability to distinguish between resolved and chronic infection [12]. The real time PCR is a highly specific and sensitive assay for measurement of serum HCV with wider detection range and good accuracy of results [6].

The monitoring of the reaction in every PCR cycle through the increment of released fluorescence rather than just the end of product allows the quantification on the early, exponential phase of the reaction. Thus, many samples can be analysed simultaneously. In addition, real-time PCR makes quantitative assay of RNA much more precise and reproducible because it relies on threshold cycle values determined during the exponential phase of PCR rather than endpoint [14, 15].

Methods

The study was carried out in Public Health Laboratory in Erbil, Kurdistan region, Iraq during the period from February 2013 to January 2014. Ethically the confidentiality and anonymity were ensured by giving a code number for each patient
after obtain permission from the scientific committee of Nurse College/ Hawler medical university.

Blood samples were collected from patients for detection of anti-HCV antibody by ELISA method, then all positive HCV antibody (445 patients) were tested for detection and quantitation of HCV RNA by artus ® HCV RG RT-PCR kit (QIAGEN, Hilden, Germany).

**ELISA assay**

The presence of HCV antibodies were assessed in clinically suspected HCV positive patients using commercial HCV Ab ELISA kit (DIALAB Gmbh, Austria), which performed based on manufacturer's instructions.

**HCV RNA detection and quantification**

All HCV positive serum samples (270 patients) were taken for extraction of viral RNA and quantitation of the viral load by real-time PCR.

**HCV RNA extraction**

The QIAamp DSP Virus Kit (QIAGEN, cat. no. 60704) was used for viral RNA purification from serum samples according to the instructions of the manufacturer provided in the kit.

**The principle of procedure**

The purification procedure was comprised of four steps: lysis, binding, washing, and elution, as followed:

1. Lysis was performed in the presence of proteinase K and lysis buffer, which together ensure digestion of viral coat proteins and inactivation of RNases enzyme.
2. Binding buffer was added to the lysed samples and adjust binding conditions. Lysates were thoroughly mixed with magnetic particles to allow optimal adsorption of viral nucleic acids and bacterial DNA to the silica surface.
3. While viral nucleic acids remain bound to the magnetic particles, contaminants were efficiently washed away during a sequence of wash steps using first wash buffer 1, then wash buffer 2, and then ethanol.
4. In a single step, highly purified viral nucleic acids were eluted in buffer AVE (contains sodium azide as a preservative). The extracted RNAs were stored at -80°C until use.
Principle of real-time PCR assay

In real-time PCR, the amplified product was detected via fluorescent dyes. These were usually linked to oligonucleotide probes that bind specifically to the amplified product. Monitoring the fluorescence intensities during the real-time PCR run allows the detection and quantitation of the accumulating product without having to re-open the reaction tubes after the PCR run.

Standard curves were generated using the 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. Sometimes, PCR inhibitors cannot be reliably removed from the sample and viral RNA may somewhat be degraded or may not be efficiently removed from the viral coat protein. The IC (internal control) serves as a control to rule out PCR inhibitors during the extraction and the amplification step [16]. A sample result was accepted only when IC was amplified. The assay amplified region of the HCV genome, which was detected by the cycling A.FAM channel. The assay also was used an internal control, which was detected by the cycling A.ROX channel.

Procedure of real-time PCR assay

The reaction mixture for RT-PCR was prepared in a single tube, which contain all of the components (buffers, reverse transcriptase, and Taq Polymerase enzyme) needed for PCR that performed as followed;

1. Thirty microliters of the reaction mixture was added into each PCR tube containing 20µl of RNA from serum sample, and RNA from a diluted standard. Correspondingly, 20µl of at least one of the quantitation standards was used as a positive control and 20µl of water as a negative control.
2. The temperature profile was created for detection of HCV RNA, then all specifications were referred to the Rotor-Gene 6000 software versions 1.7.94
3. The detection range of the fluorescence channels has to be determined according to the fluorescence intensities in the PCR tubes.
4. The gain values were determined by the channel calibration were saved automatically and were listed in the last menu window of the programming procedure.
5. After the run was finished, the data was analysed.
6. The signal was detected in fluorescence channel Cycling Green, the result of the analysis it considered positive, the sample was containing HCV RNA, while in fluorescence channel cycling green no signal was detected, it considered negative. At the same time, a signal for amplification of internal control was detected in the
cycling orange channel. On the Rotor-Gene 6000, the relevant channels were Cycling A.FAM for the positive signal and Cycling A. ROX for internal control.

The quantitation standards were defined as IU/µl. The following equation has to be applied to convert the values determined using the standard curve into IU/ml of sample material:

\[
\text{Result (IU/ml)} = \text{Result (IU/µl)} \times \frac{\text{Elution Volume (µl)}}{\text{Sample Volume (ml)}}.
\]

The threshold cycle values were plotted against the sample dilution values to produce a standard curve. With the knowledge of the threshold cycle values for the samples containing unknown quantities of HCV RNA, then the standard curve was then used to interpolate the starting RNA concentration.

Interpretation of quantitative results: If HCV RNA >34 IU/ml, the result was within the determined test range. The positive test result was statistically ensured. If HCV RNA <34 IU/ml, the result was outside the determined test range.

Statistical analysis

Viral loads were estimated by real-time PCR, statistical analysis was performed using SPSS version 20 and categorical data were analysed using the chi-squared (X²) test. P values ≤ 0.05 were considered statistically significant.

Results

Among 445 patients (275 females and 170 males) their age between 18 to 70 years had HCV positive with ELISA test, 195(43.82%) were HCV RNA positive by HCV quantitative real time PCR, and 250 (56.18%) of negative sera were excluded from further evaluation, The confirmation by real time PCR to detect false positivity of the ELISA kit was beyond the scope of this study. The observed rate of HCV RNA among females was 40.0 % (110 out of 275), while the rate was 50% (85 out of 170) among males as illustrated in Table I. Statistical analysis revealed that the differences between males and females with HCV RNA positive were statistically significant (P=0.039).

The distribution of viral load was determined in 195 HCV RNA positive samples, The highest percentage (38.97%) of viral load were10⁶, 46 (23.59%) patients had viral load 10⁵ IU/ml, 36 (18.46%) patients had the viral load below 10⁵IU/mL, and only 2 (1.2%) patients had viral load between 10⁴ to10⁵ IU/ml (Table II).
Table I. HCV RNA detection among sero positive HCV patients by real-time PCR

<table>
<thead>
<tr>
<th>RT-PCR</th>
<th>Females</th>
<th>Males</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Positive</td>
<td>110</td>
<td>40.00</td>
<td>85</td>
</tr>
<tr>
<td>Negative</td>
<td>165</td>
<td>60.00</td>
<td>85</td>
</tr>
<tr>
<td>Total numbers</td>
<td>275</td>
<td>%</td>
<td>170</td>
</tr>
</tbody>
</table>

Significant different between females and males, 
$X^2=4.27, P=0.039$

Discussion

The rate of HCV infection according to gender factor was significantly higher among males than females in this study. The progression of HCV infection is known to be worse in males than in females [17], and the rates of HCV infection in relation to gender factor were nearly twice among men than among women [18]. Despite this, the exact reasons for gender difference in the rate of HCV RNA positive are unclear. The higher rate of HCV infection among male may be referred to higher rates of lifetime related risk behaviours among males [19]. Moreover, the higher prevalence of viral clearance among women is associated with immunological factors, genetics and hormonal factor [13, 20].

Table II. Distribution of viral load in HCV RNA positive samples among females and males

<table>
<thead>
<tr>
<th>Viral load (IU/ml)</th>
<th>Females</th>
<th>Males</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>$10^3$</td>
<td>4</td>
<td>3.64</td>
<td>9</td>
</tr>
<tr>
<td>$10^4$</td>
<td>10</td>
<td>9.09</td>
<td>13</td>
</tr>
<tr>
<td>$10^5$</td>
<td>30</td>
<td>27.27</td>
<td>16</td>
</tr>
<tr>
<td>$10^6$</td>
<td>42</td>
<td>38.18</td>
<td>34</td>
</tr>
<tr>
<td>$10^7$</td>
<td>22</td>
<td>20.00</td>
<td>13</td>
</tr>
<tr>
<td>$10^8$</td>
<td>1</td>
<td>0.91</td>
<td>0</td>
</tr>
<tr>
<td>$10^9$</td>
<td>1</td>
<td>0.91</td>
<td>0</td>
</tr>
<tr>
<td>Total numbers</td>
<td>110</td>
<td>%</td>
<td>85</td>
</tr>
</tbody>
</table>

No significant different of viral load between females and males, $X^2=8.67$, $P=0.193$
The routine clinical diagnosis of HCV infection is based on the detection of anti-HCV antibodies that indicates current or past HCV infection but not viral replication [21]. Therefore, the measurement of HCV RNA levels has become an integral and increasingly important part of the management of patients with HCV infection [22].

In the present study, the viral load was detected up to $10^3$ IU/ml, because the linearity standard curve of this result permits interpolate values located below the lower limit of the dynamic range ($10^3$ IU/ml). On the other hand, the system can quantify the HCV RNA in samples with more than $10^9$ IU/ml, the high degree of sensitivity along with a considerably wide dynamic range, and allows to use a single method for the detection of the wide range of loads found with HCV [14, 16]. It has been suggested that the viral load correlates with HCV activity, and degree of liver damage [7, 23]. The analytical sensitivity of real-time PCR to HCV RNA, the smallest amount of HCV RNA were tested, and reliably quantified [24]. The detection and quantitation of HCV RNA in serum requires highly specific and sensitive assays because HCV circulates in the blood at a low copy number [25]. Moreover, Different results were observed in different studies [13, 21]. Because it has been established that viral load is relatively stable in the chronic phase of the infection, of these parameters, viral load seems to be more useful for the tailoring of treatment schedules and the monitoring of HCV replication during therapy. This dissimilarity also represents the variation of HCV infections in different geographical areas and different studies design [22, 26].

It was concluded that the RT-PCR is the technique of choice for quantitation of HCV RNA target method for the diagnosis of HCV infection, allowing serum HCV RNA determination; however, obstacles such as technical difficulties, unavailability and expenses may prevent it from being used as a routine test on a large scale of patients on a regular basis, further detailed prospective evaluation of real-time PCR assay will be useful. The influence of gender on viral load determination still needs further studies.

Acknowledgments

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References


real-time reverse transcriptase PCR. *Journal of Gastrointestinal Surgery* 16, 142-147


