Quantitation of HCV RNA using real-time PCR and fluorimetry

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Abstract

Real-time PCR technology may provide an accurate and sensitive method to quantify hepatitis C virus (HCV) RNA. So far, studies have been carried out using the Taqman technology with the ABI Prism 7700 sequence detector. An alternative and simple real-time PCR assay is described with no probe requirement, based on the SYBR Green I dye and LightCycler™ fluorimeter. Amplicon synthesis was monitored continuously by SYBR Green I dye binding to double stranded DNA during PCR of the 5’ HCV non-coding (NC) region. Specificity was verified by amplicon melting temperatures. An external standard curve was constructed with serial 10 fold dilutions of a modified synthetic HCV 5’ NC RNA. A wide range linear relationship (up to 3.7 × 10⁹ copies/ml) was observed between number of PCR cycle needed to detect a fluorescent signal and number of RNA copy. Intra- and inter-assay coefficients of variation were 0.7 to 2.1 and 3.7% respectively, indicating good reproducibility of the method. Thirty-three HCV positive sera of different genotypes were quantified by this method and gave similar but more sensitive results compared to the branched DNA (bDNA) technology. © 2001 Elsevier Science B.V. All rights reserved.

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Hepatitis C virus (HCV) is the main agent responsible for non-A non-B hepatitis and is a major health problem with more than 170 million infected people in the world (WHO, 1999). HCV infection leads to persistent liver infection in 80% of patients with the development of chronic hepatitis in 60% of cases, often resulting in cirrhosis and hepatocellular carcinoma (Alter, 1997; Colombo, 1998). Two major viral markers, HCV genotypes (Mondelli and Silini, 1999) and viral load (Hoofalse, 1999), help to evaluate the disease prognosis and the efficacy of antiviral therapy relying on recombinant interferon alpha alone or in combination with ribavirin (Davis et al.,
High viral loads before therapy are associated with lower proportion of sustained response and required longer treatment (Walsh et al., 1998; Wiley et al., 1998). Therefore, quantitation of HCV RNA in sera is an important parameter to evaluate the prognosis, determine the therapy and monitor the response to treatment.

Different quantitative HCV RNA assays have been reported using mainly two techniques based on signal amplification with the branched DNA (bDNA) technology (Urdea et al., 1991) or on target sequence amplification by reverse-transcription (RT) PCR method (Young et al., 1995; Haberhausen et al., 1998; Levis et al., 2001). Quantiplex HCV RNA 2.0 assay (Bayer Diagnostics) is based on the bDNA method and is used widely for clinical quantitation because of its accuracy and reproducibility over a large HCV RNA concentration range (Alter et al., 1995; Detmer et al., 1996). However, this assay lacks sensitivity (Hawkins et al., 1997; Ichijo et al., 1997; Hofgartner et al., 2000) with a detection threshold of 0.2 Meq/ml. In contrast, RT-PCR assays are more sensitive (Roth et al., 1998) and quantitation has been described using either an external standard curve (Simmonds et al., 1990) or an internal positive control (Becker-Andre and Hahlbrock, 1989; Kaneko et al., 1992; Hagiwara et al., 1993; Clossais-Bernard and Andre, 1994; Roth et al., 1996; Olmedo et al., 1999). Such an assay, for example the Roche Monitor assay, is also used widely for monitoring HCV replication during therapy (Hawkins et al., 1997).

Recently, quantitative, fluorescence-based real-time PCR assays have been developed in different formats (Heid et al., 1996; Wittwer et al., 1997a). The system reported most commonly for HCV RNA quantitation is the Taqman technology (Roche) using a dual labelled fluorogenic probe sensitive to the 5′–3′ exonuclease activity of Taq DNA polymerase when hybridized to the target DNA (Holland et al., 1991; Morris et al., 1996; Kawai et al., 1999; Martell et al., 1999; Mercier et al., 1999; Takeuchi et al., 1999). Another method of real-time PCR (Wittwer et al., 1997a) is described now, based on binding of the SYBR Green I dye to amplicon to quantify the 5′ non-coding region of HCV. The fluorimeter LightCycler™ (Wittwer et al., 1997b) was used to evaluate this technology by comparing the results of quantitation obtained from a panel of 33 HCV sera with those determined by the branched DNA (bDNA) assay (Kolberg et al., 1994).

The 33 HCV positive sera studied were collected at the Laboratoire de Virologie Hospices Civils de Lyon, and stored at −80°C. HCV genotypes were determined by Innolipa assay (Innogenetics) and HCV-RNA concentrations were evaluated by the Quantiplex HCV RNA 2.0 assay (Bayer Diagnostics) according to the manufacturer’s instructions. Two HCV sera from this panel, not detected as positive by the Quantiplex HCV RNA 2.0 assay, were determined positive by the qualitative HCV AMPLICOR assay, version 1 (Roche Diagnostic System).

The real-time PCR was carried out after a reverse transcription step. RNA was extracted from 200 µl of serum with the QIAamp Viral RNA kit (Qiagen), eluted in 60 µl and stored at −80°C. RNA (4 µl) was incubated with 1 pmol of a HCV 5′NC primer RC 21 (see below) for 8 min at 70°C and 5 min at 4°C. RNA template was then reverse transcribed at 60°C for 1 h with 7.5 U of Thermoscript™ Reverse Transcriptase, 20 U of RnaseOut, 10 mM DTT, 1 mM deoxyribonucleotide and cDNA buffer (50 mM Tris acetate (pH 8.4), 75 mM potassium acetate, 8 mM magnesium acetate) (GibcoBRL Life Technologies) in a final volume of 10 µl. A denaturating step was performed at 95°C for 5 min and was followed by a RNase H treatment with 1 U of E. coli RNase H (GibcoBRL Life Technologies) at 37°C for 20 min. cDNA was stored at −20°C.

The primers RC1 5′-GTC TAG CCA TGG CGT TAG TA-3′ and RC 21 5′-CTC CCG GGG CAC TCG CAA GC-3′ (Clossais-Bernard and Andre, 1994) were designed to amplify a 220 bp fragment within the 5′ non-coding region of the HCV genome. These primers match the well conserved HCV sequences among the different genotypes (Fig. 1), but do not match human frequent nucleic acid sequences according to the PC-Rare software that we previously described (Griffais et al., 1991). PC-Rare software is available by downloading at http://bioinformatics.weizmann.ac.il/in-
dex.html and selects primers according to the low frequency of their 3’ octamers in the human genome databases. The 3’ octamers of RC1 and RC21 occur less than once in $5 \times 10^5$ and $4 \times 10^6$ bases of the human genome respectively.

Real-time PCR was carried out with the LC DNA Master SYBR Green I kit (Roche Diagnostics) in a 20 µl reaction volume and was monitored after each elongation step, by SYBR Green I dye binding to amplified products using the LightCycler™ apparatus (Roche Diagnostics). The 220 bp fragment was amplified from 2 µl of cDNA with 5 pmol of RC1 and RC21. Prior to amplification, the reaction mix was treated with 1 U of uracil-DNA Glycosylase (Roche Diagnostics) to prevent DNA contamination (Longo et al., 1990), and with 0.2 µg of TaqStart™ Antibody (Clontech) to carry out a hot start PCR. An optimal MgCl2 concentration of 3 mM was determined to obtain a specific and efficient amplification. The PCR protocol consisted in an initial denaturation step at 95°C for 120 s, followed by 45 cycles of denaturation (95°C for 2 s), annealing (60°C for 5 s) and extension (72°C for 15 s). For each step, the temperature transition rate was 20°C/s.

Quantitation was carried out using an external standard curve. Standard RNA was synthesised by transcription in vitro from a modified HCV cDNA containing a 40 bp deletion in the 5’ non-coding region and cloned into pBluescript SK plasmid (Clossais-Bernard and André, 1994). Briefly, positive strand RNA was transcribed for 4 h at 37°C from 1 µg of plasmid digested by EcoRI with the T3 Ribomax™ Large Scale RNA Production System (Promega). A DNase I treatment was performed for 15 min at 37°C. After phenol-chloroform purification and ethanol precipitation, the 334 base RNA fragment was controlled by gel electrophoresis and quantified by OD 260 measurement. Synthetic RNA was tested for contaminating plasmid DNA by PCR without RT step. Standard curves were constructed from serial 10 fold dilutions of synthetic HCV RNA in lysis buffer (QIAamp Viral RNA kit, Qiagen) and extracted in a HCV negative serum. After real-time PCR was completed, logarithmic values of fluorescence (y axis) for each dilution were plotted against cycle number (x axis) (Fig. 2a). A baseline was set just above the fluorescence background and a crossing point was determined with the amplification curves obtained during the initial

Fig. 1. Sequence alignment of the 5’ HCV non-coding region corresponding to the primer positions. The two left-hand columns indicate the genotype and the Genbank accession number of HCV isolates. The sequence of the RC1 and RC21 primers is indicated below. Bold nucleotides refer to mutations observed between HCV isolates.
Fig. 2. Standard curve obtained with 10 fold dilutions of synthetic modified HCV RNA, reverse transcribed and amplified by real-time PCR. (a) Logarithmic plot of fluorescence versus cycle numbers for each dilution. The horizontal line corresponds to the baseline as defined in the text. (b) Crossing points (cycle numbers) plotted against the logarithmic concentration of the serial dilutions.

Under our conditions, 1 molecule of HCV standard RNA corresponding to 2.54 Log RNA copies/ml was detected in 10 out of 22 real-time RT-PCR reactions (45%). Therefore, 2.54 Log RNA copies/ml in serum could be detected with optimal RNA extraction and cDNA synthesis conditions.

The WHO International Standard HCV serum (Saldanha et al., 1999) (NISBC) with a titre of 100 000 IU/ml and belonging to genotype 1, was used to verify the calibration of our standard curve. In this serum, 5.52 ± 0.2 Log RNA copies/ml was measured using our protocol. This value
was in agreement with the mean titre of 100,000 IU/ml corresponding to 5.3 to 5.47 Log RNA copies/ml, as determined by 22 laboratories using different methods (Saldanha et al., 1999).

During real-time PCR, amplicon synthesis was monitored by SYBR Green I dye binding to amplified product. As SYBR Green I dye binds to any double-stranded DNA, the product specificity and the absence of non-specific amplification had to be checked to validate the quantitation assay. Highly specific primers selected by PC-Rare software (see above) and hot start PCR reduced the risk of non-specific primer annealing. The specificity of the amplified product was then determined by melting curve analysis. Melting curve acquisitions were done immediately after PCR was completed, by heating at 95°C for 2 s, cooling to 65°C for 40 s and heating slowly at 0.2°C/s until 95°C with continuous fluorescence recording. Melting curves were recorded by plotting fluorescence signal intensity versus temperature (Fig. 3a). Ampli
con melting temperatures ($T_m$) were determined by calculating the derivatives of the curve with the LightCycler software and visualized by plotting the negative derivatives against temperature (Fig. 3b). The $T_m$ of one particular PCR product, depending on length and G/C content, appeared as a single peak. As expected, $T_m$ of amplified product from HCV infected serum and synthetic HCV RNA standard differed slightly due to the 40 bp deletion in synthetic RNA (Fig. 3b). Additional peaks, that would have indicated the presence of non-specific amplification, were not observed in any of the 33 sera tested. Such non-
specific peaks were only observed with very few cases of clinical negative samples used as control (data not shown).

Reproducibility of the method was examined with an HCV positive serum extracted and amplified in 10 independent experiments. The mean concentration was 5.34 ± 0.2 Log RNA copies/ml, corresponding to a coefficient of variation of 3.7%. The intra-assay variability was also evaluated with two other HCV samples tested four times within the same experiment. The mean concentrations were 7.09 ± 0.05 Log RNA copies/ml and 5.23 ± 0.11 Log RNA copies/ml, with a coefficient of variation of 0.7 and 2.1% respectively.

33 HCV positive sera of the six major genotypes were tested by the Quantiplex HCV RNA 2.0 assay and by real-time RT-PCR amplification. As shown in Fig. 4, the slope 1.1 of the regression curve showed a good correlation between the HCV RNA levels measured by real-time PCR and those obtained by the Quantiplex HCV RNA 2.0 assay. This concordance was observed over a wide range of viral concentrations (0.3 to 88.8 Meq/ml). For 29 out of 33 sera, similar viraemia levels were obtained by the two techniques independently of the genotypes (13 sera with genotype 1; five with genotype 2; seven with genotype 3; four with genotype 4; one with genotype 5; one with genotype 6). Two sera differed significantly, one with genotype 3a was measured higher by real-
time PCR ($88.8 \times 10^6$ RNA copies/ml) compared to bDNA (41.4 Meq/ml) and the second one with genotype 1b showed a lower copy number by real-time PCR ($15.5 \times 10^6$ RNA copies/ml) than by bDNA (38.2 Meq/ml).

One advantage of real-time PCR over the bDNA technology is its higher sensitivity. Indeed, all bDNA positive sera were found positive by real-time PCR, but two sera tested negative by bDNA were found positive by real-time PCR, one with 4.17 and the other with 4.39 Log RNA copies/ml (data not shown).

Real-time PCR allows continuous monitoring of amplicon synthesis early during the reaction when amplification kinetics is exponential and before amplified product concentration reaches a plateau. This allows a good correlation between the initial number of template copies and the number of PCR cycles needed to detect amplified products, therefore leading to an accurate quanti-
tation. This constitutes a major difference with current RT-PCR for which amplicon detection and quantitation are performed at the end of the PCR, where determination of high RNA levels requires sample dilutions (Zaaijer et al., 1993). Another advantage is that the presence of polymerase inhibitors which induce underestimation of RNA concentrations, could be detected by real-time PCR. Indeed, measurement of fluorescence after each elongation step during the exponential phase, allows calculation of the slope of the amplification curve. This slope is a direct
function of the amplification efficiency and can therefore be used as an indicator of the presence of inhibitors.

The real-time PCR with the SYBR Green I format, is a simple method compared to the Taqman technology, since no probe is required to quantify amplicons, minimizing variation of quantitation related to the HCV genotypes (Martell et al., 1999), often observed in other quantitative methods (Hawkins et al., 1997; Mellor et al., 1999). Moreover, primers used in this study, were selected in highly conserved regions within the HCV 5' NC genome which do not contain mutation related to genotype. Another

![Fig. 3. Calculation of amplicon Tm. (a) Melting curves (fluorescent intensity plotted over temperature) obtained after real-time RT-PCR with wild-type viral HCV RNA and synthetic modified HCV RNA. (b) Plot of the negative derivative ($-dF/dT$) of the melting curves versus temperature; peaks indicate the $T_m$ of the two different amplified products obtained from wild-type HCV viral RNA and synthetic modified HCV RNA. Solid line with dots: wild-type viral HCV RNA; solid line: synthetic modified HCV RNA.](image-url)
point of interest in the SYBR Green I format is that non-specific amplifications which can disturb PCR efficiency are easily detected by amplicon melting temperature calculation. As already mentioned above, a wide range of RNA concentrations could be linearly quantified with a low inter- and intra-assay variation. This is of major importance as HCV virus titres vary from undetectable to more than 7 Log RNA copies/ml (Roth et al., 1996; Hawkins et al. 1997; Hofgartner et al., 2000). In conclusion, because of its sensitivity and reliability, this real-time PCR assay should provide a test suitable both for diagnosis and monitoring of HCV infection.

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References


