

Brief communication

Strand specific quantitative real-time PCR to study replication of hepatitis C virus genome

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Abstract

Qualitative detection of negative hepatitis C virus (HCV) RNA has been used widely to demonstrate HCV replication. However, relative quantitation of both positive and negative HCV RNA strands has never been reported for studying viral genome replication. A strand specific real-time PCR carried out in the highly conserved 5'-non-coding region of HCV genome and monitored either by the DNA binding dye SYBR Green I or by molecular beacons is described. Using these techniques, it was found that negative HCV RNA strand was a 100–1000 times less abundant than the positive strand in the liver of HCV infected patients.

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Hepatitis C virus (HCV) is the main etiologic agent of non-A and -B viral hepatitis leading in 60% of cases to the development of chronic hepatitis, which may progress to cirrhosis and hepatocellular carcinoma. The viral genome, a single-stranded positive RNA of 9400 nucleotides, replicates in the liver and also in extrahepatic sites (Carrozzo et al., 2002; Crovatto et al., 2000; Radkowski et al., 2000). Active replication is detected by the presence of a complementary RNA, the negative strand RNA which is supposed to be the replicative intermediate of the HCV genome. Detection of negative-strand RNA in the potential viral reservoirs gave conflicting results due to the methods used which lack strand specificity. Different events occurring during cDNA synthesis such as false priming of the incorrect strand, self priming due to the highly structured 5' UTR (Lanford et al., 1994) and random priming by small cellular nucleic acids (Gunji et al., 1994) were suggested as explanations for such low specificity. Different methods have been reported to circumvent this problem. Some improvement has been obtained using tagged primers (Lanford et al., 1994), high temperature

for cDNA synthesis (Lanford et al., 1995), the core encoding region as the amplified target (Lerat et al., 1996) and the use of exonuclease I to remove nonincorporated RT primer (Craggs et al., 2001). However, although there are numerous reports on the qualitative detection of the HCV negative strand, there is a lack of information on measurement of HCV RNA intermediate, which has been only carried out by semi-quantitative RT-PCR (Negro et al., 1998, 1999). Recently, real-time PCR has been described for viral load monitoring in serum and in liver samples (Enomoto et al., 2002; White et al., 2002) and it was shown in a previous study that this technology could be used as a very reliable and highly sensitive method to quantify the viral load of HCV in serum (Komurian-Pradel et al., 2001). This method has now been applied with some technical adaptation with the use of a tagged primer, to measure negative strand HCV RNA intermediate in the liver of HCV infected patients. The specificity of the results obtained by this method was confirmed by using molecular beacons. This method permits determination of positive and negative HCV strand ratio which is an important indicator of intrahepatic HCV replication.

First, HCV RNA positive and negative strand quantitation was carried out by real-time PCR using SYBR Green I detection, after a reverse transcription step of

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the 5' HCV non coding region as previously described (Komurian-Pradel et al., 2001). Briefly, RNA (4 μ l) was reverse-transcribed with the thermoscriptTM reverse transcriptase kit (Gibco/BRL) using either the RC21 primer (5' CTC CCG GGG CAC TCG CAA GC 3') (Besnard and Andre, 1994) or the tag-RC1 primer (5' ggc cgt cat ggt ggc gaa taa GTC TAG CCA TGG CGT TAG TA 3') for the positive and the negative strand assay, respectively. After a denaturing step at 70 °C for 8 min followed by incubation at 4 °C for 5 min, RNA template was incubated at 60 °C for 1 h with 7.5 U of thermoscriptTM reverse transcriptase and then treated with 20 U of RNaseOut for 20 min at 37 °C. Real-time PCR was carried out with 2 μ l of cDNA with 5 pmol of RC1 (5' GTC TAG CCA TGG CGT TAG TA 3') and RC21 primers for positive strand amplification and 5 pmol of tag (5' ggc cgt cat ggt ggc gaa taa 3') and RC21 primers for negative strand amplification, in a final volume of 20 μ l. The reaction was carried out with the LC FastStart DNA Master SYBR Green I kit using the LightCyclerTM apparatus (Roche diagnostics). 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⁻¹ and the monitoring by fluorescence measurement was done after each elongation step. Specificity, provided by the selected primers which do not match human nucleic acid sequences, but hybridize with the 5' NCR sequences of all HCV genotypes (Griffais et al., 1991; Komurian-Pradel et al., 2001), was determined by melting curve analysis of the amplified product.

Quantitation was carried out using an external standard curve. Positive and negative RNA standards were transcribed with T3 and T7 RiboMAXTM large scale RNA production system, respectively, (Promega) from the 5'-non-coding region cloned into pBluescript SK-HCV plasmid (Besnard and Andre, 1994). After DNase I treatment, phenol–chloroform purification and ethanol precipitation, synthetic RNA were quantified by OD 260 measurement. Strand specificity of the method was checked by carrying out the negative strand amplification protocol on the 10-fold dilutions of the positive strand synthetic RNA. No amplification could be detected until 4×10^6 RNA copies per reaction.

The strand specificity of our assay was also assessed by performing a real-time RT-PCR on genomic HCV RNA extracted from serum, using the tag-RC1 primer. The serum, containing 2.3×10^7 HCV copies i.a positive strand/ml, was tested and no amplification using the negative strand protocol was detected. Taken together, these results and those obtained with synthetic RNA demonstrate the strand specificity of the method and are in agreement with reports showing no negative strand amplification from at least 10^6 HCV genome/ml (Agnello et al., 1998; Negro et al., 1999).

The sensitivity of HCV negative strand RNA quantitation was tested in the presence of high content of positive strand RNA. Indeed, it is arguable that the formation of a very stable double stranded intermediate molecule between neg-

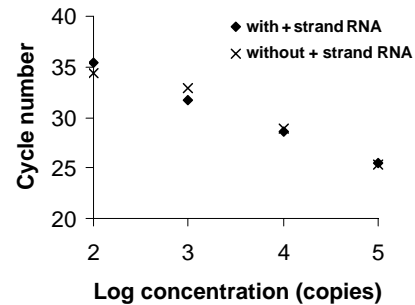


Fig. 1. Quantitation of a 10-fold dilutions of synthetic negative strand HCV RNA with or without the presence of 10^4 positive genomic HCV RNA. Cycle number (crossing points) were plotted against the logarithmic concentration of the serial dilutions.

ative and positive strands of RNA could impair the access and hybridation of the primer on the negative strand target during the reverse transcription step. To assess this point, a 10-fold dilutions of synthetic negative strand RNA were amplified and quantified in the presence of 10^4 genomic RNA extracted from HCV infected serum. As shown in Fig. 1, no inhibitory effect of positive RNA on the negative strand amplification has been observed, as the linearity of the RT-PCR reaction and the values of the crossing points were similar in the two experiments with and without genomic strand RNA. In each case, the same sensitivity was obtained with the constant detection of two Log RNA copies per reaction. Compared to the positive strand quantitation, which permits consistently the detection of one Log RNA/reaction (data not shown), the sensitivity was decreased by one Log. This lost of sensitivity of negative strand detection could be explained by the use of a tag primer during the reverse transcription step which could display less efficient accessibility to the target with a non optimal hybridation condition.

A second set of experiment for negative strand RNA quantitation was carried out by probe format using molecular beacons. Molecular beacon sequences (5' FAM GCTAGC ATT TGG GCG TGC CCC CGC IAG A GCTAGC DABCYL 3') were designed in the 220 pb 5' HCV non-coding region amplified by primer pair RC1 and RC21, and were labeled at the 5' end with the fluorophore 6-carboxyfluorescein (6-FAM) and the quencher 4-(4'-dimethylalino)phenylazo) benzoic acid (DABCYL) at the 3' end. The underlined sequences represent the stem region which does not hybridize with the HCV sequence. In the absence of target, the molecular beacon forms a hairpin loop, which places the fluorophore adjacent to the quencher, resulting in no fluorescence. But in the presence of specific amplified product, the molecular beacon hybridization during the annealing step to the template opens the hairpin and then separates the fluorophore from the quencher allowing fluorescence to occur. Real-time PCR was done by using 2 pmol of molecular beacon, 2 μ l of the same cDNA tested by SYBR Green I format analysis, the tag and RC21 primers and the LC Fast start DNA master hybridation probes (Roche diagnostics). PCR protocol consisted in an initial denaturation step at 95 °C

Table 1
Quantitation of +/- strand HCV RNA in liver tissue by real-time PCR using SYBR Green I and Beacon probe format

Patient genotype	Viral load ^a RNA (copy/ml)	SYBR Green I			Beacon probe
		+ strand RNA (copy per reaction)	- strand RNA (copy per reaction)	+/- strand ratio	- strand RNA (copy per reaction)
1b	4×10^6	8×10^4	2.2×10^2	363:1	2.4×10^2
1b	1.7×10^6	6.6×10^3	2.5×10^1	264:1	1.7×10^1
1b	4.6×10^6	4.4×10^4	4×10^1	1100:1	4.4×10^1

^a Viral load was measured from serum by real-time PCR using SYBR Green I format.

for 10 min, followed by 45 cycles of denaturation (95 °C for 10 s), annealing (55 °C for 10 s) and extension (72 °C for 10 s), with a temperature transition rate of 20 °C s⁻¹. Fluorescence measurement was recorded during each annealing step. The standard curve was generated in the same manner as SYBR Green I format and the same sensitivity of two Log copy RNA was obtained (data not shown).

We applied this method to determine the ratio of HCV positive strand RNA versus HCV negative strand in the liver. Three liver biopsies of untreated patients infected chronically with HCV were examined. The liver biopsies and the corresponding serum were provided by Dr. S. Pol (liver unit, Necker Hospital, Paris, France) and stored at -80 °C. Frozen liver tissue was disrupted and homogenized with a rotor-stator homogenizer. RNA was extracted with the RNeasy kit (Qiagen) and eluted in 50 µl RNase free water. For serum samples, RNA was extracted with the nucleospin kit (Macherey Nagel) and eluted in 50 µl RNase free water.

The measurement of viral load carried out on sera obtained at the same time as liver biopsy, show that for the three patients tested, the HCV RNA copy number was very close (1.7×10^6 to 4.6×10^6 copies/ml). For each tissue sample, the copy of positive and negative strand RNA were determined by SYBR Green I format, allowing to calculate a +/- strand ratio ranging from 264:1 to 1100:1 (Table 1). The intrahepatic negative strand HCV RNA copy number were also quantified by real-time PCR using molecular beacons, which provide a high specificity of detection. Results obtained by this approach were similar to those obtained by the SYBR Green format for the three liver samples (Table 1). Such results show marked reproducibility and accuracy between the two methods. To date, there are no quantitative data available for +/- strand ratio from HCV infected liver tissue. A semi-quantitative analysis allowed the determination of a relationship between genomic and minus strand HCV RNA titer from the liver but, without correlation with the grading/staging of the liver disease (Negro et al., 1999). In contrast, hepatocellular injury was shown to be highly correlated with the ratio of cells staining positive for HCV replicative-intermediate versus genomic RNA, by using an in situ hybridization method (Chang et al., 2000). According to our data, the production of positive strand RNA in the liver is highly favoured over the negative strand, which imply the existence of a regulatory mechanism. This has been reported in the case of Dengue virus, but with a lower magnitude as

a +/- strand ratio of 10/1 has been obtained (Cleaves et al., 1981).

A quantitative real-time PCR was developed representing a useful tool to estimate the viral replication rate in the liver and in other extrahepatic sites, which have been shown to contribute to HCV viremia in case of HCV genotype 2 infection (Negro et al., 1999). It can also be applied for understanding HCV replication in cell culture system such replicons, for which a lower +/- strand ratio (26:1 and 39:1) compared to the ratio obtained in this study from liver samples, was described recently using the Taqman assay (Gu et al., 2003).

In conclusion, this method based on the strand specific real-time PCR assay provides a suitable test to study the kinetics HCV replication in the liver and to define the replication in other viral reservoirs.

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