J. Biochem. Mol. Biol. Vol. 30, No. 3, pp. 234-236 May 31, 1997

Quantitation of Hepatitis C Viral RNA Using Direct CRT-PCR

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(Received March 24, 1997)

Abstract : Chronic hepatitis C virus (HCV) infection is associated with the rapid development of cirrhosis and hepatocellular carcinoma. It has been reported that the amount of HCV RNA may be correlated with the progression of hepatitis and may be a prognostic marker for treatment of HCV patients. The direct detection of HCV RNA by reverse transcription-polymerase chain reaction (RT-PCR) is widely used to determine the presence of circulating virions. The most relevant limit of this approach is the lack of quantitative information about the viral titer. In the present study, we developed the method for HCV quantitation using competitive reverse transcription (CRT)-PCR using the deleted HCV standard. The serially diluted standard was added in titrated amounts to the target HCV RNA. The mixture was then reverse transcribed and amplified in the same reaction tube. The methods were evaluated using over 110 HCV-PCR positive samples in Koreans. About 59% of the samples were judged to contain 10^5-10^6 copies of HCV RNA in 1 ml of serum.
Key words : CRT-PCR, hepatitis C virus, viral titer

Hepatitis C Virus (HCV), a small, positive-stranded RNA virus, is considered a major causative agent of blood-borne non-A, non-B (NANB) hepatitis (Choo *et al.*, 1989; Kato *et al.*, 1990). For clinical specimens, the absolute amount of product derived from PCR does not always bear a consistent relationship to the amount of target sequence present at the start of the reaction. Competitive PCR approaches to quantitation of nucleic acid sequences overcome the limitations of basic PCR methods for quantitation (Kaneko *et al.*, 1992; Chayama *et al.*, 1993; Manzin *et al.*, 1994; Ravaggi *et al.*, 1995; Choi *et al.*, 1996). Since the amount of HCV RNA might be correlated with the degree of severity of hepatitis and response to treatment (Kobayashi *et al.*, 1993; Hino *et al.*, 1994), we have developed a simple and direct CRT-PCR method known as competitive reverse transcription-polymerase chain reaction for quantitation of HCV RNA. Using the method, the amount of HCV RNA in serum samples obtained from 110 patients with HCV was determined.

Experimental Procedures

One hundred and ten samples were obtained from HCV patients in Korea. To prevent contamination of HCV RNA for PCR, whole blood was prepared in a vacuum container.

Direct CRT-PCR was carried out as follows. Three microliters of serum were placed in each PCR tube, boiled at 95°C for 3 min and the tube was red quickly transfer (26 μl) to ice for chilling. The RT/first PCR mixture containing 1X PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$), 200 μM dNTPs (each dATP, dTTP, dGTP and dCTP), 0.5 U of *Taq* DNA polymerase, 3 U of AMV reverse transcriptase, 15 U of RNasin and 10 pmoles outer primers (forward; 5'-CTGTGAGGAACTACTGTCTT-3', reverse; 5'-AACACTACTCGGCTAGCAGT-3') for the 5' untranslated region (UTR) of HCV (Okamoto *et al.*, 1990) was then added. To each tube, a serially diluted RNA standard (10^2-10^6 copies/μl) was subsequently added, which is constructed by the methods as described in Fig. 1. Reverse transcription was performed at 42°C for 45 min before denaturation at 95°C for 3 min. Thereafter, PCR was performed directly for 27 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C. A final extension step at 72°C for 5 min was also included. Two microliters of PCR product from the first PCR mixture were removed and transferred to a second PCR mixture containing fresh reagents and 20 pmoles of inner primers (forward; 5'-TTCA-CGCAGAAAGCGTC TAG-3', reverse; 5'-GTTGATCC-AAGAAAGGACCC-3'), which was then subjected to a

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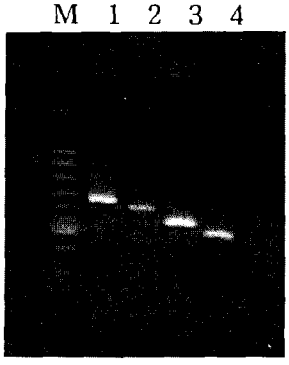
RT-PCR of HCV RNA at the 5' UTR
↓
Cloning the HCV cDNA into a plasmid pGEM7Zf
(pSCLHC, insert size; 220 bp)
↓
Delection of HCV internal 34 bp (*Sma*I site) for the pSCLHC
↓
Subcloning of the deleted HCV cDNA
(pDSCLHC, insert size; 186 bp)
↓
Synthesis of a deleted HCV RNA from the pDSCLHC
using T7 RNA polymerase
↓
Construction of standard RNA solutions by serial dilustion
(10^2 copies-10^6 copies/μl)

(A)



(B)

Fig. 1. (A) Construction of HCV standard RNA. A plasmid pDSCLHC was created by cloning a 186 bp HCV cDNA fragment containing a 34 bp deletion into pGEM7Zf. Standard RNA solutions were synthesised from pDSCLHC using T7 RNA polymerase, quantitated using the UV spectrophotometer (A_{260}), and serially diluted from 10^2 to 10^6 copies per microliter. (B) Gel electrophoresis of first and second PCR product from the cloned plasmids. M: molecular weight marker (pUCBM21/*Hpa*II & pUCMB21/*Dra* I, *Hind* III), lane 1: first product of pSCLHC (220 bp), lane 2: first product of pDSCLHC (186 bp), lane 3: second product of pSCLHC (145 bp), lane 4: second product of pDSCLHC (111 bp).

further 30 cycles of amplification. The PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The quantitation of the PCR product was estimated by comparing the intensity of the two bands of amplified DNA fragments.

Results and Discussion

In HCV infection, serum HCV RNA is the only available marker of viremia, and the circulating viral titer may be useful to evaluate the clinical evolution of the infection to gain information on antiviral therapy. The quantification of HCV RNA in clinical specimens is

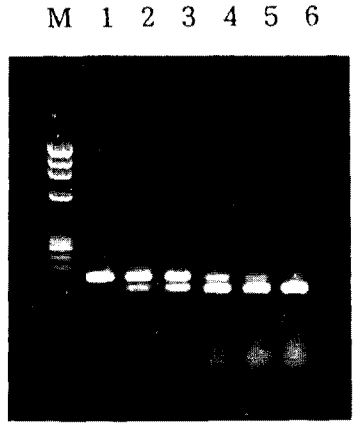


Fig. 2. Quantitation of HCV RNA derived from a serum sample of a patient. The HCV RNA was estimated to contain 3.3×10^5 copies of HCV RNA in 1 ml of serum. The DNA bands were analysed by 2% agarose gel electrophoresis and stained with ethidium bromide. Band size for patient (wild type) and standard were 145 bp and 111 bp, respectively. Size marker (M) was used with pBR322 digested *Hae* III. Lane 1: band for only 3 μl serum of a patient, lane 2: for 10^2 copies of standard RNA and 3 μl serum, lane 3: for 10^3 copies of standard RNA and 3 μl serum, lane 4: for 10^4 copies of standard RNA and 3 μl serum, lane 5: for 10^5 copies of standard RNA and 3 μl serum, lane 6: for 10^6 copies of standard RNA and 3 μl serum.

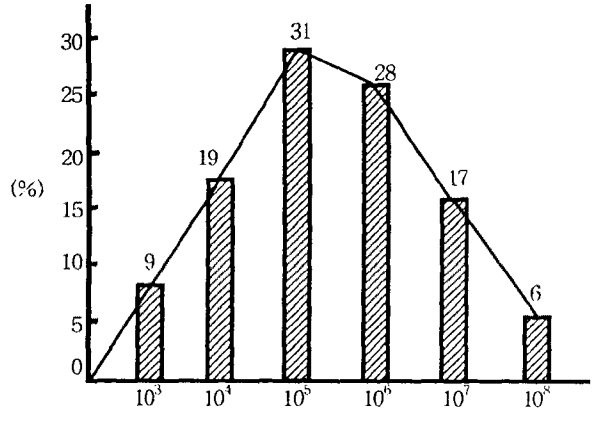


Fig. 3. Distribution of HCV copy number in 110 HCV-infected patients in Korea. About 74.5% (82 of 110) of serum samples contain more than 10^5 copies per ml of HCV. The number above stick shows cases in total 110 samples.

therefore of major interest for the understanding of the natural history of infection and for the clinical management of infected subjects. For the quantitation of HCV RNA, using only 3 ul of serum, direct CRT-PCR was carried out by reverse transcription amplification in the presence of a known amount of competitor template molecules at various concentrations that recognize the same primers. Since the standard template molecule is 34 bp shorter than the target sequence in the sample,

the initial concentration of the target RNA could be estimated by comparing the intensity of the two bands of amplified fragments (Fig. 2). The difference in the melting temperature as well as in the polymerization by Taq polymerase is thought to be negligible because the size of the deleted fragment in the standard is small. Using this method, we evaluated a total of 110 samples from Koreans with HCV infection. From the CRT-PCR, 10^3 copies of HCV RNA per 1 ml serum were found in 9 cases (8.2%), 10^4 copies in 19 cases (17.3%), 10^5 copies in 31 cases (28.2%), 10^6 copies in 17 cases (15.4%), and 10^7 copies in 6 cases (5.4%) (Fig. 3). Using this method. We found that there are large differences in the copy number of HCV RNA in serum from patient to patient (10^3-10^8). Since HCV RNA is a direct marker of viral replication, the ability to quantitate the amount of viral genome is important for monitoring responses to treatment for HCV infection. Since the direct CRT-PCR described in this paper does not require an RNA extraction step and employs direct amplification of HCV RNA by coupling RT and PCR in the same tubes, the method was very simple, rapid and reliable for monitoring of HCV RNA levels in clinical specimens from patients with an HCV infection.

Acknowledgement

This work was supported by a grants from the Seoul Medical Science Institute of Korea and we thank Dr. Kyu-Pum Lee.

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