Quantitative Measurement of Serum HCV RNA in Patients With Chronic Hepatitis C: Comparison Between Amplicor HCV Monitor System and Branched DNA Signal Amplification Assay

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Quantitative measurement of serum hepatitis C virus (HCV) RNA is important in predicting and monitoring the therapeutic effects of interferon in treating patients with chronic hepatitis C. We compared two commercial available assays, Roche Amplicor HCV Monitor test kits and Chiron branched DNA signal amplification (bDNA) assay, in quantitative measurement of serum HCV RNA in 74 patients with chronic hepatitis C. The serum HCV RNA of each of these patients was qualitatively positive by conventional reverse transcription-nested polymerase chain reaction. Serum HCV RNA was detected positive by the Amplicor test kits in 63 (85%) patients and by the bDNA assay in 58 (78%) patients ($P>0.05$). The quantitative results of HCV RNA detected by both assays showed a good linear correlation ($r=0.56$, $P<0.001$). Amplicor test kits detected 5 patients with low viremia which were below the detection limit of the bDNA assay (2.0×10^5 genome equivalents/ml).

However, the mean HCV RNA values detected by the Amplicor test kits was 1.26 log lower than that of the bDNA assay. The Amplicor test kits detected only 5 samples (8%) with a HCV RNA value greater than 5×10^6 copies/ml, while the bDNA assay detected 23 samples (40%) with a HCV RNA value greater than 5×10^6 genome equivalents/ml ($P<0.01$). HCV genotype did not affect the positive rate of HCV RNA measurement detected by both assays. However, a significantly higher mean serum HCV RNA value was noted in HCV genotype 1b as compared with the other genotypes. We concluded that the Roche Amplicor HCV Monitor test kits and the Chiron branched DNA signal amplification assay are equally sensitive in the quantitative measurement of serum HCV RNA in patients with chronic hepatitis C and can be reliably used in measuring HCV viremia clinically. *J. Clin. Lab. Anal.* 12:121–125, 1998. © 1998 Wiley-Liss, Inc.

Key words: hepatitis C virus; polymerase chain reaction; branched DNA signal amplification; Amplicor Monitor test kit

INTRODUCTION

The diagnosis of hepatitis C virus (HCV) infection is currently based on the detection of serum antibody to recombinant HCV antigens using an enzyme immunoassay (1). However, antibody detection is less sensitive in the early phase of HCV infection and cannot differentiate between active infection and resolved infectious status (2,3). Also, the lack of antibody production in persistently infected patients has been documented in some immune-compromised hosts such as patients with hemodialysis or human immune deficiency virus infection (4). Direct detection of serum HCV genome based on reverse transcription and polymerase chain reaction (RT-PCR) has solved the aforementioned problems and plays an important role in the diagnosis of HCV infection (2,5). However, the laborious home-brew PCR techniques with wide interlaboratory variations and the qualitative nature of RT-PCR results have limited its clinical use (6).

Recently, quantitative measurement of serum HCV RNA has become important in predicting and determining the therapeutic response of interferon or other antiviral agents in treating patients with chronic hepatitis C (7–10). Previous attempts to quantify HCV viremia using conventional RT-PCR with limiting dilution of extracted RNA or complementary DNA have yielded only semiquantitative results and are subject to potential error due to lack of comparative standards (11,12). Competitive RT-PCR assay, using synthetic mutant HCV RNA as a competitor and coamplified with the sequence of inter-

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est, provides a sensitive method for quantitative measurement of HCV RNA (13,14). However, the laborious home-brew PCR procedures and lack of standardization have limited its clinical use. Additionally, the difficulty in interlaboratory comparison has been noted.

Branched DNA signal amplification (bDNA, Quantiplex™, Chiron Corp., Emeryville, CA) assay has been widely used in quantitative measurement of HCV RNA. The solid-phase hybridization technique and colorimetric detection of signal amplification results successfully avoid the problem of complicated PCR procedures (7,15,16). However, the detection limit of bDNA assay (2.0×10^5 HCV genome equivalents/ml) has limited clinical use especially in detecting low levels of serum HCV RNA. Recently, the first commercially available PCR assay (Amplicor HCV Monitor test kit, Amplicor™, Roche Corp., Branchburg, NJ) using standardized procedures in RNA extraction and RT-PCR and colorimetric detection of the amplified products has been introduced in clinical use (17,18). The goal of this study is to compare the results of bDNA and Amplicor assays in quantitative measurement of serum HCV RNA.

MATERIALS AND METHODS

Seventy-four consecutive patients with chronic hepatitis C (47 males, 27 females, mean age: 57 ± 2 years) were entered into the study. They all fulfilled the following criteria: a) presence of serum anti-HCV; b) serum alanine aminotransferase levels of at least one and half times above upper normal values (> 60 IU/l) at least twice six months prior to enrollment; c) tested negative for serum hepatitis B surface antigen; d) other possible causes of hepatitis were excluded; and/or e) histological evidence of chronic hepatitis with or without cirrhosis. Patients with a prior history of interferon therapy were excluded. Serum of each patient was drawn at the time of enrollment and stored at aliquot in -70°C until analyzed for HCV RNA by RT-PCR, bDNA assay, and the Amplicor HCV Monitor test kit. Forty of 74 patients had received percutaneous liver biopsy and all showed chronic active hepatitis; 6 of them were associated with cirrhosis. In the 36 patients who had not undergone a liver biopsy, 10 were diagnosed as cirrhosis according to the clinical, biochemical, and image characteristics.

Serum anti-HCV was measured by a second generation anti-HCV enzyme immunoassay containing antigens of both the structural and the nonstructural regions of the HCV genome (Abbott Laboratories, Chicago, IL). The measurement of HCV RNA using conventional RT-PCR in our laboratory was described in detail in a previous report (19). In brief, serum RNA was extracted using the single-step method with acid guanidinium thiocyanate-phenol-chloroform, reverse transcribed to cDNA and subjected to nested PCR for amplification. Two pairs of synthetic oligonucleotide primers, synthesized from the 5' noncoding region sequences of HC-

J1 (20), were used for PCR primers. Throughout the experimental procedure, false positive PCR results were avoided by the strict application of the contamination prevention measures (21). Experiments were carried out with at least one negative control per 10 samples. No false positive results were observed in this study.

Quantitative measurements of HCV RNA using bDNA assay were performed according to the instructions of the manufacturer (Chiron). This assay is based on a specific hybridization of the viral RNA in the sample by synthetic oligonucleotides to the highly conserved 5' noncoding region and the core region of HCV immobilized on the surface of a microwell plate. To eliminate underdetection due to sequence variation, the use of oligonucleotides with minor genomic variations, but complementary to all five recognized HCV genotype variations, was incorporated. Synthetic bDNA amplifier molecules and multiple copies of an alkaline-phosphatase-linked probe were then hybridized to the immobilized complex. The complex was then incubated with a chemiluminescent substrate (dioxetane). When light emission was measured, the signal was found to be proportional to the level of the target nucleic acid. The quantity of HCV RNA in the sample was then determined from a standard curve. In all samples, standard curve members, as well as positive and negative controls were run in duplicate. The coefficient of variation of our assays was 12%. The assay gives results in numbers of copies of HCV genome equivalents per ml. The detection limit of this assay was 2.0×10^5 HCV genome equivalents/ml.

Quantitative measurements of HCV RNA using Amplicor HCV Monitor test kits were performed according to the instructions of the manufacturer (Roche). In brief, RNA was extracted from 100 μL serum samples by adding 400 μL of lysis buffer containing an internal quantification standard. After precipitating with isopropanol, the pellet was washed with 70% ethanol. One ml of specimen diluent was then added and 50 μL was taken for the following amplifications using a thermal cycler (GeneAmp PCR system 9600, Perkin-Elmer Applied Biosystems, CA). Reverse transcription was performed at 60°C for 30 min, and amplification was carried out for 2 cycles at 95°C for 15 sec and 60°C for 20 sec, and then 33 cycles at 90°C for 15 sec and 60°C for 20 sec. A thermostable and thermoactive DNA polymerase from *thermus thermophilus* were used for both RT and PCR. After adding 100 μL of denature solution to each reaction tube, 25 μL of amplification product was hybridized with HCV specific capture oligonucleotides coated on a microtiter plate for colorimetric detection. The HCV concentration was then calculated by the optical density value of HCV and internal quantification standard according to the supplier's instruction. All samples were run in duplicate and the coefficient of variation was 14% in our assays. The sensitivity of the Amplicor assay is 1000 HCV copies/ml.

HCV genotyping was performed according to a previous

report (22). The typing was based on amplification of a core gene sequence by nested PCR. The first round PCR was carried out using a pair of universal primers. The second round PCR was carried out using a universal primer (sense) and a mixture of four type-specific primers (antisense). HCV genotypes (types 1a, 1b, 2a, and 2b, according to Simmonds' classification (23)) were determined by the size of the products specific to each.

STATISTICAL ANALYSIS

Data in the text were expressed as mean $\pm$ standard error of mean. Quantitative HCV RNA results were logarithmically transformed before comparison. The results were compared using the Chi-square test with or without Yate correction, Mann-Whitney U test, Kruskal-Wallis test, or linear regression analysis depending on the type of data analyzed. A P value < 0.05 was considered statistically significant.

RESULTS

Among 74 samples whose HCV RNA was qualitatively positive by RT-nested PCR, 63 (85%) were positive by Amplicor test kits and 58 (78%) were positive by bDNA assay ($P > 0.05$). Eleven samples were negative by both assays. Amplicor test kits detected 5 samples with low viremia (2,900, 4,000, 6,500, 26,000, 30,000 copies/ml respectively) which was below the detection limit of the bDNA assay (2.0×10^5 genome equivalents/ml). The quantitative results of HCV RNA detected by both assays showed a good linear correlation ($r=0.56$, $P < 0.001$, Fig. 1). However, the mean HCV RNA values detected by the Amplicor test kits was 1.26 log lower than those of bDNA assay. The Amplicor test kits detected only 5 samples (8%) with a HCV RNA value greater than 5×10^6 copies/ml, while bDNA assay detected 23 samples (40%)

with a HCV RNA value greater than 5×10^6 genome equivalents/ml ($P < 0.01$). Thus, the quantitative results of samples with low HCV viremia (bDNA values $\leq 5.0 \times 10^6$ genome equivalents/ml) showed a significantly good correlation between both assays ($r=0.42$, $P=0.01$), while samples with high HCV viremia (bDNA values $> 5.0 \times 10^6$ genome equivalents/ml) did not ($r=0.14$, $P=0.52$).

HCV genotype was measured in 53 patients. There were 29 patients (55%) with genotype 1b, 7 patients (13%) with genotype 2a, 11 patients (21%) with genotype 2b, 4 patients (8%) with mixed genotypes 1b and 2a. Two patients were unclassified by the current method. The HCV genotype did not affect the positive rate of HCV RNA measurement detected by both the Amplicor and bDNA assays. However, a significantly higher mean serum HCV RNA value was noted in HCV genotype 1b (6.71 ± 0.72 log eq/ml) as compared with those with the other genotypes (genotype 2a: 5.56 ± 0.90 , genotype 2b: 5.86 ± 1.05 log eq/ml, $P < 0.05$, Fig. 2).

DISCUSSION

Quantitative measurement of serum HCV RNA is important in predicting and determining the therapeutic response of interferon or other antiviral agents in treating patients with chronic hepatitis C (7–10). Previous attempts to quantify HCV viremia using RT-PCR with limiting dilution of extracted RNA or complementary DNA and competitive RT-PCR assay have been limited in their clinical use due to the laborious home-brew PCR procedures, lack of standardization, and wide interlaboratory variations (6,11–14). Chiron bDNA assay has been widely used in quantitative measurement of HCV RNA recently. Although it avoids the complicated procedure of RT-PCR, the detection limit of 2.0×10^5 HCV genome equivalents/ml has led to a low sensitivity in detecting a low serum level of HCV RNA. In this study, 78% of the samples whose HCV RNA was qualitatively positive by RT-PCR was positive by bDNA assay. Our results were consistent with previous reports that the sensitivity of bDNA assay in detecting viremia of patients with chronic hepatitis C was from 70% to 91% (7,16,24–26).

The Amplicor HCV Monitor System is a newly developed method in quantitating measurement of serum HCV RNA. There are several advantages of the Amplicor HCV Monitor system: 1) standardized methods in RNA extraction, and the RT and PCR procedures overcome the interlaboratory variation of home-brew RT-PCR procedures; 2) both RT and PCR are carried out using a DNA polymerase from *thermus thermophilus*, a thermostable and thermoactive enzyme, to avoid contamination during change buffers and enzymes (27); 3) the use of Uracil-N-glycosylase to prevent the carryover of amplified DNA in the amplification steps; 4) the colorimetric detection of amplified products and the use of internal quantification standards for quantitative measurement prevents exposure of laboratory workers to radioisotope or

Fig. 1. Linear regression analysis in the quantitative measurement of serum HCV RNA detected by Amplicor HCV Monitor system and branched DNA signal amplification assay. Dot-line indicated 95% confidence interval.

Fig. 2. The mean serum HCV RNA level (log eq/ml by branched DNA signal amplification assay) was significantly higher in HCV genotype 1b as compared with the other genotypes ($P < 0.05$). Four samples whose serum

level was below the detection limit of branched DNA signal amplification assay were detected as value of the Amplicor HCV Monitor system.

ethidium bromide; and 5) a detection limit of around 1,000 HCV copies/ml is more sensitive than that of bDNA assay. Our results showed a sensitivity of 85% for Amplicor test kits in quantitative measurement of serum HCV RNA. This is more sensitive than the 78% value of bDNA assay but the difference is not statistically significant. Five samples with low serum HCV RNA level from 2,900 to 26,000 copies/ml were detected by the Amplicor test kits. These were below the detection limit of bDNA assay.

Although the quantitative results of serum HCV RNA detected by the Amplicor kits and bDNA assay showed a good linear correction, we found a lower sensitivity in the Amplicor test kits in detecting samples with high viremia. Amplicor HCV Monitor test kits detected only 5 samples (8%) with an HCV RNA value greater than 5×10^6 copies/ml, while bDNA assay detected 23 samples (40%) with an HCV RNA value greater than 5×10^6 genome equivalent/ml. Also, the mean HCV RNA values detected by the Amplicor test kits was 1.256 log lower than that of bDNA assay. Our results were also consistent with previous reports that higher values of HCV viremia are difficult to detect with the Amplicor test kits (28,29). Standardization should be further evaluated to facilitate comparison between both assays.

According to our results, HCV genotype did not affect the detection rates of serum HCV RNA by either the Amplicor test kits or bDNA assay. Our results are also consistent with previous reports that the mean serum HCV RNA concentration of HCV genotype 1b was significantly higher than that of other genotypes (7,25). In conclusion, Roche Amplicor HCV Monitor kits and the Chiron branched DNA signal amplification assay are equally sensitive in the quantitative measurement of serum HCV RNA in patients with chronic hepatitis C and can be reliably used in measuring HCV viremia clinically.

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