JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1993, p. 2679–2682
0095-1137/93/102679-04$02.00/0

Vol. 31, No. 10

Quantitative Evaluation of Hepatitis C Virus RNA in Patients with Concurrent Human Immunodeficiency Virus Infections

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Received 1 March 1993/Returned for modification 20 April 1993/Accepted 29 June 1993

Quantitation of the hepatitis C virus (HCV) provides a powerful epidemiologic and therapeutic method for the evaluation of infected patients. In this study semiquantitative reverse transcriptase polymerase chain reaction (PCR) is compared with a new branched DNA signal amplification methodology. Samples from HCV-infected patients as well as from human immunodeficiency virus-infected patients were evaluated. Reverse transcriptase PCR correlated well with the branched DNA assay ($r = 0.7036$, $P < 0.05$). HCV RNA was found to occur at significantly higher titers ($P < 0.05$) in patients coinfected with the human immunodeficiency virus compared with titers in those infected with HCV alone. Immune status as defined by the CD4$^+$ count was not associated with the observed difference in viral titer.

The isolation of a cDNA clone derived from a blood-borne non-A, non-B hepatitis virus by Choo et al. has led to the development of additional tests that enhance our understanding of the hepatitis C virus (HCV) (3). The development of reverse transcriptase polymerase chain reaction (RT-PCR) to detect specific hepatitis C viral sequences has become widely used in hepatitis C diagnosis. Numerous reports indicate that semiquantitative RT-PCR amplification can be used as an aid in defining the natural history of the disease process. Quantitation has a role in delineating physiochemical parameters such as storage stability of samples that contain HCV RNA. Additionally, there are data that suggest that the quantitative determination of HCV in serum may provide a marker of response to therapeutic interventions (2, 4, 8, 10). For this reason viral quantitation appears to be an important, though technically difficult, parameter for defining treatment modalities in the human immunodeficiency virus (HIV)-infected population.

The titer of circulating HCV may also play a role in the epidemiologic characteristics of viral transmission. It has been noted that mothers infected with HIV who also carry HCV are more likely to transmit hepatitis C to their offspring than are non-HIV-infected, HCV carrier mothers. Investigators have suggested that this may be related to a higher level of circulating HCV in the HIV-infected population, though this has not been substantiated by literature to date (5). In this study we investigate the hypothesis that individuals with coinfection of HIV and HCV have higher titers of HCV than do subjects infected with HCV alone. Quantitation was performed by a new branched DNA (bDNA) signal amplification technique which was compared with endpoint dilution RT-PCR.

MATERIALS AND METHODS

Frozen sera from 16 patients with HIV and HCV infection as well as from 32 patients with HCV infection alone were utilized. These sera were separated from the clot within 4 h of blood collection, aliquoted, and stored at −70°C in all cases. The presence of antibodies to HCV and HIV was detected by standard enzyme-linked immunosorbent assays (ELISAs) (Abbott Laboratories, North Chicago, Ill.). The diagnoses of all patients suspected of having HIV infection on the basis of the ELISA were confirmed with Western blots (immunoblots). All patients with suspected HCV infection had the diagnosis supported by a positive HCV RIBA 2.0 assay (Chiron Corp., Emeryville, Calif.). RT-PCR with endpoint dilution quantitation for the detection of HCV was performed by using a procedure adapted from that of Beach et al. (1). Phenol-chloroform extraction of nucleic acid was performed. Briefly, 50 μl of each serum sample was added to a 1.5-ml tube that contained 190 μl of the protein digestion solution (8 ml of sterile water, 1 ml of 1 M NaCl, 0.5 ml of 1 M Tris buffer [pH 8], 0.5 ml of 10% sodium dodecyl sulfate (SDS), 20 μl of 0.5 M disodium EDTA, 20 μl of tRNA from brewer's yeast) and 10 μl of proteinase K solution (20 mg/ml). The digestion solution was mixed by vortexing and incubated at 37°C for 90 min. To each tube was added 250 μl of phenol-chloroform-isoamyl alcohol (25:24:1). After being vortexed vigorously the tubes were centrifuged at 15,000 × *g* for 10 min. Then 230 μl of the aqueous phase was pipetted into new 1.5-ml tubes. A second extraction was performed, and 200 μl of the aqueous phase was transferred to new 1.5-ml tubes. Ammonium acetate (100 μl, 7.5 M) and 750 μl of cold ethanol were added to each tube, which was allowed to stand at 4°C for 30 min. The tubes were then centrifuged at 15,000 × *g* for 30 min at 4°C. The alcohol was carefully aspirated, and the remaining pellet was washed with 70% ice-cold ethanol. Ethanol was removed, and the pellets were dried by vacuum desiccation. The RNA pellet was resuspended in 10.7 μl of DEPC-treated water and transferred to a 0.5-ml PCR tube. External and internal primers from the conserved 5′ noncoding region of HCV were synthesized by the phosphoramidite method (6). RT from the avian myeloblastosis virus (Boehinger Mannheim, Indianapolis, Ind.) was used to create the cDNA product with 1 μl of the external forward and reverse primers. The reaction mixture contained 0.5 μl of RNase inhibitor. The external primers (forward, GGCGACACTCCACCATGAATC; reverse, GG TGCACGGTCTACGAGACCT) served as the RT and PCR primers for 30 cycles of amplification (94°C for 30 s, 60°C for 30 s, and 72°C for 60 s) on a Perkin-Elmer thermo-

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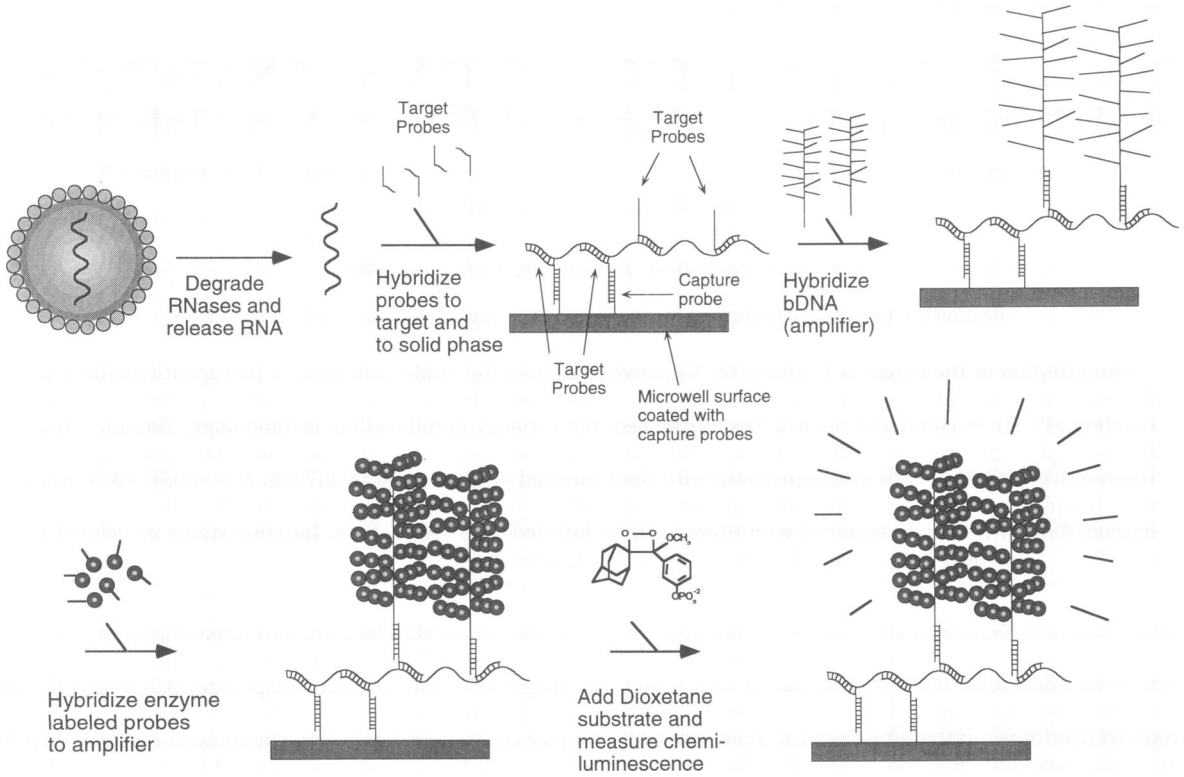

FIG. 1. bDNA signal amplification. Patient serum (50 μl) is added directly to wells of a 96-well microplate; a mixture of proteinase K, SDS, and target and capture probes is added to the wells, incubated at 63°C overnight, and washed. The bDNA amplifier is then added, incubated at 53°C, and washed; this procedure is followed by addition of alkaline phosphatase probes, another wash, and addition of a chemiluminescent substrate.

cycler. One microliter of PCR product was removed for the purpose of amplifying an area of the HCV genome incorporated within the nested primer set. The internal nested primer set (forward, CTGTGAGGAACTACTGTCTTC; reverse, CCCTATCAGGCAGTACCACAA) was then utilized for an additional 30 cycles of amplification under the same temperature cycling conditions. The final product was run on a 2% agarose gel stained with ethidium bromide to locate a 257-bp expected product. A *Hae*III digest of φX174 was used as a size marker. Semiquantitative PCR was carried out by endpoint dilution methods. Samples were serially diluted in 10-fold increments with noninfected human serum, and then 50-μl aliquots from each dilution were subjected to the HCV RT-PCR amplification described above. All samples were tested in duplicate. Endpoint dilution was also performed with a sample of chimpanzee serum which had been previously evaluated to determine the chimpanzee infectious dose (provided by Michael Beach, Center for Disease Control and Prevention, Atlanta, Ga.). Endpoint dilution samples were expressed as the reciprocal titer level. To avoid contamination, several procedures were utilized. All pipette tips had aerosol-resistant barriers. Extraction procedures were carried out in a Template-Tamer (Oncor, Gaithersburg, Md.) which was irradiated with UV light to inactivate potential DNA contaminants on all surfaces. PCR amplification was carried out in a room separate from the extraction area.

Quantitative bDNA signal amplification was also used to detect HCV RNA in human serum. The assay was performed according to the manufacturer's instruction book (Quantiplex HCV-RNA; Chiron Corp.). The assay is based on the specific hybridization of synthetic oligonucleotides (target probes) to the 5′ untranslated and core regions of HCV RNA in a crude proteinase K-SDS serum lysate. A 50-μl serum sample was lysed, hybridized, and captured, and the signal was amplified in a single well of a 96-well plate. Synthetic bDNA amplifier molecules and multiple copies of an alkaline phosphatase-linked probe were hybridized to the immobilized complex. Detection was achieved by incubating the complex with a chemiluminescent substrate and measuring the light emission (Fig. 1). Since the target is not amplified, the signal is proportional to the level of target nucleic acid. The quantity of HCV RNA in the 50-μl sample was determined from a standard curve. The standard curve was developed by comparison with the chemiluminescence signal from a cloned HCV RNA which was quantitated by phosphate determination, incorporation of [α-^{32}P]guanosine, and absorbance characteristics of intact and digested transcripts (data not shown). The curve was expressed as HCV equivalents per milliliter. The sensitivity of the assay is 350,000 HCV RNA equivalents per ml. Below this level, background signal cannot be separated from the true-positive samples. All samples were run in duplicate so that an internal coefficient of variation could be obtained (7, 9).

All $CD4^+$ counts of samples from HIV-infected patients were obtained on an Epics Profile flow cytometer (Coulter Corp., Hialeah, Fla.).

Statistical analysis was performed on the Statistix (version 4.0) program and utilized Spearman and Pearson correlations as well as one-way analysis of variance with a Scheffe

TABLE 1. Demographic and quantitative characteristics of patients used for correlation analysis

Patient no.	HIV antibody	Race[a]	HCV RNA (equivalents/ml)	PCR reciprocal titer
0083	−	B	8,176,000	100
0041	−	C	4,972,701	100
0141	−	B	10,585,000	100
0120	−	C	8,526,400	100
1602	+	B	35,770,000	1,000
2149	+	C	2,671,000	100
2440	+	C	28,105,000	100
1887	+	C	24,090,000	1,000
2928	+	B	14,600,000	100
3829	+	B	4,752,000	10
Chimpanzee 771[b]	−		23,000,000	1,000

[a] B, black; C, Caucasian. The patients were male.
[b] For the purpose of additional semiquantitative comparison, chimpanzee 771 had an estimated chimpanzee infectious dose of 10^5.

comparison of means. A two-tailed hypothesis was tested in all cases.

RESULTS

A subset of 10 patient samples and one HCV-infected chimpanzee serum sample were analyzed to determine the correlation between endpoint dilution PCR and the bDNA methodology. The endpoint dilution RT-PCR reciprocal titer ranged from 10 to 1,000 per sample (Table 1). The bDNA assay in this group yielded a range of values between 2,671,000 and 35,770,000 HCV equivalents per ml. Spearman correlation between the two methodologies revealed a significant correlation ($r = 0.7036$, $P = 0.05$). Semiquantitative endpoint dilution RT-PCR had poor within-sample consistency and required multiple runs to increase confidence in the result. In comparison, the bDNA amplification methodology revealed a high degree of internal consistency. The mean percent coefficient of variation between paired samples was 5.35 (standard error of the mean, ±1.2%). One additional RIBA-positive, RT-PCR-negative sample was included in the sample set. It provided indeterminate results on the bDNA assay. RT-PCR was more labor-intensive and required greater sample processing time than the bDNA technique.

Comparisons of HCV viral load among patients with and without coincident HIV infection were then evaluated. Thirty HCV-positive, HIV-negative patients and 13 HCV-positive, HIV-positive patients were tested and provided evaluable results. The range of HCV RNA levels among HCV-positive, HIV-positive patients was 6.6×10^5 to 2.3×10^8 HCV RNA equivalents per ml. Among HCV-positive, HIV-negative subjects the viral burden ranged from 3.89×10^5 to 3.9×10^7 HCV RNA equivalents per ml. The mean HCV RNA level among HCV-positive, HIV-negative subjects was 1.18×10^7 HCV RNA equivalents (standard error of the mean, 1.9×10^6), while HCV-positive, HIV-positive patients had a mean RNA level of 3.83×10^7 (standard error of the mean 1.7×10^6). One-way analysis of variance revealed the group means to be significantly different from each other ($P < 0.05$). $CD4^+$ counts ranged from 292 to 1,024 cells per mm^3. Pearson correlation revealed no significant relationship between $CD4^+$ count and HCV viral load. Likewise, no correlation between HCV RNA level and serum alanine aminotransferase was noted.

DISCUSSION

It is expected that the quantitation of HCV will play an increasingly important role in future attempts to elucidate key features of the epidemiology of the disease process, as well as in the evaluation of screening potential antiviral therapies. Semiquantitation of HCV by RT-PCR endpoint dilution analysis was found to be time-consuming, labor-intensive, and difficult to reproduce in a consistent manner. The resolution is low when 10-fold serial dilutions are performed. The use of increased incremental dilutions would significantly increase the workload and decrease the reproducibility over the wide range of levels at which HCV has been found to occur. In contrast, the bDNA amplification method appears to be much less labor-intensive and have more internal consistency and reproducibility.

Our data confirm the speculation that HCV titers tend to be higher in patients infected with HIV than in those patients with HCV alone, though there is considerable overlap in the ranges and mean differences can be attributed to high titers in a few HIV-infected patients. This was not associated with the degree of immunosuppression as defined by $CD4^+$ levels. If titer is the only parameter associated with HCV transmission, then it cannot be assumed that all patients with HIV infection are necessarily more infectious than their non-HIV-infected counterparts.

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