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Accurate Quantification of Hepatitis C Virus (HCV) RNA from All HCV Genotypes by Using Branched-DNA Technology

JILL DETMER, ROBERT LAGIER, JULIE FLYNN, CRYSTLE ZAYATI, JANICE KOLBERG, MARK COLLINS, MICKEY URDEA, AND RAY SÁNCHEZ-PESCADOR*

Chiron Corporation, Emeryville, California

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In studies monitoring disease progression and therapeutic response, it is essential that the method used for hepatitis C virus (HCV) quantification not be influenced by genotypic variability. The branched DNA assay provides a reliable method for the quantification of HCV RNA. A modified set of oligonucleotide probes for the branched DNA assay was developed to enhance the efficiency of binding to genotypic variants of HCV. The improved branched DNA assay (HCV RNA 2.0) yielded highly reproducible quantification of hepatitis C virus RNA and displayed a nearly 600-fold dynamic range in quantification up to 120 Meq of HCV RNA per ml. The quantification limit was set at 0.2 Meq of HCV RNA per ml to ensure a specificity of ≥95%. With this lowered quantification limit and the enhanced hybridization of the probes, the HCV RNA 2.0 assay exhibited a high level of sensitivity (96%) and was virtually unaffected by the genotypic variability of HCV. The HCV RNA 2.0 assay may be a useful tool for following HCV RNA levels throughout the course of disease, selecting patients for therapy, and evaluating therapeutic response.

Hepatitis C virus (HCV) is the major causative agent of non-A, non-B hepatitis worldwide (2, 10). A single-stranded RNA virus, HCV has a high spontaneous mutation rate, with an estimated frequency of 10^{-2} mutations per nucleotide per year (36, 37). Since the HCV genome was first cloned and sequenced (8), considerable sequence diversity among HCV isolates from various geographical regions has been reported. Genetic variants of HCV have been classified into six major genotypes, some with several subtypes, on the basis of nucleotide sequence homology and phylogenetic analysis (45). HCV genotypes 1a, 1b, 2b, and 3a are the most common types found in the United States and western Europe. HCV genotypes 1b, 2a, and 2b are prevalent in Japan and Taiwan, whereas HCV genotypes 4, 5, and 6 are predominantly found in the Middle East, South Africa, and Hong Kong, respectively (44).

In chronically infected individuals, both viral load and HCV genotype may have clinical relevance. Numerous reports have indicated that serum HCV RNA levels and HCV genotype may be predictive of a sustained response to alpha interferon therapy (5, 16, 20, 26, 27, 32, 33, 35, 56, 57). In studies exploring the clinical significance of and relationship between viral load and genotype, it is essential that the method used to quantitate viral load be unaffected by the variability of HCV.

A method for the direct quantification of HCV RNA in serum based on branched DNA (bDNA) technology has been developed for research use (25). The bDNA assay is based on the hybridization of HCV RNA to oligonucleotide probes complementary to the highly conserved 5′ untranslated region and the 5′ third of the core gene of the HCV genome. Yielding highly reproducible quantification of HCV RNA with no sample preparation, the bDNA assay allows for the direct measurement of HCV RNA in clinical samples at physiological levels. The bDNA assay has been used in the context of clinical trials to investigate the relationship between the levels of HCV RNA and the histological severity of infection (11, 19) and to predict and monitor the responses of HCV-infected individuals to therapy (3, 6, 15, 18, 26, 30, 40, 55).

Since the introduction of the first-generation bDNA assay (Quantiplex HCV RNA 1.0 assay; Chiron Corporation, Emeryville, Calif.) for the quantification of HCV RNA in clinical specimens, we have continued to explore improvements to the bDNA technology. Some of these improvements to the bDNA assay recently have been evaluated for use in a variety of clinical settings (1). Although the HCV RNA 1.0 assay included multiple probes designed to include HCV sequence diversity, it is possible that the hybridization efficiency and quantification may vary among the HCV genotypes. We now have developed a refined oligonucleotide probe set based on sequence variations among disparate HCV isolates which have been incorporated into the bDNA assay (Quantiplex HCV RNA 2.0; Chiron Corporation). In this report, we describe the performance characteristics of the HCV RNA 2.0 assay with these oligonucleotide probe modifications. We analyze the sensitivity, specificity, and reproducibility of the HCV RNA 2.0 assay for the quantification of HCV RNA in clinical specimens. We assess the linearity of the HCV RNA 2.0 assay and compare HCV RNA quantification in paired plasma and serum specimens from patients chronically infected with HCV. We also examine the sequence diversity in the probe-binding region of the HCV genome and evaluate the impact of HCV genotype variation on HCV RNA quantification by the bDNA assay.

MATERIALS AND METHODS

Clinical specimens. Plasma and serum specimens were collected from stable patients chronically infected with HCV and from healthy blood donors. Chronic HCV infection was defined by elevated serum alanine aminotransferase (ALT) values of at least 1.5 times the upper limit of normal during a period of at least 6 months and by the presence of anti-HCV antibodies as detected by enzyme-linked immunoassay (EIA-2; Ortho Diagnostic Systems, Raritan, N.J.) and was confirmed by a strip immunoblot assay (RIBA 2.0 SIA; Ortho Diagnostic Systems). Healthy blood donors showed no clinical manifestations of disease, and specimens tested negative for anti-HCV antibodies. Plasma specimens were collected by standard procedures with EDTA as the anticoagulant. Serum specimens were removed from the clot within 4 h of blood collection. All specimens were frozen and stored at −20°C or colder.

The HCV genotypes of all clinical specimens were established by the institu-

* Corresponding author. Mailing address: Nucleic Acid Systems, Chiron Corporation, 4560 Horton St., Emeryville, CA 94608-2916. Phone: (510) 601-3045. Fax: (510) 655-7733. Electronic mail address: Ray_Sanchez-Pescador@cc.chiron.com.

tions from which they were obtained. Specimens of HCV genotypes 2 and 3 were obtained from Massimo Colombo and Mariagrazia Rumi (Universitá degli Studi di Milano, Milano, Italy), Patrizia Pontisso and Alfredo Alberti (University of Padova, Padova, Italy), and Alessandro Tagger (Universitá Statatledi Milano, Milano, Italy). Specimens from patients in the United States, where HCV genotype 1 is predominant (44), were obtained from Karen Lindsay (University of Southern California, Los Angeles). HCV genotype 1 was confirmed for a subset of these specimens by using the commercially available INNO-LiPA HCV Line Probe Assay (Innogenetics N.V., Zwijndrecht, Belgium) (47, 53). Plasma and serum specimens from healthy blood donors, as well as paired plasma and serum specimens from patients chronically infected with HCV, were provided by Kenneth Kuramoto (Sacramento Medical Foundation, Sacramento, Calif.).

HCV RNA quantification by bDNA technology. HCV RNA was quantified by bDNA technology (50) by using the HCV RNA 1.0 and the HCV RNA 2.0 assays according to the manufacturer's instructions (Chiron Corporation). The HCV RNA 2.0 assay is performed in a 96-microwell format analogous to that described for the HCV RNA 1.0 assay (25). Briefly, 150 μl of specimen working reagent (627.5 mM LiCl, 157 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid); pH 7.5], 12.5 mM EDTA, 1.6% lithium lauryl sulfate, 19 μg of sonicated salmon sperm DNA per ml, 0.05% sodium azide, 0.05% Proclin 300 [Supelco, Inc., Bellefonte, Pa.], 2.2 mg of proteinase K per ml, 0.17 pmol of each HCV target probe per ml to mediate capture, and 1.34 pmol of each HCV target probe per ml to bind amplifier) was added to each oligonucleotide-modified microwell (41). Fifty microliters of serum or plasma specimens, standards, and controls was added to the appropriate microwell, in duplicate. The capture wells, which were contained in a 96-place metal microwell holder, were sealed with Mylar film and were then incubated overnight at 53°C in a heater (Chiron Heater; Chiron Corporation) in order to capture the target-probe complexes on the microwell surface. The wells were allowed to cool to room temperature for 10 min, the fluids were aspirated, and the wells then were washed twice with HCV wash A (0.1× standard saline citrate [SSC; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate, 0.05% sodium azide, 0.05% Proclin 300); this was followed by the addition of 50 μl (per well) of HCV Amplifier, which contained 2.0 pmol of bDNA amplifier per ml (bDNA [51]) in label diluent (0.5% Blocking Reagent [Boehringer Mannheim, Indianapolis, Ind.], 0.1% sodium dodecyl sulfate, 5× SSC, 0.05% sodium azide, 0.05% Proclin 300). The wells were sealed and incubated in the heater at 53°C for 30 min in order to hybridize the bDNA amplifier molecules to the target-probe complexes on the microwell surface. The wells subsequently were cooled and washed as described above; this was followed by the addition of 50 μl of HCV label working reagent (4 pmol of alkaline phosphatase-labeled probe per ml [52] in label diluent). The wells were sealed and incubated at 53°C for 15 min in order to hybridize the alkaline phosphatase probes to the immobilized bDNA amplifier molecules. The wells were cooled as described above and were washed twice with HCV wash A; this was followed by three washes with HCV wash B (0.1× SSC, 0.05% sodium azide, 0.05% Proclin 300). A 50-μl volume of chemiluminescent substrate (Lumiphos 530; Lumigen, Detroit, Mich.), an enzyme-triggerable dioxetane substrate for alkaline phosphatase (43), was added to each well; this was followed by incubation at 37°C for 25 min. Light emission, which was proportional to the amount of target present in each well, was measured in a luminometer.

The amount of HCV RNA in each specimen was quantified by using a standard curve. The assay standards contained proteinase K-digested, recombinant single-stranded bacteriophage M13 DNA (Bluescript; Stratagene, La Jolla, Calif.) containing 1,343 bases of the 5′ untranslated region and the core region of HCV (nucleotides 20 to 1363), which was carefully value assigned in comparison with the quality level 1 reference material described previously (12). Four assay standards were prepared by serial dilution of disrupted HCV recombinant phage in normal human serum (a pool of sera from healthy blood donors shown to be negative for HCV antibodies by EIA-2 [Ortho Diagnostic Systems] and HCV RNA by the HCV RNA 2.0 assay), to 120 (standard A), 12 (standard B), 1.2 (standard C), and 0.2 (standard D) Meq of HCV RNA per ml. The concentration of HCV RNA in each specimen was calculated from the standard curve, which was defined by a quadratic curve-fit function, and the results were expressed as the megaequivalents of HCV RNA per milliliter. One megaequivalent was defined as the amount of HCV RNA that generates a level of light emission equivalent to that generated by 10^6 copies of quality level 1 RNA standard.

Preparation of HCV RNA transcripts. (i) Viral origin. Plasma and serum specimens containing HCV whose genotypes had been determined were provided by Donald Smith and Peter Simmonds (Department of Medical Microbiology, University of Edinburgh, Edinburgh, United Kingdom) and Christian Bréchot (Liver Cancer and Molecular Virology, Institut National de la Santé et de la Recherche Medicale, Unité 370, Paris, France). Additional specimens were obtained from Boston Biomedica, Inc. (West Bridgewater, Mass.). The HCV genotype of the specimens was verified by nucleotide sequence analysis of the NS5 and 5′ untranslated regions (46). HCV RNA was extracted from specimens essentially as described by Bukh et al. (4) and was stored in 20 μl of diethyl pyrocarbonate-treated water at −80°C for cDNA synthesis.

(ii) RT-PCR and cloning. The synthesis of cDNA was performed at 42°C for 15 min in a 20-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM (each) the four deoxynucleoside triphosphates (dNTPs; Pharmacia, Piscataway, N.J.), 3.75 μM random hexamers (Promega, Madison, Wis.), 40 U of RNasin (from a stock solution of 40 U/μl; Promega), and 200 μl of moloney murine leukemia virus reverse transcriptase (from a stock solution of 200 U/μl; Bethesda Research Laboratories, Gaithersburg, Md.). The reaction mixture was inactivated by heating at 99°C for 5 min and was then cooled to 80°C before the addition of reaction components for subsequent amplification. PCR primers were designed on the basis of the sequence data available from a set of genotyped HCV sequences. The reverse transcription-PCR (RT-PCR) fragment had an expected size of 823 bp and included the 5′ noncoding region and core region. The PCR primers were sense (5′-GACACTC CACCATGAATCACTCCCCTG-3′; nucleotides 21 to 47) to amplify the fragments for genotypes 1 through 6, antisense, (5′-CCCTGTTGCATAGTTCACG CCGTC-3′; nucleotides 819 to 842) for genotypes 1, 5, and 6, and antisense (5′-CCCTGTTGCCGAAATTTATCCCGTC-3′; nucleotides 819 to 842) for genotypes 2, 3, and 4. The final concentrations of the reaction components for PCR were 10 mM Tris (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.4 mM (each) the four dNTPs, 0.2 μM (each) primer, and 2.5 μl of *Taq* polymerase (from a stock solution of 5 U/μl; Amplitaq; Perkin-Elmer, Norwalk, Conn.) in a 100-μl reaction volume. PCR was performed for 5 cycles of 1 min at 97°C, 1 min at 50°C, and 2 min at 72°C; this was followed by 30 cycles of 1 min at 94°C, 1 min at 50°C, and 3 min at 72°C. Final extension was performed at 72°C for 10 min. Amplification products were gel purified (Prep-A-Gene; Bio-Rad Laboratories, Hercules, Calif.) and were analyzed by agarose gel electrophoresis. The amplified 827-bp fragment was ligated into a transcription vector with a single 3′ thymidine overhang (TA cloning; Invitrogen, San Diego, Calif.).

(iii) HCV genotyping. The HCV genotype of each isolate used to generate the 827-bp transcript first was verified by nucleotide sequence analysis of the 401-bp NS5 region as described by Simmonds et al. (46) by using primers originally designed by Enomoto et al. (17). Then, cDNA synthesis and PCR amplification were performed as described above, and each amplified 401-bp fragment was purified from primers and free nucleotides by filtration in a Centricon 100 apparatus (Amicon, Beverly, Mass.). One plasmid clone per isolate was sequenced from the 827-bp 5′ untranslated and core region, as well as from the purified 401-bp amplicon from the NS5 region used to verify the genotype. The HCV DNA was sequenced by thermal cycle sequencing with *Taq* polymerase by using M13 universal and reverse primers as well as internal primers based on consensus sequences. Sequence analysis was performed with an Applied Biosystems 373A automated sequencer. Comparisons of HCV sequences were based on calculations to determine the proportion of matched nucleotides (FastDB; Intelligenetics, Mountain View, Calif.).

(iv) In vitro transcription and RNA purification. Plasmid DNA was linearized by digestion with either *Bam*HI or *Hin*dIII. In vitro transcripts were prepared from approximately 5 μg of linearized plasmid DNA using the MEGAscript in vitro transcription kit for large-scale synthesis of RNA (Ambion, Austin, Tex.), and the HCV RNA was purified by chromatography as described previously (12). α-^{32}P-labeled GTP was added as a tracer to the transcription reactions in sufficient quantity to yield a specific activity of approximately 200 cpm/ng of HCV RNA. To assess the quality of the preparations, HCV RNA transcripts were electrophoresed on 1.5% formaldehyde gels (42) which were dried and then scanned on an Ambis 4000 radioanalytic imager (Ambis, Inc., San Diego, Calif.). The size of the transcripts was determined by least-squares analysis of a plot of molecular weight versus 1/mobility. The percentage of full-length transcripts was determined by measuring the radioactivity in each rectangle of a grid placed over the dried gel and calculating the percentage of total radioactivity in the peak fractions. The preparations of HCV RNA transcripts used in the study contained <3% free nucleotides and were composed of >80% full-length transcripts.

(v) Independent quantification and serial dilution of HCV RNA transcripts. Three independent analytical methods were used for quantification of HCV RNA transcripts, including A_{260} measurement (42), phosphate determination (13), and hyperchromicity analysis (54). Preparations of HCV RNA transcripts were measured in triplicate by each of these three methods as described previously (12), and quantification results agreed within 10%. Serial dilutions of transcripts, ranging from 1.2×10^5 to 1.2×10^8 copies per ml, were prepared for quantification of HCV RNA in the bDNA assay. ^{32}P-labeled nucleotide was added as a tracer to verify the accuracies of the dilutions.

Statistical analysis. Concordance correlation coefficients (ccc's) were calculated as described by Lin (29). Linearity was evaluated by using orthogonal polynomial regression (24) and Fieller's theorem (23).

RESULTS

Principles of the bDNA assay. The bDNA assay used a solution-phase sandwich assay format in which HCV RNA from plasma or serum specimens was released from virions by treatment with proteinase K and was then hybridized in solution with oligonucleotide target probes. The HCV RNA with bound target probes was captured onto oligonucleotide-modified microwells (41) and was then hybridized with bDNA amplifier molecules (21, 51). Amplification of the HCV RNA signal was achieved by the binding of multiple alkaline phos-

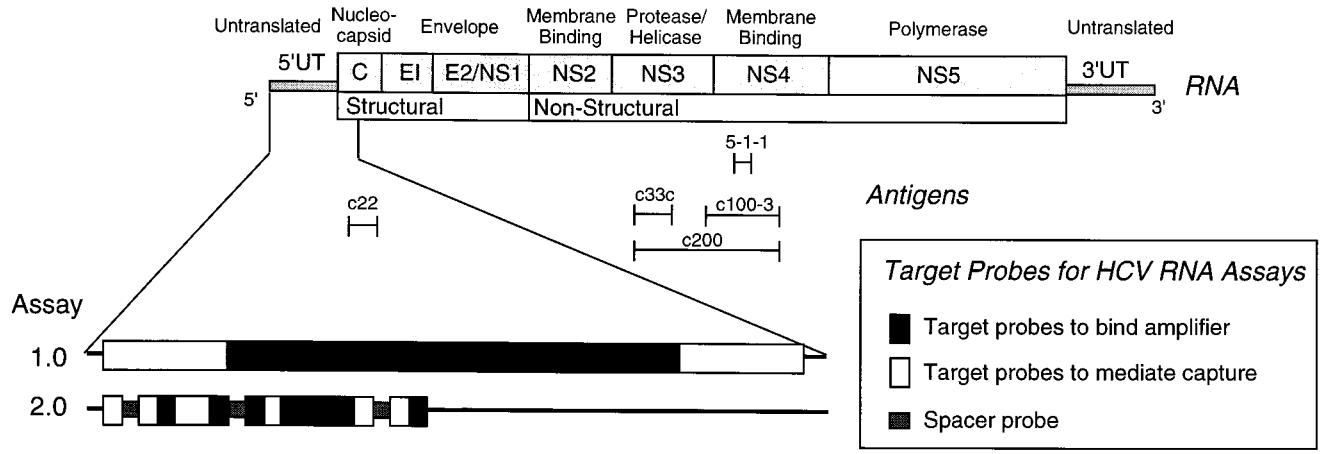

FIG. 1. Regions of the HCV genome recognized by target probes in the bDNA assay. Target probes to bind amplifier and target probes to mediate capture are homologous to the 5′ untranslated region and the 5′ third of the core gene in the HCV genome.

phatase-labeled oligonucleotide probes (52) to each bDNA amplifier molecule. A dioxetane substrate (Lumi-Phos 530; Lumigen) was added, and the resultant chemiluminescent output was quantified by photon counting in a plate luminometer (Chiron Corporation).

The design of the target probes was an important consideration in the development of the HCV RNA 2.0 assay. Both types of target probes, those that mediated capture of the HCV RNA to the microwell surface and those that mediated binding to the bDNA amplifier molecules, were designed to achieve equal quantification of the HCV genotypes. Target probes were complementary to the most conserved regions of the HCV genome, the 5′ untranslated and core regions (Fig. 1). Sequences of the target probes used in the HCV RNA 2.0 assay were based on nucleotide sequences from all six major types of HCV. Target probes contained 16 to 24 nucleotides with a calculated melting temperature of ~70°C to enhance the uniformity of hybridization and to obtain optimal specificity.

Performance characteristics. The quantification limit of the HCV RNA 2.0 assay was determined by analyzing a panel of plasma specimens from 124 healthy HCV-seronegative blood donors and serum specimens from 259 HCV-seronegative subjects, including 186 healthy blood donors, 62 patients with other diseases (human immunodeficiency virus infection, hepatitis B virus infection, autoimmune hepatitis, alcoholic hepatitis), and 11 patients with elevated ALT levels not related to HCV or other known causes of hepatitis (Table 1). Upon initial testing, 118 of 124 plasma specimens and 252 of 259 serum specimens were shown to produce signals which were lower than those calculated for 0.2 Meq of HCV RNA per ml. The quantification limit of the HCV RNA 2.0 assay therefore was set at 0.2 Meq of HCV RNA per ml in order to achieve a specificity of 95% for plasma specimens and a specificity of 97% for serum specimens. On retesting, the 13 specimens with quantification values greater than 0.2 Meq of HCV RNA per ml yielded values below the quantification limit. It should be noted that none of the 62 serum specimens from HCV-seronegative subjects with other diseases or the 11 serum specimens containing elevated ALT levels yielded values above the quantification limit. These data indicated that the HCV RNA 2.0 assay was specific only for RNA from HCV and not for RNAs from other viruses which might be found in the sera of infected individuals.

The sensitivity of the HCV RNA 2.0 assay was evaluated by testing serum specimens from 158 patients chronically infected with HCV and not undergoing antiviral therapy (Table 1). Of the 158 serum specimens, 151 yielded values greater than the quantification limit. The remaining seven serum specimens contained less than 0.2 Meq of HCV RNA per ml, and HCV RNA was not measurable by the HCV RNA 2.0 assay. Thus, the HCV RNA 2.0 assay had a 96% sensitivity (95% confidence interval, 91 to 98%) for the quantification of HCV RNA in specimens from chronically infected patients.

The dose-response linearity of the HCV RNA 2.0 assay, shown with serial dilutions of HCV RNA transcripts, revealed a linear response between HCV RNA concentration and relative luminescence over a nearly 5-$\log_{10}$ range. These results showed that the dynamic quantification range of the HCV

TABLE 1. Sensitivity and specificity of the HCV RNA 2.0 assay

Sample	No. of specimens	No. (%) of specimens with HCV RNA quantification value of:	
		>0.2 Meq/ml	<0.2 Meq/ml
Serum (HCV seronegative)	259	7 (3)	252 (97)
Plasma (HCV seronegative)	124	6 (5)	118 (95)
Serum (chronic HCV infection)	158	151 (96)	7 (4)

TABLE 2. Reproducibility of the HCV RNA 2.0 assay

Sample	Kit	No. of determinations	Mean HCV RNA level (Meq/ml)	% CV[a]
Serum (low)	1	72	0.79	16
	2	72	0.85	21
Serum (mid)	1	72	5.25	15
	2	72	5.38	13
Serum (high)	1	72	31.66	14
	2	72	30.30	9
Plasma	1	72	3.24	14
	2	66	3.43	11

[a] CV, coefficient of variance. The mean coefficient of variance was 14%.

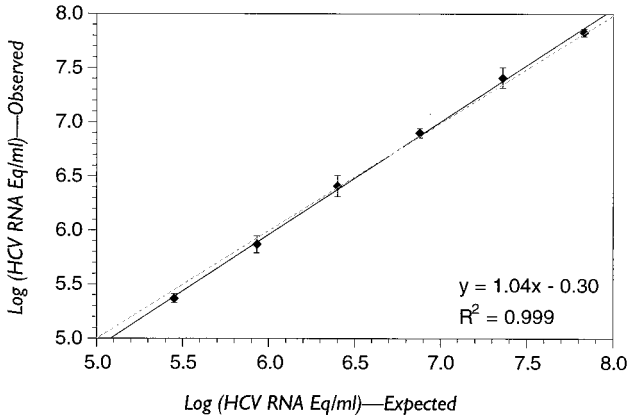

FIG. 2. Expected versus observed HCV RNA quantification values for threefold serial dilutions of serum measured by the HCV RNA 2.0 assay.

RNA 2.0 assay extended to at least 10^{10} Meq of HCV RNA per ml. Since even the most concentrated samples were measured by the HCV RNA 2.0 assay, the upper quantification limit was not reached in this dilution series. The linearity of quantification also was evaluated according to the National Committee for Clinical Laboratory Standards proposed guidelines (34) by using an orthogonal polynomial regression. A high correlation (r = 0.999) between expected and observed values for the quantification of HCV RNA in threefold serial dilutions was obtained (Fig. 2). The largest bias between observed and expected values was less than ±1.0% over the entire range of the standard curve from 0.2 to 120 Meq/ml. These results demonstrated that the HCV RNA 2.0 assay yielded accurate quantification values throughout the range of the standard curve.

The reproducibility of the HCV RNA 2.0 assay was established by testing replicates of specimen panels in 24 separate assays runs by two operators over the course of 6 days with two different lots of reagents (Table 2). The specimen panels included three HCV-positive serum specimens with quantities of HCV RNA in each of the three segments of the standard curve (low, middle, and high) and an HCV-positive plasma specimen. Mean quantification values were 0.82, 5.32, and 31.0 Meq of HCV RNA per ml for the low-, middle-, and high-titer serum specimens, respectively, and 3.34 Meq of HCV RNA per ml for the plasma specimen. The percent coefficient of variance ranged from 9 to 21%, with a mean value of 14%.

The effect of specimen type on HCV RNA quantification was examined by testing paired serum and EDTA plasma specimens obtained in parallel draws from 23 patients chronically infected with HCV. A high correlation (r = 0.97) was observed between plasma and serum specimens over the dynamic quantification range of the HCV RNA 2.0 assay. The R^2 and ccc values for the relationship between the megaequivalents of HCV RNA per milliliter in plasma and those in serum were 0.94 and 0.97, respectively. These results showed that HCV RNA in plasma and serum specimens from the same patients was quantified equally in the HCV RNA 2.0 assay and indicated that quantification of HCV was similar for plasma and serum specimens. Thus, either type of clinical specimen could be used for HCV RNA quantification to assess viral load in patients.

Effect of HCV genotypic variation on HCV RNA quantification. Nucleotide sequence variation can have a major impact on the hybridization efficiency of probe-based assays. The sequence diversity in the probe-binding region of the HCV genome was assessed by comparing the nucleotide sequences (9) from the 5′ untranslated and core regions (nucleotides 21 to 842) of isolates from each of the six major HCV genotypic groups (Table 3). Comparison of the sequences was based on the proportion of matched nucleotides in clones representing the different HCV genotypes and subtypes. The sequence similarities between HCV isolates of the same genotype ranged from 91 to 98%. The sequence similarities between HCV isolates of different genotypes ranged from 86 to 92%.

The effect of HCV genotype on HCV RNA quantification was evaluated for both the HCV RNA 1.0 and HCV RNA 2.0 assays by testing serial dilutions of quality level 1 RNA transcripts (12) representing 5′ untranslated and core sequences from each of the six major HCV genotypes. As illustrated in Fig. 3, HCV RNA from all six HCV genotypes was recognized by both bDNA assays. A threefold difference in quantification, between HCV genotypes 1 and 2, was the maximum variance observed by the HCV RNA 1.0 assay. By comparison, the HCV RNA 2.0 assay was virtually unaffected by HCV genotype. A 1.5-fold difference in quantification, between HCV genotypes 3a and 6a, was the maximum variance observed by the HCV RNA 2.0 assay.

Serum specimens from patients chronically infected with HCV genotypes 1, 2, and 3 were tested by both bDNA assays to explore further the effect of HCV genotype on HCV RNA quantification (Fig. 4). Of 69 serum specimens containing HCV genotype 1, 67 contained HCV RNA above the quantification limit of the HCV RNA 1.0 assay (0.35 Meq of HCV RNA per ml), while 68 contained HCV RNA at a level above the quantification limit of the HCV RNA 2.0 assay (0.20 Meq of HCV RNA per ml). The detection rate for specimens containing HCV genotype 1 was similar for both assays: 97% for the HCV RNA 1.0 assay and 99% for the HCV RNA 2.0 assay (P = 0.32). The ratio of quantification values for the HCV RNA 2.0 assay compared with those for the HCV RNA 1.0 assay was 1.1 for specimens containing HCV genotype 1, demonstrating that HCV RNA from HCV genotype 1 was equally quantified by both assays (ccc = 0.886). By contrast, a significant difference in the detection rate of HCV genotypes 2 and 3 in specimens was observed. Of 89 serum specimens containing HCV genotypes 2 or 3, 60 contained HCV RNA levels above the quantification limit of the HCV RNA 1.0 assay, while HCV RNA was quantified in 83 serum specimens by the

TABLE 3. Sequence homology of the 5′ untranslated region and the 5′ third of the core gene (nucleotides 21 to 842) of the HCV genome

Genotype	% Residue identity of query sequences of the following genotype being compared[a]:										
	1a	1b	2a	2a	2b	3a	3a	4a	5a	5a	6a
1a[b]	100	95	88	88	87	88	88	90	90	90	89
1b[c]		100	88	88	86	87	88	90	92	91	88
2a[c]			100	94	91	86	86	87	88	88	87
2a[c]				100	92	86	86	89	89	88	87
2b[d]					100	86	85	88	87	86	88
3a[c]						100	98	88	88	88	87
3a[c]							100	88	88	87	87
4a[d]								100	88	89	88
5a[c]									100	96	88
5a[b]										100	87
6a[d]											100

[a] Sequences were compared by using FastDB (Intelligenetics).
[b] Provided by Boston Biomedica, Inc.
[c] Provided by Christian Bréchot.
[d] Provided by Peter Simmonds and Donald Smith.

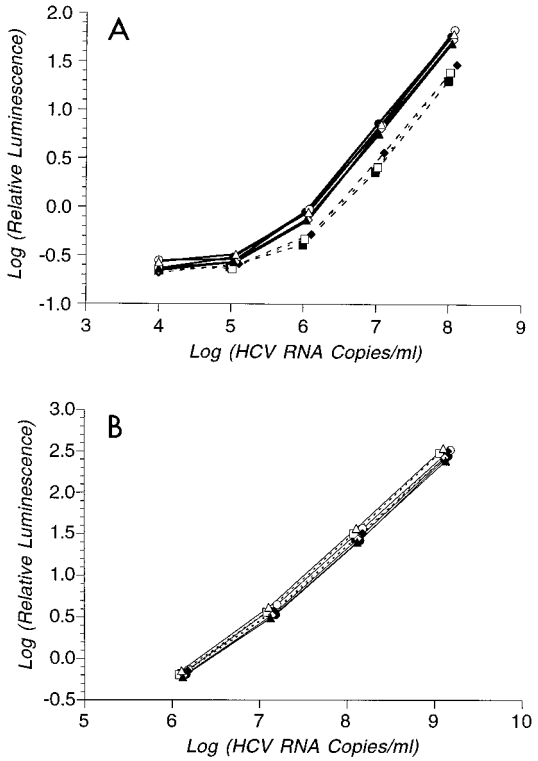

FIG. 3. Comparison of relative luminescence produced with RNA transcripts representing six genotypes of HCV. Results were obtained from both the HCV RNA 1.0 assay (A) and the HCV RNA 2.0 assay (B). HCV RNA was quantified from RNA transcripts derived from HCV isolates of genotypes 1a (●), 1b (○), 2a (■), 2b (□), 3a (◆), 4a (◇), 5a (▲), and 6a (△).

HCV RNA 2.0 assay. Whereas the HCV RNA 1.0 assay exhibited a 67% rate of detection of HCV genotype 2 and 3 in specimens, the HCV RNA 2.0 assay exhibited a detection rate of 93% ($P < 0.01$). The ratio of quantification values for the HCV RNA 2.0 assay compared with those for the HCV RNA 1.0 assay was 3.0 for specimens containing HCV genotypes 2 and 3 (ccc = 0.551). Thus, HCV RNA clearly was quantified by the HCV RNA 2.0 assay in a large number of specimens containing HCV genotypes 2 and 3, which yielded values below the quantification limit of the HCV RNA 1.0 assay. It should be noted that for all serum specimens that yielded signals below the quantification limit of the HCV RNA 2.0 assay, HCV RNA also was not quantified by the HCV RNA 1.0 assay. These results demonstrate that, whereas both bDNA assays equally quantified HCV RNA from HCV genotype 1, the HCV RNA 2.0 assay quantified HCV RNA in serum specimens containing HCV genotypes 2 and 3 more efficiently. Furthermore, these results justified the dynamic quantification range of the standard curve used in the HCV RNA 2.0 assay, since all specimens tested yielded values below 120 Meq of HCV RNA per ml.

DISCUSSION

The relationship between HCV genotype and viremia levels to disease progression and therapeutic response is not yet fully understood. Studies have demonstrated that low pretreatment HCV RNA levels are predictive of a complete and sustained response to interferon therapy in patients (14, 26, 33, 38, 56). Likewise, the HCV genotype also has been implicated as a potential factor predictive of the response to interferon therapy (7, 22, 31, 39, 49). Since HCV genotypic variability may influence the efficiency of HCV RNA quantification, the observed responsiveness to interferon therapy may reflect both genotype-specific differences and differences in viral load. To effectively evaluate these studies, it is necessary to distinguish the relative contributions of HCV RNA levels and HCV genotype.

A major obstacle to understanding the clinical and pathobiological relevance of HCV genotype and HCV viremia levels has been the lack of a reliable method for RNA quantification which is unaffected by HCV genotypic variability. Differences in detection efficiency may occur for any of the quantitative methods based on the hybridization of HCV RNA to complementary nucleotide sequences, such as the bDNA assay or RT-PCR methods. Although multiple probes in the HCV RNA 1.0 assay were designed to include the sequence variation present in the diverse HCV genotypes, we found that the hybridization efficiencies varying among HCV genotypes caused this assay to have a small but consistent bias. The efficiency of PCR amplification for some HCV genotypes may be reduced because of the presence of type-specific polymorphisms in regions targeted by amplification primers. Unfortunately, studies examining the effect of HCV genotypic variation on RT-PCR or other target amplification techniques have not yet been reported.

We have developed a modified set of oligonucleotide probes for the bDNA assay to enhance the efficiency of binding to genotypic variants of HCV. Our results demonstrated that the quantification of HCV RNA by the HCV RNA 2.0 assay, which incorporates these modified probes, was virtually unaffected by HCV genotypic variability. The improved sensitivity of the HCV RNA 2.0 assay for HCV genotypes 1 through 6 was confirmed by using both RNA transcripts as well as clinical specimens. Furthermore, the HCV RNA 2.0 assay was two- to threefold more efficient in HCV RNA quantification for HCV genotypes 2 and 3 than the HCV RNA 1.0 assay. However, HCV RNA from HCV genotype 1 was equally quantified by both bDNA assays. The resulting correction of bDNA values

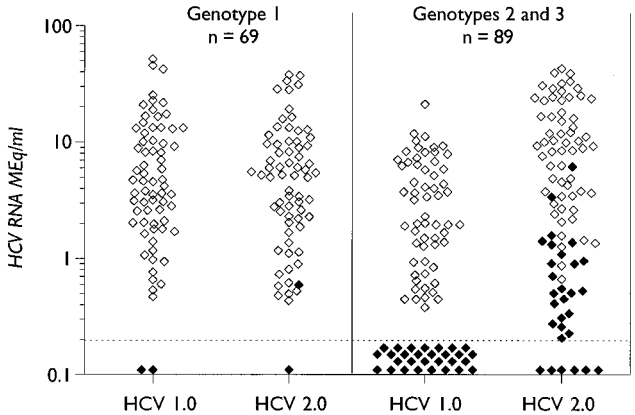

FIG. 4. Comparison of the detection rate of HCV RNA in serum specimens containing HCV genotype 1 versus that for serum specimens containing HCV genotypes 2 and 3 by the HCV RNA 1.0 and 2.0 assays. Specimens containing HCV RNA levels above the quantification limit of the HCV RNA 1.0 assay are represented as open diamonds, and those not quantified by the HCV RNA 1.0 assay are indicated as closed diamonds. A similar detection rate of HCV RNA for serum specimens containing HCV genotype 1 was observed with both the HCV RNA 1.0 (97%) and HCV RNA 2.0 (99%) assays. An improved detection rate for serum specimens containing HCV genotypes 2 and 3 was observed by the HCV RNA 2.0 assay (93%) compared with that observed by the HCV RNA 1.0 assay (67%).

on the basis of genotype does not significantly affect the predictive value of pretreatment levels of HCV RNA in patients receiving interferon (28). Continued study of HCV sequence divergence has led to the classification of new HCV genotypes, such as HCV genotypes 7, 8, and 9 recently identified in Vietnamese blood donors (48). As sequence information for the probe-binding region becomes available, it will be important to assess the quantification of HCV RNA by the HCV RNA 2.0 assay for these new genotypes.

The performance characteristics of quantitative assays must be established for use in a clinical setting. The present study demonstrated that the HCV RNA 2.0 assay accurately quantifies HCV RNA from all six major genotypes of HCV. The HCV RNA 2.0 assay yielded highly reproducible results (mean coefficient of variance, 14%) and exhibited high levels of specificity (≥95%) and sensitivity (96%). A linear response between HCV RNA concentration and relative luminescence was observed throughout the dynamic quantification range of the standard curve, from 0.2 to 120 Meq of HCV RNA per ml. Performed in a 96-well format, the HCV RNA 2.0 assay allowed up to 42 patient specimens to be included in duplicate within each assay run. There was no sample preparation time, and viral quantification was equivalent for both serum and plasma specimens. The HCV RNA 2.0 assay may provide a useful tool that will allow physicians to follow HCV RNA levels throughout the course of disease, select patients for therapy, and evaluate the efficacies of therapeutic regimens.

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