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Preclinical Evaluation of AMPLICOR Hepatitis C Virus Test for Detection of Hepatitis C Virus RNA

FREDERICK S. NOLTE,[1,2]* CATHY THURMOND,[2] AND MICHAEL W. FRIED[2,3]

Departments of Pathology and Laboratory Medicine[1] and of Medicine (Digestive Diseases),[3] Emory University School of Medicine, and the Emory Clinic,[2] Atlanta, Georgia 30322

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We compared a single-enzyme, combined reverse transcription-PCR (RT-PCR; AMPLICOR HCV Test; Roche Molecular Systems, Branchburg, N.J.) with an independent, two-enzyme, standard RT-PCR (SRT-PCR) assay for the detection of hepatitis C virus (HCV) RNA in serum and plasma. Test samples included a proficiency testing panel consisting of 10 undiluted plasma samples, three separate dilution series, and sera from 99 patients with chronic liver disease. The quantity of HCV RNA in each patient serum sample was determined by a branched DNA (bDNA) signal amplification assay (Quantiplex HCV-RNA assay; Chiron, Emeryville, Calif.). There was complete concordance between the results of the RT-PCR assays with the 10 undiluted plasma samples used for proficiency testing (3 positive and 7 negative samples). However, the analytical sensitivity of SRT-PCR was 4- to 10-fold greater than that of the AMPLICOR test in the dilution series. HCV RNA was detected in 44, 45, and 40 of the patient serum samples, by SRT-PCR, the AMPLICOR test, and the bDNA assay, respectively. There was 97% agreement between the results of the RT-PCR assays, with only three discrepancies. Review of the patients' medical records resolved all three discrepancies in favor of the AMPLICOR results (two false-negative SRT-PCR results and one false-positive SRT-PCR result). The quantity of HCV RNA in sera from five (11%) patients with viremia detected by AMPLICOR was below the bDNA assay cutoff (<3.5×10^5 RNA equivalents per ml). AMPLICOR compared favorably with SRT-PCR, with key advantages of speed, ease of use, increased sample throughput, and protection against false-positive results because of amplicon carryover.

Hepatitis C virus (HCV) is the principal cause of posttransfusion and sporadic non-A, non-B hepatitis in the world (3). HCV is a single-stranded, positive-sense RNA virus with a genome of approximately 10,000 nucleotides coding for 3,000 amino acids (9). The development of immunoserological tests for anti-HCV antibodies has facilitated the detection of HCV infection and has reduced the incidence of posttransfusion hepatitis in the United States (10).

Unfortunately, these immunoserological tests have several shortcomings. Anti-HCV antibody tests may have a prolonged window of seronegativity after acute infection, do not differentiate between active and resolved infections, and may be falsely positive in patients with hypergammaglobulinemia and other forms of chronic hepatitis such as autoimmune hepatitis (1, 5, 6). Falsely negative antibody tests may occur in as many as 10% of patients with chronic HCV infection (1). Currently, an immunological assay for the direct detection of HCV antigen in serum is not available.

Amplification of viral cDNA by reverse transcription-PCR (RT-PCR) has been shown to be a sensitive means for the direct detection of HCV (7, 8, 20). Detection of HCV RNA in serum is indicated for patients with acute hepatitis prior to seroconversion, in suspected cases of chronic HCV infection in seronegative patients, for the resolution of indeterminate immunoserological tests, and to monitor patients receiving alpha interferon therapy (9, 11, 15).

Since the genome of HCV is a single-stranded RNA molecule, PCR amplification must be preceded by a step that generates a cDNA copy. Synthesis of the cDNA is usually accomplished by using a retroviral reverse transcriptase. Amplification of the cDNA is typically done with a thermostable DNA polymerase from *Thermus aquaticus*. The need for different enzymes in the two steps initially led to the addition of new enzyme and a change of buffer conditions between the RT and DNA amplification steps. The two-step process is cumbersome and increases the opportunity for contamination of the reaction mixture with previously amplified DNA. Coupled RT-PCR assays in which retroviral reverse transcriptase, *Taq* polymerase, and both primers are present in a single buffer system have also been described (4).

A combined RT-PCR assay for HCV RNA has been developed by Roche Molecular Systems (AMPLICOR HCV test). The assay uses a single thermostable enzyme and buffer condition in a single reaction tube (21). The DNA polymerase of *Thermus thermophilus* (*rTth*) is used in the AMPLICOR test because of its enhanced RT activity in the presence of manganese (14). In addition to minimizing the possibility of contamination, the use of a thermostable enzyme in the AMPLICOR test allows for RT at elevated temperatures. This should increase the specificity of primer extension and improve the efficiency of RT through destabilization of the secondary RNA structure. In addition, the use of a thermostable enzyme for RT is compatible with the uracil-*N*-glycosylase (UNG) protocol for prevention of false-positive results because of the carryover of previously amplified DNA (13).

The branched DNA (bDNA) assay is a quantitative signal amplification method based on a series of specific hybridization reactions and chemiluminescent detection of hybridized probes in a microwell format (18). Quantitative determination of HCV RNA in serum may provide important prognostic information and a marker of response to interferon therapy (11, 12). However, the high threshold of detection of the bDNA assay (3.5×10^5 HCV RNA equivalents per ml) may limit its use in the diagnosis and management of patients with HCV infection.

In the study described here we compared the AMPLICOR HCV test with an independent, two-enzyme, standard RT-PCR assay (SRT-PCR) for the detection of HCV RNA in serum and plasma. Test samples included a proficiency testing

* Corresponding author. Mailing address: Clinical Laboratories, Emory University Hospital, 1364 Clifton Rd., N.E. Atlanta, GA 30322. Phone: (404) 712-7297. Fax: (404) 712-5567.

panel, three separate dilution series, and sera from 99 patients with chronic liver disease. The quantity of HCV RNA in each patient serum sample was also determined by the bDNA assay (Quantiplex HCV RNA assay; Chiron, Emeryville, Calif.).

MATERIALS AND METHODS

Clinical specimens. The sera included in the present evaluation were obtained from 99 patients referred to a university hepatology clinic who were undergoing evaluation for chronic liver disease. Frozen sera from our serum bank were selected for this evaluation on the basis of SRT-PCR results (44 serum samples were positive and 55 serum samples were negative for HCV RNA). All serum samples were separated from the clots within 4 h of collection, divided into aliquots, and stored at −70°C until they were tested by the AMPLICOR test and the bDNA assay. A serial 10-fold dilution series of serum from a patient (patient 844) containing 39.3×10^5 HCV RNA equivalents per ml by the bDNA assay was prepared in HCV-negative serum to compare the limits of detection of the RT-PCR-based assays.

Patients. The age, sex, diagnosis, liver histology, risk factors for HCV infection, liver function test results (alanine aminotransferase, asparatate aminotransferase, alkaline phosphatase, total bilirubin, and serum albumin), and HCV serological status of each patient were obtained by review of the patients' medical records. Forty-three (44%) patients were diagnosed as having chronic HCV infection, while the remainder were considered to have a variety of other liver diseases; cryptogenic cirrhosis, 12%; alcoholic liver disease, 10%; autoimmune hepatitis, 7%; primary biliary cirrhosis, 6%; polycystic liver disease, 3%; chronic hepatitis B virus infection, 2%; other chronic liver diseases, 10%; unspecified, 5%. The results of HCV antibody testing (enzyme immunoassay [EIA] EIA-1 or EIA-2, Abbott Laboratories, Abbott Park, Ill.) were available for 95 patients, and 45 (47%) had positive test results.

Eurohep proficiency testing panel. The second Eurohep HCV RNA proficiency testing panel was tested by both the AMPLICOR test and SRT-PCR (21). The coded test panel consisted of 10 undiluted plasma samples and two separate dilution series. The Eurohep dilution series 1 and 2 contained dilutions of HCV genotypes 1 and 3, respectively, in negative plasma.

SRT-PCR. The primer pair used in the SRT-PCR assay was selected from the highly conserved 5′-untranslated region of the HCV genome. The pair consisted of downstream primer 5PUT c1-a, 5′-CCCAACACTACTCGCCTAG-3′ (nucleotides −74 to −92), and upstream primer 5PUT 1-s, 5′-AACTACTGTCTTCACGCAG AAAGC-3′ (nucleotides −266 to −289) (2). 5PUT p1-s, 5′-GCCATGGCGTTAG TATGAGTGTC-3′ (nucleotides −238 to −260), served as a hybridization probe.

Each serum sample was thawed and divided into equal 100-μl aliquots. RNA was extracted in duplicate with RNAzol B (Biotecx Laboratories, Houston, Tex.) as described previously (17). RNA pellets were dissolved in 10 μl of RNase-free 1 mM EDTA–10 mM NaCl–10 mM Tris-HCl (pH 8.0). One-half volume (5 μl) of the dissolved RNA pellet was added to 15 μl of the RT reaction mixture (GeneAmp RNA PCR kit; Perkin-Elmer Cetus, Norwalk, Conn.) consisting of 5 mM $MgCl_2$, 1× Buffer II (Perkin-Elmer Cetus), 1 mM (each) deoxyribonucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 1 U of RNase inhibitor, 2.5 U of Moloney leukemia virus reverse transcriptase, and 0.75 μM downstream primer (5PUT c1-a). cDNA synthesis was performed under mineral oil in a GeneAmp 480 thermocycler (Perkin-Elmer Cetus) with one cycle at 42°C for 15 min, one cycle at 99°C for 5 min, and one cycle at 4°C for 5 min.

A total of 80 μl of the PCR mixture consisting of 2 mM $MgCl_2$, 1× Buffer II, 2.5 U of Amplitaq, and 0.15 μM upstream primer (5PUT 1-s) was added to each tube under the oil layer. PCR amplification proceeded with incubation at 94°C for 5 min; this was followed by 5 cycles of 94°C for 2 min, 50°C for 2 min, and 72°C for 3 min and then 30 cycles of 94°C for 1.5 min, 60°C for 2 min, and 72°C for 3 min and a final extension step at 72°C for 7 min.

After completion of the amplification reaction, 25 μl of each reaction mixture was analyzed by electrophoresis through a 2.0% agarose gel with ethidium bromide staining and Southern blotting. DNA was transferred onto Duralon (Statagene, La Jolla, Calif.) nylon membranes by alkaline transfer (19). The transferred DNA was cross-linked to the membrane by UV light (Stratalinker; Stratagene). The blots were prehybridized in a solution of 0.05 M sodium phosphate buffer (pH 7.2), 1 mM EDTA, 7% sodium dodecyl sulfate (SDS), and 1% bovine serum albumin, at 42°C for 15 min in a shaking water bath. The prehybridization buffer was removed and the blots were hybridized in the same buffer with a biotin 3′-end-labeled probe (Clontech, Palo Alto, Calif.), 5PUT p1-s, at 42°C for 1 h. The blots were washed for 20 min at 55°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.1% SDS. A chemiluminescent Southern blot procedure was used to detect the hybridized probe (Southern-Light; Tropix, Bedford, Mass.). Results were imaged on X-ray film after 30 to 60 min of exposure. If the results of duplicate tests did not agree, the samples were reextracted and reamplified in duplicate.

A negative control serum sample, a reagent blank, and a positive control serum sample containing approximately 1,000 copies of the HCV genome per ml were included with each batch of specimens. The positive and negative control sera were carried through all of the steps in the procedure from extraction to product detection. The recommended procedures were followed to prevent false-positive reactions as a result of target or amplified product contamination (16).

AMPLICOR HCV test. HCV RNA was isolated from serum by guanidinium thiocyanate lysis and isopropanol precipitation in the presence of poly(A) carrier RNA. An equivalent of 5 μl of serum was amplified in a master mixture that contained *rTth* DNA polymerase, the primers KY80 and KY78, buffer salts, UNG, dATP, dCTP, dGTP, and dUTP. dUTP is incorporated into each amplification product to serve as a substrate for UNG (AmpErase; Roche Molecular Systems) to prevent carryover contamination of previously amplified DNA. A single primer pair, including a biotinylated downstream primer (KY80, 5′-GCAGAAAGCGTCT AGCCATGGCGT, and KY78, 5′-biotinyl-CTCGCAAGCACCCTATCAGGCA GT) was used to define a 244-bp amplicon located in the 5′-untranslated region of the HCV genome. The reaction was optimized for the use of *rTth* that, in the presence of manganese, performs both RT and DNA polymerase functions, obviating the requirement for two enzymes and two separate reactions. Amplification was carried out in the GeneAmp 9600 thermocycler (Perkin-Elmer Cetus) with a program that allowed for a 2-min incubation at 50°C for optimal UNG activity; this was followed by 30 min of incubation at 60°C for the reverse transcriptase step, 40 cycles of PCR (2 cycles of 95°C for 15 s and 60°C for 20 s and 38 cycles of 90°C for 15 s and 60°C for 20 s), and a final 4-min extension at 60°C. Detection of the PCR product was accomplished through the use of a solid-phase probe, specific for HCV, that was coated onto microwell plates. The biotin-labeled PCR product was chemically denatured to form single strands, hybridized to the microwells, and detected by using an avidin-horseradish peroxidase system with a conventional microtiter plate washer and a microtiter plate reader (450 nm). Optical density readings of >0.500 were considered positive, those of <0.300 were considered negative, and those of 0.300 to 0.500 were considered equivocal.

All samples were extracted once, and a single amplification and detection were performed on each extract. One positive and three negative controls provided with the kit were run with each batch of patient specimens. The controls were not extracted with the lysis buffer. By these methods, the time required to obtain a result is approximately 6 h for a batch size of 25 samples.

bDNA assay. Quantitative bDNA signal amplification assays (Quantiplex HCV assay; Chiron) were performed according to the manufacturer's instructions as described previously (17). The limit of detection was 3.5×10^5 HCV RNA equivalents per ml. A positive and a negative control serum sample as well as the four calibration standards were run with each bDNA assay.

RESULTS

There was complete concordance between the results of the SRT-PCR and the AMPLICOR assays for the 10 undiluted plasma samples that were part of the Eurohep proficiency testing panel (3 positive and 7 negative serum samples). However, testing of three separate dilution series (Eurohep-1, Eurohep-2, and patient 844) by both RT-PCR assays showed that SRT-PCR had a greater analytical sensitivity, with a ≥4- to 10-fold difference in the limit of detection of HCV RNA between the two assays in these terminal dilution experiments. The last dilution positive by the AMPLICOR test in the dilution series prepared from patient 844 contained approximately 400 HCV RNA equivalents per ml.

HCV RNA was detected in 44 and 45 serum samples by SRT-PCR and the AMPLICOR test, respectively. No equivocal results were obtained by the AMPLICOR test. Overall, there was a 97% concordance between the results of the two RT-PCR assays, with only three discordant results. A total of 43 serum samples were positive by both SRT-PCR and the AMPLICOR test, 53 serum samples were negative by both tests, 2 serum samples were positive by the AMPLICOR test but negative by SRT-PCR, and 1 serum sample was negative by the AMPLICOR test and positive by SRT-PCR.

The resolution of the discordant results is summarized in Table 1. Patient 104 (SRT-PCR negative, AMPLICOR positive) was a 41-year-old male with elevated transaminase levels in his serum and chronic active hepatitis and cirrhosis on liver biopsy. This patient had no identifiable risk factors for HCV infection. Anti-HCV EIA and supplementary recombinant immunoblot assay (RIBA) were both positive, and other causes of liver disease were excluded by serological testing. Patient 910 (SRT-PCR negative, AMPLICOR positive) was a 37-year-old male originally thought to have cirrhosis secondary to alcohol abuse. This patient also had a history of blood transfusions and intravenous drug abuse. Anti-HCV EIA and supplementary RIBA were positive, and other causes of liver disease were excluded by serological testing. Patient 1114 (SRT-PCR positive, AMPLICOR negative) was a 57-year-old female with cryptogenic cirrhosis. She had no identifiable risk factors for HCV infection. All serological tests including anti-HCV EIA and RIBA were negative. Two additional serum samples, collected at different times, were tested for HCV RNA by SRT-

TABLE 1. Resolution of samples with discordant results by SRT-PCR and the AMPLICOR test

Patient no.[a]	EIA result	RIBA result	ALT level (U/liter)[b]	Risk factor	AMPLICOR test result	SRT-PCR result	Interpretation
104	+	+	90	None	+	−	Low-level viremia
910	+	+	73	Transfusion, IVDA[c]	+	−	Low-level viremia
1114	−	−	46	None	−	+[d]	False-positive SRT-PCR result

[a] The bDNA assay result was $<3.5 \times 10^5$ HCV RNA equivalents per ml for all patients. Histology for all patients was cirrhosis.
[b] ALT, alanine aminotransferase.
[c] IVDA, intravenous drug abuse.
[d] Date of positive SRT-PCR result was 21 July 1993; SRT-PCR assays done on 3 June and 27 September 1993 were negative.

PCR and were found to be negative. After review of the patients' charts, all three discrepant results were resolved in favor of the AMPLICOR test (two false-negative SRT-PCR results and one false-positive SRT-PCR result). The levels of viremia in both serum samples with false-negative SRT-PCR results were $<3.5 \times 10^5$ HCV RNA equivalents per ml by the bDNA assay.

Figure 1 shows the frequency distribution of the quantity of HCV RNA in the test sera which were positive by the AMPLICOR test. HCV viremia was documented by the AMPLICOR test in all sera with $\geq 3.5 \times 10^5$ HCV RNA equivalents per ml of serum and in five (11%) serum samples that contained RNA at a quantity that was below the threshold of detection by the bDNA assay. No serum sample was positive by the bDNA assay alone. The level of viremia was $>1 \times 10^6$ HCV RNA equivalents per ml in 31% of viremic patients, $>10 \times 10^6$ HCV RNA equivalents per ml in 24% of viremic patients, and $>57 \times 10^6$ HCV RNA equivalents per ml in 7% of viremic patients.

The HCV genome was detected by the AMPLICOR test in three (6%) of the anti-HCV-negative specimens. All three specimens were also positive for HCV RNA by SRT-PCR and the bDNA assay. HCV RNA was not detected in six (13%) of the anti-HCV-positive specimens by the AMPLICOR test, SRT-PCR, or the bDNA assay. For patients with a clinical diagnosis of chronic HCV infection, 89% were positive for HCV RNA by AMPLICOR and 90% were positive for anti-HCV antibodies.

DISCUSSION

The results of a recent HCV RNA proficiency testing survey illustrated the need for increased standardization (22). In that survey, one-third of the laboratories had errors in determining the status of undiluted samples, and one-half had one or more errors in the dilution series. Only 16% of participating laboratories had no errors, and even in those laboratories there was a 100-fold difference in analytical sensitivity. The development of a reliable kit assay should increase the availability, standardization, and reliability of HCV RNA detection. To date, there are no HCV RNA assays licensed by the U.S. Food and Drug Administration for diagnostic use.

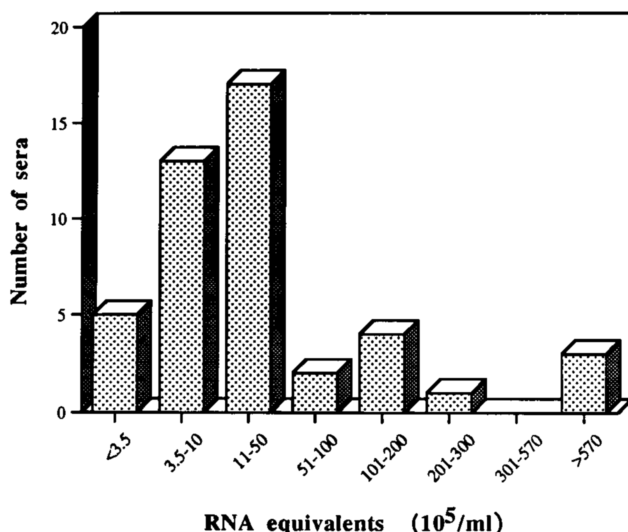

FIG. 1. Frequency distribution of the quantity of HCV RNA in 45 test serum samples which were positive by AMPLICOR.

We compared the AMPLICOR HCV test with an SRT-PCR assay for the detection of HCV RNA in a plasma proficiency testing panel and in sera from patients with chronic liver disease. The SRT-PCR assay reliably detected between 10 and 100 copies of the HCV genome per ml of serum (data not shown). We found that the SRT-PCR assay had a >4- to 10-fold higher analytical sensitivity than the AMPLICOR test in the three dilution series tested. The difference in sensitivity may be explained on the basis of the amount of RNA put into each reaction tube. RNA isolated from the equivalent of 5 μl of serum is amplified in each AMPLICOR test, whereas RNA isolated from the equivalent of 50 μl of serum is amplified in each SRT-PCR assay. By using the bDNA assay to quantitate the HCV RNA in a patient serum sample, we estimated the analytical sensitivity of the AMPLICOR test to be approximately 400 HCV genome equivalents per ml.

When the results of both RT-PCR assays were compared for the undiluted patient sera, we found 97% agreement. Despite the apparent lower analytical sensitivity of the AMPLICOR test, it detected HCV RNA in two serum samples that were negative in the SRT-PCR assay. Both patients had serological and clinical evidence of HCV infection and low-level viremia ($<3.5 \times 10^5$ HCV RNA equivalents per ml). The false-negative SRT-PCR results may have been due to inhibition of the amplification reaction. The SRT-PCR may be more prone to inhibition than the AMPLICOR test since the sample input in each reaction is larger. The inhibitors could be endogenous and patient specific or could be related to carryover of the chaotrope and organic solvents used in the RNA extraction procedure. Neither RT-PCR assay included an internal control template that could be used to assess the efficacy of each negative amplification reaction.

Zeuzem and colleagues (23) compared the AMPLICOR HCV test with a two-step, single-primer-pair RT-PCR for the detection of HCV RNA in sera obtained from 219 consecutive patients attending a hepatology clinic. Dilution of HCV RNA transcripts into control serum showed an analytical sensitivity of 5×10^3 to 1×10^4 molecules per ml of serum for the AMPLICOR test and 1×10^3 molecules per ml for the two-step RT-PCR. Despite the lower analytical sensitivity of the AMPLICOR test, no false-negative results were obtained with the AMPLICOR test with sera from 111 viremic patients (100% sensitivity). The magnitude of viremia in these patients was estimated to range from 5×10^5 to 5×10^8 genome equivalents per ml. There were three false-positive AMPLICOR test results with sera from 108 control patients (97% specificity).

The positive and negative controls for the AMPLICOR test were not subjected to the RNA extraction steps. Ideally, a positive control should contain the target nucleic acid in a matrix similar to that of the clinical samples to be tested and should be subjected to all of the steps of the assay procedure to ensure efficient sample preparation. Likewise, the negative control should be similar to the clinical samples tested and should be subjected to all of the steps to ensure that contamination did not occur.

The AMPLICOR test has two features that should limit false-positive results because of carryover of amplified product from one reaction mixture to another. It is a single-enzyme, single-tube reaction to which no reagents are added between the RT and the amplification steps. This eliminates the need to open the tube after the initial setup and reduces the possibility of contamination. Also, the reaction mixture includes UNG, which recognizes and catalyzes the destruction of dU-containing DNA. The presence of dU in amplified product renders any contaminating product susceptible to destruction by UNG prior to amplification of the target cDNA. We found one false-positive SRT-PCR result and no false-positive AMPLICOR test results in the present study.

The enzyme-linked immunosorbent assay (ELISA)-like detection format of the AMPLICOR test can potentially be another source of false-positive results (23). Unbound horseradish peroxidase could reenter the wells after washing if the wells were overfilled, and the contamination could lead to significant color development. Smaller wash volumes could reduce the occurrence of false-positive results by the detection system (23). False-positive results by the detection system were not encountered in the present study.

We tested all patient serum samples by the bDNA assay to provide a third independent RNA detection method and to determine the levels of viremia in our patient population. The magnitude of viremia was $>3.5 \times 10^5$ HCV RNA equivalents per ml in 89% of the patients in whose sera HCV RNA was detected by RT-PCR. The performance characteristics of the AMPLICOR test need to be assessed with sera from other patient groups with lower levels of viremia, especially patients receiving interferon therapy.

The format of the AMPLICOR test is better suited than the SRT-PCR assay to a clinical laboratory setting. The AMPLICOR test is less labor intensive and can be completed in approximately 6 h, whereas 48 h is required for completion of the SRT-PCR. The use of a single enzyme and buffer system for RT and cDNA amplification simplifies the RT-PCR process and reduces the opportunity for contamination. The AMPLICOR test uses familiar "ELISA" technology rather than cumbersome gel analysis and Southern blotting for the detection of amplified product.

The major limitations of the AMPLICOR test include the relatively small sample volume, the lack of an internal positive control, and failure to provide controls for the RNA extraction step. The AMPLICOR test used in the present study does not represent a final product, and it will be modified prior to large-scale clinical trials in the United States. The modifications will include a larger effective sample volume and kit controls that are carried through the RNA extraction step. We are evaluating the modified protocol.

In conclusion, the AMPLICOR test compared favorably with our SRT-PCR for the detection of HCV RNA in sera from patients with chronic liver disease in the present preclinical evaluation. The commercial development of a reliable kit assay should increase the availability and improve the standardization of testing.

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