Prospective Comparison of Whole-Blood- and Plasma-Based Hepatitis C Virus RNA Detection Systems: Improved Detection Using Whole Blood as the Source of Viral RNA

JACK T. STAPLETON, DONNA KLINZMAN, WARREN N. SCHMIDT, MICHAEL A. PFALLER, PING WU, DOUGLAS R. LABRECOUE, JIAN-QIU HAN, MARY JEANNE PERINO PHILLIPS, ROBERT WOOLSON, AND BETH ALDEN

Veterans Administration Medical Center, Iowa City, Iowa 52246, and Departments of Internal Medicine, Pathology, and Preventive Medicine, University of Iowa College of Medicine, Iowa City, Iowa 52242

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We previously demonstrated that whole blood contains significantly more hepatitis C virus (HCV) RNA than plasma. To validate the whole-blood-based HCV RNA detection method, a prospective comparison of HCV RNA detection in whole blood and plasma from 50 patients with chronic liver disease was undertaken. Whole-blood and plasma aliquots were independently tested for HCV RNA by reverse transcriptase (RT) PCR assay, and plasma was tested by the Roche Amplicor assay. HCV RNA was detected in 35 of 50 (70%) whole-blood samples by RT-PCR but in only 26 of 50 (52%) plasma samples tested by the Amplicor assay (P < 0.01). HCV RNA was detected in 85% of HCV antibody-positive patients by the whole-blood method compared with 74% of plasma samples by the Amplicor method. The five HCV antibody-positive subjects who were negative by whole-blood-based RT-PCR assay were all receiving interferon therapy and had normal transaminases at the time of testing. HCV RNA was detected in 38% of HCV antibody-negative subjects by the whole-blood-based RT-PCR assay compared with 6.25% of these patients by the Amplicor assay (P < 0.05). There were nine samples in which HCV RNA was detected in whole blood but the Amplicor test was negative. Eight of the nine RNAs prepared from these whole-blood samples tested positive in the Amplicor assay, thus confirming the specificity of our results. This study demonstrates that whole-blood-based HCV RNA detection is more sensitive than currently available commercial tests and that whole-blood RNA is suitable for use in commercial assays.

Hepatitis C virus (HCV) is a hepatotropic RNA virus responsible for the majority of cases of posttransfusion and community-acquired chronic non-A, non-B hepatitis in the United States (4, 5). It causes persistent infection in more than 90% of infected people, and up to 70% of these individuals develop progressive liver disease over a 20- to 30-year period (18, 34). An estimated 3.9 million people in the United States are currently infected with HCV, and it is the leading etiology of end stage liver disease resulting in liver transplantation in the United States (3, 9).

HCV was originally identified by cloning RNA from the liver of a chimpanzee with chronic non-A, non-B hepatitis, expressing the cDNA, and identifying cross-reactive antibodies in the original animal serum and in sera from well-characterized human patients with non-A, non-B hepatitis (5, 8, 27). Commercial immunoassays were subsequently developed to detect antibodies against structural and nonstructural viral proteins (10, 25, 38), and later improvements have increased the sensitivity and positive predictive value of HCV antibody testing (1, 7, 16, 22, 24, 26, 41).

Although current immunoassays are successful in detecting most cases of chronic HCV infection, a significant percentage of antibody-negative individuals (up to 5% of blood donors with elevated alanine aminotransferase levels) test positive for HCV RNA by serum or plasma nucleic acid amplification methods (4, 36, 39, 44). Our laboratory developed a method to detect HCV RNA in whole blood by using a cationic surfactant (Catrimox-14) to precipitate RNA from whole blood (30). We found that the amount of HCV RNA in whole blood was significantly higher than that present in plasma, and that plasma-based assays significantly underestimate the circulating HCV viral load (31, 33). Using this whole-blood-based HCV RNA detection system in patients from our liver clinic population, we found that the majority of people with unexplained chronic liver disease and negative HCV antibody tests were actually infected with HCV (32). Dries et al. recently confirmed our findings in another population of chronic liver disease patients (12). These investigators evaluated liver biopsy specimens from 44 patients with chronic, HCV antibody-negative liver disease and found that 61% of the specimens contained HCV RNA (12). These serosilent HCV infections probably contribute to the small-but-persistent risk of posttransfusion and community-acquired HCV infection.

We evaluated the distribution of HCV RNA among plasma and various cellular compartments in peripheral blood and determined that blood contains significantly more viral RNA than adjusted equivalent volumes of plasma or blood cells (33). Thus, measurement of whole-blood HCV RNA appeared to be more sensitive than measuring plasma HCV RNA (12, 33, 40). There are several potential reasons why whole-blood RNA contains a higher concentration of HCV than plasma. Although there are conflicting data regarding the replication of HCV in any of these cell types (6, 14, 19–21, 23), HCV RNA
is present among circulating lymphocytes, neutrophils, and monocytes and in the erythrocyte-platelet pellet (33). We predicted that the increased HCV RNA concentration was due to the addition of cell-associated HCV to plasma in the whole-blood preparation; however, we found that the intracellular HCV RNA accounted for only approximately half of the additional HCV RNA in the cell pellet (33). The remaining HCV RNA was removed by extensive washing of the cell pellet. Thus, we speculated that this cell-associated HCV RNA results from HCV-lipoproteins or HCV-immunoglobulin complexes that precipitate during plasma preparation (17, 33, 37).

The purpose of this study was to further validate our findings that whole-blood-based HCV RNA detection is more sensitive than plasma-based HCV RNA detection, to directly compare whole-blood-based HCV RNA detection with a widely used and validated commercial assay, and to determine if commercial assays can confirm the finding of HCV RNA in the whole blood of patients who are HCV antibody negative. To accomplish this, we prospectively studied HCV antibody-positive and HCV antibody-negative patients with chronic liver disease. We then directly compared the use of whole-blood- and plasma-based HCV RNA detection systems with a commercial HCV reverse transcriptase (RT) PCR method. Our results confirm the increased sensitivity of whole-blood-based HCV RNA detection relative to plasma-based and commercial methods and demonstrate the feasibility of utilizing whole-blood-based HCV RNA in currently available commercial assays.

MATERIALS AND METHODS

Patients and sample collection. Subjects with chronic liver disease followed by the University of Iowa Liver Clinic were invited to participate in this study. All patients had previously been tested for antibody to HCV, and subjects were selected for recruitment by using a ratio of two antibody-positive patients for each HCV antibody-negative subject. Chart review revealed that none of the patients were organ transplant recipients or clinically immunosuppressed due to known congenital or acquired immune deficiencies or medications. Five patients were receiving interferon therapy for chronic HCV infection. We then directly compared the use of whole-blood- and plasma-based HCV RNA detection systems with a commercial HCV reverse transcriptase (RT) PCR method. Our results confirm the increased sensitivity of whole-blood-based HCV RNA detection relative to plasma-based and commercial methods and demonstrate the feasibility of utilizing whole-blood-based HCV RNA in currently available commercial assays.

RESULTS

Fifty subjects (34 HCV antibody positive and 16 antibody negative) were prospectively enrolled in the study. HCV antibody-negative subjects were significantly older than HCV antibody-positive subjects (57 versus 46 years old; P = 0.003 [t test] [SigmaStat for Windows version 2.0; Jandel Scientific Software]). Males were represented significantly more frequently than females in the HCV antibody-positive group (65%) compared to the HCV antibody-negative group (25%; P = 0.005 [chi-square test with Yates correction] [SigmaStat]).

To insure that the whole-blood tests and both methods of testing plasma were performed on the same blood sample from each patient, a single tube of blood was obtained and labeled with a code to blind the laboratory investigators to the subject’s identity and plasma and whole-blood aliquots were prepared and stored under identical conditions. Samples of whole blood mixed with Catrimox and plasma mixed with Catrimox were tested by our in-house RT-PCR test. A plasma aliquot was also tested by the Roche Amplicor test by the University of Iowa Clinical Virology Laboratory. Neither laboratory knew the other laboratory’s results until all 50 patients were studied. Among HCV antibody-positive individuals, 85% of whole-blood samples, 77% of plasma-Catrimox samples, and 74% of Roche plasma assays were positive (Table 1). When we re-
viewed the clinical records of these 34 subjects, all 5 of those who were negative by the whole-blood-based test were receiving and responding to interferon therapy at the time of testing and had tested positive by both whole-blood and plasma methods prior to therapy. All five of these subjects were also negative by plasma testing.

HCV RNA was detected in the whole blood of 6 of 16 HCV antibody-negative individuals (Table 1). Only 2 of 16 subjects had HCV RNA detected by the plasma-Catrimox method, and only 1 of these was also positive by the Roche assay (Table 1). Cryoglobulins are detected in approximately 40% of individuals with chronic HCV infection (2). These large virus-antibody complexes are likely to precipitate with the cellular pellet during plasma preparation and may lead to false-negative HCV antibody tests and plasma HCV RNA determinations (17, 33).

We therefore reviewed the medical records of the antibody-negative subjects for evidence of cryoglobulinemia. Both of the subjects with HCV RNA in their plasma were found to have cryoglobulinemia. One additional subject with cryoglobulinemia was identified, and this subject tested positive by whole-blood-based HCV RNA testing, although the plasma samples tested negative by both methods. Thus, three of the six HCV antibody-negative subjects in whom HCV RNA was detected had cryoglobulins present in their plasma. Liver biopsy results were available for four of these antibody-negative, RNA-positive subjects. One biopsy was interpreted to include “autoimmune” features (in a subject with cryoglobulinemia). Another biopsy was read as primary biliary cirrhosis, and the remaining two were interpreted as hepatitis of unknown etiology.

When the HCV RNA test results of both HCV antibody-positive and -negative subjects were combined, 70% of whole-blood samples, 56% of plasma-Catrimox samples, and 52% of plasma samples tested by Amplicor were positive for HCV RNA. Using Cochran’s test for related samples to determine if there were any differences among the groups (42), an exact \( P \) value of 0.0005 was found, suggesting that the HCV RNA detection rates of the three tests (whole blood, plasma-Catrimox, and plasma-based Amplicor) were different. Using McNemar’s test for related (or correlated or matched) samples, whole blood differed from plasma-Catrimox (exact two-tailed \( P \) value, 0.0156) and whole blood differed from the Roche Amplicor test \( (P = 0.0039) \) (42). No difference was identified between plasma-Catrimox and Roche Amplicor \( (P = 0.6875) \). The whole-blood-versus-Roche and the whole-blood-versus-plasma comparisons were also statistically significant at the 0.05 level when the Bonferroni method was applied (to evaluate for multiple pairwise comparisons) (42).

In all instances where plasma samples were positive (by either the Roche test or our plasma test), whole blood was also positive. Thus, there was never discordance between our whole-blood test and a positive plasma test.

To determine if whole-blood-based RNA results could be confirmed in the Amplicor assay, and to verify that the whole-blood results represented true HCV infection and not false-positive results, we utilized RNA extracted from whole blood as the template RNA in the Roche assay. As noted above, in the Roche system RNA is prepared from 100 \( \mu \)l of plasma and then 5% of the RNA preparation is used in the assay (28).

We therefore repeated the whole-blood-based Amplicor test. Thus, upon initial analysis, the whole-blood-based Amplicor test were no different from the plasma-based Amplicor results and they did not support the hypothesis that the positive whole-blood-based HCV RNA results obtained from our in-house assay represented true infection.

Review of the protocols indicated that our in-house RT-PCR method uses a 10-fold-greater concentration of RNA than the Amplicor system. We therefore repeated the whole-blood-based RNA testing of the nine samples that were negative by the Amplicor test and positive by the whole-blood-based test and the six samples that were negative by the whole-blood- and plasma-based tests from the HCV antibody-negative group of subjects, using the increased RNA concentration. Under these conditions, eight of the nine subjects (89%) who were positive in the initial whole-blood-based testing were also positive by the Roche Amplicor system (Table 2). It is unclear why we were unable to amplify the ninth sample using the Amplicor system, although differences in PCR primers, PCR inhibition, RNA degradation, or false-positive results may account for this finding.

Importantly, all of the whole-blood RNA’s that tested negative in the in-house assay also tested negative by the Roche assay, even when RNA samples derived from high volumes of whole blood were tested \( (n = 15) \). RNA was also prepared from 50 \( \mu \)l of plasma from six of the antibody-negative subjects who were positive by the whole-blood-based tests. All six of these samples were negative by the Amplicor assay, although

### Table 1. Comparison between HCV RNA detection by Roche Amplicor and by whole blood or plasma in Catrimox tests

<table>
<thead>
<tr>
<th>Sample</th>
<th>Whole blood Catrimox</th>
<th>Plasma Catrimox</th>
<th>Amplicor</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV antibody positive</td>
<td>29/34 (85)</td>
<td>26/34 (76)</td>
<td>25/34 (74)</td>
</tr>
<tr>
<td>HCV antibody negative</td>
<td>6/16 (38)</td>
<td>2/16 (12.5)</td>
<td>1/16 (6.25)</td>
</tr>
<tr>
<td>Total</td>
<td>35/50* (70)</td>
<td>28/50* (56)</td>
<td>26/50* (52)</td>
</tr>
</tbody>
</table>

* Results are given as number positive/total number studied (%). * and † indicate comparisons that were statistically significant (see the text).

### Table 2. Use of whole-blood RNA as a template in the Roche Amplicor system

<table>
<thead>
<tr>
<th>Sample</th>
<th>Whole blood/Roche</th>
</tr>
</thead>
<tbody>
<tr>
<td>Results</td>
<td>Roche Amplicor results</td>
</tr>
<tr>
<td></td>
<td>Standard volume</td>
</tr>
<tr>
<td>Negative/negative</td>
<td>0/6</td>
</tr>
<tr>
<td>Positive/positive</td>
<td>6/6</td>
</tr>
<tr>
<td>Positive/negative</td>
<td>0/9</td>
</tr>
</tbody>
</table>

* Standard volume utilized RNA from 5 ml of plasma or whole blood (see text). High volume utilized RNA from 50 ml of whole blood. NT, not tested.
the whole-blood-based Amplicor test was positive (data not shown). These results confirm the increased sensitivity of whole blood relative to plasma.

To further confirm that these amplified products represented unique viral isolates and were unlikely to be due to laboratory contamination, we determined the nucleotide sequences of the PCR products obtained from four antibody-negative subjects. These were compared with our laboratory positive control HCV sequence and sequences from three HCV antibody-positive subjects. The HCV 5’ NTR is the genome region most highly conserved between isolates (35); hence, the sequence alignment of these eight isolates shown in Fig. 1 demonstrates that the isolates are distinct and do not represent our laboratory control strain of HCV. There were an average of seven mutations between isolates, or 3% of this highly conserved region. This degree of sequence heterogeneity also argues strongly against PCR artifact, as the expected rate of misincorporations with Taq polymerase is approximately 1 per 1,000 bases copied, or 0.01% (13).

Four of the six HCV antibody-negative subjects in whom HCV RNA was detected had previously undergone liver biopsy. Using the Mann-Whitney rank sum test and the equal variance test to compare the groups (antibody-positive RNA-positive, antibody-positive RNA-negative, antibody-negative RNA-positive, and antibody-negative RNA-negative groups), no differences in inflammation were found; however, the statistical power was poor due to the small number of biopsies present in three of the four groups, and the possibility of differences cannot be excluded (Table 3). However, significantly more fibrosis was observed in the HCV RNA-positive group compared with the HCV RNA-negative group ($P = 0.036$ [Mann-Whitney rank sum test] [SigmaStat]). This raises the possibility that antibody-negative subjects with HCV infection have more aggressive liver disease. Prospective studies are under way to address this issue.

**DISCUSSION**

We previously demonstrated that the Catrimox-14 whole-blood RNA extraction method provided high-quality RNA useful for detecting HCV RNA and that there is an increased concentration of HCV RNA in whole blood relative to plasma and individual blood cell compartments (30, 32, 33). Once mixed with Catrimox-14, HCV RNA in whole blood is stable for several days at room temperature (30). Since HCV RNA in plasma or serum is labile unless it is quickly processed and frozen at −70°C, Catrimox-14 whole-blood RNA preparation offers a practical advantage in the clinical setting over plasma assays (30).

Whole-blood-based HCV RNA detection was previously shown to be more sensitive than plasma-based detection in HCV antibody-positive patients during interferon therapy (31, 40). Patients in whom HCV RNA was detected at the end of therapy were significantly more likely to relapse than those with negative whole-blood-based HCV RNA tests (31, 40); thus, this approach may have important clinical implications.

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**TABLE 3.** Histopathologic findings among HCV antibody-negative subjects stratified by HCV RNA and antibody status

<table>
<thead>
<tr>
<th>Group and biopsy finding</th>
<th>HCV antibody negative</th>
<th>HCV antibody positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV RNA negative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammation</td>
<td>1.0 ($n = 4$)</td>
<td>1.5 ($n = 4$)</td>
<td>1.25 ($n = 8$)</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>0.5</td>
<td>0.25</td>
<td>0.375*</td>
</tr>
<tr>
<td>HCV RNA positive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammation</td>
<td>1.75 ($n = 4$)</td>
<td>1.45 ($n = 20$)</td>
<td>1.5 ($n = 24$)</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>2.75</td>
<td>1.75</td>
<td>1.92*</td>
</tr>
</tbody>
</table>

* Biopsy results were scored as follows: 0, absent; 1, mild; 2, mild to moderate; 3, moderate; 4, moderate to severe; and 5, severe. * $P = 0.036$ (Mann-Whitney rank sum test).
In this study, we confirmed the enhanced sensitivity of whole blood relative to plasma for HCV RNA detection in a mixed population of patients with liver disease, and we expanded previous findings by directly comparing the whole-blood-based assay with our in-house plasma-based assay and the well-validated Amplicor RT-PCR assay (28).

Confirmation of the specificity of our assay was accomplished by demonstrating that whole-blood RNA was suitable for amplification in the commercial assay. In all but one of the subjects with a positive whole-blood-based test but a negative plasma-based test, the Roche assay was positive when the concentration of whole-blood RNA was increased. The approach of using higher volumes of sample to prepare RNA has been proven useful in other systems, specifically in HCV (15, 16) and human immunodeficiency virus (HIV) diagnostics. For example, the only difference between the “ultrasensitive” HIV RNA detection assay and the standard HIV Amplicor test is that there is a 10-fold increase in the volume of the input serum used to prepare RNA in the former (manufacturer’s instructions; Roche Diagnostic Systems). The specificity of our whole-blood-based HCV RNA assay was demonstrated when HCV RNA-negative samples were negative in the Roche assay, even when higher concentrations of whole-blood RNA were used in the RT-PCR. We also confirmed the specificity of our results by cloning and amplifying the sequences of the PCR products from several of the subjects, demonstrating that these are independent isolates and unlikely to be the result of contamination.

We previously reported that a significant proportion of HCV RNA in whole blood segregates with the cellular pellet during the preparation of plasma or serum (33). As noted above, approximately half of the HCV in the cell pellet is adherent to the cells and not intracellular (33). This is presumably due to the binding of immune complexes or HCV-lipid complexes to cell surfaces (17, 37). Although technical issues, such as differences in PCR primers, could account for differences in sensitivity, we believe that the increased sensitivity found with whole blood is due to the increased concentration of virus in the cellular pellet. Since our lower limit of detection is approximately the same as, or slightly higher than, that of the Amplicor assay, the increased sensitivity does not appear to be inherent in the RT-PCR procedure. If the limit of detection is 200 copies per ml, as reported for Amplicor, the HCV viral load would have to be 200 copies per ml before each 5-μl sample would be predicted to contain detectable virus (Fig. 2). Consequently, in patients with low-level viremia (~200 copies per ml), increasing the volume of the initial sample or enhancing the concentration of virus within the sample is necessary to allow detection.

This study also expands our previous observations of HCV antibody-negative, RNA-positive disease (serosilent HCV infection). Six of 16 (38%) of our HCV antibody-negative patients were found to be infected with HCV, slightly lower than in our prior study of patients with HCV antibody-negative cryptogenic hepatitis (32). These results are also similar to those of Dries et al., who detected HCV RNA in liver biopsy specimens in 61% of patients with cryptogenic hepatitis (12). Although three of the six HCV RNA-positive, antibody-negative patients we identified had cryoglobulins in their sera, there were no other reasons identified to explain the failure to detect anti-HCV antibody. Thus, at least in this population, it appears that our previous findings are reproducible and HCV infection is common among individuals with chronic liver disease of undetermined etiology.

The mechanism by which the HCV isolates in these individuals avoid detection by commercial antibody tests is unclear. Antibodies may be present in the subjects, but due to extensive sequence differences between the individual isolates and the commercial antigen, the antibodies may be different enough to prevent detection in the commercial assay. Alternatively, variation in host immune responses may lead to diminished or absent antibody production in some HCV-infected individuals. Studies are under way to evaluate why antibodies are not detected in these individuals.

In summary, this study confirms and expands our previous observations of increased HCV RNA levels in whole blood relative to plasma and demonstrates that this RNA is suitable for use in commercial diagnostic assays. The studies suggest that whole-blood-based RNA detection has increased sensitivity for detecting HCV in antibody-negative patients and may therefore be important in the screening of blood donors. In addition, it has been shown to be more sensitive than plasma-based HCV RNA detection in patients receiving interferon; thus, it may prove important for the management of HCV therapy (31). Studies to determine the rates of antibody-negative, RNA-positive HCV infection in different populations of patients and to characterize the viral isolates and immune responses of these patients are under way.

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