duced substance ascorbate, at ~100-fold a normal concentration, lowered lactate slightly (by 0.3 mmol/L), this should not be significant in any samples from patients.

The main concern we have with lactate measured by the SP7 method described here is that the results, particularly at lower concentrations, appear to be ~0.3–0.4 mmol/L higher than other methods. Correcting for the average difference in water content of plasma (0.93) did not appreciably lower this bias between methods; therefore, we conclude that the calibration set points for the SP7 should be modified by the manufacturer.

The necessity to change the lactate oxidase membrane about every 6 days may be acceptable for operation in high-test-volume clinical laboratories. However, for laboratories with fewer tests for lactate, a cost of ~$14 for each membrane may be comparable with the prepackaged colorimetric (aca) test pack.

By comparing results from anticoagulated blood with results from uncoagulated blood analyzed immediately after collection, we show that heparin is a satisfactory anticoagulant and is preferred over heparinized tubes containing idoacetate. However, whole-blood samples must be kept in ice until they are either analyzed or centrifuged to obtain plasma. Whole blood appears to be stable for at least 60 min when stored in an ice bath.

With a slight adjustment of calibrator set points, the SP7 method for lactate can provide potentially accurate, precise, and linear results in an analyzer that is independent of hematocrit. Because the analyzer also measures pH, PCO₂, and PO₂, further studies are needed to establish the clinical value of this apparently logical combination of tests in critical care.

We acknowledge the financial support of Nova Biomedical in this study.

References
Non-A, non-B (NANB) hepatitis virus is considered to be the major causative agent of posttransfusion-associated hepatitis (1, 2).6 This viral type of hepatitis is not as severe as acute viral hepatitis type A or type B; however, 50–80% of the patients infected with this virus develop chronic illness. Chronic hepatitis patients occasionally develop cirrhosis or hepatoma (3–5). In 1989, the hepatitis C virus (HCV) genome was cloned from the plasma of a chimpanzee infected with etiological agents of NANB hepatitis, and HCV antigen (c100-3) was synthesized by the recombinant DNA method in yeast (6). An enzyme-linked immunosorbent assay (ELISA) based on the HCV c100-3 antigen was developed for screening to detect the circulating c100-3 antibodies to HCV (7). This diagnostic system has been valuable for clinical and epidemiological use (8, 9), but recent results suggest that the assay infrequently shows nonspecific ELISA reactivity and does not detect all HCV-infected patients (10–12). Moreover, nucleotide sequence analyses of several isolates of HCV showed genetic diversity (13–17). Recently, we isolated HCV cDNA clones from pooled plasma of Japanese patients with NANB viral hepatitis, and synthesized two recombinant HCV antigens (c11 and c7) in Escherichia coli as TrpE fusion proteins (16). The genomic locations of the c11 and c7 antigens corresponded to the HCV structural (putative core) region and the HCV nonstructural (NS3) region, respectively. Using these antigens, we developed an ELISA for measuring HCV antibodies directed to the c11 and c7 antigens. Here we report the performance and specificity of Imucheck HCV Ab (Imu[c11/c7]).

Materials and Methods

Materials. Hemoglobin, bilirubin, lipemic interferences, and rheumatoid factor were purchased as Interference Check A or Interference Check RF from International Reagents Corp., Kobe, Japan. The first-generation Ortho HCV Ab ELISA (c100-3) and the first-generation Chiron HCV RIBA™ recombinant immunoblot assay were obtained from Ortho Diagnostic System K.K., Tokyo, Japan, and the tests were performed according to the assay protocols recommended by the manufacturers.

Antigen preparation. The fusion protein for the recombinant HCV antigens c11 and c7 used in Imucheck HCV Ab included 17 amino acids of an amino-terminal E. coli TrpE protein. The c11 and c7 antigens were 22 and 33 kDa, respectively. Recombinant antigens were purified from cells of the E. coli strain carrying either plasmid. The bacterial cells were grown in M9CA medium and were disrupted enzymatically with lysozyme. The disrupted cellular material was centrifuged at low speed, and the pelleted fraction containing the antigens (c11 or c7) was solubilized in 6 mol/L urea. The solution was then centrifuged at high speed to remove insoluble materials before ion-exchange chromatography on a column of S-Sepharose and Q-Sepharose (Pharmacia, Uppsala, Sweden). The column was developed with a linear gradient of sodium chloride (0–500 mmol/L) in 6 mol/L urea to yield the purified antigens. The purity of the c11 and c7 antigens was ~90% and 95%, respectively, as determined by densitometric analysis after Coomassie Blue staining and sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Imu[c11/c7] assay. The equimolar mixture of c11 and c7 antigens was applied to microtiter plate wells. The human sera were diluted 11-fold with sample dilution buffer (0.5 mol/L NaCl, 2.5 mmol/L EDTA, and 10 g/L bovine serum albumin in 10 mmol/L phosphate buffer, pH 7.0) and were added to each well. The plates were incubated for 45 min at 30°C and then washed six times with washing buffer (0.5 mL/L Tween 20 in phosphate-buffered saline). The HCV antibodies specifically bound to the antigens were detected by a second incubation with a monoclonal antibody to human immunoglobulin conjugated to horseradish peroxidase (EC 1.11.1.7) in a second-antibody dilution buffer (0.15 mol/L NaCl, 2.5 mmol/L EDTA, and 10 g/L bovine serum albumin in 10 mmol/L phosphate buffer, pH 7.5) for 45 min at 30°C. After washing the plates six times with washing buffer, the enzyme activities were measured by using o-phenylenediamine as substrate. This enzymatic reaction was allowed to proceed for 45 min at 30°C, and stopped by 2 mol/L sulfuric acid. The absorbance of each well was measured at 492 nm, with 690 nm as the reference wavelength. These consecutive procedures were carried out by an automated ELISA system (ELSIA Auto; International Reagents Corp.).

Western blot analysis. The purified c11 and c7 antigens were separated by electrophoresis on a 15% sodium dodecyl sulfate–polyacrylamide gel by the Laemmli method (18). Electrophoretic transfer blot analysis was performed after electrophoresis of proteins from sodium dodecyl sulfate–polyacrylamide gel to nitrocellulose membranes according to the method of Towbin et al. (19).

Detection of the HCV genome by the polymerase chain reaction (PCR) method. Amplification by the polymerase chain reaction (PCR) was carried out in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) with a Gene Amp DNA amplification reagent kit (Perkin-Elmer Cetus). This procedure was performed by combining the initial reverse transcription step with the first PCR reaction and by using nested primers in a second PCR reaction. RNA was extracted from 0.1 mL of plasma with extraction buffer containing 6 mol/L guanidium thiocyanate, 5 g/L sodium laurylsarkosate, 25 mmol/L sodium citrate, and 0.1 mol/L 2-mercaptoethanol. After extraction with phenol, phenol/chloroform, and chloroform, RNA was precipitated with ethanol. Primers for reverse-transcription and first PCR were 5′-CTGTCTTCCAGGAAACGCG-3′ and 5′-CAGCAAACCGCTCATTAC-3′; for second PCR they were 5′-CTTAGGATAGTGTCTGCG-3′ and 5′-AGGACATGGAGGGTTTTACT-3′ (18). cDNA synthesis of pre-

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6 Nonstandard abbreviations: NANB, non-A, non-B; HCV, hepatitis C virus; ELISA, enzyme-linked immunosorbent assay; and PCR, polymerase chain reaction.
pared RNA was completed with 200 U of Moloney murine leukemia virus reverse transcriptase, 50 U of ribonuclease inhibitor, and 50 pmol of the first PCR primer. We carried out the first PCR as follows: 35 cycles, each at 94 °C for 1.5 min, 50 °C for 2 min, and 70 °C for 2 min. Then, using one-tenth of the first PCR product, we performed the second PCR for 35 cycles according to the same steps as the first PCR. The amplified products were separated by electrophoresis on a 3% agarose gel and detected by ethidium bromide staining.

Results

Assay precision assessment. Intra-assay precision was assessed from five measurements of five specimens of human sera (sample A, negative control serum; and samples B–E, positive control sera). The absorbance means ranged from 1.65 to 0.05 for these control sera, and the CVs ranged from 3.5% to 10.7% (Table 1). The reproducibility between runs was assessed from the same specimens. The absorbance means were 0.05–1.89 for these control sera during 4 days of measurements, and the CVs ranged from 3.4% to 6.4% (Table 2).

Interference. We assessed the influence of anticoagulants and blood elements in serum on ELISA results. The anticoagulants EDTA (≤ 5 g/L), heparin (≤ 25 IU/L), and citric acid (up to 25 g/L) showed no interference with the assay. The influence of hemoglobin, bilirubin, lipemia, and rheumatoid factor was also assessed. Various amounts of an aqueous 49 g/L hemoglobin standard were mixed with serum and assayed. Hemoglobin (up to 4.9 g/L) showed no interference in this assay system (Figure 1). Bilirubin (up to 202 mg/L) and turbidity (with lipid up to 16.5 g/L) also showed no interference.

<table>
<thead>
<tr>
<th>Day</th>
<th>Sample A</th>
<th>Sample B</th>
<th>Sample C</th>
<th>Sample D</th>
<th>Sample E</th>
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<tr>
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<td>1.807</td>
<td>0.985</td>
<td>0.521</td>
<td>0.261</td>
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<tr>
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<td>1.958</td>
<td>1.007</td>
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<tr>
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<td>1.019</td>
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<td>0.281</td>
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<tr>
<td>SD</td>
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<td>0.065</td>
<td>0.045</td>
<td>0.026</td>
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</tbody>
</table>

Table 2. Between-Run Reproducibility of Absorbance Values

<table>
<thead>
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<tr>
<td>A</td>
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<td>0.003</td>
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</table>

High concentrations of rheumatoid factor sometimes interfere with the antigen–antibody reaction. The effect of rheumatoid factor on this assay system is shown in Figure 2. Only in rare instances did rheumatoid factors have any influence.

Cutoff value. The cutoff value of Imu[c11/c7] was set according to the following formula: cutoff value = A(NC) + 0.2 × A(PC), where NC is the negative control absorbance value and PC is the positive control absorbance value. On the basis of 10 calculations, the mean (SD) cutoff for our standard positive and negative serum samples was 0.332 (0.025) A. We then applied this cutoff value to the absorbance measurements of 676 samples of normal sera. The distribution of A492/690 mm values among the 676 sera from the healthy donors is shown in Figure 3. This distribution pattern was gaussian after logarithmic transformation. Estimated from this pattern, the mean ± 3 SD was 0.020–0.372, which was compatible with the cutoff value we calculated from our formula with measurements of standard sera.

Anti-HCV testing. The presence of antibodies to HCV was assessed by Imu[c11/c7] and c100-3 ELISA tests in sera from donors with acute hepatitis, chronic hepatitis,
The serum samples were collected from patients undergoing medical examinations in the Tokyo Metropolitan Komagome Hospital.

Liver cirrhosis, hepatocellular carcinoma, autoimmune hepatitis, primary biliary cirrhosis, alcoholic liver disease, HBV carrier status, systemic lupus erythematosus, or fatty liver, and from patients undergoing hemodialysis. A comparison between the results of the two ELISA tests is summarized in Figure 4. The ratios of positive identifications of HCV antibodies to the number of samples in the group were as follows: NANB-type chronic hepatitis, 93.7% (602/642); liver cirrhosis, 93.5% (144/154); and hepatocellular carcinoma, 81.4% (188/231). Imu(c11/c7) showed the presence of antibodies in more samples for each group than did the c100-3 ELISA in the groups shown in Figure 4A. The hemodialysis patients, tending to need blood transfusion for improvement of anemia, showed 36.6% (86/235) of their samples were positive with Imu(c11/c7) and 20.9% (49/235) with the c100-3 ELISA. However, for autoimmune hepatitis and primary biliary cirrhosis, the results were positive in 21.0% (13/62) and 0% (0/10) with Imu(c11/c7) and in 32.3% (20/62) and 10% (1/10) with the c100-3 ELISA, respectively.

Confirmation with other assays. To confirm the capability of the Imu(c11/c7) assay to identify the presence of HCV antibody, we compared the results of several assays (Figure 5). In particular, we tested the capability of the Imu(c11/c7) assay to identify healthy donors who were mislabeled (false positive) with the c100-3 ELISA by comparing the results of PCR, Western blotting, and RIBA tests. The three cases for which both the Imu(c11/c7) assay and c100-3 ELISA showed HCV antibodies in sera (Figure 5, samples 1–3) were confirmed by PCR and Western blot tests (all positive). The three cases of sera (samples 4–6) that showed positive results with the Imu(c11/c7) assay and negative results with the c100-3 ELISA were all positive by the Western blot test with c11 antigen but were nonreactive by RIBA with c100-3. According to these results, these three specimens were confirmed as positive. The 11 cases (samples 7–17) that

![Graph](image-url)
were positive by the c100-3 ELISA and negative by the Imu[c11/c7] assay were also negative by PCR, Western blot test, and RIBA. The results of Imu[c11/c7] were compatible with the results of PCR and Western blot test with c11 and c7.

Seroconversion rate of HCV antibodies in acute hepatitis. We examined the transition of antibodies to HCV in 20 blood specimens from patients with acute sporadic NANB hepatitis and 33 specimens from patients with acute posttransfusion NANB hepatitis, using the Imu[c11/c7] assay and the c100-3 ELISA. In acute sporadic NANB hepatitis, the positive identification of HCV antibodies was 45% (9/20) with the Imu[c11/c7] assay and 15% (3/20) with the c100-3 ELISA after 1 month. Fifty percent (10/20) of the specimens converted to positive with the Imu[c11/c7] assay after 2 months, but only 40% (8/20) converted to positive after 6 months when measured by the c100-3 ELISA (Figure 6). In acute posttransfusion NANB hepatitis, the rate of positive identification of HCV antibodies after 1 month was 48.5% (16/33) with the Imu[c11/c7] assay and 12.1% (4/33) with the c100-3 ELISA. Whereas 81.1% (27/33) of specimens were positive after 4 months with the Imu[c11/c7] assay, only 60.6% (20/33) were positive with the c100-3 ELISA after 12 months (Figure 6). These results showed that the Imu[c11/c7] assay was able to detect antibodies to HCV at earlier stages of infection.

Discussion

We isolated HCV cDNA clones from the plasma of Japanese patients with NANB viral hepatitis and found genetic diversity among the isolates. Using these clones, we synthesized the recombinant HCV antigens in E. coli and tested their immunoreactivity. Among these recombinant antigens, the c11 (HCV structural region) and c7 (HCV nonstructural region) were shown to be highly immunoreactive with sera from chronic NANB hepatitis patients. We developed a new HCV antibody-detecting ELISA system (the Imu[c11/c7] assay) by mixing c11 and c7 antigens for blood screening, and examined its performance. The results showed that the Imu[c11/c7] assay could be used reliably for any specimens. The distribution of the cutoff values was distinct between positive and negative sera. The Imu[c11/c7] assay identified HCV antibodies at higher rates than the c100-3 ELISA in the sera from patients with NANB-type chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (Figure 4). The results were the same for the serum samples from NANB acute as for those from patients with chronic hepatitis. Moreover, Imu[c11/c7] was able to detect anti-HCV at an early stage. This performance was due to the use of both antigens, c11 and c7. It is possible that the nonstructural protein of HCV, c100-3, is a late antibody, detectable between infection and seroconversion, because, in the case of NANB acute hepatitis, we found a tendency for the antibodies to the structural region to appear during an early stage after infection, as seen with Western blot analyses. Confirmation of positive, negative, and false-positive tests was done by using the Western blot test with c11 and c7, PCR, and RIBA. The specimens that tested positive with the Imu[c11/c7] assay were all confirmed with Western blots or PCR. However, the specimens that were positive with the c100-3 test and negative with the Imu[c11/c7] assay were all negative with Western blots, PCR, and the RIBA test (Figure 5). These c100-3 ELISA false-positive samples may therefore represent nonspecific ELISA reactivity. Moreover, the c100 RIBA/c100-3 ELISA combination is less sensitive than the c100-3 ELISA alone. The results of confirmation tests were consistent with the results of the Imu[c11/c7] assay; therefore, Imu[c11/c7] enabled us to decrease the false-positive and false-negative results. The seroconversion rate in patients with acute posttransfusion NANB hepatitis after 12 months was about 80% with the Imu[c11/c7] assay. This result may be due to another etiological factor, or to non-A, non-B, and non-C hepatitis.

We conclude that by using both the c11 and c7 antigens in the new ELISA system for detecting HCV antibodies, one can detect antibodies at an earlier stage of infection than with other systems. This new system increased the sensitivity of blood donor screening and the monitoring of acute hepatitis.
Additional Keyphrases: calibration · trace elements

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Received February 24, 1992; accepted July 30, 1992.

CLINICAL CHEMISTRY, Vol. 38, No. 12, 1992 2439