JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1992, p. 750–753
0095-1137/92/030750-04$02.00/0

Vol. 30, No. 3

Effects of Anticoagulants and Storage of Blood Samples on Efficacy of the Polymerase Chain Reaction Assay for Hepatitis C Virus

JIN-TOWN WANG,[1] TEH-HONG WANG,[1]* JIN-CHUAN SHEU,[1] SY-MING LIN,[1] JAW-TOWN LIN,[1] AND DING-SHINN CHEN[2]

Department of Internal Medicine[1] and Hepatitis Research Center,[2] National Taiwan University Hospital, Taipei, Taiwan, Republic of China

Received 2 August 1991/Accepted 10 December 1991

Blood samples from 11 patients with posttransfusion hepatitis C virus infection were collected. Each sample was divided into three fractions to obtain sera, sodium-citrated plasma, and heparinized plasma and then tested for HCV RNA by a nested polymerase chain reaction (PCR). Of them, eight sodium-citrated plasma samples, seven serum samples, and no heparinized plasma samples were PCR positive. Eight PCR-positive sodium-citrated plasma samples were exposed to different physical conditions and semiquantified for HCV RNA after serial dilutions. Samples stored at −70°C showed the best preservation of HCV RNA, and storage at the other conditions resulted in only minimal loss of the PCR signal. Therefore, serum or sodium-citrated plasma specimens are satisfactory for detecting HCV RNA by PCR, but heparinized blood specimens are not.

The development of a specific antibody assay with recombinant DNA technology has made diagnosis of hepatitis C virus (HCV) infection possible (7). Seroepidemiologic studies in various parts of the world revealed that 60 to 100% of patients with chronic non-A, non-B hepatitis have circulating anti-HCV (3, 7). These studies identified HCV as the major etiologic agent of chronic posttransfusion non-A, non-B hepatitis worldwide. However, in acute non-A, non-B hepatitis, the frequency of detectable anti-HCV was much lower, ranging from 15 to 60%, with a delayed seroconversion almost 3 to 6 months after transfusion (1). These facts emphasize the need for sensitive, direct assays for viral markers. In view of the expected low titers of HCV antigens in infected individuals, antigen assays are likely to have inherent limitations in sensitivity. The direct detection of the HCV genome correlates best with the activity and infectivity of the disease (4). Because of the low titer of HCV viremia (12, 13), polymerase chain reaction (PCR) is currently the only way to detect the HCV genome in the blood, and it has become a powerful and indispensable tool for studying type C hepatitis. Thus, factors affecting the assay should be meticulously defined. Various kinds of anticoagulants are commonly used for blood sampling and cell preparation. Further, blood samples are subjected to different physical conditions in daily laboratory practice. The physical conditions of sample storage have been briefly reported to affect the efficacy of HCV PCR (14). We therefore systematically studied the effects of anticoagulants and sample storage on the HCV PCR assay in blood samples of patients with documented HCV infection.

Blood samples were collected from 11 patients who seroconverted to anti-C100 in a prospective study of posttransfusion hepatitis (16). After venipuncture, each blood sample was simultaneously divided into three fractions to obtain serum (without anticoagulant), sodium-citrated plasma (0.3 ml of 3.8% sodium citrate in 3 ml of blood), and heparinized plasma (143 USP units of heparin in 3 ml of blood) (Monoject tube; Sherwood Medical, St. Louis, Mo.). All the samples were stored at −70°C until the PCR test was performed. To detect serum HCV RNA by PCR, two primer pairs from the highly conserved noncoding region of HCV were used (sense, J004 5′ GAC ACT CCA CCA TAG ATC ACT CCC CTG TGA; antisense, J296R 5′ CAC TCG CAA GCA CCC TAT CAG GCA GTA CCA) (10). RNA from serum or plasma was extracted as described previously (4). Briefly, 100 μl of serum or plasma was incubated with 200 μg of proteinase K per ml containing 0.2 M Tris-HCl (pH 7.5)–25 mM EDTA–0.3 M NaCl–2% sodium dodecyl sulfate at 37°C for 40 min. After phenol-chloroform extraction and ethanol precipitation, the pellet was dissolved in water. RNA from 20 μl of serum was reverse transcribed in a 20-μl mixture containing the outer antisense primer (0.6 μM), 50 mM Tris-HCl (pH 7.5), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM (each) deoxynucleoside triphosphate (dNTP), 20 U of RNAsin (Bethesda Research Laboratories, Gaithersburg, Md.), and 15 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) at 37°C for 90 min.

The PCR was carried out with a 50-μl mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 2.5 U of recombinant *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), 200 μM (each) dNTP, 0.6 μM (each) outer primer, and 5 μl of the cDNA mixture. A fast temperature cycling was performed as described before to improve sensitivity (17). Briefly, 40 cycles were done at 96°C for 30 s, 56°C for 15 s, and 74°C for 30 s. The temperature was maintained at 74°C for 10 min at the end of the last cycle. Two 30-nucleotide sequences from the 5′ noncoding region were used as the inner primers (sense J021, 5′ CAC TCC CCT GTG AGG AAC TAC TGT CTT CAC; anti-sense J270R, 5′ ACC ACA CGG CCT TTC GCG ACC CAA CAC TAC) (10). One microliter of the reaction mixture was transferred to the round 2 reaction mixture, which contained 0.6 μM (each) inner primer and the buffer that was used in the first round of PCR. The temperature cycling of the second round of PCR was the same as in the first round of PCR. Samples from the same patient were tested in aliquots of the same reagents and under the same reaction conditions. An HCV cDNA fragment containing the 5′ noncoding region was cloned and serially diluted. Samples with 1 to 1,000 copies of cloned HCV cDNA fragment were

* Corresponding author.

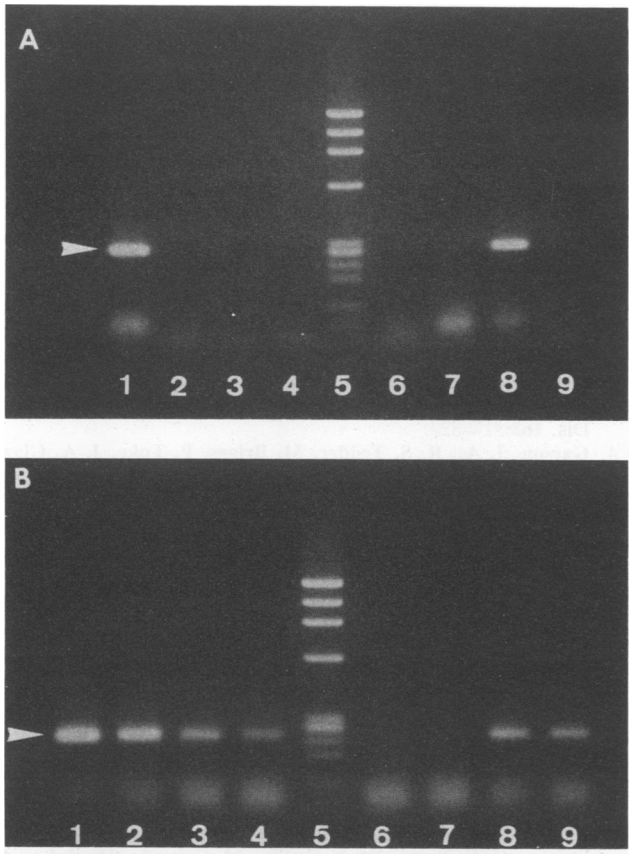

FIG. 1. (A) Gel electrophoresis of first-round PCR products. Lanes: 1 through 4, PCR products of positive controls with 1,000, 100, 10, or 1 copy of HCV cDNA, respectively; 5, ΦX174/*Hae*III; 6, plasma sample from a healthy person; 7, negative control without nucleic acid; 8, sodium-citrated plasma sample from patient 1 (Table 1); 9, sodium-citrated plasma sample from patient 2 (Table 1). Arrow indicates 293 bp. (B) Gel electrophoresis of second-round PCR products. Samples in lanes 1 through 9 are the same as those in panel A. Arrow indicates 250 bp.

used for the sensitivity assay, and serum samples from healthy persons and reagents without DNA were used as negative controls in the PCR. We followed the suggestions of Kwok and Higuchi to avoid possible contamination (8). In addition, reagents were stored in small aliquots. All the pipet tips and Eppendorf tubes were disposable. Only data which revealed no false-positive results in the negative controls and which were reproducible were adopted.

Ten microliters of the first and second rounds of PCR products was electrophoresed on a 2% agarose gel and then stained with ethidium bromide. Samples positive for HCV RNA by PCR were further divided into the following four groups: (i) stored at −70°C, (ii) stored at 4°C, (iii) stored at room temperature, and (iv) repeatedly thawed at room temperature and −70°C six times. After 48 h, these samples were semiquantified for HCV RNA. HCV cDNA of each sample was subjected to fivefold serial dilutions and then nested PCR until the results were negative at least twice.

The sensitivity of the PCR reached 100 to 1,000 copies of HCV cDNA in the positive controls after the first round of PCR, while in the second round of PCR a single copy of HCV cDNA was detected by gel electrophoresis and ethidium bromide staining (Fig. 1).

TABLE 1. HCV RNA by PCR in blood specimens of 11 patients with posttransfusion hepatitis C

Patient	Time of blood collection[a]	ALT (IU/liter)[b]	Result of sample[c]		
			Serum	Sodium-citrated plasma	Heparinized plasma
1	33 weeks	171	++	++	−
2	35 weeks	78	+	+	−
3	19 weeks	43	+	++	−
4	52 weeks	76	+	+	−
5	81 weeks	39	++	+	−
6	21 months	28	+	+	−
7	33 months	31	−	+	−
8	21 weeks	91	+	+	−
9	59 weeks	87	−	−	−
10	30 weeks	8	−	−	−
11	13 weeks	88	−	−	−

[a] Time of sample collection after the onset of hepatitis.
[b] Serum alanine aminotransferase (ALT) (normal, <31 IU/liter) at blood collection.
[c] +, positive for HCV RNA after the second round of PCR; ++, positive for HCV RNA after the first round of PCR; −, negative for HCV RNA.

For serum samples, 2 of 11 were positive after the first round of PCR and 7 were positive after the second round of PCR. In the sodium-citrated plasma, two samples were positive after the first round of PCR and eight were positive after the second round of PCR (Table 1). The eight PCR-positive sodium-citrated plasma samples were divided into four groups and semiquantified by fivefold serial dilution. Samples stored at −70°C, except those of two patients (patients 3 and 6; Table 2), yielded the highest signal ratio of the four groups. However, the differences among the four groups were minimal. Only three samples were negative after the second round of PCR. Samples positive after the first round of PCR (those of patients 1, 3, and 5; Table 1) showed relatively higher number of copies of HCV cDNA than those positive after the second round of PCR. This is assuming that the second PCR detected a single copy of the HCV genome in the plasma sample as the positive control. The viral titer was around 50 to 150,000 genomes per ml in the eight plasma samples. All the heparinized plasma samples showed negative results after the first and second rounds of PCR.

TABLE 2. Semiquantification of HCV RNA in eight plasma samples exposed to different physical conditions

Patient[a]	Signal ratio at condition[b]			
	RT[c]	4°C[d]	Thawing[e]	−70°C[f]
1	1/625	1/625	1/625	1/625
2	1/625	1/3,125	1/625	1/625
3	1	1/5	0	1
4	1/5	1/5	1	1/5
5	1/125	1/25	1/125	1/125
6	0	0	1/125	1/5
7	1/125	1/25	1/125	1/625
8	1/5	1/25	1/25	1/125

[a] Patients are the same as those listed in Table 1.
[b] Numbers indicate the highest dilution positive by PCR. Zero indicates negative by PCR for undiluted cDNA.
[c] Stored at room temperature (RT) for 48 h.
[d] Stored at 4°C for 48 h.
[e] Thawed six times in 48 h.
[f] Stored at −70°C for 48 h until test.

Currently, antibody testing for HCV establishes whether an individual has responded immunologically to exposure to viral antigens but does not determine whether the infectious agent has been eliminated or continues to persist in the host (4, 18). Studies of sequential serum samples from prospectively followed patients with HCV infection indicate a prolonged window for seroconversion (1, 16). The delay in antibody response suggests that some blood donors capable of transmitting HCV will not be detected by anti-HCV testing. A direct assay for HCV nucleic acids is thus useful in documenting HCV infection prior to anti-HCV seroconversion (11). Direct detection of HCV genomes is also useful to distinguish actively infected individuals from immune individuals. With this method, one can confidently identify viral nucleic acid in clinical samples and therefore better define the clinical course of infection and the meaning of the HCV antibodies. Perhaps the most important factor of false-negative results of HCV PCR is the existence of different strains or subtypes of HCV (5, 10, 12). Significant mismatches between PCR primers and viral sequences can markedly diminish the sensitivity of the assay or even completely eliminate the nucleic acid amplification. Fortunately, sequencing of more HCV strains has indicated that the 5′ noncoding region is one of the most conserved regions of HCV genomes (5, 10). Primers from this highly conserved region should be used and will greatly reduce false-negative results of HCV PCR (9).

Heparin is a commonly used anticoagulant and also a nonspecific ribonuclease inhibitor. However, it cannot be removed by extraction with phenol (15). Our results showed a profound interference of heparin on the HCV PCR assay. Heparin may affect not only HCV PCR but also PCR assays for other nucleic acids in the blood samples (2, 6). On the other hand, the sodium-citrated plasma samples yielded slightly better signal intensity than the serum samples.

Storage of whole blood at room temperature or at 4°C has been reported to have reduced the HCV PCR signal (14). In laboratory practice, thawing of stored serum or plasma specimens and exposure to room temperature are frequently inevitable. We found that samples frozen immediately and thawed the least spared the PCR signal. In general, after storage for 48 h under the conditions set in our study, reduction of the PCR signal was minimal. Only a few samples were negative by the nested PCR assay.

We used the dilutional endpoint titers of HCV cDNA detection by PCR to estimate the number of HCV virions and found that there were 50 to approximately 1.5×10^5 virions per ml in the eight plasma specimens. Caution should be used when interpreting these data, since PCR is, at best, semiquantitative. Although the efficacy of reverse transcription and amplification with clinical samples is not as predictable as with cloned cDNA, the semiquantitative results also confirmed the low titer of HCV viremia in the blood of patients with posttransfusion hepatitis, as previously described (12, 13).

We have confirmed that heparin interferes with the HCV PCR assay and should not be used as the anticoagulant in specimen collection when detection of HCV RNA is to be undertaken. Exposure to room temperature or 4°C or repeated thawing of plasma samples in a 48-h period causes minimal loss of the HCV PCR signal in usual laboratory practice.

This study was supported by grants from the National Science Council and Department of Health, Executive Yuan, Republic of China.

The authors thank Yi-Ying Chen, Shiu-Hui Liu, and Pao-Pao Lin for their excellent assistance.

REFERENCES

1. **Alter, H. J., R. H. Purcell, J. W. Shih, J. C. Melpolder, M. Houghton, Q. L. Choo, and G. Kuo.** 1989. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. N. Engl. J. Med. **321:**1494–1500.
2. **Beutler, E., T. Gelbart, and W. Kuhl.** 1990. Interference of heparin with polymerase chain reaction. BioTechniques **9:** 166.
3. **Chen, D. S., G. Kuo, J. L. Sung, M. Y. Lai, J. C. Sheu, P. J. Chen, P. M. Yang, H. M. Hsu, M. H. Chang, C. J. Chen, L. C. Hahn, Q. L. Choo, T. H. Wang, and M. Houghton.** 1990. Hepatitis C virus infection in an area hyperendemic for hepatitis B and chronic liver disease: the Taiwan experience. J. Infect. Dis. **162:**817–822.
4. **Garson, J. A., R. S. Tedder, M. Briggs, P. Tuke, J. A. Glazebrook, A. Trute, D. Parker, J. A. J. Barbara, M. Contreras, and S. Aloysius.** 1990. Detection of hepatitis C viral sequences in blood donations by "nested" polymerase chain reaction and prediction of infectivity. Lancet **335:**1419–1422.
5. **Han, J. H., V. Shyamala, K. H. Richman, M. J. Brauer, B. Irvine, M. S. Urdea, P. Tekamp-Olson, G. Kuo, Q. L. Choo, and M. Houghton.** 1991. Characterization of the terminal regions of hepatitis C virus RNA: identification of conserved sequences in the 5′ untranslated region and poly (A) tails at the 3′ end. Proc. Natl. Acad. Sci. USA **88:**1711–1715.
6. **Holodniy, M., S. Kim, D. Katzenstein, M. Konrad, E. Groves, and T. C. Merigan.** 1991. Inhibition of human immunodeficiency virus gene amplification by heparin. J. Clin. Microbiol. **29:**676–679.
7. **Kuo, G., Q. L. Choo, H. J. Alter, G. L. Gitnick, A. G. Redeker, R. H. Purcell, T. Miyamura, J. L. Dienstag, M. J. Alter, C. E. Stevens, G. E. Tegtmeier, F. Bonino, M. Colombo, W. S. Lee, C. Kuo, K. Berger, J. R. Shuster, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science **244:**362–364.
8. **Kwok, S., and R. Higuchi.** 1989. Avoiding false positives with PCR. Nature (London) **339:**237–238.
9. **Okamoto, H., S. Okada, Y. Sugiyama, T. Tanaka, Y. Sugai, Y. Akahane, A. Machida, S. Mishiro, H. Yoshizawa, Y. Miyakawa, and M. Mayumi.** 1990. Detection of hepatitis C virus RNA by a two-stage polymerase chain reaction with two pairs of primers deduced from the 5′-noncoding region. Jpn. J. Exp. Med. **60:**215–222.
10. **Okamoto, H., S. Okada, Y. Sugiyama, S. Yotsumoto, T. Tanaka, H. Yoshizawa, F. Tsuda, Y. Miyakawa, and M. Mayumi.** 1990. The 5′-terminal sequence of the hepatitis C virus genome. Jpn. J. Exp. Med. **60:**167–177.
11. **Shimizu, Y. K., A. J. Weiner, J. Rosenblatt, D. C. Wong, M. Shapiro, T. Popkin, M. Houghton, H. J. Alter, and R. H. Purcell.** 1990. Early events in hepatitis C virus infection of chimpanzees. Proc. Natl. Acad. Sci. USA **87:**6441–6444.
12. **Simmonds, P., L. Q. Zhang, H. G. Watson, S. Rebus, E. D. Ferguson, P. Balfe, G. H. Leadbetter, P. L. Yap, J. F. Peutherer, and C. A. Ludlam.** 1990. Hepatitis C quantification and sequencing in blood products, haemophiliacs, and drug users. Lancet **336:**1469–1472.
13. **Ulrich, P. P., J. M. Romeo, P. K. Lane, I. Kelly, L. J. Daniel, and G. N. Vyas.** 1990. Detection, semiquantitation, and genetic variation in hepatitis C virus sequences amplified from the plasma of blood donors with elevated alanine aminotransferase. J. Clin. Invest. **86:**1609–1614.
14. **van der Poel, C. L., H. T. M. Cuypers, H. W. Reesink, A. J. Weiner, S. Quan, R. D. Nello, J. J. P. van Boven, I. Winkel, D. Mulder-Folkerts, P. J. Exel-Oehlers, W. Schaasberg, A. Leentvaar-Kuypers, A. Polito, M. Houghton, and P. N. Lelie.** 1991. Confirmation of hepatitis C infection by new four-antigen recombinant immunoblot assay. Lancet **337:**317–319.
15. **Wallace, D. M.** 1987. Guide to molecular cloning technique, p.

33–40. *In* S. L. Berger and A. R. Kimmel (ed.), Methods in enzymology. Academic Press, Inc., Orlando.

16. **Wang, J. T., T. H. Wang, J. T. Lin, J. C. Sheu, J. L. Sung, and D. S. Chen.** 1990. Hepatitis C virus in a prospective study of posttransfusion non-A, non-B hepatitis in Taiwan. J. Med. Virol. **32:**83–86.
17. **Wang, J. T., T. H. Wang, J. C. Sheu, L. N. Shih, J. T. Lin, and D. S. Chen.** 1991. Detection of hepatitis B virus DNA by polymerase chain reaction in plasma of volunteer blood donors negative for hepatitis B surface antigen. J. Infect. Dis. **163:**397–399.
18. **Weiner, A. J., G. Kuo, D. W. Bradley, F. Bonino, G. Saracco, C. Lee, J. Rosenblatt, Q. L. Choo, and M. Houghton.** 1990. Detection of hepatitis C viral sequences in non-A, non-B hepatitis. Lancet **335:**1–3.