Whole-Blood Hepatitis C Virus RNA Extraction Methods

We read the article by Cook et al. (3) with interest, since they compared their findings with our previous work (4–8; D. Brashear, S. Taylor, J. Xiang, D. Klinzman, F. LaBrecque, M. Pfaller, B. Alden, D. LaBrecque, J. Stapleton, and W. Schmidt, 10th Triennial Int. Symp. Viral Hepatitis Liver Dis., abstr. C008, 2000; J. Xiang, F. LaBrecque, W. N. Schmidt, D. Klinzman, D. Brashear, D. R. LaBrecque, M. J. Perino-Phillips, and J. T. Stapleton, 10th Triennial Int. Symp. Viral Hepatitis Liver Dis., abstr. F011, 2000). Cook et al. did not find that whole blood was a more sensitive source of hepatitis C virus (HCV) RNA than plasma. Careful review of their paper revealed several potential reasons for this discrepancy, including the following. (i) A different method of extracting RNA from whole blood was used (5). We did not use Trizol following extraction with Catrimox (4, 9). The pH of the phenol-guanidinium mixture in Trizol is not published. We found that the phenol pH is critical for optimal extraction (4, Brashear et al., 10th Triennial Int. Symp. Viral Hepatitis Liver Dis.). (ii) We showed that extraction with Trizol was less sensitive than our Catrimox method (Xiang et al., 10th Triennial Int. Symp. Viral Hepatitis Liver Dis.). (iii) Cook et al. used different primers and thermocycler conditions than we did, and they used only single-round reverse transcription (RT)-PCR instead of nested RT-PCR (3, 4). (iv) Finally, the serum volume was not adjusted to the whole-blood volume (3), which we took into account (6).

As suggested by Cook et al., intracellular HCV RNA is a potential explanation for the improved sensitivity of HCV RNA detection in whole blood (3), yet we found that a significant portion of HCV RNA in the crude cellular pellet was not intracellular (6). Our study of 115 patients with HCV infection demonstrated that the improved sensitivity of whole-blood HCV RNA detection correlated with the presence and quantity of cryoglobulins (8). We also noted highly significant differences in the detection of HCV RNA for whole blood and plasma among 52 interferon-treated patients who were monitored throughout treatment (7). As was the case for the one patient studied by Cook et al. (3), among our 52 patients the clearance of intracellular virus occurred nearly simultaneously with the clearance of plasma virus during therapy (7).

The extraneous bands described by the authors (3) are not routinely present in our work (e.g., see Fig. 1, 3, and 5B in reference 4). In addition, Cook et al. stated that it was not tenable that our antibody-negative subjects were truly infected with HCV since we did not evaluate liver biopsy samples for HCV RNA (3). However, we proved that several HCV antibody-negative patients had HCV RNA present in multiple whole-blood and plasma samples collected over months and years, using primers from several different regions of the genome (4, 6). In addition, we confirmed our results by Southern blotting and sequence analysis of PCR products and by direct comparison with results obtained with the use of commercial HCV RNA assays (4, 6, 9). The finding of prolonged RNA positivity in the absence of detectable HCV antibody has been described by others (2), including after the experimental infection of chimpanzees (1). In conclusion, we believe that the difference between our work and that of Cook et al. (3) relates to differences in the whole-blood RNA extraction methodology.

REFERENCES


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Authors’ Reply

Drs. Schmidt and Stapleton think that the difference between our work (3) and theirs (6) is related to differences in whole-blood RNA extraction methods due to a presumed difference in pH of the phenol-guanidinium. The Trizol U.S. patent documents state that the pH of Trizol is 4.0, the exact pH used in their method (D. Brashear, S. Taylor, J. Xiang, D. Klinzman, F. LaBrecque, M. Pfaller, B. Alden, D. LaBrecque, J. Stapleton, and W. Schmidt, 10th Triennial Int. Symp. Viral Hepatitis Liver Dis., abstr. C008, 2000). Therefore, Trizol and the acid phenol-guanidinium mixture used by Schmidt and Stapleton are equivalent chemical environments and the fact that our data failed to confirm their findings is not explained by differences in extraction methods. Nor are the differences between PCR methodology, carrier RNA, and volume adjustment consequential. The competitive reverse transcription
(RT)-PCR method we employed was quantitative, whereas a semiquantitative method was employed by Schmidt and Stapleton. Sufficient cellular RNA is present in the whole-blood samples so that the addition of carrier RNA does not enhance the amount of hepatitis C virus (HCV) RNA isolated. We did not physically adjust the serum volume to the whole-blood volume, but when adjustment was made in the calculations (see Table 2 of reference 3), there was only a slight difference in the correlation between the serum and whole-blood results.

Extraneous bands are quite apparent in Fig. 3 and 5 of reference 6. Despite differences in the primers used in the two studies, spurious amplification appears to have occurred with both methods. As demonstrated by the sequences we detected, these extraneous bands are generated from false priming and artifactual amplification of human cellular RNA at high concentrations, when HCV RNA is absent; this cannot be circumvented entirely by increasing the stringency of the RT-PCR.

The finding of extracellular HCV RNA in the crude cellular pellet could explain differences in HCV quantification when using whole blood or serum but only when the blood or serum was stored at 4°C prior to separation as in the Schmidt et al. study (4). In routine clinical assays, the serum or plasma is not refrigerated prior to separation. Schmidt and Stapleton are correct: cryoglobulins, particularly type II cryoglobulins, can be responsible for loss of virus. Their study on cryoglobulins (5) confirms the original observation that HCV can be precipitated with cryoglobulins (1) but does not provide a rationale for routine clinical use of their method. The cryoglobulins associated HCV infection (2) are not the type II cryoglobulins that precipitate at room temperature. Hence, for routine specimens the current methodology is adequate. For patients with type II cryoglobulinemia, blood should be clotted at 37°C prior to processing.

We reiterate that the presence of HCV in blood cells without the demonstration of HCV RNA in the liver is untenable. If there were replication of HCV in blood cells, an idea that is controversial and therefore requires verification, then there must be replication in the liver. A verification of finding HCV in the blood cells but not in the serum of seronegative patients is detection of HCV RNA in the liver.

An improved method for detection of HCV RNA in blood samples would be welcomed in diagnostic clinical laboratories; unfortunately, we did not find the that the Schmidt-Stapleton methodology provided any improvement over our current method.

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