The clinical utility of using Catrimox–14-treated whole blood in detecting hepatitis C virus RNA

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Background: Measuring hepatitis C virus (HCV) RNA in serum or plasma may underestimate the true HCV burden. Extracting viral RNA from whole blood (WB) with a cationic surfactant (Catrimox–14) has resulted in HCV RNA concentrations up to 1000-fold higher than from serum or plasma in some studies, but not others. We compared the Catrimox–14 WB assay with a standard serum assay.

Methods: Seventy-two chronic HCV patients received 48 weeks of standard or pegylated interferon alpha–2b and ribavirin therapy. Catrimox–14-treated WB and corresponding serum samples were obtained at baseline and weeks 12, 24, 48 and 72. HCV RNA concentrations from WB and serum were quantified by a previously validated RT-PCR assay.

Results: Overall mean (±SD) baseline serum log10 HCV RNA concentration was 6.5 (±0.58) copies/ml. Out of 72 patients, 33 had no detectable virus at 72 weeks. Neither assay detected virus in these patients at 12 weeks and neither WB nor serum assays detected virus at end-of-treatment in the 10 patients who subsequently relapsed at 72 weeks. HCV RNA concentrations from WB and serum assays were linearly correlated (R2=0.73; P<0.001), although mean serum HCV RNA concentrations were 0.5 log10 copies/ml higher in serum than in WB [6.0 (±0.82) vs 5.5 (±0.84), respectively, P=0.12].

Conclusions: Catrimox–14-treated WB assays detect changes in HCV RNA, but do not offer clinical advantage over a conventional serum RT-PCR based assay.

Background

Chronic infection with hepatitis C virus (HCV) affects 2.7 million individuals in the USA [1] and 170 million worldwide [2]. Chronic HCV leads to cirrhosis within 30 years in 20–30% of cases and is the leading cause of liver transplants in the USA [3]. Fortunately, pegylated interferon therapy given in combination with ribavirin results in sustained virological response (SVR) in more than 50% of previously untreated patients with chronic HCV [4,5].

The majority of clinical therapeutic trials that assess hepatitis C infection quantitate HCV RNA blood concentrations by using RT-PCR or branched DNA signal amplification methods. Each commercially available assay format has a variable lower limit of sensitivity. However, plasma- and serum-based assays may underestimate true HCV RNA concentrations as they do not account for virus that may be harbouredException in white blood cells or cryoprecipitates. Thus, it has been postulated that assays utilizing whole blood (WB) instead of serum or plasma may provide a more accurate quantitation of HCV RNA.

The cationic surfactant tetradecyltrimethylammonium oxalate (Catrimox–14; Iowa Biotechnology Corp, Ames, IA, USA) lyases cells and precipitates RNA in a complex that is not degradable by RNAses. Catrimox–14 has been used to extract viral RNA from WB and faeces, which contain nucleases and RNAses. Studies from a single centre that used Catrimox–14 to extract HCV RNA from WB detected viral loads up to 1000-fold higher than in plasma or serum samples [6–8]. However, a recent study using a Catrimox–14-Trizol extraction of HCV RNA from WB found insignificant levels of virus in white blood cells, and the use of WB versus serum did not result in greater virus detection [9]. The aim of this study was to prospectively evaluate the utility of measuring HCV RNA in Catrimox–14-treated WB compared with a standard quantitative serum RT-PCR assay in assessing viral response during therapy.
Materials and methods

Patient characteristics and sample collection

Seventy-two adult patients with chronic hepatitis C were enrolled into this single-centre study. All patients received 48 weeks of combination therapy with either interferon alpha-2b (Intron A; Schering Corp, Kenilworth, NJ, USA) or peginterferon alpha-2b (Peg-Intron; Schering Corp) plus ribavirin (Rebetol; Schering Corp). The study was approved by the institutional review board at the Scripps Clinic and Research Foundation, and all patients gave written informed consent prior to enrolment. Information on baseline demographics (including age, gender, ethnicity, and mode and duration of HCV infection), laboratory results, histology and viral characteristics (genotype and serum viral load) was maintained in a Scripps Clinic database. Patients were reviewed every 4 weeks during treatment and at 24 weeks after end-of-treatment. Peripheral blood samples for HCV RNA quantification were collected at baseline and at weeks 12, 24, 48 and 72. Sustained virological response was defined as the loss of detectable serum HCV RNA by RT-PCR 24 weeks after completion of therapy. Relapse patients were defined as those who initially cleared HCV RNA at the end-of-treatment but had detectable virus during follow-up.

Peripheral WB samples were collected into one 10-ml acid-citrate-dextrose-A tube and one 10-ml non-serum separator tube (SST) tube (Vacutainer tubes; Becton, Dickson and Company, Franklin Lakes, NJ, USA). The non-SST tube was centrifuged to separate out the serum, which was stored at –70°C within 2 h of collection. Separate 0.2-ml aliquots of WB were added to 1 ml of Catrimox-14 and stored at –70°C until analysis.

RNA extraction from serum or plasma

In preparing RNA from WB using Catrimox-14, we closely followed the methods described by Schmidt et al. [6]. Briefly, 0.2 ml of WB was placed into a sterile microcentrifuge tube containing 1.0 ml of Catrimox-14 followed by the addition of 10 µg of yeast RNA (Ambion Inc, Austin, TX, USA) [10]. The sample was mixed and incubated at room temperature for 15 min and thereafter centrifuged at 12 000g for 5 min. The precipitate was washed twice with 1 ml of diethyl pyrocarbonate (DEPC)-treated water and dissolved in 0.2 ml of 4M guanidium isothiocyanate (GITC; Roche Applied Science). Then 0.2 ml of water-saturated phenol pH 4.0 (Fisher Scientific, Pittsburgh, PA, USA) was added to the sample and mixed thoroughly with 0.2 ml of chloroform. The mixture was maintained on ice for 15 min and then centrifuged at 12 000g for 5 min at 4°C. Thereafter, the aqueous supernatant was mixed with 3 µl of 20 mg/ml glycogen (Roche Applied Science) and an ethanol/ammonium acetate mixture, and allowed to precipitate overnight at 4°C. The nucleic acids were collected by centrifugation at 23 000g for 15 min at 4°C. The precipitate was washed with 1 ml of 70% ethanol, dried and redissolved in 30 µl of DEPC-treated water containing dithiothreitol (DTT) and RNase inhibitor (Roche Applied Science).

HCV RNA assay

After extraction, both the WB and serum RNA samples were treated identically. HCV RNA concentrations were determined using a quantitative, multicycle RT-PCR assay (SuperQuantTM, National Genetics Institute, Los Angeles, CA, USA) as previously described [10]. Briefly, extracted RNA was reverse transcribed to form a cDNA template. A portion of the cDNA was then amplified in four separate polymerase chain reactions at 25, 30, 35 and 45 cycles. Both the primer sets utilized bind to highly conserved regions within the 5′-untranslated region of HCV and have been demonstrated to amplify all known HCV genotypes equally. After amplification, each of the four separate PCR mixtures were loaded onto a 1.2% agarose gel, electrophoresed and then vacuum-transferred to a nylon membrane.

HCV target DNA was detected by hybridization with a non-radioactive digoxigenin-labelled DNA probe and immunostaining. The lower limit of HCV RNA detection for this assay was fewer than 100 viral copies/ml. For quantification, each Southern-blot membrane was scanned into a computer document using an automated scanner/densitometer. The resultant images were then measured for band area and mean band density. The readings were standardized to integrated band density and compared with the standard curve to obtain a viral copy number for each
band. The mean coefficient of variation (CV) for this assay is 26.3%.

Spiking experiments for assay validation
A comparison between the Catrimox-14 RNA extraction and the standard RNA extraction was performed by spiking normal plasma and normal WB samples with an in-house HCV standard to achieve final concentrations of $10^3$, $10^4$, $10^5$ and $10^6$ viral copies/ml. Three 0.1-ml aliquots of plasma at each of the four HCV RNA concentrations were prepared by the standard RNA extraction method. In addition, three 0.1-ml aliquots of WB and plasma containing HCV at each concentration were extracted using the Catrimox-14 method as outlined above, with all volumes being adjusted accordingly.

To assess the stability of HCV in stored patient samples, plasma and Catrimox-14 WB spiked with $10^3$, $10^4$ and $10^5$ viral copies/ml were analysed following storage at −70°C for 1 week. A subset of samples spiked at $10^5$ copies/ml was constructed containing different proportions of WB and plasma (PL) (0 µl WB/100 µl PL, 10 µl WB/90 µl PL, 25 µl WB/75 µl PL, 50 µl WB/50 µl PL or 0 µl WB/100 µl PL) in a final 100 µl volume before undergoing Catrimox-14 treatment. The Catrimox-14 method was followed as outlined above with the exception that yeast RNA was not added and all volumes were adjusted given the preparation of 0.1 ml of sample. For all samples, HCV RNA quantitation was performed by RT-PCR and Southern blotting using the SuperQuant Assay.

Statistical analyses
Baseline data were descriptively summarized and differences in means assessed using Student’s t-test or ANOVA. The correlation between serum and Catrimox-14-treated WB $\log_{10}$ HCV RNA concentrations was determined by using the Pearson correlation coefficient. All $P$ values were 2-tailed, and statistical significance was assessed at the $P<0.05$ level.

Results
Patient characteristics and response to treatment
Of the 72 chronic hepatitis C patients who received combination therapy with interferon and ribavirin, 46 (64%) were male and their mean (±SD) age was 45.3 (±6.3) years. Forty-seven (65%) were infected with HCV genotype 1, and the mean pretreatment serum $\log_{10}$ HCV RNA was 6.5 ±0.58 copies/ml. The overall median METAVIR fibrosis stage was 1. As judged by the serum-based RT-PCR assay, 29 patients (40%) did not respond to treatment, 33 (46%) achieved SVR and 10 (14%) relapsed following viral clearance at end-of-treatment.

Catrimox-14-treated WB versus standard serum assays
Whole blood HCV RNA quantitation was performed only in a subset of baseline (week 0) samples. For patients with detectable virus by both methods either at baseline or during therapy, there was good correlation between serum and Catrimox-14-treated WB $\log_{10}$ HCV RNA concentrations ($R^2=0.73$, $P<0.001$) (Figure 1). Yet mean HCV RNA concentration measured 0.5 $\log_{10}$ copies/ml higher in serum than in Catrimox-14-treated WB [6.0 (±0.82) vs 5.5 (±0.84), $P=0.12$] (Figure 2).

Of the 33 patients that achieved SVR, 30 had no measurable WB HCV RNA concentrations at weeks 12, 24 and 48. Results were not attainable for three of the patients given inadequate amplification due to the presence of PCR inhibitors or low RNA yield in the WB samples. As judged by both serum and Catrimox-14-treated WB assays, all 30 patients cleared HCV RNA by week 12 (Table 1). Of the 10 patients that relapsed by week 72, all were negative for HCV RNA by both serum and Catrimox-14-treated WB at the end-of-treatment (week 48).

WB and plasma spiked with HCV
To assess the sensitivity and precision of the standard and Catrimox-14 assays, normal plasma and normal WB samples were spiked with an in-house HCV control to achieve final concentrations of $10^3$, $10^4$, $10^5$ and $10^6$ viral copies/ml. The intra-assay coefficient of variation (CV) for the standard plasma preparation ranged from 7% (at the expected HCV RNA concentration of $10^3$ copies/ml) to 26% (at $10^6$ copies/ml). HCV RNA concentrations for Catrimox-14 plasma were on average sixfold lower than standard plasma and had a CV range of 19–33% (Table 2). Catrimox-14
plasma sample spikes of $10^3$ copies/ml were below detection limits of the RT-PCR assay (<100 copies/ml). Catrimox-14 WB HCV-RNA levels were lower than corresponding standard plasma fractions by three- to fourfold. The CV ranged from 6% (at $10^6$ copies/ml) to 25% (at the lower expected HCV RNA level of $10^3$ copies/ml).

Freezing at −70°C for 1 week did not significantly affect HCV RNA levels for standard plasma or Catrimox-14-treated WB at expected concentrations of $10^3$, $10^4$ and $10^5$ copies/ml (Figure 3). In keeping with prior studies, this confirmed the stability of WB RNA when stored in Catrimox-14 at −70°C [6]. Prolonged storage of Catrimox-14 WB samples at −70°C for 1.5 years did not affect HCV RNA levels significantly [fresh vs stored samples mean (±SD) HCV RNA copies/ml 32 000 (±3559) vs 29 666 (±4714); $P=0.41$].

Effect of RNA concentration on Catrimox-14 extraction efficiency

It has been reported that when using Catrimox-14 extraction, the addition of carrier RNA to plasma greatly increases the yield of HCV RNA [10]. To confirm this, gradually increasing proportions of WB in plasma spiked with HCV at $10^7$ viral copies/ml were prepared utilizing the Catrimox-14 method. Samples were prepared to an aliquot volume of 100 µl. In this case, the yeast carrier RNA was not added to the samples prior to extraction. Up to fivefold greater quantities of virus could be extracted at higher volumes of WB (mean ±SD HCV RNA copies/ml = 10 200 ±2615 vs 55 000 ±5000; $P<0.001$ for plasma samples with no added WB and 100 µl WB, respectively). For samples with 25 µl and 50 µl of added WB, mean HCV RNA was 30 000 ±6557 and 37 000 ±5567 copies/ml respectively. This demonstrates that isolating HCV RNA from plasma, which contains a relatively low nucleic acid concentration, using Catrimox-14 is inefficient unless processed in the form of WB or processed in the presence of carrier RNA.

Discussion

In this prospective cohort study, using Catrimox-14 to extract HCV RNA from WB did not demonstrate a clinical advantage over using a standard RNA extraction from serum. Although HCV RNA concentrations detected by WB and serum assays were well correlated, no residual virus was detected in the WB of relapse patients who initially achieved a virological response at the end of standard interferon and ribavirin therapy.

Table 1. Virological response to treatment as assayed by HCV RNA quantitation in standard serum or Catrimox-14-treated WB preparations

<table>
<thead>
<tr>
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<th>Relapsers, n=10</th>
<th>Sustained virological responders, n=33</th>
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<tr>
<td></td>
<td>Serum</td>
<td>Catrimox-14/WB</td>
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<tr>
<td>Week 12</td>
<td>6 (–)</td>
<td>7 (–)</td>
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<td></td>
<td>4 (+)</td>
<td>3*</td>
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<td>Week 24</td>
<td>10 (–)</td>
<td>8 (–)</td>
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<td>Week 48</td>
<td>10 (–)</td>
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*Results not available because of inadequate RNA amplification due to PCR inhibitors or low RNA yield. †For one patient, a week-48 sample was unavailable for both serum and Catrimox-14/WB; ‡two additional patient samples were not available because of inadequate RNA amplification for Catrimox-14/WB. (–), HCV RNA negative; (+), HCV RNA positive; WB, whole blood.
In addition, when virus was undetectable by the serum assay, the WB assay did not detect residual virus either. Relapse is defined as the loss of detectable serum HCV RNA during therapy followed by reappearance of virus after the end-of-treatment (usually within 12 weeks). Host characteristics predictive of relapse include being infected with HCV genotype 1 and late clearance of HCV RNA [13]. A positive WB result for HCV RNA at the end of treatment has also been proposed as a predictor of relapse [8,14]. A recent study, using qualitative and real-time RT-PCR analysis, noted residual HCV RNA in WB samples in 12 out of 18 patients who had cleared virus from serum and plasma following interferon-alpha monotherapy. All patients with detectable virus in WB subsequently relapsed, usually within the first 4 weeks [14]. Latent HCV infection in peripheral blood mononuclear cells (PBMCs) potentially explains relapse, although PBMCs and plasma clear virus at similar overall rates [8]. Furthermore, the data regarding HCV infection in PBMCs are controversial, as many studies have used non-quantitative methods for virus detection. HCV does infect PBMCs, but the level of viral replication is probably low [15]. A prior study using Catrimox-14-Trizol extraction of HCV RNA from WB found insignificant levels of virus in white blood cells [9]. Thus, detection of residual virus in PBMCs alone cannot explain the proposed increased sensitivity of WB assays compared with serum in predicting relapse. In our study, none of the 10 patients who relapsed following therapy had detectable virus by the WB assay at the end-of-treatment, and our findings confirm that the WB assay has no clinical utility in predicting relapse. Overall 46% of patients achieved SVR. These patients all cleared HCV RNA from the serum during the first 12 weeks of treatment. No residual virus was detectable in WB from these patients at any stage during treatment. Amongst the 40% of patients who did not eradicate serum HCV RNA during treatment, the WB assay also indicated non-response to therapy (data not shown). There was good correlation between the serum and WB assays in quantitating HCV, although HCV RNA concentrations were 0.5 log_{10} copies/ml lower for WB. In the clinical setting, this would certainly have negative implications for determining virological endpoints. Although there may be a role for the WB assay as a qualitative assay, its clinical utility as a quantitative measure appears limited.

<table>
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<tr>
<th>Spiked HCV RNA*</th>
<th>Standard plasma</th>
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<th>Catrimox-14/plasma</th>
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<tr>
<td></td>
<td>HCV RNA Mean (sd)</td>
<td>%CV</td>
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<td>10⁴</td>
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<td>4833 (611)</td>
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<tr>
<td>710 000</td>
<td>486 667 (30 551)</td>
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<td>123 333 (40 415)</td>
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*HCV RNA is measured as number of copies/ml, CV, coefficient of variation; HCV, hepatitis C virus; ND, not determined; sd, standard deviation; WB, whole blood.
Despite closely following Schmidt et al.’s [6,10] method for extracting HCV RNA from WB, a number of patient samples were not suitable for quantitation given the presence of PCR inhibitors or low yields of RNA, and no result was attainable. The internal RNA controls that were run during PCR amplification verified that the WB method had limitations. The corresponding plasma samples, however, gave acceptable results. The differences between the assays seen in the clinical study were also observed when standard plasma and WB samples were spiked with known quantities of HCV RNA. In these experiments, the WB assay resulted in HCV RNA concentrations three- to fourfold lower than from the standard serum assay. It is doubtful whether lysis of white blood cells leads to degradation of HCV RNA during WB storage. Catrimox-14 forms an insoluble complex with the RNA that protects it from degradation by nucleases and prior studies have also demonstrated stability of RNA in a range of clinical samples [16].

It is not entirely clear why vastly different results were obtained from those previously reported. A partial explanation could be that, in some studies, Schmidt et al. utilized Catrimox-14 to isolate HCV RNA from serum or plasma without adding carrier RNA [6–8,17,18]. The importance of adding carrier RNA has been demonstrated in our study and documented by others [10]. The WB samples in prior studies that did not have additional carrier RNA may have contained sufficient cellular RNA to aid in the precipitation of the HCV RNA. This omission may have accounted, in part, for the higher HCV RNA yields for the WB Catrimox-14 samples. In addition, a semi-quantitative PCR method was utilized when comparing HCV RNA concentrations in WB and plasma samples [6,7,19]. This method relies upon qualitative testing performed at 10-fold dilutions with the result of the assay being the last dilution to give a positive result. Testing in this manner could miscalculate concentrations by as much as 10-fold, which may have increased the sensitivity of the Catrimox-14 assay. In other experiments, Catrimox-14 WB preparation in combination with an in-house RT-PCR test was compared with a commercially available serum preparation and RT-PCR assay (Cobas Amplicor; Roche Diagnostics) [7,8,18]. In these studies, it was unclear if equal amounts of the isolated RNA from both methods were assayed in the corresponding PCR systems. It appears that, in at least one instance, the commercial assay had up to 10-fold less extraction volume added to the RT-PCR [18]. This demonstrated that the Catrimox-14 WB preparation and in-house RT-PCR was a more sensitive assay platform than the commercial test but made it difficult to comment on whether a higher concentration of HCV resides in WB versus plasma, given the unequal amounts of the extract tested. In our study, only the preparation methods were different and equal sample extraction volumes were utilized in one quantitative RT-PCR system (SuperQuant), thus allowing for a fair comparison of the extraction methods. Lastly, it is possible that our modified version of the guanidium isothiocyanate Chomczynski method utilized in this study was more sensitive than the plasma preparation methods used by Schmidt et al. [6].

In summary, this is the largest clinical study to date to validate the utility of a quantitative WB HCV RNA assay during combination interferon and ribavirin therapy for chronic hepatitis C. Our results suggest that WB extraction methods using Catrimox-14 are complex and offer no clinical advantage over simpler and validated serum-based quantitative RNA assays.

Acknowledgements

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