Distribution of Hepatitis C Virus (HCV) RNA in Whole Blood and Blood Cell Fractions: Plasma HCV RNA Analysis Underestimates Circulating Virus Load

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Previous experiments using a cationic surfactant to detect hepatitis C virus (HCV) RNA in whole blood (WB) suggested that WB was a more plentiful source of viral RNA than was plasma. The relative HCV RNA titers in WB, plasma, peripheral blood mononuclear cells (PBMC), neutrophils, and red blood cells (RBC)/platelets from 10 patients with chronic HCV infection were compared. WB contained significantly more HCV RNA than plasma, which contained more HCV RNA than PBMC, neutrophils, or RBC/platelets \((P < .001)\). To determine if this increased sensitivity was clinically relevant, results of WB and plasma HCV RNA assays were compared with commercial quantitative and qualitative plasma HCV RNA assay results obtained for patients receiving interferon therapy. WB was significantly more sensitive than commercial plasma reverse transcription–polymerase chain reaction for detecting HCV RNA \((P < .005)\). These data indicate that a significant proportion of HCV RNA in peripheral blood is not identified by standard plasma RNA detection methods.

Hepatitis C virus (HCV) is a small enveloped RNA virus that is responsible for >90% of cases of parenterally transmitted non-A, non-B hepatitis \([1–3]\). Although acute infection with HCV may be asymptomatic, the majority of patients develop persistent viremia and chronic hepatitis. About 15%–20% of HCV-infected patients progress to end-stage liver disease and are considered for orthotopic liver transplantation. Interferon–α is the only recognized treatment for chronic HCV infection. Interferon therapy leads to normalization of serum transaminase levels in ~50% of patients, but unfortunately there is a high rate of relapse after discontinuation of therapy \([4, 5]\).

Clinical diagnosis of HCV infection relies on a positive EIA result and characteristic histologic findings on liver biopsy \([3, 4, 6]\). Increasingly, viral RNA in patient plasma or serum is monitored during treatment by use of qualitative reverse transcription–polymerase chain reaction (RT-PCR) and various quantitative methods, to guide the clinical management of chronic HCV infection \([7–11]\). Although these quantitative assays accurately determine the amount of virus in plasma or serum, HCV is also found in bone marrow and peripheral blood mononuclear cells (PBMC) \([12–14]\). These cellular reservoirs of HCV are not detected by plasma RNA detection methods.

Nearly 40% of HCV-seropositive patients have measurable cryoglobulins, and a small percentage of patients develop the mixed essential type II cryoglobulinemia syndrome \([15, 16]\). Cryoprecipitates from these patients contain anti-HCV immunoglobulins, rheumatoid factor, and HCV RNA. Cryoprecipitates appear to be enriched for HCV in comparison with plasma or serum \([17, 18]\), and these complexes may be cleared during the centrifugation step used to prepare plasma or serum from whole blood (WB). Consequently, routine serum or plasma measurements of HCV RNA may not provide an accurate determination of the total HCV burden present in peripheral blood.

We recently described a method that uses a cationic surfactant (Catrimox-14) to extract HCV RNA from peripheral WB or isolated blood fractions such as plasma or blood cells \([19]\). When WB is mixed with Catrimox-14, RNA is rapidly precipitated as an insoluble complex containing the polyanionic nucleic acid on the interior, surrounded by a hydrophobic exterior \([20]\). This complex appears to protect the RNA from RNase present in WB. The method allows estimation of the total circulating HCV load and allows the relative quantitation of HCV RNA in WB, plasma, and various blood fractions. The accurate quantitation of HCV RNA is important, since high serum levels of HCV RNA appear to correlate with a poor response to interferon therapy \([11]\).

This study compared the relative concentration of HCV RNA in WB with that in plasma, PBMC, neutrophils, and red blood cells (RBC)/platelets.

Materials and Methods

Patients. Patients with chronic HCV infection were selected for study. The diagnosis was determined by a positive HCV anti-
body test (second-generation EIA and RIBAs; Abbott, Abbott Park, IL), a positive plasma HCV RT-PCR assay (see below), cyclic serum elevation of hepatic transaminases (2 to 10 times normal), and characteristic histologic findings on liver biopsy. Patients were seen during routine clinic visits. Where indicated, patients receiving therapy for chronic hepatitis secondary to HCV received 3 × 10^6 U of interferon-α2b subcutaneously three times weekly. Samples obtained for routine clinical evaluation included qualitative and quantitative plasma HCV RNA determination (Ampli
cor; Roche Molecular Systems, Branchburg, NJ; performed commercially by OncQuest Speciality Laboratories, Santa Monica, CA). Samples were prepared and treated as recommended by Onc--Quest prior to and during shipment.

Preparation of blood and blood cell fractions. WB was collected into acid-citrate-dextrose Vacutainer tubes (Becton Dickinson, Research Triangle Park, NC) and maintained at 2–4°C, and plasma or blood cell fractions were prepared within 2 h of collection. A complete blood count with white blood cell differential was obtained and used to determine the relative proportions of plasma or blood cells present in each aliquot of WB at the time of assay. Plasma was prepared (800 g for 10 min), and PBMC were prepared by ficoll-hypaque gradient centrifugation [19]. PBMC were washed three times with ice-cold Hanks’ balanced salt solution (HBSS) and, where indicated, PBMC were fractionated further on affinity columns (Biotex Laboratories, Edmonton, Canada) to prepare enriched fractions of CD4 or CD8 cells by use of the protocol supplied by the manufacturer [21]. Purity of the CD4 and CD8 cells was assessed with flow cytometry (University of Iowa Flow Cytometry Facility). Enrichment of >90% (CD4 cells) and 62% (CD8 cells) was obtained. Monocytes were prepared by overnight attachment of PBMC to tissue culture plates [22]. Highly purified neutrophils (>95%) [23] were prepared by ficoll/hypaque centrifugation, dextrose sedimentation, and hypotonic lysis of RBC. Purified neutrophils were washed three times with ice-cold excess HBSS before RNA preparation. RBC/platelet fractions were recovered from the ficoll-hypaque pellets after separation of leukocytes. Cells were counted, and aliquots of washed, purified neutrophil or RBC/platelet fractions were added directly to 1.0 mL of Catrimox in amounts adjusted to equal the number of cells found in 200 μL of WB. Control experiments verified that aliquots of the third wash buffer were free of HCV RNA sequences.

RNA preparation and RT-PCR. RNA was purified from WB, plasma, or blood cellular fractions following precipitation with Catrimox-14 as previously described [19]. Isolated plasma or purified fractions of blood cells were added directly to 1.0 mL of Catrimox in amounts adjusted to equal the respective amount found in 200 μL of WB. Where indicated, the quantity of isolated total blood RNA was determined by measurement of absorbance at 260 nm after resuspension of RNA in molecular biology-grade 5 M urea.

Primers (purchased from the University of Iowa College of Medicine DNA Core Facility) were prepared from known sequences of the highly conserved 5’-nontranslated region of HCV, and nested RT-PCR was performed as described previously [19]. Specific DNA products (250 bp) were analyzed on 1.6% agarose gels after staining with ethidium bromide. To control for known variations in PCR efficiency [24–26], rigorous standardization of the reaction was used. A standard mixture of all PCR reagents except input RNA was prepared and used to study samples obtained from individual patients. Thermocycling parameters were identical for all samples and as previously described [19]. All samples from each patient were processed together (RNA purification and limiting dilution) to minimize the effects of solvent extraction as well as technician manipulation. Replicates of RNA samples showed a coefficient of variation of <10% for mean log end point dilution titer.

RNA prepared from WB, plasma, or isolated blood cell fractions was studied. Serial dilutions of RNA were prepared in 1:2-, 1:50-, or 1:10-fold increments and assayed with nested PCR together with no-template (water) negative controls and known HCV RNA–negative patient RNA controls to assess potential contamination. If any blank value or known control was positive, the experiment was repeated; however, this rarely occurred, since the recommendations of Kwok and Higuchi [27] were rigidly followed. The highest dilution of RNA producing detectable specific product was determined and was considered to be the dilution end point [24, 25].

Results

Limiting dilution experiments have previously been evaluated for their ability to assess relative concentrations of HCV RNA in patient serum or isolated PBMC [28–32]. This methodology has been shown to be comparable to commercial assays when the RNA concentration tested is within the range that generates a linear concentration of PCR amplification products [28]. To ensure that the concentration of RNA tested in our RT-PCR assays could be accurately compared, we analyzed WB RNA obtained from various volumes of blood from 2 different HCV RNA–positive patients. The WB HCV RNA end point dilution titers (which represent the inverse of the absolute viral RNA titer) were linear with respect to the volume of WB used to prepare the RNA from each donor (r = .985, figure 1A). Furthermore, the yield of RNA from WB prepared by the Catrimox procedure was linear throughout the assay range (r = .987, figure 1B). Consequently, our method provided an accurate relative measure of input RNA, based on the volume of blood used to prepare RNA in this study.

To estimate the sensitivity of our WB assay, we determined the HCV RNA limiting dilution end point in 2 independent samples of WB RNA and tested the concentration of the same RNA preparation with a commercial test (Ampli
cor). The limit of detection for the WB and plasma RT-PCR method was ~240 genome equivalents/mL of WB or plasma (data not shown), which correlates with the limit of detection of the qualitative commercial test (200 copies/mL). The fact that WB RNA was able to generate HCV PCR products when the commercial assay was used provided additional assurance of the quality of template RNA prepared by the Catrimox procedure on WB.

To determine the relative concentration of HCV RNA in WB, plasma, PBMC, neutrophils, and RBC/platelets, limiting dilution experiments were used on RNA samples prepared from blood obtained from 10 patients with chronic HCV infection. Figure 2 demonstrates results obtained from 2 of the patients. The titer of HCV RNA sequences in 50 μL of WB was 10^6
Figure 1. Relationship between volume of RNA from whole blood (WB) and HCV RNA titers (A) and total RNA concentration (B). A. RNA prepared from various volumes of WB (10–250 μL) from 2 patients was assayed for HCV RNA by end point limiting dilution. Highest dilution of RNA producing detectable product was determined to nearest one-half dilution. Linear correlation coefficients were 0.985 for patient 1 (●) and 0.987 for patient 2 (△). B. Concentration of total RNA prepared from various amounts of WB using Catrimox procedure was determined by measurement of optical density at 260 nm. Each point represents mean of duplicate determinations from 2 separate RNA isolations. Linear correlation coefficient was 0.989.

for patient A and 10^5 for patient B. In contrast, the equivalent volume of plasma showed titers of 10^3 in both patients, and lower titers were observed for PBMC, neutrophil, and RBC/platelet preparations. HCV sequences were not seen in the neutrophil preparation from patient B.

Cumulative data for all 10 patients are shown in figure 3. The titer of HCV RNA varied among individual WB samples (10^4 to 10^8, P < .001), and the HCV RNA titer in WB ranged from 10^- to 1000-fold higher than that in any of the corresponding blood fractions (P < .001). Individual sample equivalents of plasma, PBMC, neutrophil, and RBC/platelet fractions exhibited titers of 0 to 10^4, 0 to 10^5, 0 to 10^6, and 0 to 10^7, respectively. The data in figure 3 were analyzed with a completely randomized design (with unequal group sizes) for analysis of variance with Fisher’s least significant difference test to determine the significance between grouped fractions. Data analyzed as paired comparisons within patient groups by use of a t distribution showed the same level of significance. WB contained significantly more HCV RNA than plasma (P < .001), and plasma contained significantly more HCV RNA than PBMC (P < .005), which contained more HCV RNA than neutrophils (P < .001). Neutrophil fractions demonstrated

the most variability of HCV RNA detection, with 5 of 8 patients tested having no detectable HCV RNA.

Although the data in figure 3 depict relative HCV RNA titers in the respective purified fractions, some HCV RNA could have been lost during purification and washing of the WB cellular fractions. To determine if this was the case, we assessed the HCV RNA titers in WB, plasma, and the crude cellular pellet remaining after plasma preparation from 2 patients. The HCV RNA titers of plasma plus crude pellets were about equal to that of WB (data not shown). After two washes of the pellet with balanced salt solution, 10%–50% of the original titer of viral RNA was still associated with the pellet, and subsequent washes failed to remove additional HCV RNA from the cell pellet. These experiments suggest that the excess HCV RNA found in WB relative to plasma is either intracellular virus or virus adherent to cells and pelleted during plasma preparation (or both). Large complexes of virus and specific antibody may also remain insoluble and separate with the cellular pellet.

PBMC consist of monocytes, B lymphocytes, and a variety of T lymphocytes, including CD4 and CD8 cells. We evaluated monocyte, CD4 cell–enriched, and CD8 cell–enriched fractions from 2 patients for the presence of HCV RNA. HCV
this possibility, a constant amount of HCV RNA-positive plasma was added to various amounts of HCV RNA-negative WB or HCV RNA-negative plasma. After RNA extraction, the relative titers of HCV RNA were determined. Table 1 demonstrates that the same titer of HCV RNA was detected regardless of the volume of HCV RNA-negative WB or plasma added to the HCV RNA-positive plasma. This titer was the same as that determined in the HCV RNA-positive plasma without HCV RNA-negative WB or plasma added. This demonstrates that WB does not enhance recovery of HCV RNA present in plasma. Similarly, no decrease in titer was found when HCV RNA-positive plasma was added to HCV RNA-negative plasma, excluding an inhibitory effect of plasma on RNA extraction or subsequent RT-PCR.

To compare the sensitivity of Catrimox WB and plasma HCV RNA assays with commercial quantitative and qualitative HCV RNA detection systems, we selected patients with chronic hepatitis C who were receiving standard dosages of interferon therapy ($3 \times 10^6$ U subcutaneously three times weekly for 6 months). All patients were HCV RNA-positive before interferon treatment. Blood and plasma samples were obtained for Catrimox RNA extraction during the same blood sampling for which the commercial HCV RNA assay was obtained. Patients with a positive commercial quantitative HCV RNA test, patients with negative (nondetectable) quantitative HCV RNA, and patients with a prior negative quantitative test who were further assessed with the commercial qualitative HCV RNA assay were studied (table 2). All Catrimox WB and plasma samples were positive if the commercial quantitative test was positive (group I, table 2); however, 11 (69%) of 16 patients who were negative by plasma commercial quantitative HCV RT-PCR, suggesting increased sensitivity of our plasma HCV RT-PCR assay compared with commercial quantitative HCV RNA assay; however, this was not highly significant ($P < .05$). Catrimox WB HCV RT-PCR testing were positive by Catrimox WB HCV RT-PCR (group II, table 2; $P < .005$, WB vs. commercial quantitative assay). Five of these patients were positive by Catrimox plasma HCV RT-PCR, suggesting increased sensitivity of our plasma HCV RT-PCR assay compared with commercial quantitative HCV RNA assay; however, this was not highly significant ($P < .05$).

Finally, for 19 patients who had a negative commercial quantitative HCV RNA result, a commercial qualitative HCV RNA result was obtained. Of these 19 patients, 13 (68%) were positive by Catrimox WB HCV RT-PCR but only 2 (11%) were positive by the commercial assay (group 3, table 2; $P < .005$, WB vs. qualitative commercial RT-PCR). Catrimox plasma HCV RT-PCR also detected HCV-positive patients (21%) not revealed by the qualitative assay in this group; however, the Catrimox plasma assay was not statistically different from the commercial qualitative assay. Group II and III patients (table 2) were receiving interferon therapy at the time of testing and thus had reduced concentrations of HCV RNA in plasma compared with their pretreatment studies.

**Discussion**

HCV is known to circulate in peripheral blood in a variety of forms. Virus that is presumably antibody-free (and highly

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**Figure 2.** Representative HCV RNA titers determined by RT-PCR. Whole blood (WB) was collected from 2 HCV RNA-positive patients (A and B). Individual blood fractions were adjusted so that amounts tested were equal to the amount present in patient WB aliquot. RNA prepared by Catrimox procedure was serially diluted (10-fold) ($10^{-1}$ to $10^{-6}$ or $10^{-7}$), and HCV RNA was determined by reverse transcription–polymerase chain reaction. Specific product (250 bp) was identified on 1.6% agarose gels. Each dilution series represents titer of HCV RNA present in $50 \mu L$ of WB or equivalent amount of blood fraction.

RNA titers of at least $10^2$ were determined for each of these cell fractions, suggesting that HCV is present in multiple components of PBMC (data not shown). Since these WB samples contained an HCV RNA titer of $10^5$, the lymphocyte HCV RNA does not constitute a major fraction of the total circulating HCV in WB. Since pure CD4 and CD8 lymphocyte populations could not be obtained, the relative quantity of HCV RNA present between these cell types could not be determined.

An alternate explanation for the observed increased titer of HCV RNA in WB relative to plasma is that HCV RNA recovery may be enhanced during RNA extraction. This enhancement could be due to a “carrier” effect, in which total cellular RNA coextracted with the plasma HCV RNA increases the recovery of viral RNA. In addition, the excess cellular RNA may provide increased resistance to endonucleases. To examine...
Figure 3. Summary of HCV RNA titers in whole blood (WB) or isolated blood fractions. WB was collected from 10 HCV RNA-positive patients (A–J) and fractionated. Serial 1:5 or 1:10 dilutions of RNA were assayed for HCV RNA by nested reverse transcription–polymerase chain reaction. Each bar represents $-\log_{10}$ dilution yielding HCV RNA starting with 50 $\mu$L of WB, or adjusted equivalent amount of isolated blood fractions (PBMC, peripheral blood mononuclear cells; RBC/PLT, red blood cells/platelets). WB contained significantly more HCV RNA than plasma ($P < .001$), and plasma contained significantly more HCV RNA than PBMC ($P < .005$), which contained more HCV RNA than neutrophils ($P < .001$). $F$, none detected.

infectious) and HCV-immunoglobulin immune complexes have been recovered in serum and plasma [33, 34]. Viral RNA has also been identified in PBMC [12–14], bound in large complexes of host antibody and rheumatoid factor in the form of cryoprecipitates [15–18], and may also exist engulfed in phagocytes. Studies evaluating the relative distribution of HCV RNA within the various compartments of blood have not been reported, although identification of HCV RNA sequences in these isolated blood fractions may be important in understanding the natural history, response to treatment, and pathogenesis of HCV chronic infection.

Our findings provide a relative quantification of HCV RNA in plasma and the cellular compartments of peripheral WB. The relative titer of HCV RNA was highest in unfractionated WB, with plasma containing the highest concentration of HCV RNA among the individual blood compartments. Analysis of the different blood cell types demonstrated that PBMC have significantly higher RNA titers than do neutrophil or RBC/platelet fractions and that among the cell types of PBMC, monocytes and CD4 and CD8 lymphocytes all contained HCV RNA. To our knowledge, HCV RNA has not been previously reported in purified preparations of neutrophils, yet we found low titers in 3 of 8 patients evaluated. Nevertheless, neutrophils contained an insignificant titer of HCV RNA compared with the HCV RNA titer present in WB. As might be expected, the RBC/platelet fraction also showed low or undetectable titers of viral RNA, and HCV RNA partitioning with these cells has not been previously reported.

HCV RNA may partition with peripheral blood cells by three mechanisms. The virus may simply be ingested by phagocytosis in the case of neutrophils, or HCV may be present because the

<table>
<thead>
<tr>
<th>HCV-positive plasma ($\mu$L)</th>
<th>HCV-negative whole blood ($\mu$L)</th>
<th>HCV-negative plasma ($\mu$L)</th>
<th>HCV RNA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Patient 1</td>
</tr>
<tr>
<td>50</td>
<td>200</td>
<td>0</td>
<td>$1.0 \times 10^4$</td>
</tr>
<tr>
<td>50</td>
<td>100</td>
<td>0</td>
<td>$1.0 \times 10^4$</td>
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<td>10</td>
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<td>ND</td>
</tr>
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<tr>
<td>50</td>
<td>0</td>
<td>0</td>
<td>$1.0 \times 10^4$</td>
</tr>
</tbody>
</table>

NOTE. ND = not determined.
particular cell is permissive for HCV replication. Alternatively, virus or virus-protein complexes could adhere to specific receptors on the cell surface and be pelleted during preparation of plasma. For example, virus-immunoglobulin complexes could bind Fc receptors, or virus-lipoprotein complexes could bind to specific lipoprotein receptors on circulating cells. HCV-immunoglobulin complexes, β-lipoprotein binding of HCV, and Fc binding of HCV have been previously described [33–35]. The finding of a relatively high titer of HCV RNA in PBMC is consistent with data implicating lymphocytes as a major site of extrahepatic replication of the virus. Replicative intermediates have been demonstrated in PBMC in some studies [12, 13, 36, 37] but not in others [38], and successful in vitro infection and extended passage of virus has been reported in human lymphoblastoid B and T cell lines [39, 40]. Furthermore, B lymphocytes have been proposed to be key target cells in the formation of rheumatoid factor and progression of cryoglobulinemia [15, 16]. In contrast, the low but significant titers of HCV RNA observed in some neutrophil preparations may reflect only the phagocytic function of these cells. Most, if not all, of the excess HCV RNA present in WB (compared with plasma) was accounted for by combining the HCV RNA present in plasma with the RNA found in the cellular pellet and cell washes following plasma separation. This indicates that the excess HCV RNA found in WB is likely intracellular virus, virus adhering to blood cells, or large virus-specific antibody complexes that pellet during plasma preparation (centrifugation).

Regardless of the reasons for the increased recovery of HCV RNA in WB, our studies document that WB contains significantly more HCV RNA than do plasma and individual cellular components of blood. Consequently, quantitative measurements of plasma (or serum) do not provide a complete estimate of the circulating peripheral blood HCV burden. Although quantitative serum levels of HCV RNA are commonly used to monitor the response of chronic HCV infection to interferon therapy, disappearance of HCV RNA from patient serum may not be a reliable predictor of sustained response [41–44]. This may be due to HCV contained in the peripheral blood cells or simply to the failure of current commercial plasma (or serum) assays to detect HCV RNA still present in peripheral blood (table 2).

In summary, our data demonstrate that HCV RNA can be detected throughout various fractions of peripheral blood and that WB RNA is the most sensitive source of template for HCV RNA amplification. In addition, we previously demonstrated that the HCV RNA titer in patient WB samples mixed with Catrimox does not decrease when maintained at room temperature for up to 7 days [19]. Consequently, this method offers a practical clinical advantage over current plasma HCV RNA detection systems. The present findings demonstrate the need to evaluate WB for HCV RNA in persons with unexplained chronic liver disease and suggest that epidemiologic studies of persons with normal and elevated alanine aminotransferase levels should include WB HCV RNA detection. These studies are currently underway in our laboratory. In addition, we are evaluating the prognostic benefit of measuring WB HCV RNA levels in patients receiving interferon therapy.

### Acknowledgments

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### References


### Table 2. Comparison of commercial quantitative and qualitative HCV RNA assays with Catrimox reverse transcription–polymerase chain reaction (RT-PCR).

<table>
<thead>
<tr>
<th>Group</th>
<th>Commercial assay</th>
<th>n</th>
<th>WB</th>
<th>Plasma</th>
<th>Plasma, plasma assay obtained</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>Quantitative (+)</td>
<td>38</td>
<td>38 (100)</td>
<td>38 (100)</td>
<td>ND</td>
</tr>
<tr>
<td>II</td>
<td>Quantitative (−)</td>
<td>16</td>
<td>11 (69)</td>
<td>5 (31)</td>
<td>ND</td>
</tr>
<tr>
<td>III</td>
<td>Quantitative (−) with qualitative assay obtained</td>
<td>19</td>
<td>13 (68)</td>
<td>4 (21)</td>
<td>2 (11)</td>
</tr>
</tbody>
</table>

NOTE: Patients being treated with interferon-α2b for chronic hepatitis C were followed with commercial quantitative plasma HCV RNA assay (Amplicor) and Catrimox whole blood (WB) and plasma RT-PCR assays for HCV RNA. When quantitative commercial Amplicor became negative, a follow-up qualitative commercial RT-PCR was done. ND = not determined.

* Catrimox assay with WB or plasma was performed on aliquots collected at same time as those sent for commercial assay. Results represent no. positive (%).

† Catrimox WB assay > commercial quantitative assay (P < .005), Catrimox plasma assay > commercial qualitative assay (P < .05) and WB > Catrimox plasma assay (P < .05) by χ² analysis.

‡ Catrimox WB assay > commercial qualitative RT-PCR (P < .005). Catrimox plasma assay was not significantly different from commercial qualitative RT-PCR by χ² analysis.