Hepatitis C Virus Infection in Hemodialysis Patients: Comparison of Two New Hepatitis C Antibody Assays With a Second-Generation Assay¹,²

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ABSTRACT
The performance of two new hepatitis C virus antibody (anti-HCV) assays (a third-generation immunoglobulin (Ig)G recombinant immunoblot assay (RIBA 3.0) and hepatitis C virus core IgM (HCV IgM)) in the prediction of hepatitis C viremia in hemodialysis patients was compared with that of a second-generation IgG recombinant immunoblot assay (RIBA 2.0). Forty-three patients on maintenance hemodialysis were studied. Aliquots of sera were tested prospectively for anti-HCV by RIBA 2.0, RIBA 3.0, and HCV IgM and for HCV RNA by polymerase chain reaction. Thirty-eight patients were HCV RNA positive. Among those, 7 (18%) were HCV IgM positive, 22 (58%) were RIBA 2.0 positive, and 29 (76%) were RIBA 3.0 positive. All but one viremic patients detected by HCV IgM were also detected by RIBA 2.0 and RIBA 3.0. All viremic patients detected by RIBA 2.0 were also detected by RIBA 3.0. RIBA 3.0 was more sensitive than RIBA 2.0 and HCV IgM in the detection of viremic patients (P = 0.0156 and < 0.0001, respectively). The positive predictive value for HCV IgM was 100% as compared with 96 and 97% for RIBA 2.0 and RIBA 3.0, respectively. The negative predictive value for RIBA 3.0 was 36% as compared with 24 and 14% for RIBA 2.0 and HCV IgM, respectively. At 6-months follow-up of the eight viremic patients without a detectable IgM or IgG anti-HCV response, all patients remained RIBA 2.0 nonreactive, one became RIBA 3.0 indeterminate, and one became HCV IgM positive. These data suggest that HCV IgM has poor sensitivity in the detection of hepatitis C viremia and RIBA 3.0 improves the sensitivity of IgG anti-HCV assays in the early detection of hepatitis C viremia in hemodialysis patients.

Key Words: Hepatitis C, hemodialysis, immunoglobulin M, hepatitis C virus antibody, recombinant immunoblot assay, hepatitis C virus RNA

Since the cloning of hepatitis C virus (HCV) genome in 1989 (1), serologic assays have been developed in which recombinant proteins (antigens) of the viral genome are used to detect reactive antibodies (anti-HCV) of immunoglobulin (Ig)G or IgM class, thus indicating exposure of the individual to HCV (2). HCV antibody assays of IgG class (IgG anti-HCV assays) have evolved over the past few years (first and second generations) in an effort to improve the sensitivity and specificity of anti-HCV detection (3). A second-generation IgG anti-HCV assay by enzyme-linked immunosorbent assay (ELISA 2.0) is now widely used as a screening test for HCV infection, and a second-generation IgG recombinant immunoblot assay (RIBA 2.0) is used as a supplementary assay for ELISA-positive sera (4,5).

Hemodialysis patients are recognized as a major risk group for acquiring HCV infection. A plethora of publications in the dialysis literature has amply demonstrated the high prevalence of IgG anti-HCV in this patient population. In those studies, however, second-generation assays (ELISA 2.0 and RIBA 2.0) were found to underestimate the prevalence of active viral replication (HCV viremia) (6,7). Furthermore, it was soon discovered that the absence of IgG anti-HCV by these assays cannot accurately predict the absence of hepatitis C viremia in hemodialysis patients. Indeed, 2.5 to 12% of anti-HCV-negative dialysis patients test positive for HCV RNA and only 83% of HCV RNA-positive cases have circulating anti-HCV (8,9). One possible explanation is that the currently available IgG anti-HCV assays might not be sensitive enough, either because of the low titer of the antibody or because the antigen used in the assay cannot detect the antibodies (antigen limitations) (10). Alternatively, the patient might be in the "window phase," an early stage in HCV infection when HCV RNA circulates and can be detected by polymerase chain reaction (PCR), but the antibody response is not yet manifest (10).

A third-generation RIBA (IgG anti-HCV RIBA 3.0) was recently introduced. It uses the three antigenic determinants of RIBA 2.0 (c33, c22, c100) plus a new recombinant antigen (NS-5). The coating concentrations of these antigens have been optimized to improve their "capture sensitivity" to reactive antibodies. By

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optimizing the antigen concentrations of RIBA 2.0 and adding a new antigen (NS-5), RIBA 3.0 can potentially improve the sensitivity of IgG anti-HCV detection.

In viral infections, the development of IgM antibodies usually precedes the development of IgG antibodies. Moderately high levels of IgM anti-hepatitis B core are detected in individuals with persistent hepatitis B virus infection (11). IgM anti-hepatitis B core might be the only serologic marker of ongoing infection during the "window phase" of hepatitis B virus infection (12). Similarly, studies in nondialysis patients with acute HCV hepatitis have shown the presence of IgM anti-HCV in 86 to 92% of patients (13,14). Other studies showed a correlation between HCV IgM and serum HCV RNA (indicative of HCV viremia) (15–17). This suggests that HCV IgM might improve the detection of anti-HCV during the "window or the chronic phase" of HCV infection. This investigation was undertaken to compare the performance of RIBA 3.0 and HCV IgM with that of RIBA 2.0 in the prediction of HCV viremia as determined by reverse transcriptase-PCR (RT-PCR) in hemodialysis patients.

PATIENTS AND METHODS

Patients

Patients with ESRD who had been on maintenance hemodialysis for at least 1 yr were enrolled in the study if they met the following criteria: (1) repeatedly positive anti-HCV by ELISA 2.0 and/or history of prior (>6 months) blood transfusions; and (2) negative hepatitis B surface antigen and human immunodeficiency virus reactions. A cohort of 43 patients from four dialysis units in Saudi Arabia were studied. All patients received standard hemodialysis three times weekly with high-flux or conventional membranes. Bicarbonate or acetate dialysate was used, and artificial kidneys were not reused. None of the subjects reported a history of iv drug abuse, homosexuality, or sexual promiscuity. There were 18 men and 25 women in the study population. The mean age was 38 yr (range, 16 to 64), the mean duration of hemodialysis was 55 months (range, 12 to 180), and 89% had received blood transfusions. After obtaining informed consent, blood samples were drawn; serum samples were extracted and divided into aliquots within 8 h of collection. Three aliquots were submitted to two independent laboratories (Blood Bank and Virology Section) for assay by RIBA 2.0 and RIBA 3.0 (supplied by Ortho Diagnostic Systems and Chiron Corporation, Emeryville, CA) and HCV IgM (supplied by Abbott Diagnostics Division, Wiesbaden, Germany), respectively. In these laboratories, serum samples were stored at 2 to 8°C and the tests were performed within 1 wk. A fourth aliquot was submitted to the Biological and Medical Research Department of King Faisal Specialist Hospital and Research Centre for the measurement of HCV RNA by PCR. In this laboratory, serum samples were stored at −70°C and the test was performed within 1 month. Serum samples were coded so that the staff in each laboratory were unaware of the results obtained by other staff in the same or other laboratories.

Anti-HCV Tests

The RIBA 2.0 and RIBA 3.0 were carried out according to the manufacturer's instructions. In brief, the reactivity of the specimens to each antigen was graded according to a 5-point scale (0 = no reactivity, 4+ = highest reactivity) by visual comparison of the intensity of the individual antigen band with that of the low-IgG and high-IgG internal-control bands located on each strip. A serum sample with a score of 1+ or higher in response to any two or more antigens was classified as active. A serum sample with a score of 1+ or higher in response to a single antigen was classified as preliminary indeterminate. Preliminary indeterminate samples were tested at least twice and were rated as indeterminate if they continued to show the same reaction. If the reaction changed to more than one antigen, they were rated as reactive, and if it changed to no antigen reaction, they were rated as nonreactive.

An HCV IgM enzyme immunosorbent assay was performed according to the manufacturer's instructions. Diluted patient serum was incubated with a polystyrene bead coated with recombinant HCV structural (core) antigen. The diluent contained anti-human IgG to minimize interference with anti-HCV IgG and rheumatoid factor. IgM anti-HCV (which binds to the bead) was then detected by the addition of a conjugate (anti-human antibody labeled with horseradish peroxidase). With the addition of O-phenylenediamine, the enzyme caused a color reaction proportional to the amount of IgM anti-HCV. Positive and negative controls were within acceptable limits (EIA Kit, Abbott Diagnostics Division, Wiesbaden, Germany). The results were reported as reactive or nonreactive.

RT-PCR

Serum proteins were removed by two extractions with phenol/chloroform and one with chloroform alone. Twenty micrograms of glycogen was added to the aqueous phase, and the RNA was precipitated by the addition of 2.5 vol of ice-cold absolute ethanol. After being stored at −70°C for 1 h, the RNA was pelleted in an Eppendorf centrifuge (Fisher Scientific, Springfield, NJ) (15 min at 15,800 g and 4°C). The pellet was washed once in 70% ethanol, vacuum dried, and then dissolved in 10 μL of sterile distilled water. Solutions of RNA were stored at −70°C.

Reverse transcription was performed with cloned Moloney murine leukemia virus RT (Pharmacia, Piscataway, NJ), and two-round PCR were done with native Tag DNA polymerase (Perkin Elmer Cetus, Norwalk, CT). The synthesized nested primers were based on the highly conserved (99%) 5' noncoding region of HCV genome (18). The sequences of the outer primers PT1 (sense) and PT2 (antisense) and the inner primers PT3 (sense) and PT4 (antisense) were as follows:

PT1 = 5'-CGT TAG TAT GAG TGT CGT GC-3' (90–109)
PT2 = 5'-CGG TGT ACT CAC CGG TTC C-3' (171–153)
PT3 = 5'-AGT GTG GTG CAG CCT CCA GG-3' (100–119)
PT4 = 5'-CGG TTC CGC AGA CCA CTA TG-3' (159–140)

Samples containing 10-μL samples (10 μL) of round two PCR products were analyzed by electrophoresis on 3% Nu-Sieve/1% Seakem agarose composite gel (FMC-Bioproducts, Rockland, ME). Bands were visualized by staining with ethidium bromide (1 μg/mL) and were photographed under UV light. The results were classified as positive (HCV RNA detected) or negative (HCV RNA not detected). The recommendations of Kwok and Higuchi (19) were strictly followed to avoid any contamination or false-positive results. Specificity was confirmed by the inclusion of negative controls,
which included buffer and sera from uninfected human subjects (Figure 1).

STATISTICAL ANALYSIS

Sensitivity, specificity, and positive and negative predictive values were calculated as follows: sensitivity = true positive (TP)/(TP + false negative [FN]); specificity = true negative (TN)/(TN + false positive [FP]); positive predictive value = TP/(TP + FP); negative predictive value = TN/(TN + FN). McNemar’s test was used to analyze the relative sensitivity merits of the three anti-HCV assays to predict HCV viremia and to compare the reactivity patterns of the three common antigens of RIBA 2.0 and RIBA 3.0 in viremic patients. In the final analysis, RIBA-indeterminate results were grouped with those of RIBA-nonreactive results.

RESULTS

At entry, 26 (60%) of 43 patients were ELISA 2.0 positive repeatedly. Thirty-eight of 43 patients tested positive for HCV RNA by PCR (Figure 1). Of those, 7 (18%) were HCV IgM positive, 23 (58%) were RIBA 2.0 positive, and 29 (76%) were RIBA 3.0 positive. RIBA 3.0 was found to be more sensitive than RIBA 2.0 and HCV IgM in the detection of HCV viremia (P = 0.0156, and <0.0001, respectively) (Table I). RIBA 2.0 was also more sensitive than HCV IgM (P = 0.0005). Of the seven viremic patients who were detected by HCV IgM, six were also detected by RIBA 3.0; the seventh was not detected by RIBA 3.0, ELISA 2.0, or RIBA 2.0. All HCV RNA–positive patients detected by RIBA 2.0 were also detected by RIBA 3.0 and ELISA 2.0. All HCV RNA–positive patients detected by ELISA 2.0 were also detected by RIBA 3.0. In addition, 4 of the 12 HCV RNA–positive patients not detected by ELISA 2.0 and 7 of the 16 HCV RNA–positive patients not detected by RIBA 3.0 were RIBA 2.0 positive (Table 1). In HCV RNA–positive patients, the reactive patterns of the three common antigen components of RIBA 3.0 (c33, c22, c100) were compared in each patient with those of RIBA 2.0. RIBA 3.0 detected new anti-HCV to c33 in seven patients (P = 0.0082), to c22 in five (P = 0.0253), and to c100 in nine (P = 0.0016) when compared with RIBA 2.0 (Table 2). Among the HCV RNA–positive patients, three (8%) had indeterminate results by RIBA 2.0 (all were positive by RIBA 3.0) and one (3%) had an indeterminate result by RIBA 3.0. Among the RIBA 3.0–positive patients, the new antigen (NS-5) was reactive in 13 (45%). It did not contribute to the enhanced sensitivity of this assay, however, when compared with the reactions of the other antigens on

Figure 1. Detection of HCV RNA in sera of hemodialysis patients. Agarose gel electrophoresis of PCR products after RT and amplification of cDNA. Lane 1 (M), φx 174 HaeIII molecular-weight markers. Lane 2 (P1), plasmid positive control containing cloned HCV genome. Lane 3 (P2), positive sample control. Lane 4, blank. Lanes 5 to 11, patient samples. Lane 12 (N), negative sample control. bp, base pairs.
TABLE 1. Results of HCV IgM second- and third-generation immunoblot assays according to PCR results

<table>
<thead>
<tr>
<th>PCR</th>
<th>HCV IgM</th>
<th>RIBA 2.0</th>
<th>RIBA 3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(+)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>Positive (N = 38)</td>
<td>7 (18%) (^a)</td>
<td>31</td>
<td>22 (58%) (^a)</td>
</tr>
<tr>
<td>Negative (N = 5)</td>
<td>0</td>
<td>5 (100)(^b)</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^a\) Sensitivity.  
\(^b\) Specificity.

TABLE 2. Comparison of the reactivity patterns of the three common antigen components of the recombinant immunoblot assays (RIBA 2.0 and 3.0) in viremic patients

<table>
<thead>
<tr>
<th>The Reaction of RIBA 3.0 Compared With RIBA 2.0</th>
<th>Antigens</th>
<th>RIBA 2.0</th>
<th>RIBA 3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c33</td>
<td>c22</td>
<td>c100</td>
</tr>
<tr>
<td>New Detection</td>
<td>(N = 38)</td>
<td>(N = 38)</td>
<td>(N = 38)</td>
</tr>
<tr>
<td>Concordant Positive</td>
<td>7(^a)</td>
<td>5(^a)</td>
<td>9(^a)</td>
</tr>
<tr>
<td>Result With RIBA 2.0</td>
<td>22</td>
<td>22</td>
<td>17</td>
</tr>
<tr>
<td>Concordant Negative Result With RIBA 2.0</td>
<td>9</td>
<td>11</td>
<td>12</td>
</tr>
</tbody>
</table>

\(^a\) P < 0.05.

RIBA 3.0 (all NS-5 reactive samples were also reactive to at least two other antigens on RIBA 3.0).

Among the 23 RIBA 3.0-positive and HCV IgM-negative patients, HCV IgM was detected in 19 after 6 months' follow-up (the remaining 4 patients were transplanted). HCV IgM remained negative in all but one patient at follow-up. ELISA 2.0, RIBA 2.0, RIBA 3.0, and HCV IgM were detected in the eight viremic patients who tested negative for anti-HCV by the four assays. All remained negative by ELISA 2.0 and RIBA 2.0, one became indeterminate by RIBA 3.0 (c100), and another became positive by HCV IgM.

Of the five HCV RNA-negative patients, one was anti-HCV positive by ELISA, RIBA 2.0, and RIBA 3.0, but negative by HCV IgM. The predictive value of a positive HCV IgM for the presence of viremia was 100%, compared with 96 and 97% for RIBA 2.0 and RIBA 3.0, respectively. The predictive value of a negative HCV IgM for the absence of viremia was 14% compared with 24 and 36% for RIBA 2.0 and RIBA 3.0, respectively.

**Discussion**

The prevalence of HCV RNA (88%) and anti-HCV by RIBA 2.0 and 3.0 (56 and 70%, respectively) in our cohort was higher than the average rate reported so far in hemodialysis patients (20–25). This is because, at least in part, of our inclusion criteria, by which IgG anti-HCV-positive patients by ELISA 2.0 and/or patients with two known risk factors for acquiring the infection (longer duration of hemodialysis and remote history of blood transfusion) were included.

In this study, we attempted to address the issue of “antigen limitations” in the currently available IgG anti-HCV assays and the “window phase” of the infection by introducing RIBA 3.0 and HCV IgM, respectively, as screening tests. Our data showed that the sensitivity of IgG anti-HCV detection is increased by RIBA 3.0, compared with RIBA 2.0 (see Table 1). This enhanced sensitivity appears to be the result of the better "capture sensitivity" of the three common antigens (c33, c22, c100) to reactive antibody and not the result of the addition of the new antigen (NS-5) (Table 2).

HCV IgM, on the other hand, performed poorly in the detection of viremic patients. Despite the high prevalence of viremia in our cohort, HCV IgM failed to detect 31 (86%) of 38 patients. Furthermore, at 6-months follow-up of the 19 viremic patients who were RIBA 3.0 positive and HCV IgM negative, all but 1 remained seronegative for HCV IgM. Contrary to other reports in predominantly nondialysis patients, we did not find HCV IgM to be a good marker of viral replication in either the initial (window) or the chronic phase of the infection.

All three anti-HCV assays performed well in predicting the presence of viremia (positive predictive values of 96, 97, and 100% for RIBA 2.0, RIBA 3.0, and HCV IgM, respectively) in agreement with previous reports (6,7). Therefore, when anti-HCV is detected by these assays, it indicates active viral replication. However, the prediction of the absence of viremia by these assays remained relatively poor, although improved by RIBA 3.0 (negative predictive values of 36, 24, and 14% for RIBA 3.0, RIBA 2.0, and HCV IgM, respectively). Nine (24%) of the viremic patients were seronegative by RIBA 3.0, and, eight (21%) were seronegative by RIBA 3.0 and HCV IgM combined (Table 1).

Studies in chimpanzees and immunocompetent patients with acute HCV infection have shown that HCV RNA can be detected in the first week of infection and that antibodies to c22 and c33 can usually be detected within 3 months of infection (26–31). We found that a proportion of hepatitis C viremic hemodialysis patients did not develop an IgG or IgM anti-HCV response at 6-months follow-up. This is consistent with the observations reported by Lok et al. (31), in which
hemodialysis patients were found to have a significant delay (>10 months) between the detection of HCV RNA and the development of an anti-HCV response. Our data support the notion that hemodialysis patients might have an impaired humoral immune response to viral infections. Both clinical evidence and experimental evidence strongly suggest that the humoral immune response of hemodialysis patients to a variety of antigens is less than that in nonuremic subjects (32,33). For example, studies of hepatitis B vaccination in hemodialysis patients have shown that the vaccine has to be given in more potent forms and in more frequent or additional doses in order to achieve the desired antibody response, which may not be sustained (34,35). We speculate that the impaired humoral immune response of hemodialysis patients might result in a diminished, delayed, or unsustained anti-HCV response to HCV infection.

Determining the presence or absence of HCV viremia in hemodialysis patients might be important for infection control–related issues (36) and for therapeutic decisions (the use of antiviral therapy) (37). Our data serve to emphasize the fact that, despite the better sensitivity of the new IgG anti-HCV (RIBA 3.0), we are still underestimating the true prevalence of HCV viremia in hemodialysis patients. A recent multiantigen enzyme immunoassay described by Lok et al. (31) has been reported to improve the detection of HCV infection in immunosuppressed patients (including hemodialysis patients). At present, RT-PCR must be considered the “gold standard.” Although it is currently difficult to justify its use in routine clinical practice (because it is costly and labor intensive and lacks standardization), its use as a diagnostic test appears to be justified in the hemodialysis population.

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