Chemiluminescence Assay Improves Specificity of Hepatitis C Antibody Detection

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Background: Antibodies to hepatitis C virus (anti-HCV) have typically been detected by enzyme immunoassay (EIA). A chemiluminescence assay (CA) for anti-HCV is now commercially available.

Methods: We compared the positive rate for a CA in a HCV screening program for veterans with historical rates obtained with EIA. We also compared results in 2824 samples tested by both methods and assessed the significance of low signal-to-cutoff (S/C) ratios.

Results: The frequency of CA-positive results was significantly lower than with EIA (12.6% vs 16.0%; P < 0.0001). The frequency of low S/C ratios was also significantly lower with CA (11.5% vs 20.0%; P < 0.0001). Among low-positive values, samples positive by CA were significantly less likely to be recombinant immunoblot assay (RIBA)-negative (64% vs 84%; P < 0.0005). In parallel testing, results for 111 samples (3.9%) were discrepant between the two assays; all but 6 had low S/C ratios, and confirmatory testing was performed on all but 8 samples. Of 56 EIA-positive, CA-negative samples tested by RIBA, only 1 was positive. Of 24 CA-positive, EIA-negative samples, 62% were RIBA-negative. Using a negative RIBA result as an indication of false-positive anti-HCV results, the positive predictive value of EIA-positive samples with a low signal-to-cutoff (S/C) ratio is usually found to be negative for anti-HCV antibodies by recombinant immunoblot assay (RIBA) (1–16). To minimize the likelihood of false-positive anti-HCV results, the CDC has recommended confirmation of all anti-HCV results by either RIBA or HCV RNA assays (17). We have recently shown that almost all false-positive anti-HCV results by EIA occur in samples with a S/C ratio <3.8 (18). Revised CDC guidelines recommend RIBA confirmation of EIA-positive samples with a S/C ratio <3.8 (19).

Recently, a chemiluminescence assay (CA) for anti-HCV has been developed (VITROS Eci anti-HCV assay; Ortho Clinical Diagnostics, Raritan, NJ) (20). In our initial evaluation of the method, we found that most of the samples that had low S/C ratios with EIA were negative by CA. To evaluate whether the newer assay performed better than the EIA methods, we compared the frequency of false-positive results (defined as positive for anti-HCV by the initial assay but RIBA-negative) between EIA and CA. We also compared the frequency of low- and high-positive results with historical rates in a screening program involving high-risk veterans.

Materials and Methods

Samples from a screening program for HCV among veterans in three Veterans Affairs Medical Centers (VAMCs) in the Washington–Baltimore metropolitan area and surrounding veterans centers are routinely tested at the VAMC in Washington, DC; details of this screening program have been
reported previously (18). The population screened includes predominantly (97%) males over age 40. In three cross-sectional random studies, ≈13–15% were anti-HCV-positive. From June 2000 to April 19, 2002, samples were tested with a third-generation EIA (Ortho Clinical Diagnostics) performed according to the manufacturer’s instructions on a Labotech automated microtiter plate handling system (Adaltis US Inc.). From April 22, 2002, until the end of the study period (October 21, 2002), samples were analyzed by a third-generation CA (VITROS Eci; Ortho Clinical Diagnostics) according to the manufacturer’s instructions. Results from both methods are interpreted as positive or negative based on the ratio of the signal in the sample relative to the cutoff value for the assay. The CV of the EIA method was 9.2% at a S/C ratio of 3.0, whereas that of the CA method was 6.3% at a S/C ratio of 6.3. Results of serologic testing are stored in a blinded database, along with results of supplemental testing; data for this study were extracted from that database, and the study design was approved by the Medical Center Institutional Review Board.

Additional testing was performed with a third-generation immunoblot assay (Chiron Corporation), quantitative HCV RNA by branched DNA analysis (Versant 3.0; Bayer Diagnostics), or an in-house qualitative HCV RNA assay with a lower detection limit of ≈100 copies/mL; details of these assays have been published previously (20). In the VA screening study, all but one sample with S/C ratios of 1.0–12.0 by CA were also tested by RIBA. Before August 3, 2002, HCV RNA was performed only when specifically ordered by a physician at two of the hospitals; at a third hospital, all samples positive for anti-HCV antibodies were tested with an in-house qualitative HCV RNA assay with a lower detection limit of 100 copies/mL, providing that adequate sample remained. A total of 63% of positive samples had HCV RNA testing performed. After August 3, 2002, all samples from all hospitals with a high S/C ratio or with a low S/C ratio and positive or indeterminate RIBA results were further tested by quantitative HCV RNA measurement (as long as an adequate sample volume was available); 96% of positive samples had HCV RNA testing performed.

Samples for direct comparison of the anti-HCV CA and EIA were derived from two sources. As part of its initial product evaluation, Ortho Clinical Diagnostics supported testing in four laboratories that tested 2623 samples from high-risk patients, using both a second-generation EIA (Abbott Laboratories) and the CA; details of this study have already been presented in abstract form (20). Data from this study were provided by Ortho and were analyzed for S/C ratio and correlation with results from a third-generation RIBA (Chiron Corporation). As part of the protocol, all of the samples positive for anti-HCV by CA were also tested with the RIBA; all but 11 of the samples positive by EIA but negative by CA were also tested with the RIBA. In the VAMC, 151 samples were analyzed in parallel, using the third-generation EIA test and CA. All but two of the samples with discrepant results were further tested by RIBA, qualitative HCV RNA, or both. In both the Ortho and VAMC studies, results were considered positive if equal to or above the cutoff value determined for each run; EIA-positive samples were confirmed by repeat testing in a second run and were considered positive if the results were above the cutoff for at least two of three measurements.

Differences between groups were evaluated with the χ2 test; results were considered significant at P < 0.05. Differences in means were evaluated by the two-tailed t-test for samples with unequal variances; differences were considered significant at P < 0.05.

Results

Comparison with Historical Control

There was a shift in the frequency of positive results with introduction of the CA (Table 1). Over the 6 months before introduction of the CA, 5346 samples were tested for anti-HCV by EIA; 855 (16.0%) were positive, including 684 with a S/C ratio ≥3.8 (high S/C ratio). In contrast, in the 6 months after introduction of the CA, 782 of 6222 samples tested were positive (12.5% positive; P < 0.0001 compared with EIA). Because one of the hospitals increased its screening rate in late March 2002, it is possible that the difference between the two assays was attributable to a decrease in the high- or total-positive rate as a result of increased screening of low-risk individuals. We excluded data from this site and reanalyzed the data; the high-positive rate was 13.0% for the EIA and 9.6% for the CA (P < 0.001). We therefore included data from all sites in the final analysis.

The distribution of anti-HCV S/C ratios obtained with the CA followed a bimodal distribution, as shown previously for EIAs (Fig. 1). In contrast to EIAs, however, the S/C ratios are much higher with the CA: the mean S/C ratio obtained with EIAs was 4.5, whereas the mean S/C ratio by CA was 34.4. Of the positive results, 171 of 855 (20.0%) EIA-positive samples were low-positive (S/C ratio <3.8), compared with 90 of 782 (11.5%) CA-positive samples (S/C ratio <8.0; P < 0.0001). During the period of the study, we used three different lots of CA reagent; the mean S/C ratio and the percentages of low-positive and total-positive results were not significantly different among the three lots (data not shown).

To date, HCV RNA testing has been completed in 539 of 713 consecutive veterans (76%) with positive anti-HCV by CA, excluding 69 individuals with negative RIBA results (Table 2). In those with low-positive EIA and positive or indeterminate RIBA results, 2 of 12 had positive HCV RNA results compared with 2 of 18 with low-positive CA and

| Table 1. Pattern of anti-HCV-positive results in VAMC screening program. |
|-----------------------------|-------------|--------------|
|                            | n   | High-positive, n (%) | Low-positive, n (%) |
| October 1, 2001–March 31, 2002 (EIA) | 5346 | 684 (12.8%) | 171 (3.2%) |
| April 22, 2002–October 21, 2002 (CA) | 6222 | 692 (11.1%) | 90 (1.4%) |
positive or indeterminate RIBA results. Of a total of 55 samples with S/C ratio 20, only 2 (4%) were HCV RNA-positive. HCV RNA was detected in 90.6% of EIA-positive samples with S/C ratios >3.8 and 90.3% of CA-positive samples with S/C ratios >20. In contrast, none of 25 CA-positive samples with S/C ratios between 8.1 and 20 were HCV RNA-positive (P <0.0001).

**Comparison with EIA results**

We compared the results obtained with CA and EIA in 2824 samples tested by both methods. The results were concordant in 2713 samples (1984 negative in both the EIA and CA, and 729 positive in both assays) and discordant in 111 samples (3.9%). Most of these samples (n = 86) were positive by EIA and negative by CA, whereas 25 were negative by EIA and positive by CA. To determine whether the EIA or CA results were more accurate, confirmatory tests were performed in all but 15 samples. In 56 EIA low-positive, CA-negative samples, 1 was RIBA-positive, 9 (16%) were RIBA-indeterminate, and 46 (82%) were RIBA-negative. An additional nine samples were analyzed for qualitative HCV RNA; all were negative. Of 24 samples that were low-positive by CA but negative by EIA, 15 (62%) were RIBA-negative, 6 (25%) were RIBA-indeterminate, and 3 (12%) were RIBA-positive. There were also nine samples high-positive by EIA but negative by CA; all seven tested were negative by RIBA. There were no samples that were high-positive by CA that were EIA-negative.

To determine whether there was a gradation in likelihood of positive results by RIBA in samples with low-positive S/C ratios, we compared the frequency of indeterminate and positive RIBA results at differing S/C ratios, using data from both the prospective and comparison studies. Among samples that were low-positive by EIA, we have previously shown that there is little difference in the likelihood of having positive or indeterminate RIBA results between those with the lowest and the highest S/C ratios (18). In contrast, among samples with low-positive CA results, there was a clear relationship between the S/C ratio and the likelihood of positive RIBA results, and only one sample with S/C ratio >10.0 was RIBA-negative (Table 3). The CDC has suggested selecting a S/C ratio cutoff that identifies 95% of results as RIBA-positive and below which 95% of samples are RIBA-indeterminate or -negative (19). At a CA S/C ratio cutoff of 8.0, 83 of 88 samples (94%) had negative RIBA results. In contrast, 90 of 95 samples (95%) with S/C ratios between 8.1 and 20.0 were RIBA-positive or -indeterminate, and 582 of 586 samples (99.3%) with S/C ratios >20 were RIBA-positive (the remaining 4 were RIBA-indeterminate). No samples that were high-positive by both assays were RIBA-negative. The pattern of RIBA-indeterminate reactivity in low-positive samples differed between the two assays (Table 4). In the CA, there was an increased frequency of antibodies to c22p and reduced frequency of antibodies to NS5 compared with the EIA.

Because RIBA is considered to have virtually 100% specificity for the presence of anti-HCV, we calculated the

**Table 2. HCV RNA results for samples with different S/C ratios obtained by CA.**

<table>
<thead>
<tr>
<th>S/C ratio</th>
<th>Positive, n (%)</th>
<th>Negative, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0–8.0 (n = 30)</td>
<td>2 (11%)</td>
<td>28 (89%)</td>
</tr>
<tr>
<td>8.1–20 (n = 25)</td>
<td>0 (0%)</td>
<td>25 (100%)</td>
</tr>
<tr>
<td>20.1–30 (n = 29)</td>
<td>17 (58%)</td>
<td>12 (42%)</td>
</tr>
<tr>
<td>30.1–40 (n = 172)</td>
<td>159 (92%)</td>
<td>13 (8%)</td>
</tr>
<tr>
<td>&gt;40 (n = 283)</td>
<td>261 (92%)</td>
<td>22 (8%)</td>
</tr>
</tbody>
</table>

**Table 3. RIBA results for CA low-positive samples.**

<table>
<thead>
<tr>
<th>S/C ratio</th>
<th>Negative, n (%)</th>
<th>Indeterminate, n (%)</th>
<th>Positive, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0–4.0 (n = 98)</td>
<td>69 (70%)</td>
<td>20 (20%)</td>
<td>9 (9%)</td>
</tr>
<tr>
<td>4.1–8.0 (n = 31)</td>
<td>14 (45%)</td>
<td>13 (42%)</td>
<td>4 (13%)</td>
</tr>
<tr>
<td>8.1–12.0 (n = 32)</td>
<td>5 (16%)</td>
<td>13 (41%)</td>
<td>14 (44%)</td>
</tr>
<tr>
<td>12.1–20.0 (n = 63)</td>
<td>0 (0%)</td>
<td>3 (5%)</td>
<td>60 (95%)</td>
</tr>
</tbody>
</table>

**Table 4. Comparison of RIBA reactivity in samples classified as low-positive by EIA and CA.**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>EIA-positive, RIBA-indeterminate (n = 31)</th>
<th>CA-positive, RIBA-indeterminate (n = 33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c22p</td>
<td>4 (13%)</td>
<td>15 (45%)</td>
</tr>
<tr>
<td>c33c</td>
<td>10 (32%)</td>
<td>9 (27%)</td>
</tr>
<tr>
<td>c100p</td>
<td>5 (16%)</td>
<td>3 (9%)</td>
</tr>
<tr>
<td>NS5</td>
<td>12 (38%)</td>
<td>6 (18%)</td>
</tr>
</tbody>
</table>
diagnostic performance of the CA and EIA, using RIBA results as the final determinant of antibody status. Samples that were RIBA-negative were considered false-positive results, whereas samples that were RIBA-indeterminate or -positive were considered true-positive for the purposes of this study. Of all positive RIBA results, one was negative by CA and none were negative by EIA, yielding sensitivities of 99.9% and 100%, respectively. The specificity of the EIA was 97.3%, whereas the specificity of the CA was 99.2%. The positive predictive value of anti-HCV by EIA was 93%, whereas that of CA was 98%.

Discussion
Our data show that use of the CA produces significant reductions in the frequency of positive anti-HCV results, compared with EIA methods. This led to equivalent sensitivity but much greater specificity and positive predictive value for the CA. This was true whether comparisons were made to a second-generation EIA (data from the clinical trial) or a third-generation EIA (VAMC samples) from the same manufacturer as the CA. In fact, the antigens used to detect antibodies in the EIA and the CA were identical in the two assays from the same manufacturer. A study comparing a third-generation EIA with an automated microparticle immunoassay produced by the same manufacturer similarly found that 2% of samples that were positive by EIA were negative by the microparticle assay (21).

We confirmed the results obtained with EIA in previous studies, that samples with low S/C ratios are commonly false-positive (1–16, 18). In fact, this was the major cause of differences in specificity between EIA and CA. The majority of samples with S/C ratios ≤8.0 by CA were negative by RIBA. In samples that had S/C ratios between 8.1 and 20.0 in the CA, 95% were RIBA-indeterminate or -positive. However, despite the apparent true-positive nature of these results, HCV RNA was undetectable in all 25 tested. In samples with S/C ratio between 20.1 and 30.0, only 17 of 29 (58%) were HCV RNA-positive. This was in marked contrast to samples with higher S/C ratios, where 92% were HCV RNA-positive. There were no such intermediate zones observed with EIA (18). In a study comparing two third-generation EIAIs, Goubau et al. (22) found that 98% of samples with high-positive anti-HCV by both assays were HCV RNA- or RIBA-positive. In contrast, samples with discrepant or low-positive results were frequently negative on confirmatory tests. With the CA, the S/C ratio appears more indicative of HCV RNA status than was the case for the EIA, where all results above the cutoff values were associated with the same likelihood of obtaining a positive HCV RNA result (18). Our results also support use of confirmatory testing in all CA-positive samples with low S/C ratios before reporting results.

The reasons for the differences between the EIA and CA in false-positive rates and in correlation between S/C ratio and HCV RNA are not clear. EIAIs are typically performed in microtiter plates, and it is recognized that there may be some “splashing” of sample from one well to another, which could increase the number of false-positive results. In contrast, in both the CA and microparticle immunoassays, each test is performed in a separate reaction cell, making contamination of samples much less likely. This is unlikely to explain the difference in results, however, because all EIA-positive samples must be shown to be positive on two repeat analyses in a second run before being reported as positive. A difference in antigens used in the two assays also cannot explain the difference because a similar discrepancy was shown when comparing EIA with CA or microparticle assays, using the same HCV antigens. One possible reason for the lower frequency of positive results is the apparent decreased sensitivity to the NS5 antigen in the CA. Previous studies (23–25) have shown that isolated antibody to the NS5 used in the RIBA usually represents a false-positive result. Although isolated anti-NS5 was seen in 38% of EIA-low-positive, RIBA-indeterminate samples, only 16% of CA-low-positive, RIBA-indeterminate samples had isolated antibody to NS5. There also appeared to be enhanced detection of antibody to c22p antigen, which is usually associated with HCV viremia (26). Further research into samples that are EIA-positive, CA-negative will be needed to clarify the cause of these discrepant results.

The reason for the reduced frequency of positive results in our screening program using CA is not apparent. Although some of the decrease was attributable to the lower frequency of false-positive results, high-positive results were also significantly less frequent with the CA. We considered the possibility that a contemporaneous change in screening criteria at one of the hospitals might have been responsible, but data from the other two hospitals showed a similar decrease in positive rate. This finding might suggest that the CA is less sensitive than the EIA as a screening test for anti-HCV. In direct comparison studies of almost 3000 samples from high-risk individuals, only 1 sample that was HCV RNA-positive was negative for anti-HCV by the CA and positive by the EIA. In contrast, almost 3% of samples that were high-positive by EIA were negative by CA, and all of those tested were RIBA-negative. Our data indicate that the CA has higher specificity than the EIA, rather than reduced sensitivity, and that the CA is more accurate in identifying the presence of anti-HCV than the more widely used EIIs. We therefore suspect that, although statistically significant, these results represent a gradual decrease in the number of patients tested who are truly HCV-positive as the screening program progresses because the highest risk individuals are more likely to have been identified earlier.

Our data indicate that the CA provides several advantages over EIIs. In addition to requiring only a single analysis to classify samples as positive or negative, the CA has improved specificity and positive predictive value. In addition, there are fewer low-positive samples that require confirmatory testing. Our study was of high-risk individuals; the difference in specificity is likely to be
even more important in low-risk settings, where positive predictive value differences would be magnified. The S/C ratio in CA-positive samples was also predictive of the likelihood of HCV RNA positivity, a feature we did not observe with the EIA (18). This phenomenon could be important in settings where HCV RNA tests are not routinely performed in persons positive for anti-HCV. If these findings are confirmed by other studies, it is likely that there would be reduced need for confirmatory testing and that fewer patients would be incorrectly identified as having false-positive anti-HCV results if the CA were routinely used instead of an EIA.

Ortho-Clinical Diagnostics (Raritan, NJ) kindly provided data from clinical studies that supported Food and Drug Administration clearance of their VITROS Anti-HCV assay for analysis in this study.

References


