Performance of a New-Generation Chemiluminescent Assay for Hepatitis B Surface Antigen

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Background: The usual criteria for analysis of hepatitis B surface antigen (HBsAg) are detection of HBsAg and result confirmation by antibody neutralization. We observed that with the Immulite 2000 HBsAg assay [Diagnos
tics Product Corporation (DPC)] a relatively high percentage of weakly reactive (WR) samples did not pass the neutralization step.

Methods: For each of 3 lots of Immulite 2000 HBsAg reagent (DPC), we collected and analyzed HBsAg data from ~3000 to 4000 patient blood samples and compared these data with HBsAg data from 3393 samples tested with the Abbott Auszyme assay. For 127 samples with initially WR detection signals (relative signal/cutoff index of 1.00–2.5) on the Immulite 2000 HBsAg assay, we then measured hepatitis B (HB) viral load and/or other HB serologic markers.

Results: The Immulite 2000 HBsAg assay produced more initially reactive results than the Abbott Auszyme method. Many of these reactive samples, however, were WR and did not meet the confirmation criteria in the neutralization test. Moreover, DNA PCR testing indicated that 22 of the 38 WR samples (58%) that did meet the confirmation criteria had no detectable HB viral DNA.

Conclusions: Immulite 2000 HBsAg assay results include a unique group of WR samples that are associated with both false-positive and false-negative results, regardless of neutralization status, and require careful interpretation. WR HBsAg samples should be reported as confirmed HBsAg reactive only if the samples not only meet the neutralization criteria but also are positive for other HB serologic markers such as anti-HB core total and anti-HB core IgM.

Hepatitis B virus (HBV)3 infection is a major health problem worldwide. It is estimated that more than 1 million persons in the United States are chronic HBV carriers and are potentially infectious to others. In addition, HBV chronic carriers are at high risk for liver cirrhosis and hepatocellular carcinoma (1–3). Identification of HBV carrier status in pregnant women and blood donors is critical to eliminating the transmission of HBV through blood transfusion and drastically decreasing the rate of infant infection through maternal transmission (4–6).

Hepatitis B surface antigen (HBsAg) is an HBV serologic marker that plays a major role in the diagnosis of HBV infection. A new-generation chemiluminescent HBsAg assay performed on the Immulite 2000 [Diagnostics Product Corporation (DPC)] measures HBsAg with a method that involves 2 steps: initial screening and confirmation neutralization. We observed that a relatively high percentage of weakly reactive (WR) samples did not pass the confirmation neutralization step and therefore investigated these results.

Material and Methods

We analyzed samples sent to the Bellevue Hospital laboratory for routine assessment of HBV infectivity status. Beginning in May 2003, we used the Immulite 2000 assay (DPC) to measure HBsAg. We investigated the performance of the assay during 3 different 1-month time

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3 Nonstandard abbreviations: HBV, hepatitis B virus; S/C, signal/assay cutoff; HBsAg, hepatitis B surface antigen; DPC, Diagnostic Products Corporation; WR, weakly reactive; aHBs, antibodies against hepatitis B surface antigen; aHBcT, total antibodies against hepatitis B core antigen; and aHBcIgM, IgM antibodies against hepatitis B core antigen.
periods, which corresponded to the use of 3 different lots of reagent. During each 1-month period for each reagent lot, we collected HBsAg sample data from all HBsAg tests performed for the Bellevue Hospital patient population. We also calculated the positive detection rates of HBsAg by the Immulite 2000 assay and the historical positive rate of detection by the Abbott Auszyme enzyme immunoassay in the Bellevue patient population (same data collection procedure as for Immulite 2000 assay) and used \( \chi^2 \) analysis to compare the results.

The HBsAg qualitative assay requires 2 incubation steps and a total reaction time of 65 min. Patient results were reported as reactive [sample signal/assay cutoff (S/C) index \( >1.00 \)] or nonreactive (S/C ratio \( <1.00 \)). The DPC protocol requires that samples deemed reactive must be repeated in duplicate. If the S/C ratios of 2 replicates are both \( <1.00 \), the result of this sample is reported as nonreactive. If the S/C of at least 1 of the 2 replicates is \( \geq 1.00 \), the sample is considered reactive, and the result must be confirmed by an HBsAg neutralization test, in which the percentage of HBsAg signal reduction in the presence of antibodies against HBsAg (aHBs) is measured (7). The DPC HBsAg confirmation protocol requires a signal reduction of \( \geq 50\% \) after neutralization. If the signal reduction is \( <50\% \), then the sample is considered negative for HBV (7, 8).

The Abbott Auszyme enzyme immunoassay is a semiautomatic procedure that involves 2 incubation steps at 2 different temperatures (reaction at 40 °C and color development at room temperature). The cutoff value of each run is calculated from a formula based on the negative controls from that run (9).

Qualitative assays for aHBs, total antibodies against HB core antigen (aHBcT), and IgM antibodies against HB core antigen (aHBcM) were run on the DPC Immulite 2000. The DPC-defined criteria for positive results of these assays are as follow: aHBs \( \geq 11.0 \) mIU/mL (10, 11), aHBcM \( \geq 11.0 \) IU/mL (12), and aHBcT cutoff/signal \( \geq 1.15 \) (13). We used \( \chi^2 \) statistical analysis of reactive rates among different lots (14, 15).

We used the Roche COBAS Amplicor HBV Monitor test (Ver. 2.0) to detect HBV viral DNA (16, 17). Although the quantitative detection limit is \( \geq 200 \) copies/mL, qualitatively the detection limit goes to zero. A sample with a result of 1 to 199 copies/mL is reported as “<200 copies”; a sample with 0 copies is reported as “no HBV DNA detected”.

Results

Our initial results are shown in Table 1. Samples tested with reagent lots A and B on the DPC Immulite 2000 assay yielded a fairly high percentage of initially reactive samples. Statistical analysis indicated that the initial positive rate of the DPC assay was significantly different from the initial positive rate of the Abbott assay, particularly in the case of lots A and B. After we performed the confirmation step, up to 70% of the initially reactive samples had \( \geq 50\% \) neutralization activity.

We randomly selected \( \sim 200 \) patient samples with

<table>
<thead>
<tr>
<th>Lot no.</th>
<th>Total samples, n</th>
<th>Total initially reactive samples, % (n)</th>
<th>Initially reactive (S/C ( &gt;1.00 )) and &lt;50% neutralization, % (n)</th>
<th>Initially reactive (S/C ( \geq 1.00 )) and ( \geq 50% ) neutralization, % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (August 2003.)</td>
<td>2914</td>
<td>11.7 (341)</td>
<td>8.2 (238)</td>
<td>3.5 (103)</td>
</tr>
<tr>
<td>B (October 2003.)</td>
<td>3711</td>
<td>9.9 (330)</td>
<td>4.4 (162)</td>
<td>4.5 (168)</td>
</tr>
<tr>
<td>C (August 2004.)</td>
<td>3324</td>
<td>4.4 (145)</td>
<td>1.0 (33)</td>
<td>3.4 (112)</td>
</tr>
<tr>
<td>Abbott Commander( ^a )</td>
<td>3393</td>
<td>3.4 (115)</td>
<td>NA( ^b )</td>
<td>NA</td>
</tr>
</tbody>
</table>

\( ^a \) Data on the Abbott Commander are provided as comparison.

\( ^b \) NA, not applicable.
initial reactive HBsAg results (S/C ratio ≥1.00) from lots A and B for the confirmation outcome study. Samples with unconfirmed neutralization <50% and samples with confirmed neutralization (≥50%) were grouped separately. The relationship between patient S/C result and neutralization result is illustrated in Fig. 1. It is clear that the confirmation rate correlates with the S/C ratio: the higher the ratio, the greater likelihood of being confirmed by neutralization. In group 1, 98% of samples had S/C ratios <2.5, whereas group 2 contained all of the samples with S/C ratios >10.

The high initial positivity rate and the subsequent failure at the confirmation step for a significant portion of the initial positives led us to question the clinical significance of these results and led to further investigation. To get a better understanding of the true nature of these WR samples, we used HBV viral load and other HBV serologic markers to judge the HBV infectious status.

**HBV DNA LOAD TEST**

Patient samples (n = 127) with WR HBsAg results (S/C ratio ≥2.5) measured on the DPC Immulite 2000 assay with reagents from any 1 of the 3 lots were randomly selected and tested for the viral load and other serologic HBV markers. Among the 127 samples, 38 had ≥50% neutralization activity and the other 89 had <50% neutralization activity.

The PCR results indicated that 83 of the 89 WR samples with <50% neutralization activity (93%) had no detectable HBV DNA (zero copies); 6 of the 89 had low DNA (up to 9000 copies/mL). The serologic profiles of the 6 samples with detectable HBV DNA are shown in Table 2. One sample had positive aHBcIgM (45% HBsAg confirmation and negative aHBs), suggesting early acute-phase infection; 2 samples had positive aHBcT and negative aHBs, suggesting either early or chronic infection; and 3 had negative aHBs, aHBcT, and aHBcIgM. These 3 samples could indicate a very early stage of infection, in which case HBsAg reactivity would be low, or infection with (an) HBsAg mutant strain(s) that led to neutralization failure (18). For 1 of the 6 samples (sample 3, which was negative for aHBs, HBsAg, and aHBcT), the patient had follow-up tests ordered 1 year later. The follow-up indicated that both HBsAg and aHBs were still negative. However, the lack of aHBcT data made it difficult to differentiate the likely cause of the original positive HBV viral load result.

In the group of 38 WR samples that did have ≥50% neutralization in the confirmatory step, 22 samples (58%) had no detectable DNA (zero copies), and 16 samples had detectable HBV DNA. Among this group of WR samples, the correlation between the DNA copy number and the quantitative index of the HBsAg serologic result was poor (Fig. 2).

The 22 HBV DNA-negative samples could be divided...
into 5 groups based on available serologic markers other than HBsAg (Table 3). Because group 2 samples had positive aHBs and negative aHBcT, suggesting previous immunization, their ≥50% neutralization HBsAg results were most likely false positives.

The follow-up results available for samples from groups 1 and 4 suggested that at least 2 samples had false-positive HBsAg results (follow-up with negative results for both HBsAg and aHBs). Furthermore, 1 group 1 sample from a hemodialysis patient was highly likely to be false positive because 12 subsequent HBsAg follow-up tests (1 per month) were all negative.

The serologic profile of aHBs–/aHBcT+/aHBcIgM– for group 5 samples indicated that the HBsAg-positive results were highly likely to be true positives despite the lack of detectable HBV DNA in these samples. One of the 6 samples had a follow-up result that repeated as aHBcT+/HBsAg+, which provided further evidence for the likely true HBsAg positivity of the original patient sample.

**Discussion**

Our current study (Fig. 3) and previously published data indicate that although the Immulite 2000 HBsAg assay has HBsAg detection rates similar to those of the Abbott Auzyme and Bayer Centaur (19), the specificity of the Immulite 2000 at the initial run is not as high as the other 2 assays. The occurrence of a high number of initially positive results that are not confirmed by the neutralization step is likely related to the inherent poor separation between signal and background noise of this assay. We found that as a consequence of this poor specificity, the rate of initially positive samples was 1.5- to 3.5-fold higher than for the Abbott assay (see Table 1). Although we observed this situation over multiple reagent lots during the last 3 years, we also found that the quality of the assay reagent has improved (see lot C in Table 1). However, a reasonable and accurate report format is still needed for the ~1% of samples that are WR, regardless of whether they pass confirmation criteria. The Immulite 2000 assay instructions state that, “the patient appears to not be infected with HBV” if the neutralization is ≥50%, whereas any sample with neutralization ≥50% should be reported as “confirmed reactive for HBsAg” (8). Our results, however, suggest that this approach may lead to both false-positive and false-negative results and that, therefore, a mor e careful and thorough protocol is needed to deal with this issue.

To avoid the reporting of false-negative HBsAg results, we suggest that any sample, from any manufacturer, with a low-positive result that does not pass the assay’s confirmation test not be designated “nonreactive” until other HBV serologic markers are measured to provide a clearer picture of the patient’s status (Fig. 4). For these samples, laboratories should consider reflex testing to aHBcT, aHBs, and aHBcIgM, if these tests were not ordered by the physician together with the HBsAg test, and review by the laboratory director.

In a similar vein, our data also indicate that at least some of the samples (6 of the 22) that were WR (S/C ratios, 1.00–2.50) and did pass neutralization criteria for positive results did not have evidence of HBV infection (Table 3). This finding is understandable because the impact of the inherent high CV (~10%) and poor separation between signal and noise of this assay (19) can give poor separation between true negatives and false positives.

Although the DPC assay instructions advise laboratories to rely entirely on the neutralization result to decide the HBsAg outcome, our results suggest that further testing is needed before WR samples are reported as “confirmed HBsAg reactive”. Again, we would recommend that other HBV serologic markers be tested and that final results reported as confirmed HBsAg reactive only if the sample is also positive for aHBcT and/or aHBcIgM. Otherwise, the test should be repeated in 2 weeks (Fig. 4). This approach is similar to some other test procedures recommended by the CDC. In the guidelines for laboratory testing and result reporting of HCV antibody, the CDC recommends that a reflex test be performed before HCV positives with low S/C ratios are reported (20).

Although we investigated only the DPC Immulite assay, we suspect that similar low positive results with questionable status might occur with other HBsAg assays, and we recommend study of the issue by each laboratory.

In conclusion, our analysis of the performance of the DPC Immulite 2000 chemiluminescent HBsAg test at Bellevue Hospital indicates that samples identified by this assay as WR (S/C ratios, 1.00–2.50) need additional testing and review, regardless of neutralization status. Laboratories

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**Table 3. Serologic profile and follow-up results for the 22 HBV DNA–negative samples with confirmed HBsAg neutralization results.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample no.</th>
<th>Serologic marker profile</th>
<th>Follow-up availability</th>
<th>Follow-up result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>aHBs+/aHBcT+/aHBcIgM+</td>
<td>3 of 8</td>
<td>2 HBsAg+; 1 HBsAg+/aHBs+</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>aHBs+/aHBcT+/aHBcIgM+</td>
<td>NA*</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>aHBs (NA)/aHBcT (NA)/aHBcIgM (NA)</td>
<td>1 of 2</td>
<td>HBsAg+</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>aHBs+/aHBcT (NA)</td>
<td>1 of 2</td>
<td>HBsAg+/aHBcT+</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>aHBs+/aHBcT+/aHBcIgM+</td>
<td>1 of 6</td>
<td></td>
</tr>
</tbody>
</table>

* NA, not available.
performing HBsAg testing should determine the significance of WR assay results. When interpreting WR samples, one should look at the whole profile of HBV tests, including the HBsAg neutralization test and assays of other hepatitis serologic markers, to provide the most accurate status of HBV infection to clinicians. Laboratories
should establish HBV test panel(s) to allow the reflex test(s) such as aHBcT and aHBcIgM to be performed in these cases.

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