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Multilaboratory Evaluation of Real-Time PCR Tests for Hepatitis B Virus DNA Quantification

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The performance characteristics of four different assays for hepatitis B virus (HBV) quantification were assessed: the Abbott RealTime HBV IUO, the Roche Cobas AmpliPrep/Cobas TaqMan HBV test, the Roche Cobas TaqMan HBV test with HighPure system, and the Qiagen artus HBV TM ASR. Limit of detection (LOD), linear range, reproducibility, and agreement were determined using a serially diluted plasma sample from a single chronically infected subject. Each assay was tested by at least three laboratories. The LOD of the RealTime and two TaqMan assays was approximately 1.0 log10 IU/ml for artus HBV (which used the lowest volume of extracted DNA), it was approximately 1.5 log10 IU/ml. The linear range spanned 1.0 to at least 7.0 log10 IU/ml for all assays. Median values were consistently lowest for artus HBV and highest for Cobas AmpliPrep/Cobas TaqMan HBV. Assays incorporating automated nucleic acid extraction were the most reproducible; however, the overall variability was minor since the standard deviations for the means of all tested concentrations were ≤0.32 log10 IU/ml for all assays. False-positive results were observed with all assays; the highest rates occurred with tests using manual nucleic acid extraction. The performance characteristics of these assays suggest that they are useful for management and therapeutic monitoring of chronic HBV infection.

Viremia in chronic HBV infection varies from very low or undetectable to >10⁸ copies/ml. Effective quantitative assays must therefore measure a wide range of viral DNA concentrations. Commercially available quantitative assays utilize a variety of different detection methods, including signal amplification (Versant HBV bDNA; Siemens Healthcare Diagnostics), conventional PCR (Amplicor HBV Monitor test; Roche Diagnostics), and real-time PCR (Cobas AmpliPrep/Cobas TaqMan HBV test [Roche Diagnostics] and RealTime HBV assay [Abbott Molecular]). Of these methods, only real-time PCR is able to cover the wide dynamic range required for quantification of the virus in all stages of infection.

Reports of studies of real-time assays have mainly focused on the performance of individual tests compared to signal amplification tests rather than the comparative performance of multiple real-time PCR tests (2, 3, 6, 9). The present study assessed the limit of detection, linear range, reproducibility, and agreement among four commercially available real-time PCR HBV viral load tests: the Abbott RealTime HBV IUO, the Roche Cobas AmpliPrep/Cobas TaqMan HBV test, the Roche Cobas TaqMan HBV test with HighPure system, and the Qiagen artus HBV TM ASR. The Abbott m2000sp and Roche AmpliPrep protocols were performed using automated extraction methods, while the extractions for the Roche HighPure and Qiagen ASR were performed manually.

Hepatitis B virus (HBV) has infected an estimated 400 million persons worldwide; cirrhosis and hepatocellular carcinoma, the major sequelae of chronic hepatitis B, result in over a half million deaths annually (4). HBV viremia is a critical risk factor for progression of chronic HBV infection (1); accordingly, quantification of HBV DNA in blood has become a critical tool in the assessment and management of chronic infection. In addition to serologic tests for HBV and measurement of serum transaminases, HBV viral load testing is used to determine the phase of chronic HBV infection (8) and is particularly useful in distinguishing active from inactive disease in individuals with no detectable HBeAg. A number of antiviral drugs have been introduced recently for the treatment of chronic HBV infection, and viremia is an important component in the decision to initiate treatment and in monitoring therapeutic response (5, 7).

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‡‡A.M.C. and A.V. are co-first authors and contributed equally to the manuscript.

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TABLE 1. HBV panel composition

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>Nominal concn</th>
<th>Log_{10} IU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IU/ml</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
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</tr>
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</tr>
<tr>
<td>5</td>
<td>50,000,000</td>
<td>7.7</td>
</tr>
</tbody>
</table>

TABLE 2. Detection rate of the HBV viral load assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>No. detected/no. of valid results at each nominal concn (log_{10} IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>HighPure TaqMan HBV</td>
<td>2/28</td>
</tr>
</tbody>
</table>

$^a$ Four samples invalid or not extracted.  
$^b$ Three samples invalid or not extracted.
IU/ml) and were reported as such by the TaqMan instrument (Fig. 1).

The assay bias, expressed as \( \log_{10} \) IU/ml viral load values minus the nominal concentration, is shown in Fig. 2. The RealTime assay showed very consistent bias for samples greater than or equal to 3.0 \( \log_{10} \) IU/ml. The AmpliPrep TaqMan assay consistently gave the highest viral load values, and there was variable bias throughout the linear range of the assay. A similar pattern of bias was seen with the HighPure TaqMan assay, although the values were lower than those seen with the AmpliPrep TaqMan assay. The \textit{artus} HBV assay showed consistent bias for samples above 2.0 \( \log_{10} \) IU/ml and overall showed the lowest viral load values.

### DISCUSSION

This comparison of four real-time PCR assays demonstrated that they have very similar performance characteristics, although some differences were noted. For example, the two TaqMan and the RealTime assays had a lower limit of detection of 1.0 \( \log_{10} \) IU/ml, compared to 1.5 \( \log_{10} \) IU/ml for the \textit{artus} HBV assay. This minor difference is likely due to plasma input volume differences between \textit{artus} HBV (200 \( \mu l \)) and the other assays (500 \( \mu l \)). In addition, a lower percentage of the extracted DNA was added to the master mix with the \textit{artus} HBV assay compared to the other three assays (33% versus ~70%). Despite this analytical difference, all assays had detection limits within the necessary range for clinical decision making.

Another difference that was observed pertained to false-positive rates. Assays that relied on manual extraction (HighPure TaqMan and \textit{artus} HBV) had higher false-positive rates than did those that employed automated extraction (RealTime and AmpliPrep TaqMan assays). The occurrence of false-positive results with automated extraction platforms is an important observation that may reflect the performance expected in the clinical laboratory, where samples with viral load values in excess of 8.0 \( \log_{10} \) IU/ml may be tested alongside samples containing no virus. These data suggest that regardless of the extraction method employed, careful attention to good laboratory practices will be needed to avoid false-positive results due to the extraordinarily high viral loads that occur in chronic HBV infections.

All four assays demonstrated a broad linear range of approximately 7 \( \log_{10} \) IU/ml; we were unable to obtain a large-volume sample with a higher viral load to better define the upper limits of linearity of the assays. Quantification of the 7.7-\( \log_{10} \) IU/ml sample resulted in concentrations that exceeded the upper limit of the AmpliPrep TaqMan assay but not those of the other three assays. According to the AmpliPrep TaqMan package insert, it would have been acceptable to dilute these high-concentration samples up to 1:100 in order to report out a value up to 10.23 \( \log_{10} \) IU/ml; however, specimen dilution was not part of the study protocol and, therefore, it was not performed. This approach is appropriate for all of the assays as long as the dilution process is validated by the laboratory.

The reproducibility of the assays was similar to that seen with other real-time PCR tests. For viral load values in the middle of the linear range where the standard deviations are 0.05 to 0.10 \( \log_{10} \) IU/ml, a change in viral load that is greater than 3-fold would be interpreted to be a significant difference. For viral load values less than 2.0 \( \log_{10} \) IU/ml, where the standard deviation is higher, 5-fold changes would be significant. Overall, the \textit{artus} HBV assay was the least precise of the four assays evaluated; this may in part reflect the manual extraction method.

The differences in the median viral load values obtained with
the four assays ranged from $0.29 \log_{10} \text{IU/ml}$ to $0.56 \log_{10} \text{IU/ml}$. The smallest difference was seen for the $6.0 \log_{10} \text{IU/ml}$ sample, and the largest difference was seen for the $3.0 \log_{10} \text{IU/ml}$ sample. The most consistent bias (difference between nominal concentration and observed concentration) was seen with the \textit{artus} HBV assay for values of $\geq 2.0 \log_{10} \text{IU/ml}$ and with the RealTime assay for values of $\geq 3.0 \log_{10} \text{IU/ml}$. Both TaqMan assays showed an inconsistent bias throughout the linear range of the assays. One limitation of this study is that only a genotype A sample was studied, so it is not possible to determine if any of these assays has a genotype bias.

The availability and regulatory status of the investigated assays have changed since the conclusion of experimentation. The \textit{artus} HBV assay is available outside the United States as a Conformité Européenne (CE)-marked product. The Real-Time and TaqMan reagents have been approved for use by the U.S. Food and Drug Administration (U.S. FDA) and are CE marked. Plasma volumes identical to those used in this study are used in the U.S. FDA-approved assays. The RealTime assay is additionally approved for use with 200 $\mu$l of plasma. Comparative performance of the assay with this reduced volume was not investigated in this study.

Though there is reasonable agreement in viral load values across the four assays, the intra-assay and between-assay variability are such that patients should be monitored with a single assay. False positives were observed on all platforms, and care-
ful attention should be paid to avoid cross-contamination by samples that may contain extremely high concentrations of this virus.

In summary, all four assays are similarly sensitive and have a broad linear range, providing clinical utility for both diagnostic testing and therapeutic monitoring.

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