Sequence Conservation of the Region Targeted by the Abbott RealTime HBV Viral Load Assay in Clinical Specimens

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The Abbott RealTime HBV assay targets the N-terminal region of the S gene. Here we analyzed the sequence variability of the assay target region from >2,100 clinical specimens. Thermodynamic modeling of the percentage of bound primer/probe at the assay annealing temperature was performed to assess the potential effect of sequence variability.

Hepatitis B virus (HBV) is one of the most widely distributed viruses that infect humankind. Approximately one-third of the world’s population has serological evidence of past or present infection with HBV, and 350 million people are chronically infected. HBV-related end-stage liver disease or hepatocellular carcinoma is responsible for over 1 million deaths per year (1). Distinct clinical and virological characteristics of HBV infection have been reported in different geographical parts of the world and are increasingly associated with genetic diversity of the infecting virus (2). HBV replicates through an RNA intermediate involving the reverse transcriptase (RT) activity of its DNA polymerase (3). During replication, HBV production may approach 10¹¹ molecules/day, although during peak activity this rate may increase 100 to 1,000 times (4). The HBV DNA polymerase lacks fidelity and proofreading function partly because exonuclease activity is either absent or deficient (4). In addition to naturally occurring polymorphisms, the introduction of nucleoside analogues, such as lamivudine, with a low barrier to resistance brought about a selective pressure leading to the rapid emergence of drug-resistant variants. Newer therapies such as entecavir and tenofovir have a high genetic barrier to resistance, and as such, variants resistant to these compounds are rare (<2% over 5 years on therapy).

All real-time PCR tests require target-specific primers and probes. Natural polymorphisms occurring within the primer and/or probe sites have the potential to abolish or reduce the efficacy of hybridization, resulting in reduced analytical sensitivity and accuracy (5). The Abbott RealTime HBV viral load assay targets the highly conserved N-terminal region of the S gene of the HBV viral genome. This region was selected because it is essential for the assembly and secretion of subviral particles and acts as the surface antigen membrane anchor. Therefore, the N-terminal domain of the S gene is vital for HBV and changes in this domain are not likely to be tolerated by the virus (6). Nonetheless, it is important that the manufacturers of molecular diagnostic assays monitor the performance of their tests against the ever-changing genetic landscape of the HBV epidemic. The objective of this study was to investigate whether naturally occurring polymorphisms in the HBV genome could have any discernible impact on the performance of the Abbott RealTime HBV viral load assay.

Sequences from the HBV S gene comprising the Abbott RealTime assay target regions were obtained from two sources, Evivar Medical (East Melbourne, Australia) and Abbott Diagnostics (Abbott Park, IL). Sequence data from 1,916 unique to fully identified clinical specimens submitted for resistance testing to Evivar and 245 to 259 (depending on the region studied) sequences from the Abbott Global Viral Surveillance program were aligned with RealTime assay primer and probe sequences, and sequence variability relative to the reference sequence at each nucleotide position was assessed (Abbott sequences were collected from Cameroon, Congo, Thailand, Pakistan, and the United States; a subset of samples from unknown origins outside the United States were provided by a U.S.-based reference laboratory; and Evivar sequences are a compilation from various geographical locations, but not all sequence origins are available). Variants of sequences in the primer/probe target regions were identified, and changes versus reference occurring at greater than 0.1% frequency were characterized. Thermodynamic modeling of the percentage of bound primer/probe at the assay annealing temperature was performed to assess the potential impact of sequence variability on the performance of the Abbott RealTime HBV viral load assay. This analysis was performed using the TM Mismatch feature of the OligoAnalyzer 3.1 online primer design program (Integrated DNA Technologies, Iowa City, IA). This method accounts for PCR conditions (target type and oligonucleotide, deoxynucleoside triphosphate [dNTP], monovalent, and divalent cation concentrations) to determine the melting temperature (Tm) of the mismatched primer or probe and the percent bound at the PCR annealing/fluorescence readout temperature.

Ninety-four percent of sequences belonged to genotypes A (16%), B (26%), C (31%), D (16%), and E (4%). Sequence variability compared to the reference strain is presented in Tables 1 to 3 according to target site (forward primer, probe, or reverse primer, respectively). Perfect homology with the forward primer, probe, and reverse primer was observed in 96.8%, 97.5%, and 96.3% of the sequences, respectively. Differences were most often observed within the reverse primer binding region, with a prevalence of 2.2% of sequences containing a single mismatch (Table 3), while sequences in the forward primer binding region were the most conserved, with a prevalence of 1.6% of sequences con-

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ated mutations (RAMs) that may interfere with accurate quantification for assays whose primers and probes have binding sites in targets of diverse virus specimens (24). An additional source of concern with virus that have divergent sequence in critical genome regions rely on reactivity of synthetic primers and probes with the target quickly to new environmental pressures. The resulting evolution at each site, binding efficiency ranged from 81.4 to 94.5%. At the probe binding site, binding efficiency ranged from 81.4 to 94.5%.

Based on thermodynamic modeling, the predicted observed; no change was closer than 7 bases from the 3’ end of either primer. Based on results from the Evivar database, there are numerous reports in the literature that describe cases of underestimation of viral load in patients infected with virus that have divergent sequence in critical genome regions (7–23). The Abbott RealTime HBV assay has performed well in studies of diverse virus specimens (24). An additional source of concern for assays whose primers and probes have binding sites in targets of antiviral drug therapy is the development of resistance-associated mutations (RAMs) that may interfere with accurate quantification or detection. Since the Abbott RealTime HBV assay targets the N-terminal region of the S gene, a highly conserved region of the HBV genome upstream of any antiviral drug targets, this is less likely to occur (6).

The study performed here represents a sequence analysis with a large number of data derived from clinical specimens. There is no discernible impact on performance of the RealTime viral load assay based on naturally occurring polymorphisms available for analysis. Furthermore, based on results from the Evivar database, there continue to be no resistance-associated mutations located within the RealTime HBV assay primer or probe binding sites.

**ACKNOWLEDGMENTS**

We are grateful to Evivar and the Abbott Global Viral Surveillance program for providing sequence data. G.A.C., J.R., V.H., and C.M. are employees and stock holders of Abbott Laboratories. L.Y. is an employee of Evivar Medical Pty. Ltd. T.P.Y. is a former employee and stock holder of Abbott Laboratories. N.T.P. has no conflict of interest to declare.


**REFERENCES**


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**TABLE 1 Forward primer haplotypes**

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<thead>
<tr>
<th>Sequence</th>
<th>Count</th>
<th>%</th>
<th>No. of differences</th>
<th>Predicted % bound at annealing temp</th>
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**TABLE 2 Probe haplotypes**

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**TABLE 3 Reverse primer haplotypes**

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**REFERENCES**


