**HBV RNA co-amplification may influence HBV DNA viral load determination**

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**List of abbreviations:** HBV, hepatitis B virus; NA, nucleos(t)ide analogue; TMA, transcription-mediated amplification; RT, reverse transcription; HPS, High Pure System; LLOQ, lower limit of

quantification; cccDNA, covalently closed circular DNA ; IQR, interquartile range; ETV, entecavir; TDF, tenofovir disoproxil fumarate ; UL, upper limit; LL, lower limit; OLS, ordinary least squares; CI, confidence interval

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**Abstract**

Despite effective hepatitis B virus (HBV)-DNA suppression, HBV-RNA can circulate in patients receiving nucleoside/nucleotide analogues (NAs). Current assays quantify HBV-DNA by either real-time PCR, which employs DNA-polymerase, or transcription-mediated amplification (TMA), which employs reverse transcriptase (RT) and RNA-polymerase. We assessed the impact of RT capability on HBV-DNA quantification in samples from three cohorts, including patients with quantified HBV-RNA. We compared HBV-DNA levels by real-time PCR (cobas HBV, Roche 6800/8800; Xpert HBV, Cepheid), TMA (Aptima HBV, Hologic) and real-time PCR with added RT capability (cobas HBV+RT). In the first cohort (n=45), followed over 192 weeks of NA therapy, on-treatment HBV-DNA levels were higher with cobas HBV+RT than cobas HBV (mean: 0.89 log10 IU/ml). In a second cohort (n=50) followed over 96 weeks of NA therapy, HBV-DNA viral load was significantly higher with the cobas HBV+RT and Aptima HBV compared to the cobas HBV test at all time points after initiation of NA therapy (mean difference: 0.65-0.93 log10 IU/ml). A clinically significant difference was not detected between the assays at baseline. In a third cohort (n=53), after a median of 2.2 years of NA therapy, we detected HBV-RNA (median 5.6 log10 copies/ml) in 23 patients (43.4%). Median HBV-DNA levels by Aptima HBV were 2.4 versus <1 log10 IU/ml in samples with HBV-RNA and without HBV-RNA, respectively (p=0.0006). In treated patients with HBV RNA, Aptima HBV measured higher HBV-DNA levels than Xpert HBV and cobas HBV. ***Conclusions:*** Tests including an RT step may overestimate HBV-DNA, particularly in samples with low viral loads as a result of NA therapy. This overestimation is likely to be due to amplification of HBV-RNA and may have an impact on clinical decisions.

**Introduction**

Current guidelines for the management of chronic hepatitis B virus (HBV) infection recommend quantitative measurement of circulating HBV DNA to guide therapeutic decisions and monitor response to antiviral therapy.1,2 The main endpoint of current treatment strategies is the induction of long-term and complete suppression of HBV replication.1 Detection of low, residual HBV DNA levels during nucleos(t)ide analogue (NA) therapy may have significant clinical consequences, including a higher estimated risk of hepatocellular carcinoma.3 Repeatedly confirmed undetectable (or at least not quantifiable) levels of HBV DNA may identify patients in whom NA treatment can be discontinued under specific circumstances.1,4 Inaccurate or inconsistent measurement of viral load could adversely affect patient care, with some patients potentially not receiving the medication they need, and others getting antiviral treatment that might no longer be required.1,4 Overestimation of viral load may erroneously suggest adherence issues or virological failure, resulting in further inconclusive resistance testing and potential changes to treatment that may be associated with risk of toxicity, additional cost and emotional distress for the patient.

To date, general agreement has been reported when comparing commercial HBV tests across different platforms.5−9 However, in studies comparing HBV viral load quantification using analytical performance panels, underestimation of values at high viral loads and a lack of linearity in performance across the viral load range has been reported.10 Available assays for HBV DNA quantification operate according to one of two main designs. Historically, most tests used real-time PCR formats, which employ different types of DNA polymerases to amplify the target DNA. Real-time PCR can also be used to quantify RNA; however, this requires the inclusion of an extra step of reverse transcription (RT) prior to DNA amplification and RT activity of the polymerase. Alternative nucleic acid amplification technologies recently introduced for HBV include real-time transcription-mediated amplification (TMA).11 Due to its excellent sensitivity TMA has an established track record in the context of blood safety12 and is also applied to the quantification of human immunodeficiency virus RNA13 and hepatitis C virus RNA.14 The TMA assay incorporates two enzymes for nucleic acid amplification, Moloney murine leukemia virus reverse transcriptase and T7 RNA polymerase; this makes RT of RNA into DNA potentially part of any amplification process.11 As a result, it seems likely that a TMA assay may be prone to amplifying not only HBV DNA, but potentially also HBV RNA, particularly in those receiving NA therapy.

Following infection, the HBV genome forms a covalently closed circular DNA (cccDNA) episome in the nucleus of hepatocytes, which functions as the viral transcriptional template. The pregenomic HBV RNA transcribed from cccDNA must be converted into DNA by the viral polymerase to allow production of new virus particles for export. By targeting the viral polymerase, NAs effectively block production of infectious viruses. In patients with high levels of cccDNA transcription, excess pregenomic HBV RNA can bypass RT (and inhibition by NAs) and be exported in HBV RNA-containing particles, which are thought to be non-infectious.15 Thus, patients receiving antiviral therapy can have circulating HBV RNA despite the NA effectively blocking HBV DNA synthesis and virus replication.

To date, the magnitude of any possible overestimation of HBV DNA as a result of amplification of HBV RNA, and whether clinically relevant discrepancies need to be taken into consideration, has not been investigated. In this study we used samples from three independent cohorts to compare HBV DNA measurements obtained by TMA and real-time PCR, to explore the impact of adding an RT step to real-time PCR, and to relate the findings to the duration of NA therapy and, in a subset of patients, the direct quantification of circulating HBV RNA.

**Materials and Methods**

**Study populations**

All studies were performed in accordance with the International Conference on Harmonisation Good Clinical Practice Guidelines, the Declaration of Helsinki, and relevant local legislation. Ethical approval was obtained from the relevant Institutional Review Board/Independent Ethics Committee. All study cohorts and evaluated HBV DNA tests are summarized in **Table 1**.

*Hannover cohort: HBV DNA quantification by real-time PCR (cobas HBV) and by real-time PCR plus reverse transcription (cobas HBV+RT)*

In a previous study conducted at three sites in Germany (Hannover Medical School), Switzerland, and Korea, we demonstrated the concordance between the real-time PCR cobas® HBV DNA assay for use on the cobas® 6800/8800 Systems (henceforth described as cobas HBV) and the real-time PCR COBAS® TaqMan® HBV Test for use with the High Pure System (henceforth described as cobas HPS) (Roche Molecular Diagnostics, Pleasanton, CA, USA) (**Supplementary Figure S1**). The materials and methods, as well as the results for this study have been published previously.8 In an exploratory evaluation, all available samples (n=191) from 45 patients (**Supplementary Table S1**) under NA therapy (principally entecavir and tenofovir disoproxil fumarate; 78%) from the Hannover Medical School (Germany), were used to explore the contribution of circulating HBV RNA to any viral load difference obtained with the standard cobas HBV software and a non-standard total nucleic acid software (cobas HBV+RT), which includes an RT step in the PCR profile, allowing for the amplification of HBV RNA that otherwise would not be amplified at a meaningful level. The need for written informed consent was waived by the Institutional Review Board.

*DEFINE cohort: HBV DNA quantification by TMA (Aptima HBV), real-time PCR (cobas HBV), and real-time PCR plus reverse transcription (cobas HBV+RT)*

Based on the results of the first study, a second study was conducted at two sites in Germany and Spain to compare HBV DNA quantification by cobas HBV, cobas HBV+RT, and the TMA Aptima® HBV Quant assay (henceforth described as Aptima HBV) for use with the Hologic Panther system (Hologic Inc., Marlborough, MA, USA). A total of 346 plasma samples collected from 50 lamivudine-resistant adult patients (≥18 years of age) starting salvage therapy with various NA combinations were collected from baseline to Week 96 of treatment. Detailed information on the design of the DEFINE study and the study population has been published elsewhere.16 Viral load concentrations ranged from undetectable to >9 log10 IU/mL, overlapping the medical decision points (≥20,000 IU/mL, ≥2,000 IU/mL, <2,000 IU/mL, and <50 IU/mL) used to direct treatment during the study. A total of 271 samples with enough remaining volume generated valid results for quantitative HBV DNA for both cobas HBV and Aptima HBV and were considered for further statistical analysis. All samples were anonymised by an Independent Ethics Committee-approved procedure. All specimens were derived from archived samples that had been stored at -20°C or lower for a maximum of 10 years (DEFINE trial,16 (all patients had provided informed consent)). Each sample was divided into sufficient aliquots to allow at least single-replicate testing with each test. HBV DNA results from the original test (cobas HPS) at the time of collection were available for each sample (referred to as nominal viral load).

*Liverpool cohort: HBV DNA quantification by TMA (Aptima HBV) and real-time PCR (Xpert HBV and cobas HBV) in patients with circulating HBV RNA*

As part of an ongoing study evaluating HBV RNA detection during chronic HBV infection at the University of Liverpool, UK (Research Ethics Committee Approval 18/YH/0286, July 2018), plasma samples from 101 patients underwent HBV RNA quantification by an in-house real-time PCR assay that applies the method described by van Bömmel et al17 and was performed at DDL Diagnostic Laboratory (Rijswijk, The Netherlands). The assay reported detection/quantification range that spans 2.5/4.0-9.5 log10 copies/ml. Aptima HBV is the test used for routine care in the accredited National Health Service (NHS) diagnostic laboratory of the Royal Liverpool University Hospital. To determine whether there was any overestimation of HBV DNA by Aptima HBV, plasma samples from 26 patients that had both quantifiable HBV RNAand quantifiable HBV DNAby Aptima HBV were also tested for HBV DNA by real-time PCR, using Xpert® HBV Viral Load (henceforth described as Xpert HBV) (Cepheid, Maurens-Scopont, France). Xpert HBV was performed in the local diagnostic laboratory,andcobas HBV was performed either at a referral NHS laboratory or at Roche Diagnostics.

**HBV DNA quantification**

All tests were performed by trained operators in accordance with the manufacturers’ specifications.18-20 Runs were considered valid if both positive and negative controls were valid and no protocol deviations or incidents occurred that might affect the validity of the data. If a run was considered invalid, all samples included in that run were re-tested wherever possible. The cobas HBV+RT test was performed according to an in-house protocol as described above.

**Analysis and statistical methods**

All statistical analyses were carried out using the SAS System software v9.4 through the SAS Enterprise Guide software v7.12 or higher. Results were log10-transformed and compared according to Clinical and Laboratory Standards Institute guidance EP09-A.21 HBV viral loads were compared between assays using the Student’s *t*-testand *s*catter plots were overlaid with the Deming regression lines used to assess correlation; Bland−Altman plots were used to estimate bias. Longitudinal plots were used to present viral loads at different time points for individual subjects and combined means. For individual subject graphs, the viral load trajectory was measured using the slope of the regression line for each test. When comparing HBV DNA levels, results below the lower limit of quantification (LLOQ) were assigned a value of 0.5 x LLOQ(IU/ml) if the assay reported qualitative target detection, and a value of 0.0 log10 IU/ml if the target was not detected. The characteristics of patients with or without detectable HBV RNA were compared by chi-square or Fisher's exact test for categorical variables and Kruskal-Wallis test for continuous variables.

**Results**

*Impact of an RT step on the measurement of HBV DNA*

To investigate the potential impact of an RT step on the measurement of HBV DNA we tested 191 clinical samples taken from 45 patients under treatment with NAs for up to 48 months with the cobas HBV and cobas HBV+RT tests (Hannover cohort). All samples (N=191) generated valid results (**Supplementary Table S2**). Testing these samples with the cobas HBV+RT test resulted in a proportionally biased positive linear correlation, with viral load results that were up to 4 log10 IU/ml higher than those detected with the standard cobas HBV test (**Figure 1A**; mean ± SD, 0.89 log10 IU/ml ± 1.33; paired Student’s *t-*test for mean difference [min, max] 0.14 log10 IU/ml [-0.70, 4.25], p<0.0001). Bland−Altman bias analysis confirmed these observations (**Figure 1B**), with differences being particularly evident in samples with low HBV DNA levels. A total of 92/191 samples tested had HBV viral loads <LLOQ (detectable or undetectable) [**Supplementary Table S2**]) with the cobas HBV test. Of note, 38 (41%) of these samples yielded levels ≥LLOQ when tested with the cobas HBV+RT step, with viral loads ranging from 1.06-4.43 log10 IU/mL (**Supplementary Table S3A**).

To confirm the results above we tested samples from a second, independent patient cohort. This unique cohort consisted of lamivudine-resistant patients starting salvage therapy with entecavir, entecavir + adefovir, or adefovir + lamivudine (DEFINE cohort).18 A total of 346 samples from seven selected time points (baseline, and Weeks 12, 24, 48, 72, 84, and 96) were tested with the cobas HBV and cobas HBV+RT tests. Data from 271 samples that had enough volume and generated valid complete paired observations were included in the analysis. As was the case in the previous analysis, HBV DNA levels were consistently higher with the cobas HBV+RT test, particularly at lower HBV DNA levels (**Figure 2A+B)**. Fourteen samples had viral levels <LLOQ with the cobas HBV test. Of these, five (36%) yielded levels ≥LLOQ with the cobas HBV+RT test (range: 1.12–3.07 log10 IU/mL [**Supplementary Table S3B**]).

To further evaluate the impact of RT in tests developed to quantify HBV DNA levels, samples from the DEFINE cohort were additionally tested with the Aptima HBV test, a TMA-based assay that includes an RT step. Of the samples available, 253 were within the overlapping linear range (1-9 log10 IU/mL) for quantitative HBV DNA for both the cobas HBV and Aptima HBV tests. Six samples that generated results <LLOQ with the cobas HBV test (five undetectable and one detectable but <LLOQ) generated results ≥LLOQ with the Aptima HBV test, with levels ranging from 1.11-2.92 log10 IU/mL (**Supplementary Table S3C**). Comparison of these results similarly indicated a proportionally biased positive linear correlation between the viral loads quantified with each test (**Figure 3A**). In line with previous findings, Bland−Altman bias analysis showed that the Aptima HBV quantified higher HBV DNA levels than the cobas HBV test (relative to the unity line) at the lower end of the test range, where HBV DNA is being suppressed by treatment, progressing closer to the unity line towards the upper end of the test range (**Figure 3B**).

Finally, the two tests incorporating the RT step, cobas HBV+RT and Aptima HBV, were compared to each other (**Figure 4**; **Supplementary Table S4**) and each was compared versus cobas HBV (without RT). Across each of the study time points, the differences between the cobas HBV test versus the cobas HBV+RT, and the cobas HBV versus Aptima HBV, were similar (**Table 2**). Comparison of samples showing quantification of HBV DNA with both the cobas HBV+RT and Aptima HBV indicated a near 1:1 positive linear correlation in HBV DNA viral load (**Figure 4A**). Bland−Altman bias analysis further revealed minimal alterations between the two different tests that include the RT step (**Figure 4B**).

*Longitudinal comparison of tests with and without an RT step*

As the documented differences between the HBV tests with and without the RT step were most prominent in samples with suppressed HBV DNA levels to <LLOQ, we decided to investigate the impact of NA treatment in more detail. For this purpose, we first compared HBV DNA results at baseline and after initiation of salvage therapy in the DEFINE cohort (Weeks 12, 24, 48, 72, 84, and 96).

Mean viral loads detected by the cobas HBV, cobas HBV+RT, and Aptima HBV tests at each time point are shown in **Figure 5**, along with nominal HBV DNA levels measured by cobas HPS. At baseline, discrepancies between the cobas HBV and the two tests that include RT were low (mean differences 0.13 and 0.09 log10 IU/m,l for cobas HBV+RT and Aptima HBV, respectively). In contrast, there were significant differences in the HBV viral load determined using the assays with RT versus no RT after only 12 weeks of NA therapy, ranging from 0.65-0.93 log10 IU/ml (p<0.0001). The difference between Aptima HBV and cobas HBV increased from 0.65 log10 IU/ml (p>0.0001) at Week 12 to a maximum of 1.08 log10 IU/ml (p<0.0001) at Week 84 of therapy. The difference between cobas HBV and cobas HBV+RT increased from 0.93 log10 IU/ml (p<0.001) at Week 12 to a maximum of 1.18 log10 IU/ml (p<0.0001) at treatment Week 48. Differences between the assays slightly decreased at Week 96 yielding 0.92 log10 IU/ml (p<0.0001) and 0.9 log10 IU/ml (p<0.0001) with the cobas HBV and the Aptima HBV, and the cobas HBV and the cobas HBV+RT, respectively. Of note, no statistically significant difference was documented between the cobas HBV+RT and the Aptima HBV (RT assays), with the exception of Week 24 (p=0.0426). Similarly, no significant differences were observed between the cobas HBV and the cobas HPS (no RT assays) at Weeks 72, 84 and 96 of therapy (**Table 2)**.

A subset of eight patients had samples available at all seven selected time points that were tested across all platforms. Descriptive statistics of HBV DNA levels across these patients for all tests are presented in the supplementary file (**Supplementary Table S5**) along with the mean and median log differences between tests (**Supplementary Table S6**). Analyses demonstrated that results were comparable for all four tests at baseline and at Week 96 (**Table 3**). Results for cobas HBV+RT vs Aptima HBV were comparable throughout all time points and the cobas HBV and HPS test results were comparable at all time points (apart from Week 12; **Table 3**). However, from Week 12 until Week 84, the Aptima HBV test results were significantly different from the cobas HPS (mean difference: 0.58-0.99 log10 IU/ml) and cobas HBV results (mean difference: 0.94-1.2 log10 IU/ml). Similarly, results with cobas HBV were significantly different from those with cobas HBV+RT (mean difference: 1.13-1.42 log10 IU/ml). Longitudinal assessment of the clinical samples throughout the treatment of these eight patients generally corroborated this picture (**Supplementary Figures S2A−S2H**). Longitudinal assessment of samples from all other patients with cobas HBV and Aptima HBV is provided in **Supplementary Figure S3**.

Similar results were documented when comparing the cobas HBV and the cobas HBV+RT tests the Hannover cohort. In the overall analyses, differences were evident from Week 12 until Week 192 (end of follow-up [**Supplementary Figure S4**]). Longitudinal data for individual patients are presented in **Supplementary Figure S5**.

**Difference of HBV RNA levels between samples with and without overestimation of circulating HBV DNA by TMA based assays**

To further evaluate whether the observed differences could be attributed to the amplification of HBV RNA, we accessed a third independent patient population (Liverpool cohort). In this particular cohort, HBV RNA was amplified by directly applying a specific PCR-based protocol. The cross-sectional Liverpool cohort comprised 101 patients, of whom 41 (40.6%) had detectable HBV RNA at a median level of 5.6 log10 copies/ml (interquartile range (IQR) 2.9-6.8 log10 copies/ml). Patients with detectable HBV RNA were more frequently females, of Asian ethnicity, HBeAg positive, and had higher HBV DNA levels by Aptima HBV than patients without HBV RNA (**Table 4**). Among the 101 patients, there were 53 on antiviral treatment and, at the time of sampling, they had received NA therapy for a median of 2.2 years (IQR 1.1-4.5). Of the treated patients, 23 (43.4%) had detectable HBV RNA at a median level of 5.6 log10 copies/ml (IQR 3.4-6.3 log10 copies/ml), and treatment duration was slightly shorter in patients with detectable HBV RNA relative to those without (**Table 4**). Among treated patients, median HBV DNA levels were 2.4 log10 IU/ml (ranging from <1 to 9.4 log10 IU/ml) in patients with HBV RNA versus <1 log10 IU/ml (ranging from <1 to 3.5 log10 IU/ml) in those without HBV RNA (p=0.0006). A total of 26 samples from patients with both detectable HBV RNA and quantifiable HBV DNA underwent repeat HBV DNA testing using real-time PCR (**Figure 6**). Overall, HBV DNA levels were higher with Aptima HBV than Xpert HBV, and the greatest difference was seen in patients on antiviral therapy (up to 1.5 log10 IU/mL). Details of ten treated patients who showed both detectable HBV RNA and quantifiable HBV DNA are shown in **Table 5**. After a median of 1.7 years of antiviral therapy, median HBV RNA levels were 5.9 log10 copies/ml and median HBV DNA levels by Aptima HBV were 3.5 log10 IU/ml. HBV DNA levels were a median of 1.0 log10 IU/ml higher by Aptima HBV relative to Xpert HBV, with similar results obtained with cobas HBV.

A review of medical records indicated that in three of these patients, after continuous NA therapy for between 2.8 and 4.1 years, persistent quantification of HBV DNA by Aptima HBV (the local routine test) led to a diagnosis of suboptimal treatment responses, increased frequency of follow-up, failed attempts at drug resistance testing by HBV DNA sequencing, unsuccessful treatment intensification to dual tenofovir/entecavir therapy, and distress for the patients when their adherence to treatment was repeatedly questioned. These three cases were resolved once testing by real-time PCR demonstrated effective HBV DNA suppression; details of one of the cases, with results of retrospective HBV DNA testing by cobas HBV and cobas HBV+RT, are shown in **Supplementary Figure S6**.

# Discussion

Accurate quantification of HBV DNA levels guides the clinical management of chronic HBV infection1,2. We investigated HBV DNA quantification in three independent cohorts of patients with the specific aim of assessing how inclusion of RT capability in the viral load assay influenced the results. The key finding was that tests with an RT step (cobas HBV+RT and Aptima HBV) differed in their quantification of HBV DNA for patients undergoing treatment with NAs compared with those without an RT step (cobas HBV, cobas HPS and Xpert HBV). While the cobas HBV+RT and Aptima HBV tests demonstrated good agreement with tests without the RT step at baseline, they appeared to consistently overestimate HBV DNA viral load in samples collected during NA therapy when HBV DNA is declining or even completely suppressed. Our data indicate that the difference is dependent on both the persistence of circulating HBV RNA during treatment and the decreasing HBV DNA to HBV RNA ratio. Subsequently, HBV DNA levels were fully or almost fully suppressed by real-time PCR but quantifiable by Aptima HBV in some patients.

HBV tests operate using different designs; tests such as cobas HBV or Xpert HBV use real-time PCR amplification,18,20 whereas tests such as Aptima HBV use TMA.19 TMA is not a DNA-specific detection tool since it includes intrinsic RT capability that can amplify HBV RNA once the target is detected, a process that creates RNA and DNA fragments.11 Most previous studies comparing labelled HBV DNA tests on different platforms have found overall correlation between the viral loads.5−9 However, an overestimation with the Aptima HBV test later in NA treatment compared with the cobas HPS test has been previously reported, but the authors suggested that further analysis into the samples would be needed to understand the effect.6 Our data indicate that presence of circulating HBV RNA in a subset of NA-treated patients is likely to be responsible for these reported discrepancies. It should be noted that the cobas HBV assay uses a DNA polymerase also capable of RT. Whilst this may theoretically also allow amplification of RNA in the standard setup of the cobas assay, estimated levels of HBV DNA were far higher by cobas HBV+RT and Aptima HBV. However, HBV DNA levels measured by cobas HBV were not different from those measured by Xpert HBV or the cobas HPS, which was the test widely used to determine currently recommended HBV DNA thresholds for the initiation and monitoring of NA therapy. Thus, we conclude that a theoretically low-level quantification of HBV RNA by cobas HBV would not be of clinical significance.

In our longitudinal evaluations, the difference in HBV DNA levels measured by assays with and without RT capability became clearly evident after the start of NA therapy, persisting in consecutive samples collected over long-term follow-up. In comparison with the marked effect of NA therapy on levels of HBV DNA, its influence on HBV RNA is limited.Differences in viral kinetics between HBV DNA and HBV RNA have been reported previously, with serum HBV RNA being reported as pre-genomic RNA in virus-like particles.17,22 At baseline HBV RNA levels have been described to be 1.2 to 2.2 log10 IU/mL lower than HBV DNA.17 This may explain why all tests evaluated quantified HBV DNA levels comparably at baseline. However, NA treatment inhibits the viral polymerase preventing the formation of HBV DNA from the pre-genomic HBV RNA template already encapsidated that can be enveloped and then secreted in plasma as virus-like particles that are resistant to plasma RNAse.17,23 Consequently, the balance becomes shifted towards RNA-containing particles, which become predominant as DNA levels decline over time. As a result, relatively higher HBV RNA levels are likely leading to the enhanced difference between HBV tests with and without an RT step during NA therapy, as documented in our study. This is clinically important because medical decisions during NA therapy are based on HBV DNA levels.1,2,4 Changes in viral load of 1 log10 IU/mL are used to define different levels of response or virological breakthrough, depending on the treatment guidelines. Therefore, overestimations >1 log10 IU/mL may lead to the misclassification of a patient and have a meaningful impact on their treatment. Undetectable, or at least not quantifiable, HBV DNA is considered as the definition of complete response to NA treatment and the detection of HBV RNA particles influencing the reported result can lead to an HBV DNA suppressed patient being classified as failing therapy, as it was the case in at least three patients of the Liverpool cohort.

Circulating HBV RNA has recently been suggested as a potential surrogate of transcriptionally active hepatic covalently closed circular DNA (cccDNA).15,24,25 While HBV DNA suppression through treatment with NAs is the main endpoint, undetectable HBV DNA does not indicate suppressed cccDNA activity.17 Although hepatitis B surface antigen (HBsAg) levels correlate better with cccDNA levels in this population, some of the HBsAg present in plasma could originate from integrated HBV DNA24,25; this makes HBsAg an unreliable surrogate for cccDNA. A more reliable surrogate could be HBV RNA. The difference in HBV RNA decline relative to HBV DNA suggests that cccDNA activity persists and is of interest for the development of new therapies that target clinical cure in chronic hepatitis B. Monitoring of HBV RNA could be useful in the assessment of treatment response to therapies such as core protein allosteric modulators, with the potential to predict sustained response that would be classified as clinical cure when monitoring response from therapies targeting the cccDNA or other steps in the viral life cycle.17 However, to achieve this an ability to distinguish between DNA and RNA would be critical. Most recently, undetectable HBV RNA has been suggested as indicator for a safe cessation of currently available NA therapy. The evaluation of tests with an RT step as well as the direct detection in a subset of patients (Liverpool cohort) afforded us the opportunity to investigate HBV RNA kinetics during NA therapy in more detail. It is clear from our study that HBV RNA persists in many patients and is likely to be detectable for several months on treatment. Data from the Hannover cohort support the persistence of HBV RNA in HBV DNA suppressed patients being treated with the most potent regimens (entecavir or tenofovir disoproxil fumarate) for up to 196 weeks. This is in line with recently published data reporting detectable HBV RNA in 30% and 14% of patients after 3 and 5 years of NA therapy, respectively. However, longitudinal data from our patients indicate two patterns of HBV RNA kinetics; one where HBV RNA persists despite treatment with NAs and another where HBV RNA declines with HBV DNA. This observation indicates the need for further studies.

Our study has several limitations. In the current study, the results generated with the cobas 6800 and Panther platforms were compared to historical results that were used for medical decision making and considered the benchmark, since there was not enough volume to retest samples in parallel with the reference method used in these original studies. Whilst samples from across the HBV genotypes were assessed it was beyond the scope of this analysis to determine if any of the effects were genotype specific. In addition, the conduct of the exploratory analysis with the Hannover cohort samples predates the availability of the Aptima HBV test so testing with this test on these samples was not possible.

Comparison of HBV DNA tests in this study suggests that HBV DNA levels are consistently overestimated, on average, with those tests that incorporate an RT step throughout the first 96 weeks of therapy as HBV RNA as well as HBV DNA can be amplified. Of note, this affects widely used tests that are based on TMA. The overestimation of viral load due to the interference of HBV RNA may lead to the misclassification of treatment responses with subsequent unnecessary clinical interventions.

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**Legends to the Figures**

**Figure 1. Comparison of cobas HBV and cobas HBV+RT (Hannover cohort).**

(A) Deming regression analysis of viral load quantification for cobas HBV vs. cobas HBV+RT and (B) Bland−Altman bias plot for measurement of HBV with both tests. UL, upper limit; LL, lower limit; OLS, ordinary least squares; CI, confidence interval

**Figure 2. Comparison of cobas HBV and cobas HBV+RT (DEFINE cohort).**

(A) Deming regression analysis of viral load quantification for cobas HBV vs. cobas HBV+RT and (B) Bland−Altman bias plot for measurement of HBV with both tests. Of 346 longitudinal observations available from seven selected time points (Day 1, Weeks 12, 24, 48, 72, 84, and 96), data from 241 samples were within the linear range of both tests and are included in these plots. UL, upper limit; LL, lower limit; OLS, ordinary least squares; CI, confidence interval

**Figure 3. Comparison of Aptima HBV vs. cobas HBV (DEFINE cohort).**

(A) Deming regression analysis of viral load quantification for Aptima HBV vs. cobas HBV and (B) Bland−Altman bias plot for measurement of HBV with Aptima and cobas HBV tests. Of 346 longitudinal observations available from seven selected time points (Day 1, Weeks 12, 24, 48, 72, 84, and 96), data from 253 samples were within the linear range of both tests and are included in these plots. UL, upper limit; LL, lower limit; OLS, ordinary least squares; CI, confidence interval

**Figure 4. Comparison of Aptima HBV vs. cobas HBV+RT (DEFINE cohort).**(A) Deming regression analysis of viral load quantification for Aptima HBV vs. cobas HBV+RT and (B) Bland−Altman bias plot for measurement of HBV with Aptima and cobas HBV+RT tests. Of 346 longitudinal observations available from seven selected time points (Day 1, Weeks 12, 24, 48, 72, 84, and 96), data from 281 samples were within the linear range of both tests and are included in these plots. UL, upper limit; LL, lower limit; OLS, ordinary least squares; CI, confidence interval

**Figure 5. Longitudinal assessment of mean on-treatment viral load.**

Longitudinal plot of mean viral load (across patients) from baseline to Week 96 of treatment with NAs for cobas HBV, Aptima HBV, cobas HBV+RT, and historical viral load.

**Figure 6. Difference in HBV DNA levels quantified by TMA (Aptima HBV) and real-time PCR (Xpert HBV)**

Each dot indicates a single patient (n=26). The solid dots indicate patients who were receiving antiviral therapy at the time of sampling (n=10) whose characteristics are detailed in Table 3.