# Hepatitis B Virus RNA Detection and a Mindful Use of Serum Hepatitis B Virus DNA and Hepatitis B Surface Antigen Measures in Clinical Practice

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In recent years, the diagnosis of hepatitis B virus (HBV) infection and disease has improved significantly thanks to the diagnostic performance of new molecular biology or immunometric assays that warrant better sensitivities and ranges of quantification.[1]

*Abbreviations: anti-HBe, antibody to hepatitis B e antigen; cccDNA, covalently closed circular DNA; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; NA, nucleos(t)ide analogue; PCR, polymerase chain reaction; pgRNA, pregenomic RNA; RT, reverse transcription; TMA, transcription-mediated amplification.*

*Received April 29, 2020; accepted May 20, 2020.*



*View this article online at wileyonlinelibrary.com.*
*DOI 10.1002/hep4.1554*

*Potential conflict of interest: Dr. Brunetto advises, is on the speakers' bureau for, and received grants from AbbVie; she advises Abbott, Janssen, and Roche and is on the speakers' bureau for and received grants from Gilead and MSD; she received grants from BMS.*

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Since the measure of serum HBV DNA became available, viral load has proved very useful to identify clinical settings of chronic HBV infection with different prognoses and outcomes and to tailor antiviral treatment according to individual response.[1] Hepatitis B surface antigen (HBsAg) serum levels were shown to vary during the different phases of chronic HBV infection, with the lowest levels in hepatitis B e antigen (HBeAg)-negative infection and their progressive decrease as the hallmark of response to antiviral therapy.[2] Furthermore, circulating HBsAg was proposed as a surrogate marker of covalently closed circular DNA (cccDNA) transcriptional activity; however, the hypothesis that HBV DNA sequences, which are integrated into the host genome, might be an alternative source of HBsAg production raised concerns about the usefulness of serum HBsAg quantification in clinical practice.[2] Actually, the occurrence of HBV DNA integration has been a well-known event for decades and is mainly associated with HBV oncogenesis.[3] A recent study accurately traced the *in vitro* dynamics of HBV DNA integration, showing its early occurrence at the onset of HBV infection at a rate (approximately 1 in $10^3$ cells) that would justify a large burden of HBV DNA integration (approximately 500 hepatocytes containing an HBV DNA integrant for any given gene).[4] These findings are in agreement with previous reports in animal models and humans; however, in the last few years the attention has shifted from the oncogenic potential of HBV integrants to their potential major role in HBsAg production. Wooddell et al.[5] investigated the cause of the poor response in terms of HBsAg decline in patients with HBeAg-negative chronic hepatitis B (CHB) treated with an RNA interference-based agent targeting HBV transcripts (small interfering RNA [siRNA]). The authors showed that HBsAg circulating in antibodies to HBeAg (anti-HBe)-positive chimpanzees

was mainly non-cccDNA derived and that both total liver HBV DNA and precore/pregenomic RNA (pgRNA) transcripts were lower in HBeAg-negative animals compared to HBeAg-positive animals. In addition, full-length HBV transcripts contained HBV sequences fused to the chimp genome, suggesting their origin from integrated HBV DNA. Finally, the administration of siRNAs targeting integrated HBV DNA sequences resulted in significant HBsAg declines.[5] The paper by Prakash et al.[6] in this issue of *Hepatology Communications* further explores this topic with an alternative technical methodology that provides an accurate analysis of the different intrahepatic HBV RNA species in a cohort of 76 individuals with chronic HBsAg who underwent liver biopsy.

# Could HBV RNA Detection in the Liver Contribute to a Better Understanding of HBV Biology?

The authors demonstrated that the ratios between RNA forms only derived from cccDNA (core RNA and 3′ precore RNA) and total intrahepatic HBV RNA reflect and correlate strongly with the ratios between HBV DNA and HBsAg serum levels. Furthermore, they showed that in patients who were HBeAg negative, HBV RNA resulting from the S RNA region was more than 1.6 $\log_{10}$ units higher than the core and 3′ end redundancy regions, suggesting that more than 90% of S RNA was derived from integrated HBV DNA rather than cccDNA transcription. The authors explained these findings as a consequence of the predominance of HBV DNA integration over cccDNA in HBeAg-negative livers, with significantly lower levels of cccDNA in patients who were HBeAg negative compared to patients who were HBeAg positive (0.01 vs. 1.4 copies/cell; $P < 0.001$).[7] A factor that could further explain the variation of the ratio between infected cells and hepatocytes with HBV integrates could be the result of their selective clonal expansion during the liver turnover that follows the typical alanine aminotransferase flares preceding HBeAg to anti-HBe seroconversion.[8] Together, these interpretations are consistent with the modest reduction of HBsAg serum levels reported after HBeAg to anti-HBe seroconversion despite a concurrent major decline of serum HBV DNA. However, in the HBeAg-negative phase, HBsAg serum levels are significantly lower (1.8 log) in individuals with HBeAg-negative infection than in patients with CHB; in low viremic HBeAg-negative/anti-HBe-positive carriers, the clinical outcome was better with lower HBsAg levels.[2] Future studies should explain whether this difference depends on the lower rate of hepatocytes with integrated HBV DNA and/or the reduction of hepatocytes with florid HBV infection or transcriptionally active cccDNA. Therefore, measuring not only intrahepatic cccDNA but also its transcriptional activity would improve the classification of HBsAg carriers. Although major efforts are aimed at developing accurate and standardized quantitative techniques, the need to perform a liver biopsy will limit its use in clinical practice.

# What About Serum?

Circulating, noninvasive, surrogate markers of cccDNA remain an unmet need, and detection of hepatitis B core-related antigen and HBV RNA is under investigation as a potential new diagnostic tool.[1] Serum HBV RNA results from a mixture of intact, spliced, and polyA-free pgRNA, and its composition varies depending on the phase of HBV infection and antiviral treatment.[1] Recent data showed that HBV RNA serum levels are correlated with intrahepatic HBV RNA, total HBV DNA, and cccDNA, suggesting that serum HBV RNA reflects cccDNA activity.[1] The most compelling data were those on serum HBV RNA kinetics during antiviral treatment, suggesting its potential role in predicting treatment outcome.[1,9] Particularly during nucleos(t)ide analogue (NA) treatment, the kinetics of serum HBV DNA and HBV RNA appear dissociated. Despite the rapid decline of serum HBV DNA after starting treatment, reduction of HBV RNA is slower and the HBV RNA to HBV DNA ratio increases significantly.[9] This finding results from the persistence of transcriptionally active cccDNA producing the pgRNA that is not reversely transcribed in HBV DNA because of the inhibition of HBV DNA polymerase induced by NAs. Accordingly, serum HBV RNA correlates with intrahepatic HBV RNA and with the ratio between intrahepatic HBV RNA and cccDNA, which is indicative of viral transcriptional activity.[9] Therefore, the persistence of serum HBV RNA during long-term

treatment is indicative of a transcriptionally active infection and associated with inevitable relapse in the case of treatment discontinuation.

## Does the Presence of Serum HBV RNA Interfere With HBV DNA Detection?

The presence of serum HBV RNA should not interfere with HBV DNA detection, but it depends on when (on or off NA treatment) and how (which assay is issued) HBV DNA is measured. This is consistently shown by Maasoumy et al.[10] in this issue of *Hepatology Communications* in their excellent methodology manuscript that addresses the issue of the specificity of quantitation of circulating HBV DNA by nucleic acid amplification techniques, which use different amplification strategies. The authors demonstrated that the inclusion of a reverse transcriptase step in the amplification process causes an overestimation of HBV DNA copies due to the detection of circulating HBV RNA. This inconvenience becomes particularly evident in subjects undergoing antiviral therapy with NAs when the imbalance between pgRNA production and its reverse transcription (RT) prompts the release of HBsAg particles containing HBV RNA into the blood. Accordingly, in samples obtained at baseline and before starting NAs, measurements of HBV DNA serum levels were comparable in standard real-time polymerase chain reaction (PCR) assays (such as cobas HBV [Roche Molecular Diagnostics, Pleasanton, CA] or Xpert HBV [Cepheid, Maurens-Scopont, France]) and assays able to co-amplify circulating HBV RNA, either by adding an extra step of RT (cobas HBV plus RT) to real-time PCR or using a real-time transcription-mediated amplification (TMA) (HBV Quant Assay; Hologic, Inc., Marlborough, MA). On the contrary, once the effective inhibition of HBV DNA production by NAs is evident, the two different methodologic approaches for serum HBV DNA amplification result in a significantly different quantification of viral load, with an overall overestimation of HBV DNA levels when HBV RNA co-amplification occurs. The abundance of pgRNA serves as a template during the RT amplification step, resulting in the nonspecific overestimation of real HBV DNA copy equivalents. Therefore, the use of the RT step that provides an overboosting of the overall viral load assay sensitivity taking advantage of the additional amplification of pregenomic HBV RNA forms has an advantage in some specific diagnostic settings, such as blood screening in blood banking. However, such a technical gimmick that increases sensitivity by HBV-RNA amplification becomes a boomerang in the specific contest of monitoring viremia during NA antiviral therapy. In fact, in this case, the acritical use of a positive result obtained by these assays would lead to the misclassification of patients, resulting in inappropriate clinical decisions. Nevertheless, the evidence that median HBV DNA serum levels by TMA during long-term NA treatment were significantly higher in patients with detectable HBV RNA compared to those with undetectable HBV RNA (median HBV DNA, 2.4 [range, <1-9.4] vs. median HBV DNA, <1 [range, <1-3.5] $\log_{10}$ IU/mL, respectively; $P = 0.0006$) suggests that a mindful combined use of both assays would allow the identification of two subgroups of patients treated with NAs (HBV RNA positive and HBV RNA negative) who might eventually benefit from different therapeutic approaches. Future studies should further investigate the potential additional information provided by TMA or real-time PCR plus RT to optimize the management of patients treated with NAs, provided the results of the assays are adequately interpreted.

In conclusion, both manuscripts[6,10] in this issue of *Hepatology Communication*s provide compelling evidence that emphasizes once again the clinical importance of the mandatory role of laboratory medical experts who help to identify the more appropriate assays needed to address specific clinic-pathologic questions of medical practice, such as the ones raised by monitoring the response to NAs.

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