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Quantification of HBsAg: Basic virology for clinical practice

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Author contributions: Ahn SH designed this article and revised it critically; Lee JM acquired information and wrote this article.

Supported by The Grant of the Bilateral International Collaborative R&D Program from the Ministry of Knowledge Economy and the Good Health R&D Project from the Ministry for Health, Welfare and Family Affairs, South Korea (A050021)

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Received: February 22, 2010 Revised: March 23, 2010

Accepted: March 30, 2010

Published online: January 21, 2011

Abstract

Hepatitis B surface antigen (HBsAg) is produced and secreted through a complex mechanism that is still not fully understood. In clinical fields, HBsAg has long served as a qualitative diagnostic marker for hepatitis B virus infection. Notably, advances have been made in the development of quantitative HBsAg assays, which have allowed viral replication monitoring, and there is an opportunity to make maximal use of quantitative HBsAg to elucidate its role in clinical fields. Yet, it needs to be underscored that a further understanding of HBsAg, not only from clinical point of view but also from a virologic point of view, would enable us to deepen our insights, so that we could more widely expand and apply its utility. It is also important to be familiar with HBsAg variants and their clinical consequences in terms of immune escape mutants, issues resulting from overlap with corresponding mutation in the *P* gene, and detection problems for the HBsAg variants. In this article, we review current concepts and issues on the quantification

of HBsAg titers with respect to their biologic nature, method principles, and clinically relevant topics.

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Key words: Hepatitis B virus; Hepatitis B surface antigen; Quantitative assay; Virology

Peer reviewers: Yukihiro Shimizu, MD, PhD, Kyoto Katsura Hospital, 17 Yamada-Hirao, Nishikyo, Kyoto 615-8256, Japan; Yogesh K Chawla, Professor, Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Lee JM, Ahn SH. Quantification of HBsAg: Basic virology for clinical practice. *World J Gastroenterol* 2011; 17(3): 283-289
Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i3/283.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i3.283>

INTRODUCTION

Hepatitis B virus (HBV) causes a wide range of clinical consequences, from acute and chronic infection to cirrhosis and hepatocellular carcinoma, and represents a global public health problem^[1,2]. Historically, HBV dates to 1967 when an unknown antigen in Australia was recognized to be associated with hepatitis type B, which was later referred to as the hepatitis B surface antigen (HBsAg)^[3]. Since then, HBsAg has served as a qualitative diagnostic marker for HBV infection. Notably, advances have been made in the development of quantitative HBsAg assays, which have allowed viral replication monitoring. A number of clinical studies have evaluated the clinical utility of HBsAg and suggested its potential roles. Yet, it needs to be underscored that a further understanding of HBsAg, not only from a clinical point of view but also from a virologic point of view, would enable us to deepen our insights, so that we could more widely expand and apply its utility. Therefore, in this article, we review current concepts and issues on the quantification of HBsAg titers

(qHBsAg) with respect to their biologic nature, method principles, and clinically relevant topics.

STRUCTURE AND MOLECULAR VIROLOGY OF HBsAg

Components of the viral structure

HBV belongs to *Hepadnaviridae* and is composed of the envelope, core, DNA genome, and viral polymerase. It has a circular form of partially double-stranded DNA and is approximately 3200 nucleotides in length^[4,5]. A 42-45 nm long HBV spherical form (Dane particle), which is the full virion with infectivity, can be visualized (Figure 1) under electron microscopy. It has two-layered shells. The outer shell is the envelope protein referred to as hepatitis B surface (HBs) protein, which is further divided into small, middle, and large HBs proteins (SHBs, MHBs and LHBs proteins, respectively), and the inner shell is a core protein referred to as the hepatitis B core protein in which viral polymerase and the HBV genome is enclosed. In addition to the abovementioned full virion, smaller non-infectious subviral particles are present in the serum; 17-25 nm spherical particles, mainly composed of SHBs protein, constitute the most abundant form, which is as much as 10000-fold in excess of the full infectious virion^[4,6]. Filamentous (or tubular) particles are another form, with a 20 nm diameter and variable length, and are composed of SHBs, MHBs, and the LHBs protein. The form of the HBV particles appears to be determined by the proportion of LHBs protein^[7]. All three forms can be detected in serum with commercial assays and are collectively referred to as HBsAg.

Synthesis and secretion

HBV has four distinct open reading frames (ORFs) that encode the envelope, core, polymerase, and X proteins. ORF S has three internal AUG codons encoding the SHBs, MHBs, and LHBs proteins, which correspond to the S, preS2 + S, and preS1 + preS2 + S domains, respectively (Figure 2). These proteins have a common carboxyl end but different amino ends^[8].

Like all other proteins, mRNA transcription is the first event to occur. Two 2.1 kb mRNAs for the M/SHBs proteins and a 2.4 kb mRNA for the LHBs protein are formed, and take a separate pathway from viral replication. Diverse transcription factors are involved and act on promoters, enhancers, and other regulatory elements, such as the glucocorticoid responsive element^[9,10]. LHBs and M/SHBs expression are thought to be independently regulated with different promoters; a typical TATA box is present in the LHBs promoter (S promoter I, SPI), whereas the TATA-less promoter, which usually has multiple initiation sites, is associated with the M/SHBs promoter, thus accounting for synthesis of distinct proteins from one mRNA. In patients with active viral replication, the protein expression pattern shows a predominance of the M/SHBs protein in contrast to a predominance of the LHBs protein in inactive carriers^[11]. After transcription, protein synthesis and glycosylation follows at the endoplasmic reticulum (ER) membrane resulting in a 226

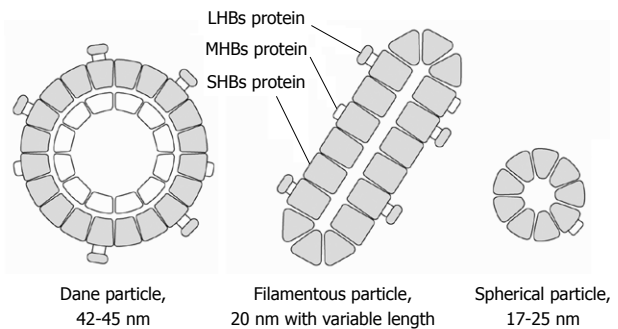


Figure 1 Schematic model of hepatitis B surface antigen structure. Three forms of hepatitis B surface (HBs) antigen (Dane particle, filamentous particle, and spherical particle) are visualized in serum by electron microscopy. These are composed of small, middle, and large hepatitis B surface proteins. LHBs: Large HBs proteins; MHBs: Middle HBs proteins; SHBs: Small HBs proteins.

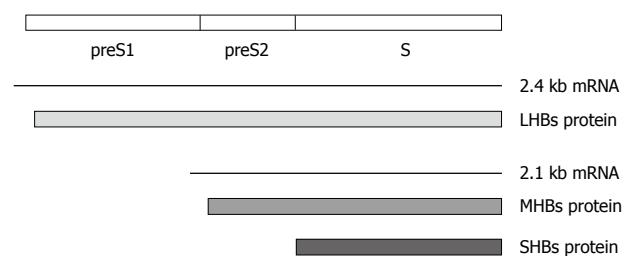


Figure 2 Schematic presentation of the S/preS1/preS2 gene, RNA transcripts, and translational products. Opening reading frame S has three internal AUG codons. Transcription to produce the 2.1 kb and 2.4 kb mRNAs first occurs after translation into small hepatitis B surface proteins (SHBs), middle hepatitis B surface proteins (MHBs), and large hepatitis B surface proteins (LHBs) ensues with different promoters.

amino acid SHBs protein, the MHBs protein with an additional 55 amino acids, and the LHBs protein with an additional 108-119 amino acids. Although the LHBs mRNA includes the M/SHBs sequence, it does not translate into the M/SHBs protein, and the ratio between the MHBs and SHBs protein is controlled by a complex mechanism, which is not fully understood^[12]. To form a full virion, a mixture of HBs proteins in a well-balanced ratio is utilized to envelop core particles in which SHBs and LHBs protein are indispensable^[13]. The virion is transported to the cell membrane through vesicles, and several conditions must be satisfied for successful secretion, because excess SHBs protein is required, whereas excess LHBs protein prevents secretion and causes dilatation of the ER with a ground-glass appearance^[14-16].

Function

The primary function of the HBs protein as a virologic structure is to enclose the viral components. It also plays a major role in cell membrane attachment to initiate the infection process. Several studies have confirmed the idea that the peptide in the preS1 domain is essential in this process, showing that it specifically binds to the human liver plasma membrane and can be inhibited by a monoclonal antibody^[17,18]. However, participation of the SHBs protein in attachment has also been suggested following identification of hepatocyte-bound endonexin II, which

specifically binds the SHBs protein^[19]. Additionally, from the host perspective, the HBs protein has the major antigenic components, including the *a* determinant, which is important for host-activated immunity. However, from a virologic perspective, it is postulated that excess HBs protein may divert such neutralizing antibody immune function away from the infectious virion^[20].

QUANTITATIVE HBsAg ASSAYS

Methods to detect HBsAg were first described in the 1970s using radioimmunoassays and enzyme immunoassays^[21,22]. Since then, various diagnostic techniques have been developed, which are mostly confined to qualitatively diagnose HBV in clinical practice. Recently, quantitative assay of HBsAg has been developed, and two commercially available assays will be briefly introduced here.

The Architect HBsAg QT (Abbott Diagnostic, Wiesbaden, Germany) is a chemiluminescent microparticle immunoassay, which is currently the method most widely used in clinical studies^[23]. The Architect HBsAg QT assay is a two-step immunoassay with flexible assay protocols, referred to as Chemiflex, for quantitatively determining human serum and plasma HBsAg concentrations. In the first step, the sample and hepatitis B surface antigen antibody (anti-HBs) coated with paramagnetic microparticles are combined. HBsAg present in the sample binds to the anti-HBs coated microparticles. After washing, acridinium-labeled anti-HBs conjugate is added. Following another wash cycle, pre-trigger and trigger solutions are added to the reaction mixture. The resulting chemiluminescent reaction is measured as relative light units (RLUs). A direct relationship exists between the amount of HBsAg in the sample and the RLUs detected by the Architect Immunoassay System optics. The Architect HBsAg is a fully automated system and can detect as low as 0.2 ng/mL of HBsAg with a dynamic range of 0.05-250.0 IU/mL^[24].

Elecsys HBsAg II (Roche Diagnostics, Indianapolis, IN, USA) is another method for quantitatively determining HBsAg^[25]. In the first incubation step, the antigen in the sample reacts with two biotinylated monoclonal HBsAg-specific antibodies and a monoclonal/polyclonal (sheep) HBsAg-specific antibody, labeled with a ruthenium complex, to form a sandwich complex. In the second step, streptavidin-coated microparticles are added, and the complex binds to the solid phase *via* interaction with biotin and streptavidin. The results are reported as a cutoff index (signal sample/cutoff), and the sample is considered reactive if the index is greater than 1.0.

CLINICAL APPLICATION OF QUANTITATIVE HBsAg

Correlation with serum HBV DNA

Although measuring serum HBV DNA is the gold standard for monitoring viral load, it is relatively expensive and not yet readily available in some areas. By contrast, the technique for detecting qHBsAg is fairly easy and inexpensive, and the primary aim of initial clinical studies

was to determine the relationship between qHBsAg and serum HBV DNA (Table 1). In 2004, Deguchi *et al.*^[23] first reported the clinical significance of a high qHBsAg in patients who were hepatitis B e antigen (HBeAg) positive as opposed to those with an antibody positive to the hepatitis B e antigen (anti-HBe), and that qHBsAg correlated well with the serum HBV DNA level ($r = 0.862$). Although there are some contradicting results on whether qHBsAg is correlated with serum HBV DNA^[26,27], it seems that they are correlated based on a number of studies^[28-33]. Further studies are required to investigate the possibility of using qHBsAg as an aid, if not an alternative, for HBV DNA.

Correlation with covalently closed circular DNA

An important qHBsAg issue is its association with covalently closed circular DNA (cccDNA). cccDNA is a minichromosome and acts as a viral template and replenishing pool for maintaining a chronic HBV infection^[34]. Therefore, it is essential to understand the biology of cccDNA when considering HBV therapy. However, to examine cccDNA, an invasive procedure is required, and qHBsAg has been suggested as a surrogate marker for cccDNA. Werle-Lapostolle *et al.*^[29] reported a significant decrease in cccDNA, qHBsAg, and serum HBV DNA with adefovir (ADV) therapy, and that there was a strong correlation between cccDNA and other variables. This observation was supported by subsequent studies; Wursthorn *et al.*^[35] and Chan *et al.*^[36] also showed that cccDNA was significantly correlated with qHBsAg, suggesting that serial monitoring of qHBsAg might act as an additional marker to evaluate treatment response during antiviral therapy.

Prediction of response to antiviral therapy

After the accumulation of data confirming that qHBsAg can be utilized as a viral monitor, qHBsAg has been evaluated as a predictor of virologic response. In a study by Chan *et al.*^[36] the sensitivity, specificity, and positive and negative predictive values for sustained virologic response (SVR) in patients treated with pegylated interferon (Peg-IFN) + lamivudine (LAM) were 86%, 56%, 43%, and 92%, respectively, with baseline qHBsAg concentrations less than 10000 IU/mL. According to the data of Manesis *et al.*^[31] achieving the complete elimination of HBsAg would probably require 10.6 years of effective LAM therapy or 5.4 years of a sustained response to interferon. Recently, the clinical usefulness of on-treatment qHBsAg in patients treated with Peg-IFN ± LAM has been suggested in both HBeAg positive and negative patients; a decline in qHBsAg of > 1 log IU/mL or specifically 0.5 and 1.0 log IU/mL at weeks 12 and 24, respectively, had high predictive value for SVR, and on-treatment HBsAg levels could be used as an early predictor of durable off-treatment response to Peg-IFN-based therapy^[32,33,37]. Of note is a long-term study by Marcellin *et al.*^[38] in which 35% of patients who had qHBsAg < 1500 IU/mL at week 12 eventually cleared the HBsAg by 4 years post-treatment, which supports the clinical utility of qHBsAg. Furthermore, qHBsAg was superior to cccDNA and serum HBV DNA for predicting SVR in patients undergoing Peg-IFN-based therapy with receiver

Table 1 Recent clinical studies with quantification of hepatitis B surface antigen titers in hepatitis B virus infection

Author	Antiviral therapy	Correlation	Prediction	Clinical results
Deguchi <i>et al</i> ^[23]	-	HBV DNA	-	qHBsAg is higher in HBeAg(+)
Chen <i>et al</i> ^[28]	-	HBV DNA	-	qHBsAg is higher in HBeAg(+) and high HBV DNA levels, whereas qHBsAg is low in low HBV DNA level CHB
Werle-Lapostolle <i>et al</i> ^[29]	ADV	cccDNA, HBV DNA	-	HBsAg and cccDNA decrease with ADV
Kohmoto <i>et al</i> ^[30]	LAM	HBV DNA	-	qHBsAg is helpful for early detection of drug resistant strains
Wursthorn <i>et al</i> ^[35]	Peg-IFN + ADV	cccDNA	-	Peg-IFN + ADV decreases cccDNA and HBsAg, which are well correlated
Chan <i>et al</i> ^[36]	Peg-IFN + LAM	cccDNA	Low baseline qHBsAg can predict SVR	Peg-IFN + LAM decreases cccDNA and HBsAg, which are well correlated
Manesis <i>et al</i> ^[31]	IFN vs LAM	HBV DNA	Low baseline qHBsAg can predict SVR	IFN induces sharper decrease in qHBsAg than LAM
Wiegand <i>et al</i> ^[27]	FAM ± LAM	HBV DNA (not correlated)	Decline of qHBsAg can predict HBsAg loss	2 log drop to below 100 IU/mL is associated with HBsAg clearance
Moucarri <i>et al</i> ^[32]	Peg-IFN	HBV DNA	Early qHBsAg drop can predict SVR	qHBsAg may be useful to optimize Peg-IFN therapy
Brunetto <i>et al</i> ^[33]	Peg-IFN ± LAM vs LAM	HBV DNA	On-treatment qHBsAg decline can predict sustained HBsAg loss	qHBsAg < 10 IU/mL at week 48 and 1 log decline predict sustained HBsAg clearance to optimize treatment strategy
Lau <i>et al</i> ^[37]	Peg-IFN ± LAM	-	On-treatment qHBsAg can be used as an early predictor of SVR	In HBeAg(+) patients, qHBsAg reduction through weeks 12, 24 and 48 were higher in patients with HBeAg seroconversion
Marcellin <i>et al</i> ^[38]	Peg-IFN ± LAM	-	qHBsAg at week 12 can predict long-term HBsAg clearance	35% of patients who had qHBsAg < 1500 IU/mL at week 12 cleared up HBsAg by 4 yr post-treatment
Lu <i>et al</i> ^[39]	Peg-IFN ± LAM	cccDNA	qHBsAg was superior to cccDNA and serum HBV DNA in predicting SVR	Area under ROC curve with qHBsAg, cccDNA and HBV DNA was 0.769, 0.734, and 0.714, respectively, for predicting SVR
Brunetto <i>et al</i> ^[65]	Peg-IFN ± LAM	-	On-treatment qHBsAg can be used as an early predictor of SVR	On-treatment decline in HBsAg appears to be genotype dependent. Genotype B patients showed the most rapid and pronounced decline
Hou <i>et al</i> ^[66]	Peg-IFN vs LAM	-	-	Peg-IFN was superior to ADV in HBeAg seroconversion and qHBsAg decline in LAM-resistant patients

HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; qHBsAg: Quantification of HBsAg titers; HBeAg: Hepatitis B e antigen; CHB: chronic hepatitis B; ADV: Adefovir; LAM: Lamivudine; Peg-IFN: Pegylated interferon; cccDNA: Covalently closed circular DNA; FAM: Famciclovir; SVR: Sustained virologic response; ROC: Receiver operating characteristic.

operating characteristic (ROC) curves of 0.769, 0.734, and 0.714, respectively^[39].

MOLECULAR HBsAg VARIANTS

Much of our understanding of the biologic nature of the HBs protein has been gathered from various mutation and truncated protein experimental models^[40,41], and it is worthwhile to address the relevance and consequences of HBsAg variants from a clinical point of view. Besides the lack of HBV proof-reading capacity^[42], the development of an HBsAg mutation can be attributed to immune pressure from extensive vaccination programs, injections of hepatitis B immunoglobulin (HBIG) following liver transplantation, and the overlap with a mutation in the corresponding *P* gene.

Immune escape mutants

Since the introduction of an extensive vaccination program, concerns about HBsAg variants have increased after an HBV infection occurred in infants who had received an HBV vaccination and who had mounted an adequate anti-HBs response. This was presumed to be caused by immune selection pressure, because the HBsAg *a* determinant is the major epitope for HBV vaccination^[43,44]. Changes in the amino acids within the *a* determinant, particularly between 137-147, disable surface antigen domain

recognition by neutralizing antibodies. Of importance is the G145R mutant, because it is the most common and is replication competent with stability^[45]. In a Taiwanese epidemiological study, it was reported that the prevalence of the *a* determinant mutation had increased from 7.8% to 28.1%, after 15 years of a universal vaccination program^[46]. Fortunately, in the following years, neither the percentage increase nor any significantly adverse events with an outbreak of HBV infection actually occurred; thus, a mass vaccination program is continuing with adequate justification^[47].

In addition to the extensive vaccination program, the wide use of HBIG following liver transplantation adds selection pressure to HBV. Ten of 20 patients who developed recurrent HBV infection despite hepatitis B immunoglobulin prophylaxis had amino acid substitutions involving the *a* determinant, which were mostly absent in pretransplantation clones^[48].

Overlap and mutation in the *P* gene

A mutation in the *P* gene from prolonged oral nucleos(t)ide therapy can cause an altered sequence in the corresponding *S* gene due to overlap of the two genes^[49], which is summarized in Table 2^[50]. The nucleotide at rt204 in the *P* gene is associated with resistance to LAM, telbivudine (LdT), and entecavir (ETV), and the rtM204V/I mutation typically results in a sI195M, sW196S, sW196L or a termi-

Table 2 Mutations in viral polymerase gene induced by oral antiviral agents and corresponding changes in hepatitis B surface antigen

Polymerase domain	Mutation in polymerase	Oral antiviral agents	Corresponding change in HBsAg	
B	rtI169T	ETV	sF161H/L	
	rtL180M	LAM, LdT	No change	
	rtA181T	ADV, TFV, LAM, LdT	sW172 ¹	
	rtA181T	ADV, TFV, LAM, LdT	sW172L	
	rtA181V	ADV, TFV, LdT	sL173F	
	rtT184A	ETV	No change	
	rtT184C	ETV	sL175F + sL176V	
	rtT184I	ETV	No change	
	rtT184G	ETV	sL176V	
	rtT184S	ETV	sL175F	
	rtT184M	ETV	sL176 ¹	
	rtT184L	ETV	sL175F	
	C	rtS202C	ETV	No change/sS193F
		rtS202I	ETV	sV194F/S
rtS202G		ETV	No change/sS193L	
rtM204V		LAM	sI195M	
rtM204I		LAM, LdT	sW196 ¹ /S/L	
D	rtN236T	ADV, TFV	After end of HBsAg	
E	rtM250I	ETV	After end of HBsAg	
	rtM250V	ETV	After end of HBsAg	

¹Stop codon. Modified from reference^[50]. HBsAg: Hepatitis B surface antigen; ETV: Entecavir; LAM: Lamivudine; LdT: Telbivudine; ADV: Adefovir; TFV: Tenofovir.

nal codon in the overlapping *S* gene^[50]. In previous studies, LAM selected HBsAg mutants with reduced anti-HBs binding capacity, and secretion of HBsAg was prevented with a mutant strain due to the stop codon^[51,52]. rtI181 is another important site that confers resistance to ADV and/or LAM/LdT. Recently, Warner and Locarnini demonstrated that rtA181T caused a secretory defect and had a negative effect on secretion of the wild-type HBV virion because of a concomitant change in the envelope protein at sW172^[53]. Similarly, ETV-associated rtI169T/sF161L leads to a decrease in HBsAg immunoreactivity^[54].

The clinical significance of overlap and a common mutational substitution in the *S* and *P* gene was further extended by Kamili *et al.*^[55] who demonstrated a successful experimental infection with the rtV173L, rtL180M, and rtM204V HBV mutants that resulted in sE164D and sI195M despite high anti-HBs levels in chimpanzees^[55]. Furthermore, the possibility of a *vice versa* phenomenon with respect to an extensive vaccination program might be postulated in that HBsAg mutants from selection pressure might harbor the corresponding *P* gene mutation, resulting in primary resistance to antiviral agents and therapy failure with these agents.

Detection and variants of HBsAg

As described above, an HBsAg mutation leads to diverse effects, such as decreased secretion and reduced binding capacity to anti-HBs. Of note is that not only a mutation in the *a* determinant but also in the *S* promoter or a deletion in the preS region can cause such effects^[56,57]. These effects may hamper the diagnostic performance of commercial assays, and several reports have pointed to the

problem of not being able to detect HBV with an *a* determinant mutation^[58,59].

An occult HBV infection is defined as the persistence of the HBV genome in HBsAg negative individuals, and one of the explanations for occult HBV infection is a mutation in HBsAg and undetectability by available assays^[60]. Both the Architect HBsAg QT and Elecsys HBsAg II seem to reliably detect HBsAg mutants with high sensitivity and specificity^[24,25,61]. However, further studies are needed to validate such detection ability, because new or complex combinations of mutations can arise in this era of antiviral agents and extensive vaccination.

FUTURE PERSPECTIVES

Despite progress on qHBsAg, a number of unanswered questions still remain. Precise control mechanisms for HBsAg production in HBV are poorly understood. A discrepancy between qHBsAg and serum HBV DNA exists, although a correlation has been documented. Further research on the virologic nature of HBV could answer these two questions. Meanwhile, the role of qHBsAg in the clinical field is being actively investigated, especially as a predictor to virologic response. Of particular interest is the potential role of qHBsAg for defining the end point of oral antiviral therapy. Current American Association for the Study of Liver Diseases and European Association for the Study of the Liver guidelines with respect to an end point for therapy are unsatisfactory, because reversion to HBeAg positivity does occur after terminating therapy, and the loss of HBsAg is infrequently encountered^[62,63]. In this regard, qHBsAg might be particularly helpful in patients with undetectable HBV DNA, even with a highly sensitive polymerase chain reaction assay^[64]. In contrast to undetectable HBV DNA, which provides no further information for the virologic responders, HBsAg is continuously shed and detected and, based on the observations of previous studies, qHBsAg with serial monitoring in patients with undetectable HBV DNA may be utilized to determine the end point of therapy and validate the durability of antiviral agents.

CONCLUSION

HBsAg is produced and secreted through a complex mechanism that is still not fully understood. Nevertheless, quantification of serum HBsAg is currently available and there is an opportunity to make maximal use of qHBsAg to elucidate its role in clinical fields. However, a deep understanding of the virology is necessary, and it is also important to be familiar with HBsAg variants and their clinical consequences in terms of immune escape mutants, issues resulting from overlap with corresponding mutation in the *P* gene, and detection problems for the HBsAg variants. Unanswered questions need to be resolved through further qHBsAg research.

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S- Editor Wang JL L- Editor O'Neill M E- Editor Lin YP