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 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. 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

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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.
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\[43\]. 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\[44\]. 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\[45\]. 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\[46\].

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\[50\]. 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 1, 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 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 proteins are indispensible\[13\]. 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. 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.

QUANTITATIVE HBsAg ASSAYS

Methods to detect HBsAg were first described in the 1970s using radioimmunoassays and enzyme immunoassays. 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. 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.

Elecsys HBsAg II (Roche Diagnostics, Indianapolis, IN, USA) is another method for quantitatively determining HBsAg. 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. 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, it seems that they are correlated based on a number of studies. 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. 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. 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. and Chan et al. 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., 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. 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 HBsAg positive and negative patients; a decline in qHBsAg of $\geq 1$ 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. Note is a long-term study by Marcellin et al. 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...
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, 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, 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. 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 replication competent with stability. 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. 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.

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.

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, which is summarized in Table 2. The nucleotide at rt204 in the P gene is associated with resistance to LAM, telbivudine (LT), and entecavir (ETV), and the rtM204V/I mutation typically results in a s195M, 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

<table>
<thead>
<tr>
<th>Polymerase domain</th>
<th>Mutation in polymerase</th>
<th>Oral antiviral agents</th>
<th>Corresponding change in HBsAg</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>rtI69T</td>
<td>ETV</td>
<td>sF161H/L</td>
</tr>
<tr>
<td></td>
<td>rtL180M</td>
<td>LAM, LdT</td>
<td>No change</td>
</tr>
<tr>
<td></td>
<td>rtA181T</td>
<td>ADV, TFV, LAM, LdT</td>
<td>sW172</td>
</tr>
<tr>
<td></td>
<td>rtA181T</td>
<td>ADV, TFV, LAM, LdT</td>
<td>sW172L</td>
</tr>
<tr>
<td></td>
<td>rtA181V</td>
<td>ADV, TFV, LdT</td>
<td>sI175F</td>
</tr>
<tr>
<td></td>
<td>rtT184A</td>
<td>ETV</td>
<td>No change</td>
</tr>
<tr>
<td></td>
<td>rtT184C</td>
<td>ETV</td>
<td>sL175F + sL176V</td>
</tr>
<tr>
<td></td>
<td>rtT184I</td>
<td>ETV</td>
<td>No change</td>
</tr>
<tr>
<td></td>
<td>rtT184G</td>
<td>ETV</td>
<td>sL176V</td>
</tr>
<tr>
<td></td>
<td>rtT184S</td>
<td>ETV</td>
<td>sL175F</td>
</tr>
<tr>
<td></td>
<td>rtT184M</td>
<td>ETV</td>
<td>sL176</td>
</tr>
<tr>
<td></td>
<td>rtT184L</td>
<td>ETV</td>
<td>sL175F</td>
</tr>
<tr>
<td>C</td>
<td>rtS202C</td>
<td>ETV</td>
<td>No change/sI195F</td>
</tr>
<tr>
<td></td>
<td>rtS202i</td>
<td>ETV</td>
<td>sV194F/S</td>
</tr>
<tr>
<td></td>
<td>rtS202G</td>
<td>ETV</td>
<td>No change/sI193L</td>
</tr>
<tr>
<td></td>
<td>rtM204V</td>
<td>LAM</td>
<td>sI195M</td>
</tr>
<tr>
<td></td>
<td>rtM204I</td>
<td>LAM, LdT</td>
<td>sW196/S/L</td>
</tr>
<tr>
<td>D</td>
<td>rtN236T</td>
<td>ADV, TFV</td>
<td>After end of HBsAg</td>
</tr>
<tr>
<td>E</td>
<td>rtM230I</td>
<td>ETV</td>
<td>After end of HBsAg</td>
</tr>
<tr>
<td></td>
<td>rtM230V</td>
<td>ETV</td>
<td>After end of HBsAg</td>
</tr>
</tbody>
</table>

Stop codon. Modified from reference[60]. HBsAg: Hepatitis B surface antigen; ETV: Entecavir; LAM: Lamivudine; LdT: Telbivudine; ADV: Adefovir; TFV: Tenofovir.

problem of not being able to detect HBV with an a determinant mutation[66,69].

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[24,25]. 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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January 21, 2011 | Volume 17 | Issue 3

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