SIEMENS

www.siemens.com/diagnostics

Detection of Hepatitis B Virus Surface Antigen (HBsAg) Mutants by IMMULITE® 2000 HBsAg Assay

Jason Lei, Ph.D.
Senior Scientist, Department of Molecular Biology, Research and Development
Bruce A. Campbell, Ph.D.
Director, Department of Molecular Biology, Research and Development
Carol Cervantes, B.S.
Director, Product Support Infectious Diseases
Liza Bronstein, M.S.
Associate Director, Product Support Infectious Diseases
Dickson Hong, Ph.D.
Director, Department of Infectious Diseases, Research and Development
Dennis Sustarsic, Ph.D.
Vice President, Research and Development

Answers for life.

Introduction

Synopsis

Recently, it has been shown that hepatitis B virus surface antigen (HBsAg) mutants can cause false-negative results when evaluated by commercial immunoassays. In the study reported here, patient sera and recombinant proteins representing different HBsAg mutations were evaluated by the DPC IMMULITE® 2000 HBsAg assay. The results show that the IMMULITE 2000 HBsAg assay can detect both wild-type and mutant HBsAg. The IMMULITE 2000 HBsAg is a robust assay for the detection of hepatitis B virus infection.

Epidemiology

Hepatitis B virus (HBV) has infected over two billion people worldwide.[1] Most people (more than 95 percent) recover from the infection with the development of immunity.[2] About one to five percent of the infections result in the virus carrier state for the life of the individual (chronic infection).[2] There are approximately 350 million HBV carriers worldwide,[1,3] and the world can be categorized into areas of high, intermediate and low prevalence of chronic infection.[1]

In highly endemic areas such as Southeast Asia, China, sub-Saharan Africa and parts of South America, over eight percent of the population are chronic carriers of HBV.[1] In moderately endemic areas, such as Eastern and southern Europe, the Middle East, Japan, and parts of South America, two to seven percent of the population are chronic carriers.[1] In contrast, where HBV infection is low, such as in North America, Northern and Western Europe, and Australia, only one-half to two percent of the population are chronic carriers.[1] HBV chronic carriers are the main source for HBV infection. Detection of infection through blood screening and prevention of transmission through vaccination are therefore critical for reducing the impact of HBV infection on public health.

HBV is very heterogeneous at the DNA level and can be classified into eight genotypes designated A through H (on the basis of a greater than eight percent DNA sequence difference between genotypes).[1,4] It is well documented that HBV genotypes are associated with distinct geographical regions. For example, genotypes B and C predominate in Asia, genotype E in West Africa, genotype F in Central and South America, and genotype G in the US and France.[1] In addition, HBV genotypes may have different biological characteristics and different patterns of clinical infection.[1] For example, genotypes B and C have been correlated with different amounts of HBV e antigen clearance and liver damage during infection, while genotype A has been shown to be more responsive to standard interferon therapy.[1]

Viral particle structure

HBV is a small DNA virus with a partially double-stranded genome of about 3,200 nucleotides.[5] The genome is extremely compact and contains sequences for four overlapping genes that encode structural and nonstructural proteins of the virus particles (Figure 1).[6] Most of the infectious virus particles are spherical, approximately 43 nm in diameter with a complex structure (Figure 2).[7] The core particle consists of HBV genomic DNA, viral polymerase and other proteins enclosed in the core protein coat.[8] The core particle is surrounded by a membrane containing large, medium, and small hepatitis B virus surface antigen (HBsAg) proteins. The small HBsAg (Figure 3) is the most abundant HBV protein. It has 226 amino acid residues[6] and is the most important diagnostic marker of HBV infection. The main antigenic determinants (epitopes) on the external surface of HBV particles are primarily within the major hydrophilic region (MHR) from amino acids 100 to 160 of HBsAg. Within the MHR is a highly conserved domain from amino acids 124 to 147 known as the "a" determinant (Figure 3).[9] Numerous subtype-specific determinants within the MHR make HBV very heterogeneous, and it has been differentiated into at least nine serologic subtypes (ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4, adrq+ and adrq-).[10] Despite this heterogeneity, the conserved "a" determinant enables the detection of HBsAg and thus HBV infection by most immunoassays.[9]

HBsAg mutants

The heterogeneity of the HBV at the level of DNA and protein is attributable to the infidelity of the HBV polymerase during replication of the viral genome. In fact, the polymerase error rate has been reported to be about one per 10^7 bases[11] During an active HBV infection, which can generate as many as 10^{11} viral particles per day, this high error rate can produce as many as 10^7 base errors per day.[11] Due in part to the overlapping nature of the HBV protein reading frames (Figure 1), some of the mutants generated cannot survive or replicate as efficiently

as the wild type. Nevertheless, an increase in HBsAg mutants has been associated with the selective pressure exerted by the available hepatitis B treatments (e.g., active immunization and passive immunoprophylaxis).[11-13] The MHR is the major region involved in this immunoselection, and many strains with mutations in this region survive. These MHR mutations can also affect detection by commercial immunoassays because the MHR region which includes the "a" determinant contains the epitopes targeted by the antibodies used in many HBV immunodiagnostic tests.

The most commonly reported mutation in HBsAg occurs at amino acid residue 145, where the wild-type glycine is replaced by an arginine (G145R) (Figure 4).[11-14] The presence of this G145R mutation has allowed some patient specimens to escape detection by certain commercial immunoassays.[11] Occasionally, mutants carrying other amino acid changes within the "a" determinant (e.g., G130D, T131N, T143L, T143M, D144A) have also been reported to evade detection by some commercial HBsAg immunoassays (Figure 4).[11, 15, 16]

This report details the performance of the IMMULITE 2000 HBsAg assay for the detection of natural and recombinant HBsAg proteins that contain the most stable and common mutation: the G145R mutation. In addition, other recombinant HBsAg mutants with single or multiple amino acid substitutions or multiple amino acid insertions were evaluated. The results clearly demonstrate that the IMMULITE 2000 HBsAg assay can detect wild-type HBsAg and a variety of HBsAg mutants within the critical "a" determinant and other MHR regions.

Figure 1. HBV genome and open reading frames for the corresponding encoded proteins.

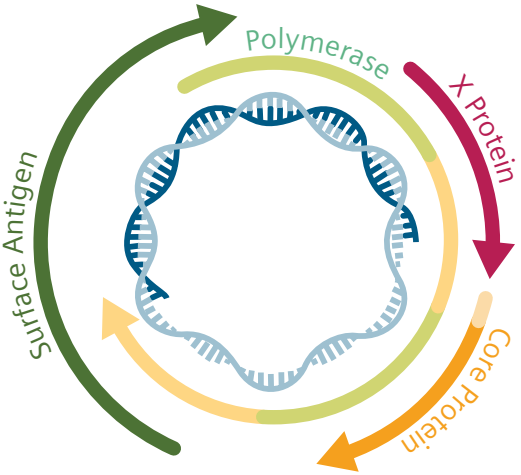


Figure 2. Structure of the HBV particle.

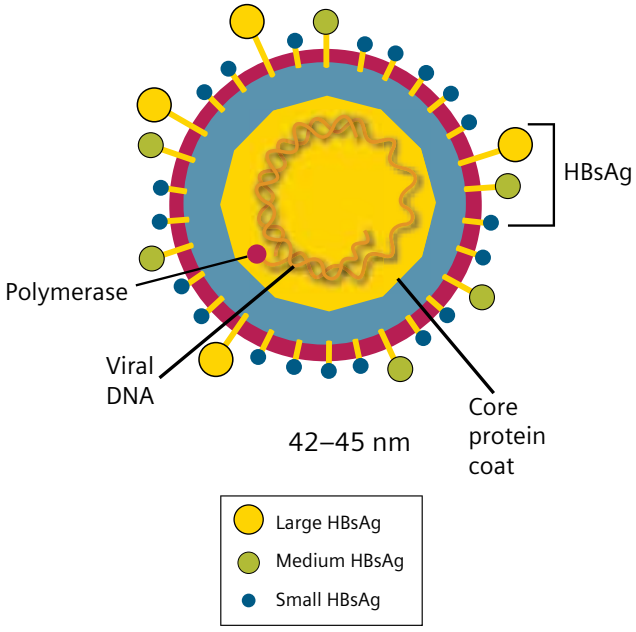
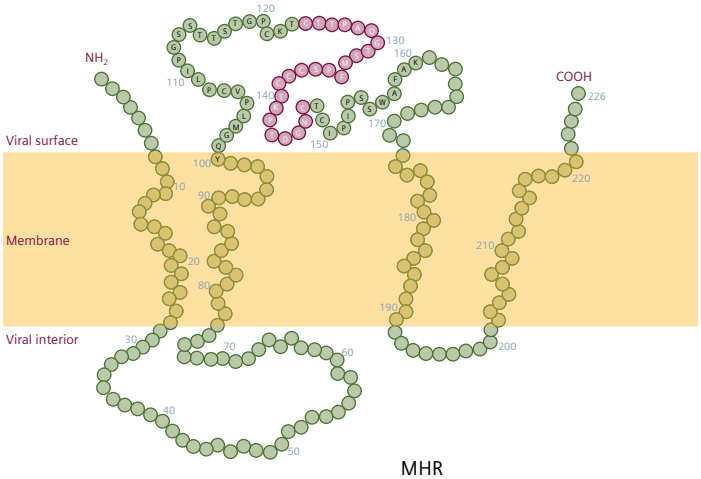
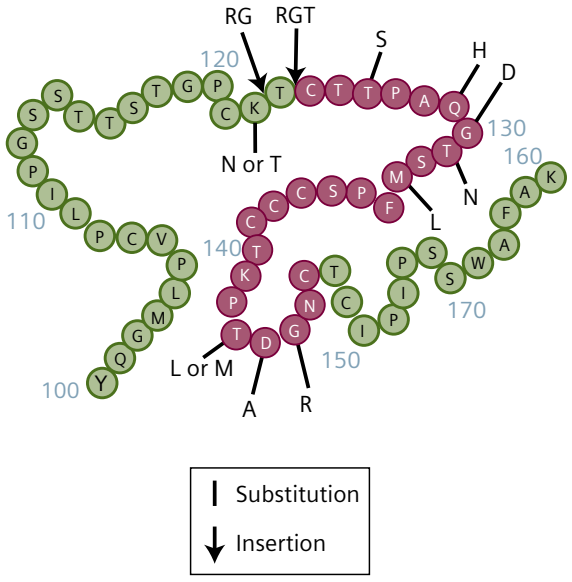


Figure 3. Model for the protein structure of small HBsAg. Amino acids are represented by circles. Circles with letters represent the MHR (amino acids 100 to 160) and the black circles with letters represent the "a" determinant (amino acids 124 to 147).



MHR
(amino acids 100 to 160)
"a" determinant
(amino acids 124 to 147)

Figure 4. The MHR (amino acids 100 to 160) and the "a" determinant (amino acids 124 to 147). The letters in circles denote HBsAg wild-type amino acids, and the letters outside circles are the mutations evaluated in this study. The mutations are indicated by type.



Methods

The IMMULITE 2000 HBsAg assay is a qualitative assay available for use on the IMMULITE 2000 automated analyzer with an analytical sensitivity of 0.063 IU/mL (WHO IS for HBsAg, NIBSC 80/549). It is a two-step, solid-phase chemiluminescent enzyme immunoassay. The solid phase consists of a polystyrene bead coated with an antibody directed against the HBsAg. The patient specimen is added to a reaction tube containing the coated bead, and then undergoes a 30-minute incubation at 37°C with intermittent agitation. During this time, HBsAg in the sample binds to the HBsAg antibody–coated bead. After the incubation, a centrifugal wash removes unbound material, and an alkaline phosphatase–labeled HBsAg antibody is introduced for a second 30-minute incubation. The labeled HBsAg antibody binds to the immobilized HBsAg in the sample. Unbound antibody is then removed by another centrifugal wash, chemiluminescent substrate is added, and the reaction tube is incubated with agitation for 5 minutes. During this final incubation, the chemiluminescent substrate undergoes hydrolysis in the presence of alkaline phosphatase. The photon output measured by the luminometer is correlated to the presence of HBsAg in the sample.

The IMMULITE 2000 HBsAg assay has been shown to be effective in detecting HBsAg mutants. Results in one study showed that it was better than competitor assays at detecting mutant HBsAg.[17] The number of mutants was too low, however, to predict performance with a higher number of mutants. Consequently, the current study included a larger number and variety of HBsAg mutants. Two approaches were used. First, known HBV-positive patient specimens from Taiwan (where the number of HBV carriers is high and HBsAg mutants can be common) were evaluated by the IMMULITE 2000 HBsAg assay. In parallel, HBV DNA sequences were amplified from the positive patient specimens and evaluated by DNA sequence analysis. Specimens suspected of being below the limit of detection for the IMMULITE 2000 HBsAg assay were further evaluated by recombinant DNA technology and quantitative PCR. Secondly, using recombinant DNA techniques, a series of documented, naturally occurring HBsAg mutants was produced in vitro and analyzed with the IMMULITE 2000 HBsAg assay.

Results

HBV patient specimens evaluated by IMMULITE 2000 HBsAg assay A total of 13 HBV-positive patient serum specimens from Taiwan were evaluated by the IMMULITE 2000 HBsAg assay. The data (Table 1) show that 12 of the 13 specimens tested positive in the assay; one (specimen 2) tested negative. This specimen was also negative when evaluated by BioRad Genetic Systems HBsAg EIA 2.0 (Hercules, CA).

Table 1. IMMULITE 2000 HBsAg assay results for HBV-positive patient specimens.

Specimen	Result
1	Positive
2	Negative
3	Positive
4	Positive
5	Positive
6	Positive
7	Positive
8	Positive
9	Positive
10	Positive
11	Positive
12	Positive
13	Positive

DNA sequence analysis of MHR domain amplified from patient specimens

The HBV DNA corresponding to the MHR region of all 13 HBV-positive patient specimens was evaluated by DNA sequence analysis to look for nucleotide changes that resulted in amino acid mutations. HBV sequences were amplified from patient specimens by polymerase chain reaction (PCR), and five independent clones were evaluated from each specimen. The DNA sequences from the clones were used to deduce the MHR region amino acid sequences via computer analysis (data not shown). The observed mutations were localized to two amino acids within the MHR and are summarized in Table 2. Four specimens (1 through 4) had a mutation resulting in a change at amino acid 145: the G145R mutation. In contrast, all but one of the 45 clones sequenced from the remaining nine specimens had the wild-type amino acid at this position. In addition, specimens 1 through 4 seemed to be mixed infections because some of the clones from these specimens also had a mutation at residue 122 (K122N or K122T). Mixed infections in HBV patients (including those containing the K122N mutation) have been documented in the literature.[18]

Table 2. Summary of amino acid sequences deduced from HBsAg DNA sequences from the 13 HBV specimens studied.

Specimen	Amino acid 122 (No. of clones)*	Amino acid 145 (No. of clones)*
1	K† (4) T (1)	R (5)
2	K (4) N (1)	R (5)
3	K (2) N (3)	R (5)
4	K (2) N (3)	R (5)
5–13	K (45)	G† (44) R (1)

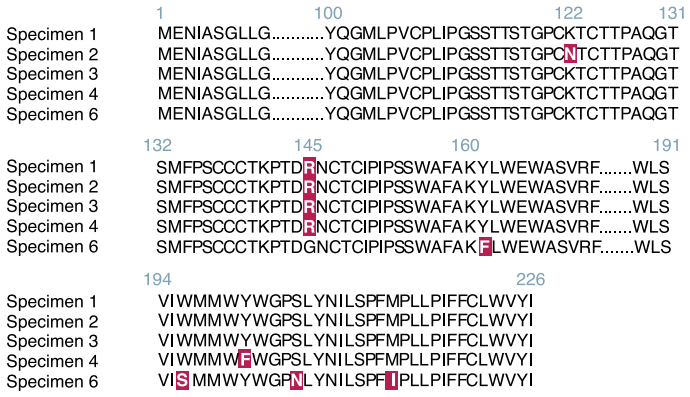
*Five clones were sequenced per specimen.
†Wild-type amino acid.

Recombinant HBsAg G145R mutant detection by IMMULITE 2000 HBsAg assay

The IMMULITE 2000 HBsAg assay readily detected three of the four HBV-positive patient serum specimens containing HBsAg with the G145R mutation, while one (specimen 2) was negative. As mentioned previously, this specimen was also negative when evaluated by the BioRad Genetic Systems HBsAg EIA 2.0 assay. In addition, PCR amplification of HBV DNA (to isolate MHR DNA) from this specimen was very difficult (data not shown). This difficulty suggested that specimen 2 had a low HBV titer and tested negative because it was below the limit of detection for both immunoassays. To test this hypothesis, two studies were undertaken. First, the HBsAg sequences from all G145R mutant specimens (specimens 1 through 4) and a wild type (specimen 6) were cloned for expression of recombinant HBsAg. Second, the HBV viral titer in specimen 2 was measured using quantitative PCR.

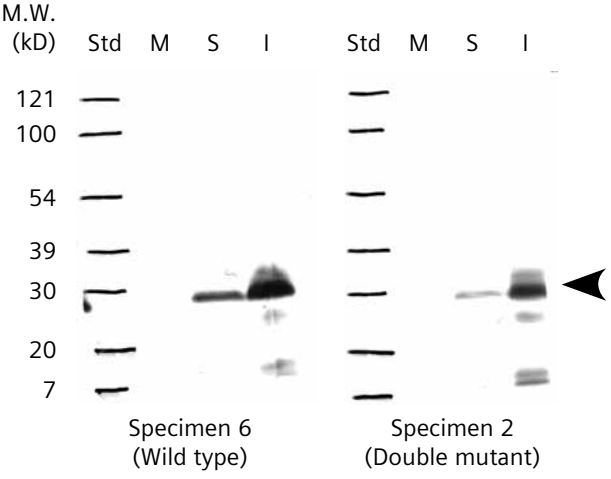
The entire HBsAg DNA sequence was amplified from the five patient specimens (specimens 1 through 4 and 6) using PCR. These HBsAg DNA sequences were cloned into a vector for expression of recombinant HBsAg in insect cells. Sequence analysis of the cloned HBsAg DNA (Figure 5) revealed that isolates from specimens 1 and 3 had only the G145R mutation. In contrast, specimen 2 contained the K122N mutation in addition to the G145R mutation. Changes in amino acids between residues 160 and 214 lie outside of the MHR (e.g., phenylalanine [F] at residue 200 in specimen 4) and are not thought to change to the conformation of this highly antigenic region (MHR) and are consequently not likely to affect immunoassay results.

Figure 5. Amino acid sequences of HBsAg from selected patient specimens. Amino acid changes are highlighted.



Expression of recombinant HBsAg proteins in insect cell cultures was confirmed for all clones by Western blot analysis using a polyclonal antiserum specific for HBsAg. The expression pattern was identical for all clones, and two representative blots are shown in Figure 6.

Figure 6. Western blot analysis of HBsAg protein expressed from insect cells. The arrowhead indicates recombinant HBsAg. Std: Molecular mass standards; M: Media; S: Soluble Fraction; I: Insoluble Fraction.



The insect cell protein extracts containing the mutant or wild-type recombinant HBsAg proteins were evaluated by the IMMULITE 2000 HBsAg assay. The results (Table 3) show that the IMMULITE 2000 HBsAg assay detected the mutant recombinant HBsAg proteins derived from specimens 1, 3, and 4, which had been positive in the initial analysis (Table 1). In addition, the recombinant protein generated from specimen 2 (double mutant that initially had tested negative) was also positive. This positive result for specimen 2 supports the hypothesis that the specimen was negative in the initial analysis because its HBV titer was too low for immunoassay detection and not because of the presence of either the G145R or K122N mutation.

Table 3. IMMULITE 2000 HBsAg assay results for recombinant HBsAg proteins.

Specimen	Recombinant HBsAg	Result
1	G145R mutant	Positive
2	G145R + K122N mutant	Positive
3	G145R mutant	Positive
4	G145R mutant	Positive
6	Wild type	Positive
Negative Control	None	Negative

Quantitative PCR of patient HBV DNA

The positive IMMULITE 2000 HBsAg result with recombinant HBsAg from specimen 2 suggested that the original analysis was negative because the HBsAg titer in this patient specimen was below the limit of detection for the immunoassay, and not because of its G145R or the K122N mutation. To measure the titer, quantitative PCR was performed on HBV DNA isolated from specimen 2 and specimen 12 (wild type). The HBV DNA titer was determined by comparison to a standard curve using HBV copy standards included in the quantitative PCR kit (Qiagen RealArt™ HBV TM PCR ASR).

The low HBV DNA titer for specimen 2 was confirmed by a high threshold cycle number of 39.3, which corresponded to a titer of less than 100 genomes/mL. Specimen 12, which was positive in the IMMULITE 2000 HBsAg assay, had a titer of 1.6×10^5 genomes/mL (Table 4).

Table 4. Quantitative PCR results for patient specimens 2 and 12.

Sample	Threshold cycle number	HBV DNA titer (genomes/mL)
Quantitation Standard A	30.9	1.0×10^4
Quantitation Standard B	28.1	1.0×10^5
Quantitation Standard C	24.8	1.0×10^6
Specimen 2 (mutant)	39.3	Very Low (< 100)
Specimen 12 (wild type)	27.3	1.6×10^5

Detection of other recombinant HBsAg mutants by the IMMULITE 2000 HBsAg assay

The IMMULITE 2000 HBsAg assay is clearly robust enough to detect recombinant and patient HBsAg protein with mutations at amino acid positions 145 (G145R) and 122 (K122N). Additional studies were undertaken to evaluate other naturally occurring HBsAg MHR mutations using recombinant HBsAg. Published reports show that many of these mutant viruses escape detection in some commercial immunoassays.[11, 15, 16, 18]

All mutant constructs were created from the cloned HBsAg DNA sequence isolated from the wild-type specimen 6. PCR was used to create nucleotide changes using mutagenic primers. A total of eight discrete HBsAg single mutant sequences were produced: T126S, Q129H, G130D, T131N, M133L, T143L, T143M, and D144A (Figure 4). In addition, using the same PCR strategy, three more complex HBsAg mutants were generated: T126S + Q129H + M133L, 122RG (R and G inserted between amino acids 122 and 123), and 123RGT (R, G, and T inserted between amino acids 123 and 124).

Siemens Healthcare Diagnostics, a global leader in clinical diagnostics, provides healthcare professionals in hospital, reference, and physician office laboratories and point-of-care settings with the vital information required to accurately diagnose, treat, and monitor patients. Our innovative portfolio of performance-driven solutions and personalized customer care combine to streamline workflow, enhance operational efficiency, and support improved patient outcomes.

Order No. A91DX-0700662-XC3-4A00
09-2011 | All rights reserved

Global Siemens Headquarters
Siemens AG
Wittelsbacherplatz 2
80333 Muenchen
Germany

Global Siemens Healthcare Headquarters
Siemens AG
Healthcare Sector
Henkestrasse 127
91052 Erlangen
Telephone: +49 9131 84-0
Germany
www.siemens.com/healthcare

Global Division
Siemens Healthcare Diagnostics Inc.
511 Benedict Avenue
Tarrytown, NY 10591-5005
USA
www.siemens.com/diagnostics

www.siemens.com/diagnostics

These eleven mutant sequences were cloned into transfer vectors. All sequences were evaluated by DNA sequence analysis to confirm that the desired mutations were present. The confirmed HBsAg mutant sequences were then used to create recombinant baculoviruses for expression of the mutant recombinant HBsAg proteins in insect cells. The mutant recombinant HBsAg proteins were extracted from insect cells and evaluated by the IMMULITE 2000 HBsAg assay. All of the recombinant mutant HBsAg proteins were detected (Table 5).

Table 5. IMMULITE 2000 HBsAg assay results for other recombinant HBsAg mutants.

Mutant	Result
T126S	Positive
Q129H	Positive
G130D	Positive
T131N	Positive
M133L	Positive
T143L	Positive
T143M	Positive
D144A	Positive
T126S + Q129H + M133L	Positive
122RG	Positive
123RGT	Positive
Negative Control	Negative

Summary

- The DPC IMMULITE 2000 HBsAg assay detected patient and recombinant HBsAg with the most common and frequently missed mutation—G145R.
- One of the HBsAg G145R patient specimens was negative in the original analysis because its titer was too low for immunoassay detection and not because of the presence of mutations. This is supported by the low HBV titer (< 100 genomes/mL) and by the positive IMMULITE 2000 HBsAg assay result from the recombinant protein derived from this specimen.
- The IMMULITE 2000 HBsAg assay was able to detect recombinant HBsAg proteins that carry mutations at other locations within the MHR domain. They are T126S, Q129H, G130D, T131N, M133L, T143L, T143M, D144A, T126S + Q129H + M133L, 122RG and 123RGT mutants.

Conclusion

The IMMULITE 2000 HBsAg assay is able to detect HBsAg mutants that escape detection by some commercial immunoassays and is therefore a robust assay for the diagnosis of HBV infection.

References:

1. Hou J, Liu Z, Gu F. Epidemiology and prevention of hepatitis B virus infection. Int J Med Sci. 2005;2(1):50-57.
2. Fagan EA, Harrison TJ. Viral hepatitis. A handbook for clinicians and scientists. New York: Springer Verlag; 2000.
3. Allen MI, Gauthier J, DesLauriers M, Bourne EJ, Carrick KM, Baldanti F, et al. Two sensitive PCR-based methods for detection of hepatitis B virus variants associated with reduced susceptibility to Lamivudine. J Clin Microbiol. 1999 Oct;37(10):3338-47.
4. Weber B. Genetic variability of the S gene of hepatitis B virus: clinical and diagnostic impact. J Clin Virol. 2005 Feb;32(2):102-12.
5. Stuyver L, de Gendt S, van Geyt C, Zoulim F, Fried M, Schinazi RF, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol. 2000;81:67-74.
6. Offensperger W, Wahl S, Neurath AR, Price P, Strick N, Kent SBH, et al. Expression in *Escherichia coli* of a cloned DNA sequence encoding the pre-S2 region of hepatitis B virus. Proc Natl Acad Sci USA. 1985 Nov;82:7540-44.
7. Hitzeman RA, Chen CY, Hagie FE, Patzer EJ, Liu C, Estell DA, et al. Expression of hepatitis B virus surface antigen in yeast. Nucl Acids Res. 1983;11(9):2745-63.
8. Fujiyama A, Miyanohara A, Nozaki C, Yoneyama T, Ohtomo N, Matsubara K. Cloning and structural analyses of hepatitis B virus DNAs, subtype adr. Nucl Acids Res. 1983;11(13):4601-10.
9. Carman WF, van Deursen FJ, Mimms LT, Hardie D, Coppola R, Decker R, et al. The prevalence of surface antigen variants of hepatitis B virus in Papua New Guinea, South Africa, and Sardinia. Hepatology. 1997 Dec;26(6):1658-66.
10. Norder H, Hammas B, Lofdahl S, Courouce A, Magnius LO. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. J Gen Virol. 1992;73:1201-8.
11. Coleman PF. Detecting hepatitis B surface antigen mutants. Emerg Infect Dis 2006;12:198-203. Available from http://www.cdc.gov/ncidod/EID/vol12no02/05-0038.htm.
12. Gerlich WH. Diagnostic problems caused by HBsAg mutants—a consensus report of an expert meeting.Intervirology. 2004;47:310-13.
13. Grethe S, Monazahian M, Bohme I, Thomssen R. Characterization of unusual escape variants of hepatitis B virus isolated from a hepatitis B surface antigen-negative subject. J Virol. 1998 Sept;72(9):7692-96.
14. Waters JA, Kennedy M, Voet P, Hauser P, Petre J, Carman W, et al. Loss of the common "a" determinant of hepatitis B surface antigen by a vaccine-induced escape mutant. J Clin Invest. 1992 Dec;90:2543-47.
15. Chen WN, Oon CJ. Changes in the antigenicity of a hepatitis B virus mutant steming from lamivudine therapy. Antimicrob Agents Chemother. 2000 Jun;44(6):1765.
16. Chen WN, Oon CJ. Hepatitis B virus surface antigen (HBsAg) mutants in Singapore adults and vaccinated children with high anti-hepatitis B virus antibody levels but negative for HBsAg. J Clin Microbiol. 2000 Jul;38(7):2793-4
17. Weber B, Dengler T, Berger A, Doerr HW, Rabenan H. Evaluation of two new automated assays for hepatitis B virus surface antigen (HBsAg) detection: IMMULITE HBsAg and IMMULITE 2000 HBsAg. J Clin Microbiol. 2003 Jan;41(1):135-43.
18. Hou J, Wang Z, Cheng J, Lin Y, Lau GKK, Sun J, et al. Prevalence of naturally occurring surface gene variants of hepatitis B virus in nonimmunized surface antigen-negative Chinese carriers. Hepatology. 2001 Nov;34(5):1027-34.