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# Sensitivities of Four New Commercial Hepatitis B Virus Surface Antigen (HBsAg) Assays in Detection of HBsAg Mutant Forms

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**Mutations in hepatitis B virus surface antigen (HBsAg) involving amino acid substitution within the immunodominant "a" determinant may affect the performance of commercial HBsAg assays. The performances of four HBsAg assays that recently received Conformité Européene marking, Advia Centaur HBsAg (Bayer), Monolisa HBsAg Ultra (Bio-Rad), Liaison HBsAg (Dia Sorin), and Vidas HBsAg Ultra (bioMérieux), were compared with that of the routinely used HBsAg assay AxSYM HBsAg V2 (Abbott). Assays were evaluated for (i) analytical sensitivity performance with a national reference HBsAg panel (including 10 samples with calibrated HBsAg concentrations from 0.04 to 2.24 ng/ml) and (ii) the detection of HBsAg mutants by studying a panel of 35 HBsAg mutants (23 collected from patients and 12 recombinant mutants). The limits of detection of these assays were <0.15 ng/ml (from 0.089 to 0.121 ng/ml). The sensitivity performances for mutant virus detection varied, ranging from 37.1% to 91.4%. The lack of detection of these mutants by commercial assays was probably due to the epitope recognition of the anti-HBs assay reagents in the capture phase and in the conjugates. The prevalence and clinical impact of HBsAg mutants are under investigation. However, the manufacturers must be vigilant in the design of the assays in order to reduce the risk of missing a broad range of described S gene mutants.**

Hepatitis B virus (HBV) infection is a global health problem. Two billion people have been infected worldwide; 360 million suffer from chronic HBV infection, and over 520,000 die each year (50,000 from acute hepatitis B and 470,000 from cirrhosis or liver cancer) (15). HBV surface antigen (HBsAg) is the established serological marker used routinely for the diagnosis of acute or chronic HBV infection, the screening of blood or organ donors, and the surveillance of persons at risk of acquiring or transmitting HBV. HBsAg can induce a strong immunogenic response by neutralizing antibodies. The main antigenic determinant ("a" determinant) is located between amino acids 124 and 147 within the major hydrophilic loop of HBsAg (amino acids 100 to 170) (10). Antigenic variation of the "a" determinant occurs naturally due to HBV genetic heterogeneity. HBV has been classified into eight genotypes, designated A to H, based upon genetic divergence of 8% or more in the complete nucleotide sequence (17). Further divergence by S gene mutations has been detected in vaccinated children (4, 11, 12, 34), in liver transplant recipients receiving anti-HBs immunoprophylaxis (23, 27, 33), and in chronic carriers (9, 24). There are several reports of HBsAg-negative virus carriers (HBV DNA positive) with immunosilent infections (1, 3, 4, 5, 8, 16). Natural variation and mutations can induce HBsAg conformational changes. Since many HBsAg immunoassays use monoclonal antibodies with epitopes directed against the major hydrophilic region, in particular against the "a" determinant, amino acid substitution in this region may account for false-negative results in immunoassays (6, 14, 18, 19, 30).

Newly developed HBsAg assays show a performance increase in terms of specificity and sensitivity, allowing the detection of <0.15 ng/ml of HBsAg (2). The prevalence of HBV mutations in the general population is assumed to be low, but selection pressures such as new antiviral drugs or large vaccination campaigns may change the situation (29). In this study, we analyzed the HBsAg mutant detection capabilities of four newly launched HBsAg assays in comparison to the HBsAg assay routinely used in our laboratory.

## MATERIALS AND METHODS

**Panels.** All specimens were stored at −20°C in aliquots. One aliquot was thawed before testing.

**HBsAg panel for sensitivity testing.** A national reference HBsAg panel (SFTS 2004; French Society of Blood Transfusion), including 10 sera with decreasing concentrations of a mix of adw2 and ayw3 subtypes ranging from 2.24 to 0.04 ng/ml, was used to assess analytical sensitivity. The values for HBsAg detection limits of the five assays were calculated by using statistical regression ($y = ax + b$), where the limit is represented by a sample/cutoff ratio of 1.0.

**HBsAg mutants.** The following two categories of HBsAg mutants were analyzed: (i) 12 recombinant HBsAgs representing vaccine escape mutants obtained by site-directed mutagenesis, including recombinants A to I, provided by Abbott (T126S, Q129H, M133L, P142L, P142S, D144A, and G145R), and K to M, with single (T126S) or double (T123N and T143S) point mutations, provided by Bio-Rad; and (ii) 23 samples from patients infected with mutants, including 6 diluted natural HBsAg mutants provided by Abbott (a to d) and Dia-Sorin (e and f) and 17 undiluted samples from 17 patients. Three of the patients were coinfected with human immunodeficiency virus (HIV) and received antiviral therapy at the time of sample collection (patients 4, 14, and 15). For the 17 patients, HBV viral loads were quantified by using a Cobas Amplicor HBV Monitor kit (Roche Diagnostics, Meylan, France), which has a detection threshold of 200 copies/ml. HBsAg sequencing was performed by two procedures. One was based on the

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TABLE 1. Virological and clinical features of patients infected by natural HBV mutants

| Patient no. | HBV genotype | HBsAg mutation(s) | Presence of HBeAg/HBeAb | Viral load (log copies/ml) | Clinical status |
|---|---|---|---|---|---|
| 1 | D | S204R/K, L205V, C/Y206S D/S207N, I208T | −/+ | 5.5 | Chronic hepatitis |
| 2 | D | P120T, S132F, Y134N E/D144G | −/+ | 5.2 | Chronic hepatitis |
| 3 | D | S143L | −/+ | 3.1 | Chronic hepatitis |
| 4 | A | D144E, G145R | +/− | 6.0 | Acute hepatitis, HIV-coinfected patient, lamivudine (3TC)-resistant HBV delta virus superinfection |
| 5 | B | T126A, M133I | −/+ | 3.3 | Chronic hepatitis |
| 6 | D | S143L | −/+ | 4.4 | Moroccan blood donor |
| 7 | D | I110M, T116N, S117T, T118S, T140S, S143L | −/+ | 1.7 | Moroccan blood donor |
| 8 | D | P127T, G145R | −/+ | 5.3 | Liver transplant patient, HBV recurrence after 2 yr of HBIG escape |
| 9 | D | F134V, D144G | −/+ | 6.3 | Liver transplant patient, HBV recurrence after 2 yr of HBIG escape |
| 10 | D | T126, I Y134H, P142L, G145R | −/+ | 6.0 | Liver transplant patient, HBV recurrence after 1.5 yr of HBIG escape but HBIG maintenance |
| 11 | A | P120S, D144E, G145R, T189I | −/+ | 5.0 | Liver transplant patient, HBIG escape |
| 12 | E | G145A | −/+ | 6.0 | Liver transplant patient, HBIG escape |
| 13 | G | P108H, S113T | −/+ | 4.4 | Chronic hepatitis 6 mo after 3TC initiation |
| 14 | D | M133I, Y134H, S143M | +/− | 6.6 | HIV-coinfected patient, 3TC-resistant HBV |
| 15 | D | L109I, G112K/R, S113A, P120T, F/Y134S | −/+ | 7.5 | HIV-coinfected patient, HBV reactivation from anti-HBc alone (previous status) |
| 16 | F | S143L | −/+ | 6.2 | Renal transplant patient, HBV reactivation |
| 17 | C | I/L110R, S117, I G119R, T123N, C124R, P203R | −/+ | 5.4 | Liver transplant patient, HBV recurrence after 2 yr of HBIG escape, 18 mo after adefovir initiation |

Trugene HBV assay (Bayer Diagnostics), which was used according to the manufacturer's instructions: HBsAg sequences from amino acids 102 to 226 were obtained and compared to 168 sequences of the complete genome (17). The second procedure was an in-house method based on a consensus PCR assay using primers HBV256 (5′-TCGTGGTGGACTTCTCTC) and HBV725 (5′-AC AGTGGGGGAAAGCCC), allowing us to analyze HBsAg sequences from amino acids 100 to 178. Nucleotide sequences were determined for both strands (forward and reverse strands). Several reference sequences of HBV genotypes (A to H) retrieved from GenBank were included in the data bank for comparison. The sample sequences were aligned using ClustalW software (28). Phylogenetic analysis was performed by using the Phylip package. Distances between sequences were analyzed using the neighbor-joining algorithm, based on the Kimura two-parameter distance estimation method for nucleotides and the Dayhoff PAM matrix for amino acids (22). The characteristics of the patients, especially descriptions of the S gene mutations present in each sample, are detailed in Table 1. The 17 undiluted samples were classified into the following four categories according to the context of their inclusion in the study. (i) Five sera were from five HBV-positive chronic hepatitis patients with discordant HBsAg screening results with at least one negative HBsAg result. Patient 1 was negative for HBsAg with the ACCESS HBsAg assay (Bio-Rad), patients 2, 3, and 4 were negative with the Monolisa HBsAg Plus assay (Bio-Rad), and patient 5 was negative with the Centaur Advia HBsAg Assay (Bayer). (ii) Two sera were from two blood donors (patients 6 and 7) who were HBsAg negative with the assay used for blood screening (Monolisa HBsAg Plus assay; Bio-Rad). These two blood donations collected by a Moroccan blood bank were subsequently discovered to be HBV positive by a French laboratory for plasma fractionation (Le Laboratoire Français du Fractionnement et des Biotechnologies) which has responsibility for the fractionation of plasmas collected in Morocco. (iii) Five sera were from five liver-transplanted patients with HBV-related disease who escaped anti-HBs (HBIG) prophylaxis (patients 8, 9, 10, 11, and 12). Sequencing was performed shortly after escape diagnosis. (iv) Five sera were from five chronic carrier patients (13, 14, 15, 16, and 17) with known HBsAg mutations included in a previous study (25).

**Immunoassays.** The following five HBsAg assays were studied: (i) Advia Centaur HBsAg (Bayer, Marburg, Germany), (ii) Liaison HBsAg (Dia-Sorin, Saluggia, Italy), (iii) Monolisa HBsAg Ultra (Bio-Rad, Marnes La Coquette, France), (iv) Vidas HBsAg Ultra (bioMérieux, Marcy l'Etoile, France), and (v) AxSYM HBsAg V2 (Abbott Diagnostics, Delkenheim, Germany). The specific features of the different assays used are summarized in Table 2. All tests were performed and interpreted in accordance with the manufacturer's recommendations.

**AxSYM HBsAg V2.** The AxSYM HBsAg V2 assay is based on microparticle enzyme immunoassay technology. Briefly, the sample (150 μl), anti-HBs (one monoclonal antibody)-coated microparticles, and biotinylated anti-HBs (poly-

TABLE 2. Characteristics of HBsAg assays

| Characteristic | Description or value for assay | | | | |
|---|---|---|---|---|---|
| | Advia Centaur HBsAg | Monolisa HBsAg Ultra | Liaison HBsAg | Vidas HBsAg Ultra | AxSym HBsAg V2 |
| Company | Bayer | Bio-Rad | Dia-Sorin | bioMérieux | Abbott |
| Yr of CE mark | 2004 | 2003 | 2003 | 2004 | 1994[b] |
| Turn-around time (min) | 29 | 120 | 30 | 80 | 30 |
| Sample volume (μl) | 100 | 100 | 150 | 150 | 150 |
| Assay format[a] | | | | | |
| Capture antibody | 1 AbMo | 3 AbMo | 3 AbMo | 2 AbMo | 1 AbMo |
| Conjugate antibody | 1 AbMo | Ab poly and 1 AbMo | Ab poly | Ab poly | Ab poly |
| Method | Chemiluminescence | Enzyme immunoassay | Chemiluminescence | Enzyme immunoassay | Enzyme immunoassay |

[a] AbMo, monoclonal antibody; Ab poly, polyclonal antibody.
[b] Product placed on the market in 1994 following national regulations but prior to CE markings.

clonal antibody) are combined and incubated in one reaction vessel. This step is followed by dispensing of the microparticles and sample onto a matrix cell. The anti-biotin–alkaline phosphatase conjugate is then dispensed onto the matrix cell, followed by buffer washes. The alkaline phosphatase activity is determined by the addition of a substrate, 4-methylumbelliferyl phosphate, which is converted to methylumbelliferone and is quantitated based on the fluorescent signal measured by the AxSYM instrument. Results are automatically calculated against the stored AxSYM HBsAg index calibration curve. A test value above a signal/negative-value ratio of 2 is considered positive.

**Advia Centaur HBsAg.** The Advia Centaur HBsAg assay is a direct "sandwich" chemiluminescence immunoassay. The minimum volume required is 250 μl (100 μl specimen plus 150 μl dead volume). During the first step, HBsAg present in the sample binds to a biotinylated monoclonal antibody and an acridinium ester-conjugated monoclonal antibody. Streptavidin-coated paramagnetic microparticles are then added to bind the antibody-antigen complexes. After being washed, the microparticles are flashed by acid/base addition. The resulting signal is read against a stored standard curve, and the result is expressed as an index value, with a positive cutoff of 1.0.

**Liaison HBsAg.** The Liaison HBsAg method for qualitative determination of HBsAg is a direct sandwich chemiluminescence immunoassay. The minimum volume required is 300 μl (150 μl specimen plus 150 μl dead volume). During the first incubation, HBsAg present in calibrators, samples, or controls binds to the solid phase (magnetic particles) coated with three mouse monoclonal antibodies. After a first washing step and during the second incubation, a sheep polyclonal isoluminol-antibody conjugate reacts with HBsAg already bound to the solid phase. After the second incubation, the unbound material is removed with a wash cycle. Subsequently, the starter reagents are added, and a flash chemiluminescence reaction is thus induced. The light signal, and hence the amount of isoluminol-polyclonal antibody conjugate, is measured by a photomultiplier in relative light units and is indicative of the HBsAg concentration present in calibrators, samples, or controls. The analyzer automatically calculates HBsAg levels, expressed as index values. Samples with HBsAg levels of ≥1.1 and <0.9 should be graded reactive and negative, respectively.

**Monolisa HBsAg Ultra.** Monolisa HBsAg Ultra is a one-step sandwich enzyme immunoassay using a solid phase coated with monoclonal antibodies (three). In the first step, the sample (100 μl) is incubated with a peroxidase conjugate (monoclonal antibody from mouse and polyclonal antibody from goat) in a microtiter plate. After a washing step, a tetramethylbenzidine substrate solution is added. The presence of HBsAg is proportional to binding of the conjugate and peroxidase activity. The colorimetric reaction is stopped, and the optical density is measured by bichromatic reading (450/620 nm). The cutoff value is calculated as the mean negative control value plus 0.050. Samples with a signal/cutoff ratio of ≥1.0 are reactive, and those with a signal/cutoff ratio of <0.9 are negative.

**Vidas HBsAg Ultra (short protocol).** The Vidas HBsAg Ultra assay is performed on a Vidas instrument, an automated immunoassay system. After a preliminary washing step, the HBsAg present in the sample (the volume required is 150 μl) will bind simultaneously to the monoclonal antibodies (two) coating the interior of the solid-phase receptacle and to a polyclonal antibody conjugated with biotin. Unbound sample components are washed away. The HBsAg bound to the solid phase and to the biotinylated antibody is in contact with streptavidin conjugated with alkaline phosphatase, which will bind with biotin. Another wash step follows and removes unbound components. During the final detection step, the substrate (4-methylumbelliferyl phosphate) is cycled in and out of the solid-phase receptacle. The conjugate enzyme catalyzes the hydrolysis of the substrate into a fluorescent product (4-methylumbelliferone), whose fluorescence is measured at 450 nm. The intensity of the fluorescence is proportional to the concentration of HBsAg present in the sample. At the end of the assay, results are automatically calculated by the Vidas instrument in relation to the standards and printed. Test values above 0.13 are considered positive.

## RESULTS

**Analytical sensitivity.** The detection limit was calculated as 0.08 ng/ml for the Monolisa Ultra test, 0.09 ng/ml for the VIDAS HBsAg Ultra test, 0.12 ng/ml for the Advia Centaur HBsAg test, 0.11 ng/ml for the Liaison HBsAg test, and 0.16 ng/ml for the AxSYM HBsAg V2 test.

**Mutant HBsAg detection.** The results are presented in Table 3.

**(i) Recombinant mutant samples ($n = 12$).** Centaur was unable to detect 7 of the 12 tested samples (5 harbor the G145R mutation and 2 are T123N mutants). AxSYM missed two samples (both exhibited a T123N mutation). Liaison detected all samples, with one in the gray zone (a D144A/G145R double mutant). Vidas Ultra and Monolisa Ultra detected all recombinant mutants.

**(ii) Natural undiluted samples ($n = 17$).** Centaur was unable to detect 8 of the 17 natural undiluted samples (no. 3, 4, 6, 7, 9, 10, 11, and 17). Liaison detected all samples with high ratios, except for one (no. 10) which carried the following additional mutations: T126I, Y134H, P142L, and G145R. Monolisa Ultra, Vidas Ultra, and AxSYM detected all samples with high ratios, except sample 17, which harbored I/L110R, S117I, G119R, T123N, C124R, and P203R mutations. The G145A mutant was detected by all tested assays.

**(iii) Natural diluted samples ($n = 6$).** None of the natural diluted samples was detected by Centaur, including two without the G145R mutation (d and f). Liaison, Monolisa Ultra, and Vidas Ultra missed the same three of six diluted samples (b, d, and e). AxSYM detected all six diluted mutants.

## DISCUSSION

Commercial assays utilize monoclonal antibodies raised against the immunodominant "a" determinant of HBsAg to detect wild-type HBV infections at very low analyte levels in patient samples. However, the "a" determinant is also a target of anti-HBs selection pressure. The emergence of a vaccine escape mutant with an altered "a" determinant was first described in Italy some 15 years ago (34). Amino acid replacement of the glycine residue at position 145 by arginine (G145R) was caused by a mutation in the viral genome (guanine to adenine at nucleotide position 587). This mutation allowed the variant virus to emerge as the dominant viral form despite the presence of neutralizing anti-HBs. The G145R mutant is the most commonly described HBsAg mutant in the literature (35). It is stable over time and may retain its ability to replicate at a high level for several years (4). Moreover, horizontal transmission of this variant was recently described (21). Many HBV S gene mutants have been reported, affecting nearly all amino acid positions of the "a" determinant. In Taiwan, the frequency of "a" determinant variants in children and adolescents markedly increased after the introduction of universal vaccination (11, 12). Although "a" determinant variants, including the G145R mutant, are more frequent in vaccinated patients, they also occur naturally in nonvaccinated chronic carriers (12, 33). The G145R, K141E, and T131I substitutions and an insertion of three amino acids between residues 123 and 124 have been shown to markedly affect binding to various monoclonal antibodies (26). These mutations may also lead to evasion of the neutralizing antibody response and to reduced detection by immunoassays. HBsAg mutants are probably more frequent than previously believed (21), but there are no prospective and extensive sequencing data on HBV mutant isolates. The need for such information was recently underscored by a panel of experts in the field (7). The impact of HBsAg amino acid changes on immunoassay detection has been reported by several authors (6, 13, 14, 16, 18, 19, 20, 30). However, these studies used diluted natural samples or recombinant antigens to reflect mutations described in the

TABLE 3. Results obtained with five HBsAg assays for HBs mutants

| Mutant or parameter | Mutation(s) | Index value[a] | | | | |
|---|---|---|---|---|---|---|
| | | Centaur (cutoff = 1) | Liaison (cutoff = 1.1) | Monolisa Ultra (cutoff = 1) | Vidas Ultra (cutoff = 0.13) | AxSYM (cutoff = 2) |
| Recombinant mutants | | | | | | |
| A | T126S | 20.12 | 15 | 12.73 | 1.73 | 7.47 |
| B | Q129H | 16.51 | 17 | 16.31 | 1.33 | 8.15 |
| C | M133L | 12.62 | 15 | 12.41 | 1.61 | 8.33 |
| D | D144A | 5.89 | 6.2 | 12.31 | 1.34 | 6.32 |
| E | G145R | **<0.10** | 4 | 11.03 | 1.09 | 6.4 |
| F | T126S, G145R | **0.12** | 3 | 9.21 | 1.37 | 4.7 |
| G | P142L, G145R | **0.12** | 3.7 | 10.48 | 1.06 | 6.51 |
| H | P142S, G145R | **<0.10** | 5 | 10.57 | 1.15 | 6.19 |
| I | D144A, G145R | **<0.10** | **1**[b] | 10.71 | 0.38 | 5.21 |
| K | T123N, T143S | **0.3** | 5.8 | 1.51 | 0.49 | **1.29** |
| L | T123N | **0.56** | 6.5 | 4.6 | 1.16 | **0.99** |
| M | T126S | 1.73 | 4.6 | 3.05 | 0.2 | 2.2 |
| Samples with natural HBsAg mutants | | | | | | |
| 1 | S204R/K, L205V, C/Y206S, D/S207N, I208T | >1,000 | >240 | >30 | >17 | 354 |
| 2 | P120T, I150T, S132F, F134N, P135A, D144G, | >1,000 | >240 | >30 | >17 | 220 |
| 3 | S143L | **0.9** | >240 | >30 | 16.5 | 326 |
| 4 | D144E, G145R | **0.75** | 99 | >30 | >17 | 342 |
| 5 | T126A, M133I | 123 | 5.1 | 2.37 | 0.47 | 12 |
| 6 | S143L | **0.43** | >240 | >30 | >17 | 238.6 |
| 7 | I110M, T116N, S117T, T118S, T140S, S143L | **0.87** | >240 | >30 | >17 | 392.4 |
| 8 | P127T, G145R | 594.5 | >240 | >30 | >17 | 163.5 |
| 9 | F134V, D144G | **<0.10** | >240 | >30 | >17 | 229 |
| 10 | T126I, Y134H, P142L, G145R | **<0.1** | **0.4** | 8.7 | 2.9 | 28.8 |
| 11 | P120S, T189I, D144E, G145R | **<0.10** | 54 | >30 | >17 | 164.34 |
| 12 | G145A | 889.7 | >240 | >30 | >17 | 224.7 |
| 13 | P108H, S113T | >1,000 | >240 | >30 | >17 | 381.8 |
| 14 | M133I, Y134H, S143M | 164.7 | >240 | >30 | >17 | 338 |
| 15 | L109I, G112K/R, S113A, P120T, F/Y134S | >1,000 | >240 | >30 | >17 | 400 |
| 16 | S143L | 3.31 | >240 | >30 | >17 | 360 |
| 17 | I/L110R, S117I, G119R, T123N, C124R, P203R | **0.02** | 51 | **0.29** | **0.1** | **1.09** |
| Diluted natural HBsAg mutants (dilution) | | | | | | |
| a (1/10) | G145R | **<0.10** | 1.42 | 26.43 | 3.09 | 12.84 |
| b (1/10) | P120Q, T131K, G145R | **<0.10** | **0.8** | **0.29** | **0.02** | 3.1 |
| c (1/10) | T118V, M133I, F134N, P142S, T143L, G145R | **<0.10** | 1.82 | 26.99 | 3.25 | 28.66 |
| d (1/10) | T115N, P120L, M133I, F134H, D144V, S154P | **<0.10** | **0.47** | **0.41** | **0.02** | 4.1 |
| e (1/100) | G145R | **<0.10** | **1.03**[b] | **0.76** | **0.02** | 4.37 |
| f (1/100) | C147Y, C149Y | **<0.10** | 9.3 | >30 | 14.56 | 169.24 |
| Score[c] | | | | | | |
| Recombinant samples | | 42 | 92 | 100 | 100 | 83 |
| Natural undiluted samples | | 53 | 94 | 94 | 94 | 94 |
| Natural diluted samples | | 0 | 50 | 50 | 50 | 100 |
| Total | | 40 | 86 | 89 | 89 | 91 |

[a] Results shown in bold are negative reactions.
[b] Gray-zone result.
[c] The score is a percentage derived by comparing the number of positive samples to the number of samples tested.

literature, as sufficient volumes of the natural samples are difficult to obtain.

The present study was performed with a large panel of HBsAg mutants, including a significant number of natural HBsAg mutants ($n$ = 17), and showed discrepant results when tested with that recently received Conformité Européen (CE) marking. HBsAg immunoassays. The assay scores varied from 40% (Centaur) to 91% (AxSYM). The G145R mutant seems to induce an epitope conformation which is undetectable by Centaur. Indeed, 12 of the 13 samples presenting this mutation

alone (3 samples) or in association with one (5 samples) or more (5 samples) mutations were negative in this assay, irrespective of the sample origin. These results confirmed the detection failure of Centaur with the G145R mutants found by Moerman (20) and Weber (30). Surprisingly, two "natural" mutants (patients 8 [P127T and G145R mutations] and patient 16 [S143L mutation]) were detected by Centaur. These two cases might be explained by the quasispecies structure of circulating HBV virions. Wild-type HBV could coexist with mutant HBV in a proportion not detectable by direct sequencing. It is usually assumed that direct sequencing does not detect a second population if it represents $<20\%$ of the sample viral burden. In this case, the HBsAg assay could give a positive result by detecting this minor wild-type population. This hypothesis could be proven by sequencing a significant number of clones from these patient samples. We show also that the nature of the amino acid substitution is more important for mutant detection than the position of the substitution. For example, although the G145R mutant (no. 12) was missed by the Centaur assay, it was accurately detected by all other assays tested. Indeed, the replacement of glycine by alanine at position 145 has little demonstrated impact on HBsAg antigenicity. One natural sample (from patient 17) with multiple mutations was only detected by Liaison. Three samples (K, L, and 17) which presented the T123N mutation alone or in association were missed by AxSYM, highlighting the fact that this amino acid residue is crucial for the detection of HBsAg with this assay. The antigen end-point analytical sensitivity also has to be taken into account when evaluating assay performance. Even with appropriate capture and detection antibodies, the HBsAg concentration can fall below the threshold for assay detection. Some mutations show only a partial reduction in immunodetection. For example, sample 16 (S143L mutant) was detected by Centaur with a low ratio (3.31), but when the sample was diluted 10 times, the result was negative. In contrast, the other assays tested positive with the same dilution (data not shown). The performance differences between assays highlight the importance of the epitopes targeted by the capture and detection antibodies. Vidas Ultra, Liaison, and Monolisa Ultra use two or three anti-HBs monoclonal antibodies coating the solid phase to capture HBsAg, with polyclonal antibodies as the detection conjugate, except for Monolisa Ultra, which uses a combination of monoclonal and polyclonal antibodies as the detection conjugate. Both Centaur and AxSYM use only one monoclonal antibody for HBsAg capture but have different detection conjugates: Centaur relies on a monoclonal/monoclonal format, whereas AxSYM uses a polyclonal antibody conjugate (Table 2). Moreover, the monoclonal antibody (H166) in the AxSYM assay, which binds amino acids 121 to 124 (6) in a conformation-dependent manner, is directed against an epitope located outside the classical "a" determinant (amino acid residues 124 to 147).

Immunoassay failure to detect HBsAg mutants could impact the diagnosis of HBV infection and therefore compromise blood safety if an appropriate screening algorithm is not used. Regarding blood safety, two cases of blood donations harboring diagnostic escape mutants have been reported. The first reported case was a blood donation of Surinam origin containing an HBV strain with double mutations at positions 129 and 133 (14), although these mutants were subsequently shown to be detected in recombinant form (6). The second case was from a Slovenian donor with five mutations, at positions 114, 122, 131, 134, and 143 (18). These results highlight the observation that the use of HBsAg screening assays capable of detecting common mutants could preclude the transfusion transmission of such viruses. However, an HBV screening algorithm that includes anti-HBc testing (positive in the two previously described cases) in combination with HBsAg testing is able to prevent the transfusion of such a donation, especially in situations where HBV DNA detection by nucleic acid amplification technology is not implemented.

In clinical practice, two diagnostic circumstances may present with HBsAg false-negative results due to the presence of mutants. First, when the patient's HBV status is unknown, the situation is similar to the blood donor screening problem, and anti-HBc testing is recommended in combination with HBsAg testing for HBV diagnosis. Second, when the patient is undergoing antiviral treatment, the impact of an HBsAg-negative result due to a mutant is lessened since the infection was previously diagnosed.

HBsAg mutant investigation should be considered when unusual serologic profiles occur, e.g., for (i) individuals with isolated anti-HBc reactivity, (ii) patients with discordant results between HBsAg assays, (iii) patients seronegative for HBsAg but positive for HBeAg, and (iv) individuals with the presence of both HBsAg and anti-HBs (mostly at low titers of $<100$ mIU/ml). Moreover, we suggest that since they do not detect the more common G145R and S143L mutants, HBsAg assays using a single monoclonal antibody directed against the second loop of the "a" determinant (amino acids 139 to 147) cannot be used (i) for HBV infection screening of the blood donor population or of organ and tissue donors when anti-HBc testing is not performed or (ii) for systematic screening of HBV infection in pregnant women due to the risk of mutants causing false-negative results.

The frequency of HBsAg mutants is not well established. However, a mathematical model has anticipated that under the pressure of large vaccination campaigns, the G145R mutant may emerge as the common HBV circulating form in 100 years time in infants born to carrier mothers (32). Thus, there is a need for more complete epidemiological data on the prevalence of HBsAg mutants and strategies for the differential screening of mutants.

By increasing the number of HBsAg epitopes recognized, three of the new assays evaluated were capable of detecting most of the mutated viruses tested. Despite the limited number of natural undiluted mutants ($n = 17$), the results of the present study show that some natural samples may escape detection by commercial HBsAg kits, even the new assays now available on the international market. Moreover, with the demonstration of antiviral resistance mutations against lamivudine and adefovir (licensed drugs for HBV) causing a corresponding mutation in the overlapping S gene sequence (29, 31), a new threat of a combined drug and vaccine escape mutant may emerge to further challenge HBsAg immunoassay surveillance. These factors must be considered by the clinician when developing an HBV screening algorithm.

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