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Coexistence of circulating HBsAg and anti-HBs antibodies in chronic hepatitis B carriers is not a simple analytical artifact and does not influence HBsAg quantification

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Abstract

Background Presence at the same time of HBsAg and anti-HBs antibodies (HBsAg/Ab) is an entity sometimes encountered in chronic hepatitis B (CHB) carriers.

Objectives This study was designed to characterize such serological profiles and to assess the reliability of serological marker quantification by three commercially available assays in this setting.

Study design Among 2578 CHB identified patients, 129 (5%) had an HBsAg/Ab profile as determined by Abbott Architect. After exclusion of co-infections (HIV, HCV, HDV), HBV reactivation or HBIG treatment, 101 samples from 62 patients were tested for HBsAg and anti-HBs quantification using Architect, DiaSorin Liaison-XL and Roche Modular-Cobas. Influence of genotype and HBsAg variants was studied in 31 samples with HBV replication.

Results HBsAg detection was confirmed with the 3 techniques for 98% (n=99) of the samples while the HBsAg/Ab profile was concordant between all techniques for 65% of them. The overall correlation between the 3 HBsAg quantification techniques was good (r²: 0.94-0.97). The median HBsAg concentration was comparable for the 99 samples whatever the used technique but a bias of -0.11 and 0.02 log IU/mL were noticed for DiaSorin and Roche compared to Abbott, respectively. Anti-HBs quantifications were poorly correlated between techniques with major discrepancies observed. Genotype and substitutions within the "a" determinant showed an impact on HBsAg quantification.

Conclusions The double HBsAg/Ab profile is not an analytical artifact and is confirmed on all commercially available techniques. While such profile does not influence HBsAg quantification, differences of HBsAg quantification were noticed according to HBV genotype or HBsAg variant.

Keywords: HBsAg quantification – HBsAg variant – Genotype – immune complexes - interference
**Highlights**

- HBsAg and anti-HBs double positive serological profile is found in 5% of CHB
- Concomitant detection of both markers is confirmed by 3 assays in 65% of the cases
- HBsAg quantification is not affected by the presence of detectable anti-HBs
- HBsAg quantification varies according to the assay, the genotype and Ag substitutions
Background

HBsAg is a key marker for the diagnosis of acute or chronic HBV infection. Available qualitative or quantitative assays detect all circulating HBsAg containing particles, mainly infectious virions (Dane particles) but in a large excess non infectious/defective particles (1). While chronic infection is defined by the persistence of HBsAg over 6 months, HBsAg loss and appearance of anti-HBs Ab (anti-HBs) usually sign viral clearance. Yet, occurrence of anti-HBs is sometimes observed despite persistence of HBsAg and viral replication. The prevalence of this entity has been reported in less than 5 % to more than 25 % of CHB cases, depending on the studies (2–5).

The molecular and immunologic mechanisms leading to this serologic profile remain unclear but could be due to the selection of HBV escape mutants by immune pressure (6–8). In this scenario, the detected circulating antibodies are directed against an HBV strain not present anymore. Indeed, several reports have shown an association of this pattern with an increased proportion of HBsAg mutants characterized by specific substitution or deletion in the preS/S gene, especially in the "a" determinant region. Amino acid substitutions within this epitope could modify the antigenic conformation of HBsAg and alter the binding of both monoclonal and polyclonal anti-HBs antibodies; the main consequences being the emergence of an immune escape variant and an alteration of HBsAg detection assay (3,4,9–13).

Most recently, the amount of circulating HBsAg has been recognized as a potential biomarker linked to the liver disease stage, the risk of HCC or the chance to respond to interferon therapy (14–17). Beside the most widely used Architect assay (Abbott), two other CE marked assays are also available for the quantification of HBsAg, the Elecsys platform (Roche) and the Liaison-XL from DiaSorin. These assays use both monoclonal and/or polyclonal antibodies to enhance recognition of HBsAg and HBsAg mutants. Several studies have convincingly demonstrated the comparable performances of Roche and Abbott assays (18–20). More recently, the DiaSorin assay was shown to perform comparably to the Architect assay in sequential serum samples from 14 HBe-negative patients (21).

Objectives

The aims of our study were first to document the performances of the DiaSorin assay compared to our routine Abbott's Architect HBsAg quantification. Secondly, the robustness of HBsAg quantification using the three CE marked available assays on a panel of CHB seras containing both HBsAg and anti-
HBs was challenged. One main issue was to determine whether concomitant detection of both HBsAg/anti-HBs could solely be an analytical artifact linked to a specific assay. Likewise, a further goal was to assess the ability of these assays to quantify potential variants more prone to be selected in these peculiar serological profiles.

**Study design**

*Patients and sera*

In the first part of the study comparing Abbott and DiaSorin on HBsAg positive samples, 227 sera successively tested for HBsAg quantification between September and November 2012 on Architect (Abbott, Rungis, France) were tested on DiaSorin Liaison-XL (Antony, France).

One hundred and one sera corresponding to 62 CHB patients identified HBsAg/anti-HBs positive on Architect between January 2008 and June 2012 and stored at -20°C were selected. Exclusion criteria were any HIV, hepatitis C or hepatitis D coinfection, HBlg passive immunization or reactivation of past HBV infection. All sera were subsequently tested for HBsAg and anti-HBs on the Roche Elecsys system (Meylan, France) and DiaSorin Liaison-XL, according to each manufacturer's recommendation. The measurements were performed within 5 days on all systems.

Among the 62 selected patients, 31 had also an HBV viral load above 100 IU/mL on the same day as HBsAg quantification and sequencing of the HBsAg gene could be performed on -80°C stored plasma.

Unless specified, HBsAg quantification was reported in log_{10} IU/mL and anti-HBs in IU/L. The study general outline is presented on figure 1.

*HBsAg gene sequencing method*

HBV-DNA was purified from 500µL of plasma using Nuclisens EasyMAG (Biomérieux, Marcilly, France) and resuspended in 70µL of sterile water. Amplification of part of the HBsAg coding region (nt. 186-1196, aa 100-227) was performed as previously described (4). Sequences were aligned and compared to selected Genbank reference sequences of known genotype using SeqScape (Applied Biosytems) and Mega (v5) softwares (22). For analysis purpose, HBsAg was divided into 3 regions: residues 100-123, 124-147 ("a" determinant) and 148-221. Substitutions were determined manually and with the help of two online tools (http://hbv.bioinf.mpi-inf.mpg.de/ and http://www.hiv-grade.de).

*Statistical analysis*
Statistical analysis, were performed using Analyze-it software. Chisquare and Fischer tests were used for categorical variables. Mean and medians were analyzed with Mann Whitney and Kruskal tests. Bland-Altman analysis was used to compare methods. Significance was defined by a P value below 0.05.

Results

Overall performances of Liaison-XL HBsAg quantification on 227 HBsAg positive samples

On these 227 samples, there was a strong correlation (p<0.001) between both Abbott and DiaSorin systems with an $r^2$ of 0.96 (fig. 2A) despite a mean quantification difference of $-0.16 \log_{10} \text{IU/mL}$ (95% CI: -0.19; -0.14) throughout the quantification range (fig. 2B). The results provided by the Liaison-XL were constantly and significantly lower than those by the Architect assay (p=0.04).

Characteristics of patients carrying both HBsAg and anti-HBs

Among 2578 chronic HBsAg carriers followed between 2008 and 2012, 129 (5%) patients were concomitantly HBsAg/anti-HBs positive and 62 (2.4%) were included in the study (fig. 1). Except from above mentioned inclusion criteria, available stored sample volume also restricted inclusion into the study. The main characteristics of the patients selected for the principal and molecular studies are summarized in table 1. Noteworthy, most of the samples had an anti-HBs concentration lower than 50 IU/L (median: 24 IU/L) but 31 (30.7%) and 14 (13.9%) had values above 50 and 100 IU/L, respectively. No correlation was found between the concentration of HBsAg and anti-HBs (data not shown). The median viral load of the patients selected for S-gene sequencing was 3.6 log IU/mL; the best performance for sequencing being usually obtained above 1000 IU/mL.

Qualitative analysis of the HBsAg results obtained with the three assays

Concurrent HBsAg and anti-HBs reactivity was confirmed unequivocally with the three techniques for 65% of the 101 samples (table 2). The concordance between all techniques was better for HBsAg than for anti-HBs. Noteworthy, the median anti-HBs values obtained with Abbott for concordant (32.5 IU/L; IQR 69.4) versus discordant (19 IU/L; IQR 21.8) results were statistically different (p=0.01).

The HBsAg discordant samples (1 for DiaSorin and 2 for Roche) belong to the same patient who was followed at several visits and had persistently very low HBsAg levels: 0.07 and 0.05 IU/mL on
Architect (table 3). Noteworthy, a third sample with a value of 0.1 IU/mL with Abbott was correctly detected with Roche and DiaSorin, respectively at 0.041 UI/mL and 0.057 IU/mL.

Quantitative analysis of HBsAg results obtained with the three assays

All HBsAg positive data for HBsAg/anti-HBs samples were considered in the quantitative assessment of these assays.

The overall correlation of all assays was satisfactory with $r^2$ ranging from 0.93 for DiaSorin (n=100) and Roche (n=99) to 0.97 for Abbott and Roche assays (fig. 3). The best concordance was obtained between Roche and Abbott with a mean bias of 0.022 log IU/mL (95% CI: -0.024 to 0.067) in favor of Roche while the most divergent quantification was observed between DiaSorin and Roche (bias of 0.130 log IU/mL in favor of Roche with 95% CI from 0.048 to 0.212). The mean bias between Architect and Liaison-XL was -0.113 log IU/mL; the lower values being reported by DiaSorin. A comparison of the biases was performed between samples concordant for the detection of both Ag and Ab on all techniques (n=66) to the discordant ones (n=33); no statistically significant difference was observed according to the discordant status.

Quantitative analysis of the results obtained with the three assays for anti-HBs antibodies

To limit any bias, only samples reactive (anti-HBs > 10 IU/L) with the three techniques (n=68) were taken into consideration for this analysis. Each technique value was compared to the obtained mean value on the three techniques. The best correlation was obtained with DiaSorin assay ($R^2 = 0.81$). The mean differences (95% CI) to the mean for Abbott, DiaSorin and Roche were, -18.9 (-31 to -6.8); 21.2 (8.4 to 33.9) and -2.30 (-12.1 to 7.5) IU/L, respectively (data not shown).

Variability of HBsAg quantification according to genotypic features

Thirty one samples could be sequenced for genotype determination and HBsAg substitution analysis (table 1). Ten genotype C, 8 D, 7 E, 4 B, and 1 each genotype A and F were detected. To compare HBsAg quantification between all techniques, the mean value obtained by the 3 techniques for each sample was calculated. Then, the percentage of HBsAg quantification over the mean was calculated for each technique and analyzed according to the genotype or the presence of substitution.
Sequence analyses showed that 77% (24/31 cases) of the HBsAg/anti HBs samples harbored amino acid substitutions on residues 100-221. Substitutions previously described to modify HBsAg antigenicity were closely examined (23). Forty five percent (n=14) of the strains had such substitutions, with the following amino-acid changes: sT126I (n=3), sQ129G (n=1), sG130N (n=1), sN131H (n=1), sF134S (n=1), sC137S (n=1), sS143L (n=3), sG145R (n=2) (table 4). The most described sG145R substitution was detected in 2 cases (6%). All but one genotype D strains carried S substitution, while only 3 (30%) variants were found among the 10 genotype C strains.

Quantification differences between the three assays according to genotype and "a" determinant substitutions were specifically analyzed on the three systems (figure 4 and 5). Although the size of our population was limited, we found a slight influence of HBV genotype on HBV quantification whatever the considered technique. Regarding the "a" determinant substitutions, Roche and DiaSorin techniques seemed to be the most affected. Noteworthy, all variants were correctly detected but DiaSorin tended to underestimate the variant concentration while Roche gave higher than expected values.

**DISCUSSION**

HBsAg concentration measurement has become a clinically relevant marker in addition to the well standardized panel of serological and molecular markers. As for HBV viral load, standardization of both the measurement unit and the techniques is mandatory to provide clinicians with comparable quantitative HBsAg values whatever the utilized method. Both Roche and Abbott HBsAg quantification techniques have been extensively studied with the conclusion that both techniques are overall very comparable and can be used indifferently to monitor patients (18,19,24). By contrast, the newest DiaSorin assay has only been studied in two small studies comparing its performance to the Abbott system (21,25). Our study first confirms these previous results and indicates that the Architect values are always 0.16 log IU/mL higher than those from the Liaison-XL. From all published studies, one may conclude that a patient could be followed indifferently with Abbott or Roche assay but an adjustment may be required if the Liaison-XL is used during follow up.

Simultaneous detection of both HBsAg and Ab is often puzzling in clinical situations. Indeed, although this peculiar profile has been described by several authors in different settings, one may wonder whether it corresponds to a true analytical observation (3–5,26–29). Overall, we show that the
qualitative agreement is 65% for the coexistence of both Ag and Ab, providing a strong argument that these detections are not a simple artifact. These data are comparable to the 78.6% concordance rate reported in a small study using only two first generation qualitative assays (30). Each of these techniques uses different combination of Ag and antibodies in different format and confirmation by three independent techniques is reassuring. According to our experience, Ag/Ab samples may sometimes pose problem when performing an HBsAg neutralization assay as proposed by manufacturers. One should stress that a thorough selection was performed in our population to not include clinical situations where such profiles may simply be a consequence of therapeutic interventions. Indeed, coexistence of both Ag and Ab markers is common during HBV reactivation (occurrence of HBsAg in a patient with resolved HBV infection) or administration of immunoglobulins; in these settings coexistence of both markers are usually only transient. In our set of samples, the agreement for anti-HBs detection screened by Abbott, is not as convincing as for HBsAg but reach 86% and 71% with DiaSorin and Roche assays, respectively. In terms of correlation between anti-HBs measurement, the results are concordant with what observed on classical samples, anti-HBs positive but HBsAg negative (31). Taking into account the mean of the three techniques for anti-HBs determination, it should be pointed out that 15.8% (n=16) samples were above 100 IU/L, a threshold often considered as highly protective in exposed persons. Yet, it emphasizes the risk of considering someone protected against HBV infection if anti-HBs are solely looked for in a screening program; obviously, anti-HBs should not be used as a single screening test as it may falsely misdiagnose a vaccine status in a patient with an active HBV infection! (32)

Correlations between HBsAg measurement assays were very satisfactory even in these peculiar sera where anti-HBs could potentially interfere with measurement (fig. 3). Astonishingly, all assays showed performances very similar to what observed in classical HBsAg positive samples by us and others (18,21,24,25,33). The concordance for HBsAg detection was also very strong and only 2 samples at the limit of detection by all techniques were not detected on the Elecsys. The above conclusions, regarding the lower values obtained on the Liaison-XL, were still valid on these Ag/Ab positive samples. One rationale for testing such Ag/Ab samples was the likelihood to artificially enrich our set with HBsAg variants (7,34). Actually, variant strains were detected in 77% (24/31) of the samples and 45% (14/31) of the substitutions could potentially modify HBsAg antigenicity (23). Although our study was not primarily designed to demonstrate an influence of HBV genotype on HBsAg quantification and
the tested numbers are rather limited, some unexpected differences between techniques for the most represented genotypes, B, C, D and E were observed. This observation is currently being further documented in a collaborative study on more samples. Many studies have shown that "a" determinant variants were incorrectly detected or quantified by commercially available assays in the past (13). The attractive strategy of protein unfolding proposed by DiaSorin did not seem to significantly enhance the detection of variants and the Architect system was the less affected by the substitutions present on our samples (fig. 5) (25). Noteworthy, the 2 samples carrying the sG145R variant were quantified to the same level by all three techniques. Overall, it was not possible to identify a specific protein pattern that would particularly affect one technique and the most divergent results were not specifically observed for HBsAg variants.

In summary, this study demonstrates the overall good performance of quantitative assays for HBsAg even in patients with coexisting HBs and anti-HBs. Although, the precise reasons for this entity remain elusive, confirmation on three techniques tend to prove that a technical artifact is not to be blamed in the majority of the cases. The difference observed between techniques should remind us that sequential monitoring should ideally be performed with a unique assay even though all techniques provide well correlated quantification.
Funding: Reagents and technical support were kindly provided by DiaSorin for the Liaison-XL and Roche for the Elecsys.

Competing interests: All authors but VT declare to have no conflict of interest in the presented work. VT declares to have received speaker fees and travel support from Abbott, DiaSorin and Roche.

Ethical approval: this non interventional study has been performed according to the national current health regulations and in accordance with the ethics principles of the Declaration of Helsinki.

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Figure legends

Figure 1: General outline of the study

Figure 2: Correlation between HBsAg quantification using Abbott and DiaSorin assays (n=227) (A) and Bland Altman representation (B). The mean bias between both techniques was -0.162 logIU/mL.

Figure 3: Correlation between the mean HBsAg values obtained by the three techniques (x axis) and values obtained by each technique (y axis) on 99 samples detected by all techniques. Abbott: closed circles, plain line (\( y = 0.9724x + 0.0361, R^2 = 0.9877 \)); DiaSorin: open squares, interrupted line (\( y = 1.0457x - 0.2432, R^2 = 0.9576 \)); Roche: closed triangles, dotted line: (\( y = 1.0042x - 0.0031, R^2 = 0.9801 \))

Figure 4: Box plot representation of HBsAg quantification difference according to HBV genotype. HBsAg values expressed as IU/mL were expressed as a percentage to the mean values obtained with the 3 assays and grouped according to the genotype B (n=4), C (n=10), D (n=8) and E (n=7). Statistically significant differences according to genotype are indicated for each assay.

Figure 5: Box plot representation of HBsAg quantification difference according to the presence of "a" determinant substitution. HBsAg values expressed as IU/mL were expressed as a percentage to the mean values obtained with the 3 assays and grouped according to the presence (Y, n=11) or absence (N, n=20), of "a" substitution. Statistically significant differences according to "a" substitution are indicated for each assay.
Table 1: Studied population main characteristics

<table>
<thead>
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<th>Parameters: median (range)</th>
<th>Main cohort</th>
<th>Molecular study selected patients</th>
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<td>Sex ratio M/F</td>
<td>37/25</td>
<td>18/13</td>
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<tr>
<td>Age (y)</td>
<td>44.9 (22.1-79.6)</td>
<td>45.2 (22.1-72.2)</td>
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<td>HBsAg Abbott (IU/mL)</td>
<td>556 (0.1-233 185)</td>
<td>1545 (4.7-233 185)</td>
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<tr>
<td>Anti-HBs Ab Abbott IU/mL</td>
<td>24 (11-257)</td>
<td>22 (11-115)</td>
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Table 2: qualitative agreement between all techniques (n=101)

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<thead>
<tr>
<th>Abbott Reactivity</th>
<th>DiaSorin</th>
<th>Roche</th>
<th>DiaSorin and Roche agreement</th>
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<tr>
<td>HBsAg</td>
<td>100 (99%)</td>
<td>99 (98%)</td>
<td>99 (98%)</td>
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<tr>
<td>Anti-HBs</td>
<td>87 (86%)</td>
<td>72 (71%)</td>
<td>68 (67%)</td>
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<tr>
<td>Ag/Ab</td>
<td>86 (85%)</td>
<td>70 (69%)</td>
<td>66 (65%)</td>
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Table 3: Main results for the two HBsAg discrepant samples (samples below detection limit are underlined)

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<th>Sample</th>
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<td>HBsAg (IU/mL)</td>
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<td>Anti-HBs (IU/L)</td>
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<td>91</td>
<td>HBsAg (IU/mL)</td>
<td>0.05</td>
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<td>32</td>
<td>57</td>
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Table 4: Main substitutions known to influence HBsAg antigenicity detected on each strain

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HBsAg residues
**Chronic hepatitis B carriers [2008-2012]**

- **n=2578**
  - HBsAg/anti-HBs profile
    - **n=129 [5%]**
  - AgHBs/anti-HBs profile
    - **n=62 [2.4%]**

**Exclusion criteria:**
- Co-infection (HIV, HDV, HCV)
- Treated with HBIg
- Reactivation of past infection
  - **n=67 [52%]**

**Samples tested for HBsAg and anti-HBs [Abbott/Roche/DiaSorin]**
- **n=101**

**Patients with HBV-VL>100 IU/mL**
- **n=31**

**HBsAg gene sequencing**
Figure 2

A

\[ y = 1.023x - 0.2377 \]
\[ R^2 = 0.9626 \]

B
Figure 3: Mean of the 3 technique HBsAg values (log IU/mL) compared to each technique individual HBsAg values (log IU/mL).
Fig. 5

Determinant "a" variant

Abbott | DiaSorin | Roche

Y | N | Y | N | Y | N

% difference to the mean

$p=ns$  $p=0.0002$  $p<0.0001$