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**European collaborative evaluation of Enzygnost HBsAg 6.0:
performance on hepatitis B virus surface antigen variants**

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Table 1. Nature, HBV genotype, and number of mutations predicting amino acid changes in HBsAg and/or pre-S proteins with 201 samples used in the study.

Genotype	Subtype	HBsAg variants				Pre-S variants only	Total
		1-3 AA changes		>3 AA changes ^a		Native samples	
		Native samples	Recombinant samples	Native samples	Recombinant samples		
A	adw2	16	26	5	6	1	54
	adw4q+	1	-	-	-	-	1
	ayw1	1	-	-	-	-	1
	ayw2	1	-	-	-	-	1
	?	1	-	-	-	-	1
B	adw2	1	8	-	-	-	9
	ayw1	1	-	-	-	-	1
C	adr	8	4	-	-	-	12
	ayr	1	-	-	-	-	1
	? ^b	-	-	1	-	-	1
D	ayw2	31	3	3	6	3	46
	ayw3	22	-	1	-	-	23
	ayw4	4	1	-	-	-	5
	ayw2/3 ^c	1	-	-	-	-	1
	? ^b	6	-	1	-	-	7
D/E ^d	ayw? ^b	1	-	-	-	-	1
E	ayw4	9	-	1	-	16	26
	ayw2/4 ^c	1	-	-	-	-	1
	n.a.	2	-	-	-	-	2
F	adw4q+	2	-	1	-	-	3
	adw4q-	2	-	-	-	-	2
G	adw2	2	-	-	-	-	2
Total		114	42	13	12	20	201

^a: Range: 4-11 changes; ^b: Unpredictable subtype because of amino acid changes in residues involved in subtype specificity; ^c: Quasispecies; ^d: Recombinant strain.

Table 2. HBsAg variants in residues involved in difficulties of diagnosis included in the study. Substitutions reported previously in association with such difficulties are highlighted in bold (see [Avellón and Echevarría, 2006] for full references).

AA residue	Wild type	Variants included	AA residue	Wild type	Variants included
103	M	I	133	M	I, T, L
104	S	T	134	F/Y	L, N, S
105	P	L, R	136	S	Y
109	L	V, R	138	C	R
110	I	M, L	140	T/S	S
112	G	V	141	K	E
113	S	P, T	142	P	S, L,
114	T/S	K, P, A	143	T/S	M, L, W
115	T	N, R, I	144	D	A, G, E, V
116	T	S, N	145	G	R, A
117	S	N, I	146	N	D
118	T/A	M, R, P, V	148	T	I
119	G	E, R, D	150	I	F
120	P	T, S, Q, L	154	S	P, L
122	K/R	I	159	G	A
124	C	R, Y, S	160	K	R, N
125	T/M	M, R	161	Y	F
126	T/I	I, N, S	164	E	D, G, V
127	P/T/L	A, S, I, L, T	168	V	A
128	A	V	166	A	G
129	Q	H, R	170	F	S
130	G	R, S, N	172	W	STOP
131	T/N	A, I, K	173	L	P
132	S	F, C			

Table 3. Nature, S/CO values, and amino acid changes in selected samples.

a) Samples testing S/CO ≥ 25.0 by Enzygnost[®] HBsAg 6.0, and border line positive (S/CO <2.0) or negative (bold) (S/CO <1.0) in Enzygnost[®] HBsAg 5.0.

Type	Sample Id	HBsAg reactivity (S/CO) by			AA changes
		E6.0 (dilution tested)	E5.0 (dilution tested)	Other tests (dilution tested)	
Natural	53	55.6 (1:40)	0.83 (1:40)	1854 ^a (1:40) 347 ^b (1:40) 6.3 ^c (undil.) 441 ^d (1:40)	T115N+P120Q+N131K+ D144A
Recombinant (all samples tested undiluted)	215	52.9	0.78	8.3 ^e	T115R+P120Q+S154L+ E164V+Q181R
	218	56.3	0.99	43.3 ^e	M133I+F134N+P142S+ T143L+G145R
	219	55.0	1.0	67.8 ^e	T118V+M133I+F134N+ P142S+T143L+G145R
	222	52.3	1.2	1.2 ^e	V96A+M103I+S114A+ T115I+T116N+S117N+ T118R+P120Q+P127T +Q129H+S136Y
	225	53.7	0.86	0.34^e	Q101H+S117N+T118R+ P120Q+S143L
	226	51.3	0.86	0.88^e	S117N+T118R+P120Q
	227	56.3	0.75	1.2 ^e	Q101H+S117N+T118R+ P120Q
	204	47.4	0.95	2.4 ^e	P120Q+N131K+G145R
221	41.4	0.82	0.44^e	Y100C+P105R+T118R+ P120L+R122K+P142L+ K160N+L173P	

b) Samples displaying S/CO <25.0 or negative (bold) results (S/CO <1.0) by Enzygnost[®] HBsAg 5.0 and/or Enzygnost[®] HBsAg 6.0.

Type	Sample Id	HBsAg reactivity (S/CO) by			AA changes
		E6.0 (dilution tested)	E5.0 (dilution tested)	Other tests (dilution tested)	
Natural	105	17,6 (1:2)	11,2 (1:2)	11,3 ^a (1:10) 11,2 ^b (1:10) 23,4 ^f (1:10)	A184V+ 48T+Q82P+ P94L (preS1)
	154	4,6 (1:10)	3,4 (1:10)	n.a.	M133I+S207N
	111	1,6 (1:2)	1,2 (1:2)	4,6 ^a (1:10) 1,2 ^b (1:10) 1,6 ^f (1:10)	E54D (preS1)
	109	1,5 (1:2)	1,1 (1:2)	6,2 ^a (1:10) 1,2 ^b (1:10)	A184V

				1,6 ^f (1:10)	
	48	1,2 (1:10)	0,64 (1:40)	1,6 ^a (undil.) 0,6^b (1:40) 0,1^c (undil.) 1,2 ^d (1:40)	G145R
	170	6,1 (undil.)	5,3 (undil.)	1,6 ^a (undil.)	L127P+F134Y+S/T140
Recombinant (all samples tested undiluted)	193	21,5	1,3	27,2 ^e	D144A+G145R
	207	7,7	0,93	10,0 ^e	P142S+G145R+N146D
	206	4,3	1,1	0,44^e	P105L+T125M+D144E
	178	12,1	6,4	11,5 ^e	K141E

^a AxSYM[®] HBsAg V2 (Abbott Diagnostics, Delkenheim, Germany)

^b Elecsys[®] HBsAg (Roche Diagnostics, Penzberg, Germany)

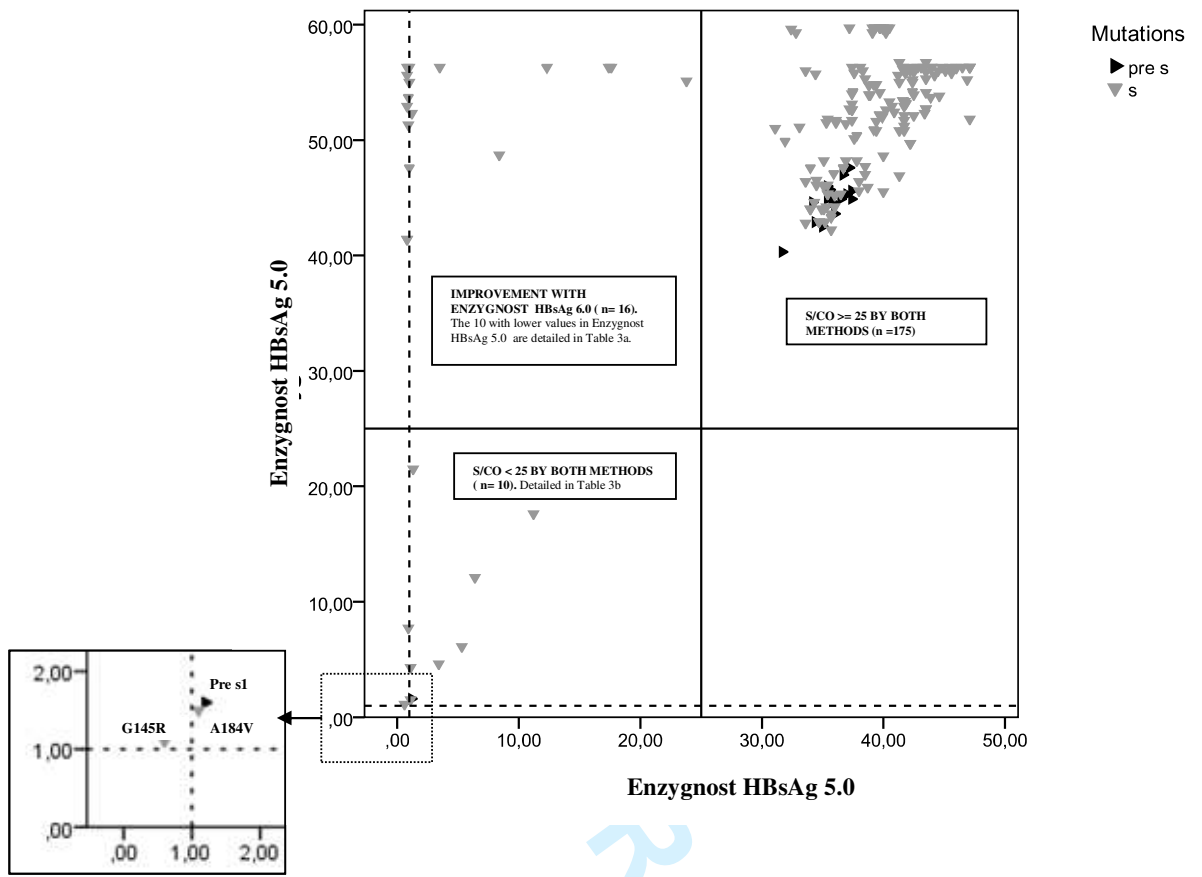
^c VITROS[®] ECi HBsAg (Ortho-Clinical Diagnostics, High Wycombe, Buckinghamshire, UK)

^d VITROS[®] ECi HBsAg ES (Ortho-Clinical Diagnostics, High Wycombe, Buckinghamshire, UK)

^e MONOLISA[®] HBs Ag ULTRA (Bio-Rad, München, Germany)

^f VIDAS[®] HBsAg ULTRA (Biomérieux, Marcy-l'Etoile, France)

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3 **European collaborative evaluation of the Enzygnost HBsAg 6.0 assay:**
4 **performance on hepatitis B virus surface antigen variants**
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Abstract

Amino acid changes within the major antigenic determinant of the hepatitis B virus (HBV) surface antigen (HBsAg) may modify eventually the antigenic properties of the protein and may have impact on the sensitivity of diagnostic assays. Modifications in the design of an assay can, however, improve significantly its ability to detect HBV mutants. One-hundred and forty-seven clinical samples containing HBsAg variants, and 54 supernatants of cells expressing recombinant HBsAg mutants were tested by two generations of a commercial HBsAg test (Enzygnost® HBsAg 5.0 and 6.0, Siemens Healthcare Diagnostics Products, Marburg, Germany), and the results were compared. A significant improvement was demonstrated for the second test by comparing the mean and individual sample/cut-off values, as well as by the detection of several samples displaying amino acid changes in residues 120 and 145 of the HBsAg which were recorded as negative by the former test. The results showed that modifications in design of the assay improved considerably the ability of the test to detect HBsAg mutants, and that difficulties in detecting such HBV variants should not be expected with the routine use of the test in diagnostic laboratories and in blood transfusion centres.

1. Introduction

Involvement of reverse transcriptase activity in the replication of the hepatitis B virus (HBV) genome leads to a higher mutation rate and to a broader genetic variability than in most DNA viruses. Random mutations originating in polymerase errors give rise to viral mutants, which may emerge as major viral populations under the pressure of host factors, and some of these mutants display amino acid changes within the major antigenic determinant of the HBV surface antigen (HBsAg). These changes may modify the antigenic properties of the protein and may have impact on relevant issues, such as the immunity to re-infection by the mutant or the sensitivity of diagnostic assays for detecting the mutated form of the antigen [Echevarría and Avellón, 2006; Weber, 2006].

Although amino acid changes resulting in decreased binding to specific antibody have been found in many residues of the HBsAg molecule, particular changes in residues 126, 144 and 145 are of special significance, since they may lead to false negative results in diagnostic assays [La'ulu and Roberts, 2006; Louisirirochanakul et al., 2006; Ly et al., 2006; Muhlbacher et al., 2008; Scheiblauer et al., 2006; Weber et al., 2006]. Modifications in the design of an assay can, however, improve significantly its ability to detect these HBsAg mutants [Echevarría and Avellón, 2008; Weber et al., 2006]. Assessing the success of such changes requires collaborative studies involving, ideally, both clinical samples containing natural variants and samples displaying recombinant mutant proteins, and the performance of such studies has been strongly recommended [Gerlich, 2004].

Enzygnost® HBsAg 5.0 (E5.0) is an enzyme-immunoassay (EIA) used widely for the diagnosis of the HBV infection for many years. A novel version of the test (Enzygnost® HBsAg 6.0, Siemens Healthcare Diagnostics Products, Marburg, Germany) (E6.0) has been developed recently by the manufacturer, and changes have been introduced in order to improve the detection of HBsAg variants. The aim of the present collaborative study was to assess the HBsAg mutant detection ability of the E6.0 assay in comparison with the former version of the test (E5.0). Clinical samples containing HBsAg variants and supernatants of cells expressing recombinant HBsAg mutants were used in the study.

2. Materials and Methods

Samples containing HBsAg and Pre-S variants

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3 The study involved four European laboratories and a total of 201 HBV mutant samples
4 of natural (n=147) or recombinant (n=54) origin (Table 1). The panel spanned mutations
5 predicting amino acid (AA) substitutions between positions 103 to 173 of HBsAg, most
6 involving residues from the major hydrophilic region (AA 124 to 147) that may impair
7 the detection of the antigen [Avellón and Echevarría, 2006; Gerlich, 2004; Weber,
8 2006] (Table 2). All samples were aliquoted and stored at -20°C prior to testing.
9 Multiple freeze/thaw cycles were avoided. Samples were tested undiluted or, in case of
10 limited amounts, diluted as described below. Dilutions were prepared in E6.0 negative
11 control solution for samples tested by Enzygnost or diluted in fetal calf serum, or as
12 recommended by each manufacturer, for samples tested with the remaining assays.
13 Sixty-five samples of the panel were from Spanish HBV carriers displaying predicted
14 AA substitutions at positions 112 to 145, plus one sample with a predicted stop codon at
15 position 196 [Avellón and Echevarría, 2006]. All the samples were tested at a 1/40
16 dilution, and again at a 1/10 dilution at least by the E6.0 assay if an absorbance value
17 below the cut-off was obtained. Retesting at a lower dilution by the E5.0 assay was not
18 done when the volume of the sample available was small. The results obtained on
19 undiluted aliquots by the Vitros[®] ECi HBsAg and HBsAg ES (Ortho-Clinical
20 Diagnostics, High Wycombe, Buckinghamshire, UK) [Echevarría and Avellón, 2008],
21 Axsym[®] HBsAg V2 (Abbott Diagnostics, Delkenheim, Germany) and the Elecsys[®]
22 HBsAg (Roche Diagnostics, Penzberg, Germany) were available for comparison.
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39 Thirty-five samples, supplied by the German National Consulting Laboratory for HBV
40 (NCL), displayed AA changes leading to difficulty in HBsAg detection [Gerlich, 2004;
41 Westhoff et al., 2003]. Samples were tested undiluted or in dilutions ranging from
42 1/3.16 to 1/25. The results obtained by the Axsym[®] HBsAg V2 and by the Prism[®]
43 HBsAg (Abbott Diagnostics, Wiesbaden, Germany) were available for comparison.
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48 Forty-seven samples were provided by Laboratoires Réunis Junglister, Luxemburg.
49 Twenty-two samples were from Luxembourgian HBV carriers [Weber, 2006]. The
50 remaining 25 were from Nigerian patients with liver disease. Samples were tested in
51 dilutions ranging from 1/2 to 1/100. The results obtained by the Axsym[®] HBsAg V2
52 (Abbott Diagnostics, Delkenheim, Germany), the Elecsys[®] HBsAg (Roche Diagnostics,
53 Penzberg, Germany) and by the Vidas[®] HBsAg Ultra (Biomérieux, Marcy-l'Etoile,
54 France) were available for comparison.
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60 Fifty-four recombinant HBV mutant samples, comprising HBV genotypes A to D and
displaying AA substitutions and insertions between residues 96 to 181 of HBsAg, were

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3 provided by Siemens Healthcare Diagnostics. The mutants were constructed by site-
4 directed mutagenesis of wild-type sequences and were expressed in transfected Huh7
5 cells. All the samples consisted of cell culture supernatants. The HBsAg concentration
6 was not determined. Results obtained by the Monolisa[®] HBsAg Ultra (Bio-Rad,
7 München, Germany) were available for comparison.
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12 13 14 2.1 HBsAg testing

15 All the samples were tested by the E5.0 and E6.0 assays. The latter test uses sheep
16 polyclonal antibody to HBsAg (anti-HBs) on the solid phase, and a mixture of three
17 monoclonal anti-HBs, targeting different HBsAg epitopes, in the conjugate. Tests were
18 performed following the manufacturer's guidelines and using an automatic processor
19 provided and maintained by the manufacturer. The results were recorded as a signal to
20 cut-off ratio (S/CO). The samples displaying S/CO lower than 1.0 were recorded
21 negative, and the remaining was recorded positive. The samples were not distributed to
22 all participants for testing.
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31 32 2.2 Statistics

33 Comparison of mean S/CO values was made by the t student test (SPSS software).
34 Statistical significance was considered for $p < 0.005$.
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39 40 3. Results

41 S/CO values obtained by the E6.0 assay were always equal to or higher than 1.0. Figure
42 1 shows a dispersion chart comparing the S/CO values obtained by the E6.0 and E5.0
43 assays.
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45 Dots arranged on the chart displaying one main and two minor clusters defined by
46 $S/CO=25$ in both tests (inner lines). Considering that the cut-off value was usually set
47 close to an optical density (OD) value of 0.080 absorbance units in both assays, the
48 S/CO range from 0 to 25.0 would reflect the full range of OD values within the linear
49 range of the tests. Samples placed on the upper right quadrant of the chart ($S/CO \geq 25.0$
50 in both assays) were considered as detected correctly by both methods, and were
51 classified into category 1. Samples placed on the lower left quadrant ($S/CO < 25.0$ in
52 both assays) drew category 2, which included samples displaying false negative results
53 ($S/CO < 1.0$) by either one or both tests. The remaining samples (category 3) placed on
54 the upper left quadrant ($S/CO \geq 25.0$ by E6.0, < 25.0 by E5.0), and were thought to reflect
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3 differences in performance favouring the E6.0 assay. Samples in category 2 were also
4 thought to represent significant differences between tests when the S/CO value recorded
5 by one test was at least 1.5 times higher than the value recorded by the other test. No
6 samples placed in the lower right quadrant of the chart were found.
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10 One hundred and seventy-five samples (87.0%) were classified into category 1.
11 Eighty-one displayed single amino acid substitutions in residues 120, 126, 129, 133,
12 134, 140, 142, 143 or 145, and 41 displayed multiple substitutions involving at least one
13 of these residues. The remaining samples presented either insertions (n=2), substitutions
14 involving the pre-S region only (n=19), or substitutions involving other residues of the
15 HBsAg (n=32). The mean S/CO value found with these samples was significantly
16 higher with the E6.0 assay (51.7, S.D.: 4.8 vs. 39.3, S.D: 3.6; F=16.7, p<0.005).
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23 Sixteen samples (7.9%) were classified into category 3. Ten samples were borderline
24 positive (S/CO <2.0) or negative by the former assay (Table 3a), and one was a natural
25 sample. Multiple amino acid substitutions were recorded in all these ten samples, and
26 eight shared the substitution P120Q.
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30 Finally, 10 samples (4.9%) were classified into category 2 (Table 3b). Six were natural
31 samples, and three of them displayed borderline reactivity by the E6.0 assay (S/CO
32 <2.0) and in some other assays in association with single amino acid changes in the
33 HBsAg (G145R, A184V) or in the pre-S1 protein.
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37 Both natural and recombinant samples with amino acid changes in residues 126, 129,
38 134, 138, 140, 143 and 148 were strong positive by the E6.0 assay despite the additional
39 occurrence of other relevant changes in some of them. Substitutions in residues 144
40 were associated with reactivity S/CO <25.0 on recombinant samples when additional
41 changes were present. A single substitution K141E lead to S/CO value of 12.1 in a
42 recombinant sample, but substitutions in residue 142 had no particular effects in
43 absence of additional changes. Amino acid changes in these two residues are
44 uncommon among HBV carriers [Avellon and Echevarria, 2006], and no natural
45 samples with such changes could be tested.
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53 As shown in Table 3b, changes in residues 145 and 184 in natural samples were
54 associated with S/CO values < 2 by the E6.0 assay, similarly to some of the additional
55 assays tested. Single changes in residue 145 had effects both on natural and
56 recombinant samples. The substitution A184V, either alone or in association to other
57 changes, had also effect on the level of reactivity (samples 105 and 109, Table 3b).
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3 The pre-S region was studied in 47 samples, and amino acid substitutions were found
4 in 31 samples. The pre-S1 region displayed changes in 14 samples, the pre-S2 region in
5 eight samples, and both regions in nine samples. Twenty samples with pre-S variants
6 displayed a wild-type sequence for HBsAg, and only one (sample 111, table 3b)
7 displayed a borderline S/CO value by the E6.0 assay, paralleling the results obtained
8 with other tests.
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10 11 12 13 14 15 16 **4. Discussion**

17 Warning on the emergence of HBV variants involved potentially in diagnostic failures
18 [Gerlich, 2004] led many companies to modify their assays in order to overcome the
19 problem, and some significant improvements have been noted [Echevarría and Avellón,
20 2008]. The results of the present study show that the new Enzygnost® HBsAg 6.0 test
21 improves the characteristics of the former with regard to detection of such HBsAg
22 variants.
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28 The improvement was demonstrated by comparing the mean and individual S/CO
29 values, as well as by the detection of several samples displaying amino acid changes in
30 residues 120 and 145 that were recorded negative by the former test. Difficulties with
31 current commercial tests for detecting HBsAg variants with substitutions in positions
32 143 to 145 have been recognised widely [Echevarría and Avellón, 2008; Ly et al., 2006;
33 Mühlbacher et al., 2008; Weber et al., 2006]. Substitutions in residue 120 were
34 associated in the past with false negative results by diagnostic assays [Coleman et al.,
35 1999], but do not involve such difficulties for the current, modified tests [Mühlbacher et
36 al., 2008]. Similar results were obtained with two recombinant samples displaying the
37 substitution Q101H, which correspond to an HBsAg residue placed outside the “a”
38 antigenic determinant. A relative sensitivity reduction in HBsAg testing has been
39 reported recently in association with this substitution, and has been shown to affect a
40 significant proportion of the 70 commercial tests studied [Scheiblauer et al., 2010].
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51 Just ten samples from the present study rendered S/CO values < 25 by the E6.0 assay.
52 All were, however, positive despite dilution, and all displayed weak reactivity, or
53 negative results, by tests used for comparison. As reported for other tests, these samples
54 displayed often multiple amino acid substitutions [Avellón and Echevarría, 2006]. A
55 low HBsAg concentration, either alone or in association with relevant amino acid
56 substitutions, could also contribute to weak reactivity or negative results by diagnostic
57 tests. The sample 170, which stems from a patient after antiviral therapy, might display
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3 a low concentration of the antigen. Evaluating the real HBsAg concentration in samples
4 containing variants that may impair binding to capture antibody is, however, a difficult
5 task, and the hypothesis is barely testable in practice. Prior reports suggest, in addition,
6 that difficulty in HBsAg detection in samples containing relevant HBsAg variants are in
7 relation to a major participation of the variant in the quasispecies viral population, and
8 this is a likely explanation for low reactivity by the E6.0 assay of sample 48 [Avellón
9 and Echevarría, 2006].

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16 This study suggests, in addition, that amino acid changes in the pre-S1 region might
17 also be responsible for low reactivity, since one sample displaying a wild type HBsAg
18 sequence and a single AA change (E54D) in the pre-S1 region was low-reactive by five
19 different tests, including the E5.0 and E6.0 assays. The reduced number of samples
20 available for testing hampered, however, the significance of this finding, which could be
21 due to an unusually low HBsAg concentration in the sample.

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26 The results obtained in the present collaborative study show, in conclusion, that
27 modifications introduced in the design of E6.0 have improved significantly the ability of
28 the test to detect HBsAg mutants. After such modifications, the new version displays a
29 high profile of performance in comparison with other assays, and difficulty in detecting
30 HBsAg variants should not be expected with the routine use of the test in diagnostic
31 laboratories or blood transfusion centres.

32 33 34 35 36 37 38 **Conflict of interest**

39 Michael Weik and Uwe Schobel are employees of Siemens Healthcare Diagnostic
40 Products GmbH. The remaining authors state that no financial or personal relationship
41 exists with other people or organisations that could inappropriately influence their work
42 in regard to the issues of the present report.
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4 **Legend for figure**

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6 Figure 1. Dispersion chart of S/CO values. Comparison of Enzygnost HBsAg 6.0 vs.
7 Enzygnost HBsAg 5.0. In black, samples displaying mutations only in the pre-S region.
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