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Improved detection of early acute, late acute, and occult Hepatitis B infections by an increased sensitivity HBsAg assay

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ABSTRACT

Background: Hepatitis B surface antigen (HBsAg) is the primary marker for diagnosis of acute and chronic hepatitis B. Although HBsAg assays have undergone continuous improvement, gaps remain in the detection of early and late acute infection and occult hepatitis B infection (OBI).
Objectives: The performance of a prototype, improved sensitivity HBsAg assay run on the ARCHITECT and Alinity instruments was evaluated for detection of early and late acute infection and OBI.
Study design: Seventy seven early acute samples [positive only for hepatitis B viral DNA (HBV DNA)], twelve seroconversion panels spanning late acute infection, and 101 occult samples (HBsAg negative, positive for HBV DNA and anti-HBc) were tested with the prototype assay and ARCHITECT HBsAg Qualitative II. HBsAg gene sequencing was performed to determine genotype and mutations in the immunodominant region.
Results: Compared with ARCHITECT HBsAg Qualitative II, the prototype assay showed increased detection of NAT yield samples (28/77, 36.4%,), late acute samples (≥13 days longer detection of HBsAg for 6/12 panels), and OBI samples (11/101, 10.9%). HBsAg sequence data were obtained for 62 samples. Genotypes represented were A1, A2, B2, B4, C1, C2, C5, D3, E, and H. HBsAg escape mutations were found in 4.8% of NAT yield and 38.9% of OBI samples sequenced. Prototype assay values for 188 samples were equivalent on the ARCHITECT and Alinity instruments.
Conclusions: The new prototype HBsAg assay will be of diagnostic value in providing improved detection of early acute, late acute, and occult HBV infections.

1. Background and objectives

Globally, more than 257 million people are chronically infected with the hepatitis B virus (HBV) [1]. Hepatitis B surface antigen (HBsAg) remains the mainstay for diagnosis of acute and chronic hepatitis B as well as for screening blood and blood products. Although HBsAg assays have undergone continuous improvement, gaps remain in the identification of some phases of HBV infection [2–5].

During the early phase of acute infection, hepatitis B viral DNA (HBV DNA) is the only serum viral marker detectable prior to the appearance of HBsAg. The introduction of HBV DNA nucleic acid testing (NAT) into blood screening allowed the identification of these HBV DNA positive samples (HBV "NAT yield") among blood donors [3]. The late phase of acute infection is marked by the presence of antibody to hepatitis B core antigen (anti-HBc) and loss of detectable serum HBsAg. During this phase, HBV DNA is present at low/declining levels prior to becoming undetectable in serum as the infection resolves and protective antibody to HBsAg (anti-HBs) appears. Improvements in the sensitivity of HBsAg assays have shortened but not closed the periods during the early and late phases of acute infection when HBsAg may be undetectable.

Occult hepatitis B infection (OBI) is defined as the presence of

Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; OBI, occult hepatitis B infection; HBV DNA, hepatitis B viral DNA; Anti-HBc, antibody to hepatitis B core antigen; NAT, nucleic acid testing; Anti-HBs, antibody to hepatitis B surface antigen

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detectable HBV DNA in liver or plasma with undetectable HBsAg (excluding the pre-seroconversion window) with or without anti-HBc or anti-HBs. The HBV DNA level in serum is usually very low (< 200 IU/ml) [6,7]. Lack of HBsAg detection in OBI may be due to HBsAg levels below the detection limits of the immunoassays used, mutations in the HBsAg gene, or masking of HBsAg by anti-HBs [4,6].

We previously reported on the development of a new prototype HBsAg assay with improved sensitivity [8]. In the current study, we evaluated the performance of the prototype assay in the detection of HBV NAT yield samples from blood donors, seroconversion panels spanning the late acute phase of infection, and anti-HBc positive OBI samples. Samples were characterized for viral load, genotype, presence of anti-HBs, and mutations that may affect HBsAg detection.

2. Study design

2.1. Samples

Hepatitis B seroconversion panels with members spanning the late acute phase of HBV infection were purchased from commercial vendors. Panels 6508, 6512, 6514, 6528, 6535, 6537, and 6546 were purchased from Zeptometrix Corporation (Franklin, MA). Panels 13867-3482, 1807–3463, 26022-14518, 43527-3453, and 26982-14399 were purchased from North American Biologicals, Inc. (Boca Raton, FL). Detailed information on the panels is shown in Table 3. OBI samples (HBsAg negative, anti-HBc positive, HBV DNA positive) and HBV NAT yield samples (HBsAg negative, anti-HBc negative, HBV DNA positive) were purchased from the American Red Cross (Gaithersburg, MD), the South African National Blood Service (Boksburg, South Africa), BocaBiolistics (Pompano Beach, FL), and PromedDx (Norton, MA). For the purposes of this study, samples were considered HBV DNA positive if at least two determinations were positive using Abbott RealTi*m*e HBV, Procleix Ultrio Discriminatory Assay for HBV (vendor-provided data), or PCR of the preS-S region (method described below).

2.2. Serologic and molecular testing

Samples were tested for HBsAg using ARCHITECT HBsAg Qualitative II (analytical sensitivity 0.017 – 0.022 IU/ml, Abbott, Sligo Ireland) and with a new prototype qualitative HBsAg assay with improved analytical sensitivity of 0.0052 IU/ml [8]. The new assay is a one-step chemiluminescent microparticle immunoassay with two monoclonal antibodies coated on the microparticles and a goat anti-HBs conjugate. The assay uses 75 ul of specimen (the same as ARCHITECT Qualitative II) with no sample pretreatment. The prototype assay is fully automated and is performed on the Abbott ARCHITECT and Alinity instruments equipped with heat induction probes to eliminate sample carry-over. Ancillary wash buffer is added in a second incubation step so the instrument performs a two-step assay protocol. Samples repeatedly reactive in the new assay were confirmed by neutralization with sheep anti-HBs in a new HBsAg confirmatory assay designed for use with the prototype assay. Anti-HBc and quantitative anti-HBs were tested using ARCHITECT assays (Abbott, Sligo, Ireland or Wiesbaden, Germany). HBV DNA levels were quantitated with Abbott RealTi*m*e HBV (Abbott Molecular, Des Plaines, IL).

2.3. HBV gene sequencing

HBV DNA was extracted from 0.5 ml of plasma using automated protocol DNA-protK-500-50 (research use only) on the *m*2000*sp* system (Abbott Molecular, Des Plaines, IL). First- and second-round PCR were performed to amplify the preS1-S region using Amplitaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA). First-round primers were HBV2813 F (5′- TCATTTTGTGGGTCACCATATT-3′, nt 2811–2832) and 18R (5′−CCCATGAAGTTTAGGGAATAAC-3′, nt 860–881); second-round primers were HBV-2822 F (5′- GGGTCACCAT ATTCTTGGGAAC-3′, nt 2820–2841) and 19R (5′- GTTAGGGTTTAAAT GTATACCC-3′, nt 822–843) amplifying a 1245 base pair fragment. The 50 µl PCR reaction (first and second rounds) contained 0.4 µM of each primer, 2.5 mM $MgCl_2$, 0.8 µM dNTP mix and 25 µl extracted DNA for first-round PCR, or 2 µl of first-round PCR as template for the second-round. First- and second-round amplifications consisted of preincubation at 95 °C (10 min), 40 cycles at 95 °C (20 s), annealing at 50 °C (45 s), extension at 66 °C (2.5 min for the first-round or 1.5 min for the second round), and final extension at 72 °C (10 min). Both strands of purified amplification products were sequenced directly using the BigDye® Terminator v3.1 Cycle Sequencing kit and the ABI 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence data were assembled and edited using Sequencher software (version 5.4.6; Gene Codes Corporation, Ann Arbor, MI). Positions with sequence ambiguities were assigned the appropriate IUPAC designation. Genotype was determined by phylogenetic analysis using the PHYLIP v3.5c software package (J. Felsenstein, University of Washington, Seattle, Washington). Nucleotide sequences were aligned with the reference sequences representing genotypes A–I using BioEdit 7.0.4.1 [9].

2.4. Mutation analysis

Atypical amino acid substitutions were determined by comparing the specimen sequences to the genotype consensus sequence created in BioEdit from alignment of genotype sequences downloaded from https://hbvdb.ibcp.fr [10].

3. Results

3.1. Detection of HBV NAT yield samples

Seventy-seven HBV NAT yield blood donor samples (ARCHITECT HBsAg Qualitative II negative, anti-HBc negative, HBV DNA positive), were tested with the prototype HBsAg assay. Viral loads ranged from < 1 to 3 log IU/ml. 28/77 (36.4%) were repeatedly reactive and confirmed positive by the neutralization assay (Table 1). HBsAg gene sequences were obtained on all 28 prototype positives and fourteen prototype negatives selected for sequencing based on the presence of anti-HBs (Table 2) Two samples had an HBsAg escape mutation.

Table 1
Incremental Detection of HBV in NAT Yield and OBI Samples by the New Prototype HBsAg Assay.

Sample Group	N	ARCHITECT HBsAg Qualitative II	Anti-HBc	HBV DNA	Incremental Detection by Prototype HBsAg Assay*	N prototype (+) with Anti-HBs ≥10 mIU/ml
NAT Yield	77	–	–	+	28 (36.4%)	4[a]
Occult	101	–	+	+	11 (10.9%)	2[b]

* Compared to ARCHITECT HBsAg Qualitative II.
[a] 11.95, 16.43, 34.43, 53.13 mIU/ml.
[b] 24.63 and 91.98 mIU/ml.

Table 2
Features of HBV NAT Yield Samples Relative to Detection by the New Prototype HBsAg Assay.

	Genotype	N	HBsAg Escape Mutations	Anti-HBs mIU/ml (number of samples)
Prototype Assay (+)	A1	11	none	< 10 (n = 10), 11.95 (n = 1)
	A2	11	none	< 10 (n = 10), 53.13 (n = 1)
	C1	1	none	16.43 (n = 1)
	C2	2	none	< 10 (n = 2)
	C5	1	I126T	< 10 (n = 1)
	E	2	none	< 10 (n = 1), 34.43 (n = 1)
Prototype Assay (-)	A1	5	none	16.03 – 98.75 (n = 5)
	A1	1	T143M	< 10 (n = 1)
	A2	5	none	26.2 – 466.72 (n = 5)
	B2	1	none	90.12 (n = 1)
	E	1	none	98.99 (n = 1)
	H	1	none	17.99 (n = 1)

Surface antigen gene sequences were obtained for 28 / 28 NAT yield samples detected by the prototype assay and 14/ 49 NAT yield samples negative by the prototype assay. Only non-mixed substitutions are listed.

3.2. Detection of late acute HBV samples

Samples from 12 HBV seroconversion panels representing the late phase of acute infection were tested with ARCHITECT Qualitative II and the prototype assay (Table 3). The prototype assay detected HBsAg ≥13 days longer than ARCHITECT HBsAg Qualitative II in six of twelve panels. The incrementally detected samples were repeatedly reactive and confirmed positive by the neutralization assay. Viral loads were low in the incrementally detected samples. One prototype positive sample was negative by RealTime HBV and PCR of the preS-S region, although a later sample had detectable HBV DNA. No HBsAg escape mutations were found in five of the panels (all genotype A2). Two panels were genotype D2 and carried HBsAg escape mutations: P127 T, A128 V (panel 6546) and A128 V (panel 6535).

3.3. Detection of occult HBV

101 blood donor samples were identified as ARCHITECT HBsAg Qualitative II negative, anti-HBc positive, and HBV DNA positive (OBI). Viral loads for 100 of the samples ranged from < 10 IU/ml to 229 IU/ml (one sample was 6.2×10^4 IU/ml). Eleven (10.9%) were repeatedly reactive and confirmed positive with the prototype HBsAg assay (Table 1). Viral loads were low for all eleven samples with nine of them having less than ten IU/ml HBV DNA.

Sequence data were obtained for 6/11 prototype positive and 12/90 prototype negative OBI samples (Table 4). Three of the six prototype positive and four of the twelve prototype negative samples had HBsAg escape mutations. The sample with 91.98 mIU/ml anti-HBs appeared to be a mixed infection with one strain having D144 N. The viral load in the OBI sample with 24.63 mIU/ml anti-HBs (Table 1) was too low to be sequenced.

Table 4
Features of Occult HBV Samples Relative to Detection by the New Prototype HBsAg Assay.

	Genotype	HBsAg Escape Mutations	Anti-HBs mIU/ml
Prototype Assay (+)	A2	none	< 10
	B2	M133L	< 10
	B4	M133 L, F134V	< 10
	A1	none	< 10
	A2	none	< 10
	A2	D144N[a]	91.98
Prototype Assay (-)	A1	none	< 10
	D3	none	< 10
	A2	none	< 10
	A2	G145A	< 10
	A1	M133T	11.35
	A1	F134 V, P142 L, D144A	22.52
	A2	none	42.77
	A2	N131 K, M133I, D144E	43.28
	A1	none	46.24
	A2	none	91.26
	A1	none	103.18
	A2	none	105.5

Surface antigen sequences were obtained for 6/11 occult HBV samples detected by the prototype assay and 12/90 occult samples negative by the prototype assay. Two prototype negative samples had apparent insertions at amino acids 117/118 but poor sequence quality beyond that point. 18 occult samples had insufficient DNA for sequencing. Only non-mixed substitutions are listed.
[a] A second strain within the same specimen had the W182* stop codon.

3.4. Comparison of prototype HBsAg results on ARCHITECT and Alinity

The new prototype HBsAg assay was designed for both the Abbott ARCHITECT and the new Abbott Alinity instrument. Fig. 1 summarizes

Table 3
Detection of Seroconversion Panels Spanning the Late Phase of Acute HBV Infection.

Panel	Panel Members (Total Days)	Last Qual II(+) Panel Member (Day)	Follow-up After Last Qual II(+): N members (Days)	Last Prototype(+) Panel Member (Day)	Days Longer Detection by Prototype[a]	HBV DNA log IU/ml[b]
6508	15 (223)	6 (70)	9 (153)	6 (70)	0	Detected, < 1
6535	12 (154)	4 (42)	8 (112)	4 (42)	0	Detected, < 1
6546	12 (154)	5 (56)	7 (98)	5 (56)	0	1.16
13867-3482	31 (128)	13 (43)	18 (85)	13 (43)	0	4.45
26022-14518	31 (135)	9 (42)	22 (93)	9 (42)	0	1.4
26982-14399	25 (96)	6 (18)	19 (78)	6 (18)	0	3.58
6528	12 (154)	6 (71)	6 (83)	7 (84)	13	Not detected
6512	15 (223)	4 (42)	11 (181)	5 (56)	14	1.31
6514	17 (260)	5(56)	12 (204)	6 (70)	14	Detected, < 1
6537	12 (153)	6 (69)	6 (84)	7 (83)	14	Detected, < 1
43527-3453	27 (365)	21 (258)	6 (107)	23 (293)	35	Detected, < 1
1807-3463	25 (237)	19 (85)	6 (152)	20 (159)	74	1.17

Twelve HBV seroconversion panels that span the late phase of acute infection were tested with Abbott ARCHITECT HBsAg Qualitative II and the prototype HBsAg assay. Table 3 focuses on the results during the late acute phase after the HBsAg peak and decline as determined by current assays. All panels seroconverted to anti-HBc positivity; the last Qualitative II positive and the last prototype assay positive members in all panels were anti-HBc positive. For the last prototype positive panel members, anti-HBs was < 10 mIU/ml in all panels except 6528 (last prototype positive had 100 mIU/ml anti-HBs). Assay S/CO's (mean of four determinations) ranged from 1.04 to 2.31 (median 2.09) for panel members incrementally detected by the prototype assay.
[a] In comparison with ARCHITECT HBsAg Qualitative II.
[b] Result for last prototype(+) sample.

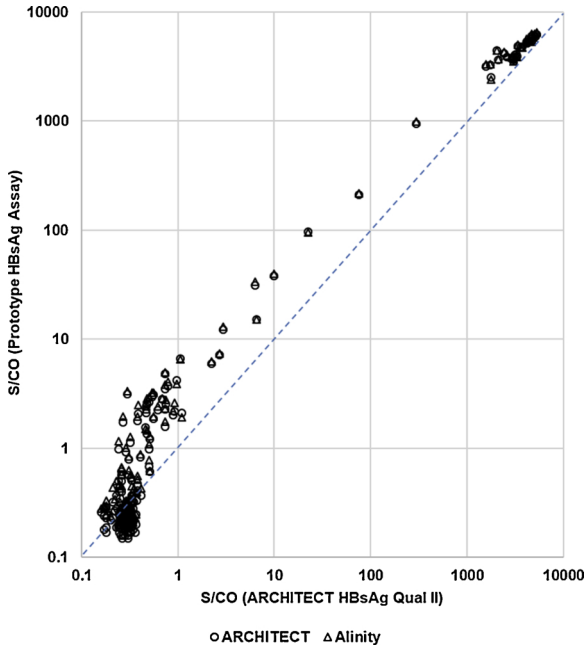

Fig. 1. S/CO values for the new prototype HBsAg assay performed on the ARCHITECT and Alinity instruments compared with the ARCHITECT HBsAg Qualitative II assay. 188 samples representing late acute HBV infection, occult HBV infection, and HBV NAT yield samples were tested. An S/CO ≥1.0 is considered reactive by all three assays. Dotted line represents the line of identity.

results for both platforms for 188 samples representing the NAT yield, late acute, and OBI samples. The data demonstrate the enhanced signal and improved sensitivity of the prototype assay on both platforms.

4. Discussion

We previously reported on the development of a new prototype HBsAg assay with analytical sensitivity of 0.0052 IU/ml, approximately four to fourteen fold better than comparator assays [8]. The new assay was shown to be more sensitive in detection of HBV genotypes (A–H) and in detection of native and recombinant HBsAg mutants compared with current assays, including ARCHITECT Qualitative II, without compromising specificity [8]. The new assay also has improved detection of vaccine breakthrough infections and more sensitive detection of HBsAg in the presence of anti-HBs compared to current assays [12].

In the current study, we evaluated the ability of the prototype assay to detect HBsAg in 77 NAT yield and 101 OBI samples and in twelve seroconversion panels representing late acute infection. All samples repeatedly reactive in the prototype assay were confirmed positive by the neutralization assay. HBV DNA and anti-HBs levels were determined for all samples and HBsAg gene sequence data were obtained for 62 samples, allowing genotypic classification and identification of HBsAg escape mutations that might affect immunoassay detection.

In acute HBV infection, HBV DNA is the first marker detectable in peripheral blood, followed by HBsAg and anti-HBc. The time period before detectability of HBsAg is often referred to as the early window period. Improved HBsAg assay sensitivity can shorten this window. The prototype assay was previously shown to shorten the early window by a median of 4.1 days compared to ARCHITECT Qualitative II [8]. In the current study, we show that this window period reduction translates to an incremental detection of 36.4% of NAT yield samples. In four of these samples, the prototype assay was able to detect HBsAg in the presence of anti-HBs levels ≥10 mIU/ml (the anti-HBs level considered protective).

We extended the evaluation of acute hepatitis B to include twelve longitudinal panels spanning the late phase of acute infection, after HBsAg has peaked, declined, and become undetectable by current assays. During this phase of resolving infection, anti-HBc remains positive while HBV DNA levels decline and eventually become undetectable and anti-HBs appears. The panels in the current study included extensive follow-up spanning 78–204 days after HBsAg loss according to the current HBsAg assay. The prototype assay demonstrated improved HBsAg detection of late acute infection in six of the 12 panels, extending the time of HBsAg positivity by ≥13 days. These data, combined with the early acute window period reduction reported previously, demonstrate that the new assay increases the overall length of time that HBsAg can be detected during acute infection.

In one panel, HBsAg was detected in an HBV DNA negative sample. A low level of HBV DNA was detected in a subsequent sample, suggesting that the viral load in these late acute samples was near the limit of detection of the HBV DNA assay. DNA levels may be extremely low during the late acute phase and detection by HBV DNA assays may require testing of multiple replicates and/or extraction of larger sample volumes. The increased detection of HBsAg by the new assay during spontaneous resolution of infection in seroconversion panels was also reflected in a study of antiviral therapy patients with apparent HBsAg loss [13]. The new assay detected 24/48 samples from 13 patients with apparent HBsAg loss by ARCHITECT HBsAg Qualitative II. However, the clinical significance of this observation relative to therapeutic endpoints is unclear at this time.

Diagnosis of OBI depends on the sensitivities of the HBsAg and HBV DNA assays used. Recent reports indicate that there may be a substantial number of OBI patients missed by current testing including patients presumed to be immune. These OBI patients may be at risk for chronic liver disease or reactivation of infection if immunosuppressed. HBV DNA testing and improved HBsAg assays would help identify these patients [5,11]. However, the complexity and expense of HBV DNA testing may limit its use in testing for OBI.

The prototype HBsAg assay detected 11 (10.9%) of the 101 OBI samples in this study. All prototype repeatedly reactive samples were confirmed positive by the neutralization assay, demonstrating the specificity of the new assay in this population. Although the incremental detection of OBI was relatively modest in this study where the majority of samples were genotype A1/A2, preliminary data indicate that detection by the new assay may be substantially higher (detection rates of 40%–50%) in OBI patients from highly endemic areas with genotypes B, C, and E (publication in preparation).

Sequencing of the HBsAg gene from prototype positive and negative OBI samples was performed to determine the presence of HBsAg escape mutations that might affect immunoassay detection. Three prototype positive OBI samples had escape mutations, including one with protective levels of anti-HBs (91.98 mIU/ml) and a D144 N mutation, a rare mutation associated with viral escape from vaccine induced anti-HBs [12,14]. Four prototype negative OBI samples had escape mutations. All of the escape mutations observed in this report were shown to be detected by the prototype assay in a previous study [8]. Thus the prototype negative results for the OBI samples shown in Table 4 are most likely due to HBsAg levels below the detection limit of the new assay rather than the presence of HBsAg mutations.

Limitations of this study included testing of a small population of OBI samples (N = 101) from blood donors from only two geographic regions, limiting the genotype representation to genotypes A, B, and D. Studies are needed to evaluate the ability of the new assay to detect OBI among the major genotypes A–H, and in diagnostic populations representing low, medium, and high endemicity. In addition, analysis of genome variability was limited to the S gene on a subset of samples with sufficient HBV DNA for sequencing. Sequencing of the whole genome would provide information on genetic variability that may affect HBsAg levels.

Furthermore, studies are needed to address the clinical utility of detecting very low levels of HBsAg that were previously out of reach

using the available HBsAg assays. The new assay may represent an additional tool for optimizing risk assessment in a variety of settings, including stratification of HBV − HCV coinfected patients undergoing direct acting antiviral therapy, in reactivation associated with immunosuppression, and in the liver transplant setting where use of grafts from anti-HBc positive donors is relatively common. Although further improvements in HBsAg assays are required to fully close the gaps in detection of all phases and settings of HBV infection, the new prototype assay represents an advancement in HBsAg testing, providing not only increased analytical sensitivity but improved detection of early acute, late acute, and occult HBV infections.

Ethical approval

None

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CRediT authorship contribution statement

Mary C. Kuhns: Conceptualization, Data curation, Formal analysis, Supervision, Writing - original draft, Writing - review & editing. **Vera Holzmayer:** Data curation, Formal analysis, Methodology, Writing - review & editing. **Anne L. McNamara:** Data curation, Formal analysis, Methodology, Writing - review & editing. **Eva Sickinger:** Methodology, Data curation, Writing - review & editing. **Jan Schultess:** Methodology, Data curation, Writing - review & editing. **Gavin A. Cloherty:** Writing - review & editing.

Declaration of Competing Interest

MCK, VH, and GAC are employees and stockholders of Abbott Laboratories; ALM is retired from Abbott Laboratories; ES and JS are employees of Abbott Laboratories.

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