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Evaluation of *in vitro* screening and diagnostic kits for hepatitis B virus infection

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ABSTRACT

Background: For the diagnosis of hepatitis B virus (HBV) infection, the detection and quantification of hepatitis B surface antigen (HBsAg) and HBV DNA are used. Several kits are available for this purpose, and there is a growing need for the evaluation of these kits because their performance may be affected by HBV genotype- or strain-specific polymorphisms.
Objectives and study design: In this study, we used International Standards and the established regional reference panel to evaluate the performance of two HBV DNA quantitative kits, five HBsAg qualitative kits, seven HBsAg quantitative kits and three rapid immune-chromatographic tests for HBsAg.
Results: The quantification values of two HBV DNA quantitative kits exhibited excellent correlation. In the evaluation of HBsAg qualitative and quantitative kits, the titers of several specimens in the HBV-positive panel were below the detection limits of a few kits, and the specimens were determined as HBV-negative. Notably, the quantitative kit results exhibited low correlation values. However, when these data were analyzed for each genotype, the correlations improved. These results suggest that the HBsAg quantification data are influenced by HBV genotypes. The novel rapid immune-chromatographic test exhibited the comparable level of sensitivity to the HBsAg quantitative kits.
Conclusions: We evaluated the performance of kits for the detection of HBV infection. The HBV DNA quantification data correlated with an excellent agreement, whereas the HBsAg quantification data were affected by HBV genotype. Such evaluations will be useful for estimating the quality of currently available and new HBV assay kits, and for the quality control of these kits.

1. Background

Hepatitis B virus (HBV) infection is a major cause of chronic liver disease, and the global prevalence is estimated to over 290 million [1]. HBV is transmitted *via* blood or other body fluids, and the infection occurs through sexual contact or by sharing needles or syringes, or it can be passed from mother to infant at birth. After infection with this virus, some people cannot eliminate it and become chronically infected, even when infected in adults. Chronic HBV infection can lead to serious liver diseases, including cirrhosis and hepatocellular carcinoma. Currently, treatment for chronic hepatitis B is based on the lifelong administration of nucleos(t)ide analogs that keep HBV DNA at undetectable levels [2]. The current research aims are the discovery of antiviral reagents or strategies that lead to the clearance of the hepatitis B surface antigen (HBsAg) and seroconversion of the anti-HBs antibody, which is the so-called functional cure of HBV infection [3,4]. Therefore, for the purpose of diagnosis of HBV infection, the evaluation of the pathological condition of patients and the therapeutic effects on HBV infection, the detection and quantification of HBV DNA and HBsAg are indispensable. For this purpose, several kits for HBsAg and HBV DNA are available in Japan, and there is a growing need for the evaluation of these kits because their performance may be affected by HBV genotype- or strain-specific polymorphisms.

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2. Objectives

In this study, we evaluated the performance of two quantitative kits for HBV DNA, and five qualitative kits, seven quantitative kits, and three rapid immune-chromatographic tests for HBsAg. To evaluate these kits, we used International Standards issued by the World Health Organization (WHO) and a regional reference panel with HBV-positive and HBV-negative plasma specimens that were collected in Japan and provided by the Japanese Red Cross Society.

3. Study Design

3.1. International standards for HBV DNA and HBsAg

International Standards are used to calibrate the international unit (IU). The 3rd WHO International Standard for HBV DNA (DNA-IS; code: 10/264) and the 3rd WHO International Standard for HBsAg (HBs-IS; code: 12/226) were obtained from the National Institute of Biological Standards and Control (NIBSC, UK). These International Standards were serially diluted and stored at −80 °C until use.

3.2. Establishment of HBV reference panel

The HBV regional reference panel was established with 53 HBsAg-negative and 80 HBsAg-positive plasma specimens that were provided by the Japanese Red Cross Blood Centers. These specimens were collected from January 2013 to May 2014 in Japan and were determined to be ineligible for transfusion. The HBV genotypes were determined by IMMUNIS HBV Genotype EIA (Institute of Immunology, Tokyo, Japan) [5]. In the HBV-positive panel, specimens of HBV genotype- (GT-) A (12; 15.0%), GT-B (24; 30.0%), and GT-C (41; 51.3%) were included, and the genotype was undetermined in the 3 remaining samples. These genotypes comprise the predominant genotypes in Japan [6,7]. The specimens were aliquoted in 1.0 mL volumes into 1.5-mL screw-cap tubes after centrifugation to exclude agglutinates or clots and then stored at −80 °C until use.

3.3. Evaluation of in vitro diagnostics kits for HBV infection

In this study, two HBV DNA quantitative kits were included: Abbott RealTime HBV assay (ART HBV; Abbott Japan, Tokyo, Japan) and COBAS AmpliPrep/COBAS TaqMan HBV Test v2.0 (CAP/CTM HBV v2.0; Roche Diagnostics, Tokyo, Japan). The targets of amplification of these kits are different; ART HBV targets the HBs region, and CAP/CTM HBV v2.0 targets the HBc region. The dynamic ranges of ART HBV and CAP/CTM HBV v2.0 are 1.00–9.00 log IU/mL and 1.30–8.23 log IU/mL, respectively, according to the manufacturer's instructions. Five HBsAg qualitative kits were included: ADVIA Centaur HBsAg II (Centaur; SIEMENS, Tokyo, Japan), Enzygnost HBsAg 6.0 (Enzygnost; SIEMENS), Elecsys HBsAg II (Elecsys; Roche Diagnostics), STACIA CLEIA HBsAg (STACIA; LSI Medience, Tokyo, Japan), and VITROS HBsAg ES (VITROS; Ortho-Clinical Diagnostics, Tokyo, Japan). Seven HBsAg quantitative kits were included: ARCHITECT·HBsAg QT (ARCHITECT; Abbott Japan) [8,9], Accuraseed HBsAg (Accuraseed; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), BLEIA 'Eiken' HBsAg (BLEIA; Eiken Chemical, Tokyo, Japan), Elecsys HBsAg II quant II (Elecsys quant; Roche Diagnostics), ST AIA-PACK HBsAg (AIA; TOSOH, Tokyo, Japan), HISCL HBsAg Assay Kit (HISCL; Sysmex, Kobe, Japan) [10], and Lumipulse Presto HBsAg-HQ (Lumipulse-HQ; Fujirebio, Tokyo, Japan) [9]. Three rapid immune-chromatographic tests (ICT) for HBsAg were included: ESPLINE HBsAg (ESPLINE; Fujirebio), Alere Determine HBsAg (Determine; Alere Medical, Tokyo, Japan), and Determine HBsAg 2 (Determine 2; Alere Medical). All tests except the ICTs were performed by the respective manufacturers at their research laboratories.

Table 1
Measurement of HBV DNA in WHO International Standard by HBV DNA quantitative kits.

International Standard	Theoretical titer (log IU/mL)	ART HBV		CAP/CTM HBV v2.0	
		Titer (log IU/mL)	Difference from theoretical titer (%)	Titer (log IU/mL)	Difference from theoretical titer (%)
DNA-IS 1	4.00	4.03	0.750	4.02	0.500
DNA-IS 2	3.48	3.46	0.575	3.62	4.02
DNA-IS 3	3.00	2.97	1.00	3.14	4.67
DNA-IS 4	2.48	2.44	1.61	2.66	7.26
DNA-IS 5	2.00	2.08	4.00	2.15	7.50
DNA-IS 6	1.48	1.53	3.38	1.56	5.41
DNA-IS 7	1.00	1.13	13.0	1.34	34.0
Diluent	0	ND	–	ND	–

DNA-IS: WHO International Standard for HBV DNA, ND: not detected.

3.4. Statistical analysis

The correlation between the test data was assessed by means of regression analysis using the least squares method, and the regression equation coefficient and the value of the coefficient of determination (R^2) were calculated by GraphPad PRISM 7 (GraphPad Software, San Diego, CA). Statistical analysis among GTs was performed using one-way factorial ANOVA and multiple comparison tests, and *P*-values < 0.05 were considered statistically significant.

4. Results

4.1. Evaluation of HBV DNA quantitative kits

In this study, two HBV DNA quantitative kits were evaluated: ART HBV and CAP/CTM HBV v2.0. The DNA-IS was serially diluted in a half-log fashion and subjected to evaluation by the two kits. The HBV DNA titers measured by both kits were near the theoretical titer of each DNA-IS, and the differences from the assigned HBV DNA titers were ≤10.0%, except for the lowest concentration (Table 1). Of the 53 negative specimens in the HBV reference panel, both kits could determine all specimen as negative. Of the 80 positive specimens in the HBV reference panel, 68 (85.0%) were quantifiable by both kits (Table 2). The HBV DNA titers of the 80 positive specimens were distributed extensively (Fig. 1). HBV DNA titers were significantly correlated between CAP/CTM HBV v2.0 and ART HBV ($p < 0.0001$). The regression equation was $y = 1.01x - 0.153$ ($R^2 = 0.9665$). Genotype dependency was not observed in this correlation, and the correlation equation coefficient for each genotype was nearly identical: 0.951, 1.05, and 1.00 for GT-A, -B, and -C, respectively.

4.2. Evaluation of HBsAg qualitative kits

Five HBsAg qualitative kits were evaluated in this study by using the serially diluted HBs-IS. As shown in Table 3, all but one kit detected HBsAg of the HBs-IS 5 at a concentration of 100 mIU/mL as the lowest

Table 2
Measurement of HBV DNA in HBV-positive panel specimens by HBV DNA quantitative kits.

HBV-positive panel (N = 80)		ART HBV		
		Quantified	Detected	Not detected
CAP/CTM HBV v2.0	Quantified	68 (85.0%)	0	0
	Detected	5 (6.3%)	3 (3.8%)	1 (1.3%)
	Not detected	0	2 (2.5%)	1 (1.3%)

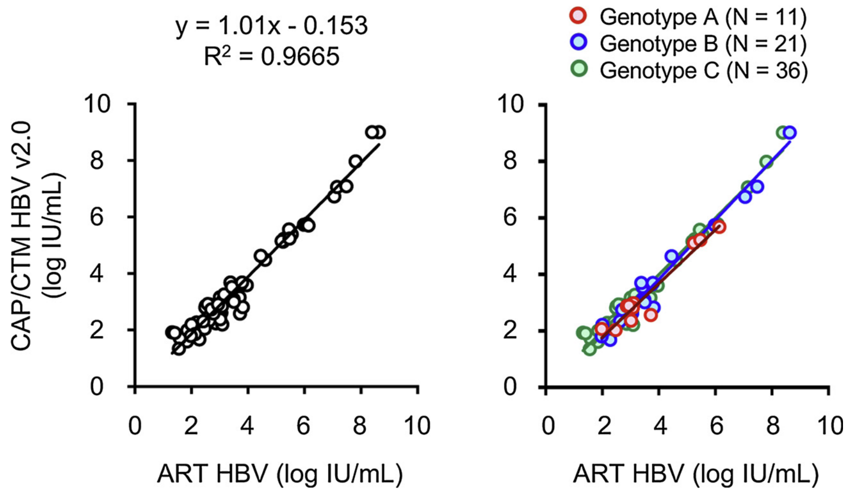

Fig. 1. Correlation of HBV DNA titers of specimens in the HBV-positive panel.
HBV DNA titers were quantified by CAP/CTM HBV v2.0 and ART HBV. The correlation equation and the value of the coefficient of determination are indicated at the top of the left panel. In the right panel, the correlation of each genotype is indicated.

Table 3
Detection of HBsAg in WHO International Standard by HBsAg qualitative kits.

International Standard	Theoretical titer (mIU/mL)	Centaur (COI)	Elecsys (COI)	Enzygnost (COI)	STACIA (COI)	VITROS (COI)
HBs-IS 1	10,000	203	43.8	734	259	213
HBs-IS 2	3000	60.1	45.1	204	79.0	63.8
HBs-IS 3	1000	20.6	20.4	67.9	27.3	22.2
HBs-IS 4	300	6.33	6.68	20.9	7.60	6.09
HBs-IS 5	100	2.43	2.70	5.98	2.90	1.71
HBs-IS 6	30	UDL	UDL	1.65	UDL	UDL
HBs-IS 7	10	UDL	UDL	UDL	UDL	UDL
Diluent	0	UDL	UDL	UDL	UDL	UDL

HBs-IS: WHO International Standard for HBsAg, COI: cut off index, UDL: under the detection limit.

Table 4
Detection of HBsAg in HBV reference panel specimens by HBsAg qualitative kits.

HBV reference panel	Determined as	Centaur	Elecsys	Enzygnost	STACIA	VITROS
HBV-negative panel (N = 53)	Negative	53	53	53	53	53
HBV-positive panel (N = 80)	Positive	80	80	80	77 (96.3%)	78 (97.5%)

Table 5
Measurement of HBsAg in WHO International Standard by HBsAg quantitative kits.

International Standard (Theoretical titer: mIU/mL)	ARCHITECT (mIU/mL) LDL: 50	Accuraseed (mIU/mL) LDL: 50	BLEIA (mIU/mL) LDL: 5	Elecsys quant (mIU/mL) LDL: 50	AIA (mIU/mL) LDL: 50	HISCL (mIU/mL) LDL: 30	Lumipulse- HQ (mIU/mL) LDL: 5
HBs-IS 1 (10,000)	11,360	10,020	12,099	9,200	8,170	12,030	10,432
HBs-IS 2 (3,000)	3,740	2,760	3,574	2,760	2,670	3,680	3,010
HBs-IS 3 (1,000)	1,340	830	1,184	941	860	1,160	967
HBs-IS 4 (300)	430	300	365	269	250	320	318
HBs-IS 5 (100)	162	100	130	93	90	110	108
HBs-IS 6 (30)	59	UDL	35	UDL	50	UDL	34
HBs-IS 7 (10)	UDL	UDL	12	UDL	UDL	UDL	12
Diluent (0)	UDL	UDL	UDL	UDL	UDL	UDL	UDL

HBs-IS: WHO International Standard for HBsAg, LDL: Lower detection limit, UDL: under the detection limit.

concentration. One kit (Enzygnost) detected HBs-IS 6 at a concentration of 30 mIU/mL. The performance of these kits was also evaluated by measuring the specimens in the HBV reference panel. All specimens in the HBV-negative panel (N = 53) were correctly determined to be negative by all kits (Table 4). However, when measuring specimens in the HBV-positive panel (N = 80), two of the five kits could not detect all of the positive specimens, and a few specimens were determined to be negative.

4.3. Evaluation of HBsAg quantitative kits

Seven kits for HBsAg quantitative were evaluated. As with the evaluation of the HBsAg qualitative kits, HBs-IS was serially diluted and analyzed by these quantitative kits. Three of the seven kits detected HBsAg at the concentration of 100 mIU/mL (Table 5). Two kits, ARCHITECT and AIA, detected HBsAg at the concentration of 30 mIU/mL, and two kits, BLEIA and Lumipulse-HQ, detected HBsAg at the

Table 6
Measurement of HBsAg in HBV reference panel specimens by HBsAg quantitative kits.

HBV reference panel	Determined as	ARCHITECT (mIU/mL)	Accuraseed (mIU/mL)	BLEIA (mIU/mL)	Elecsys quant (mIU/mL)	AIA (mIU/mL)	HISCL (mIU/mL)	Lumipulse- HQ (mIU/mL)
HBV-negative panel (N = 53)	Negative	53	53	53	53	53	53	53
HBV-positive panel (N = 80)	Positive	80	79 (98.8%)	80	80	77 (96.3%)	80	80

concentration of 10 mIU/mL. The HBV reference panel was also used to evaluate these HBsAg quantitative kits. Of the 53 HBV-negative specimens, all specimens were determined to be negative by these kits (Table 6). On the other hand, of the 80 HBV-positive samples, not all specimens were correctly identified by two kits. The detection rates were 98.8% and 96.3% for the Accuraseed and AIA, respectively. The correlations of quantified HBsAg levels among these seven kits were also investigated. When comparing ARCHITECT to other quantitative kits, the regression equation coefficient ranged from 0.747 to 2.11, and the R^2 value ranged from 0.868 to 0.979 (Fig. 2 and Table 7). These data suggested that the data generated by these kits were not consistent with each other. However, when correlations were analyzed in each genotype, the values of the coefficient of determination were increased and reached approximately 1. For instance, in a comparison between ARCHITECT and Accuraseed, the regression equation coefficient of all samples was 1.22, and the R^2 value was 0.914. However, in the assessment of each genotype, the regression equation coefficients were much different at 0.540, 1.33 and 1.31 for GT-A, -B, and -C, respectively, and the R^2 values of each genotype increased to 0.987, 0.978 and 0.949 for GT-A, -B, and -C, respectively. In comparison between ARCHITECT and others, this genotype-dependent difference was most pronounced in comparison between Lumipulse-HQ and ARCHITECT (Table 7). These observations were also detected in comparisons between other kit combinations (Supplementary Table S1 and Supplementary Figure S1).

4.4. Evaluation of ICTs for HBsAg

Finally, we evaluated ICTs for HBsAg. We evaluated two conventional ICTs, ESPLINE and Determine, and the novel Determine 2. By assaying the serially diluted International Standard with conventional ICTs, HBsAg was detected at the concentration of 1000 mIU/mL. However, by assaying with the novel ICT, HBsAg was detected at the concentration of 30 mIU/mL (Table 8). For the evaluation of 53 HBV-negative specimens in the HBV reference panel, all ICTs determined the HBV status correctly. For the evaluation of 80 HBV-positive specimens, the accurate detection rate of the two conventional ICTs was 88.8%, whereas the novel ICT identified all positive specimens (Table 9).

5. Discussion

The detection of HBsAg and HBV DNA is important for diagnosing HBV infection, and the quantified values of these parameters are used to understand the clinical status of patients with chronic hepatitis B and to monitor the therapeutic effects of anti-HBV reagents. Thus, it is necessary to assess the performance of diagnostic kits for clinical use. Although HBV is a DNA virus, it has a high mutation rate because it has an RNA stage in its life cycle [11]. The polymorphisms of nucleotides or amino acids at the target regions of HBV diagnostics kits are known to affect the quantification values or sensitivities [12–14]. To evaluate the performance of these kits, the International Standard and the regional reference panel are available. The International Standard is issued by WHO to be used as a master calibrator of the IU and is used as the reference material. The regional reference panel consists of specimens obtained in each region and is useful for assessing the detection of regional endemic genotypes or strains. We established a regional reference panel for hepatitis C virus and used it to assess *in vitro* diagnostic kits for HCV infection [15,16]. In this study, we established an HBV reference panel and assessed the performance of *in vitro* diagnostic kits for HBV infection by using this regional panel and the International Standard.

In the assessment of HBV DNA quantitative kits, two kits exhibited comparable performance and could detect DNA-IS 7 at a concentration of 10 IU/mL. Although the quantified data deviations for most DNA-ISs were low, deviations at the lowest DNA-IS concentration were greater than 10%. These data imply that the quantification around the lower

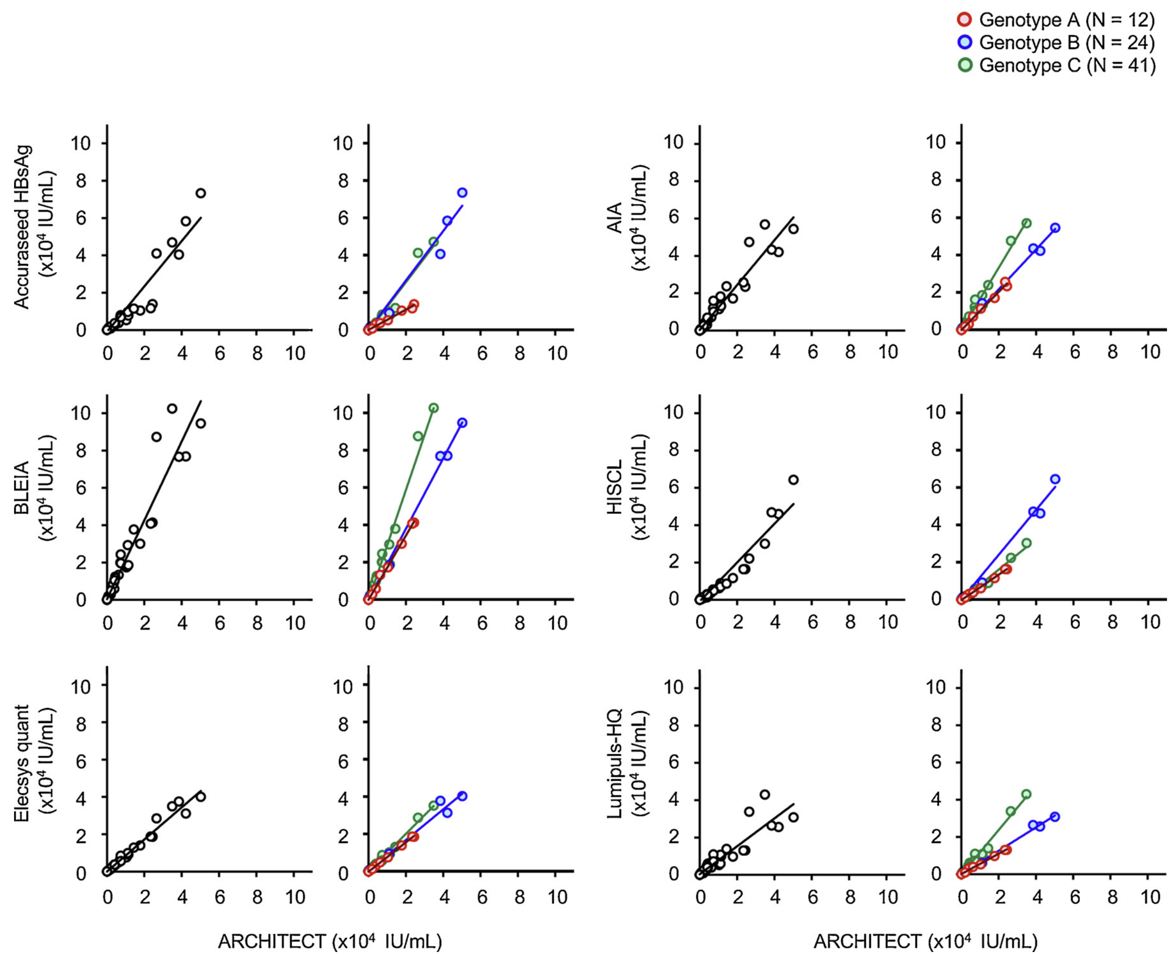

Fig. 2. Correlations of quantified HBsAg values in HBV-positive panel specimens.
HBsAg in the HBV-positive panel specimens was quantified by the indicated HBsAg quantitative kits. The correlations of all samples (left panel) and of each genotype (right panel) are shown.

Table 7
Correlations of quantified values of HBsAg in HBV-positive panel specimens.

Compared with ARCHITECT	Parameter	All	Genotypes		
			GT-A	GT-B	GT-C
Accuraseed	coefficient	1.22	0.540	1.33	1.31
	R^2	0.914	0.987	0.978	0.949
BLEIA	coefficient	2.11	1.72	1.89	2.96
	R^2	0.933	0.997	0.998	0.981
Elecsys quant	coefficient	0.856	0.783	0.823	1.00
	R^2	0.979	0.999	0.986	0.991
AIA	coefficient	1.20	1.02	1.07	1.67
	R^2	0.939	0.991	0.997	0.988
HISCL	coefficient	1.04	0.679	1.20	0.810
	R^2	0.941	0.997	0.992	0.980
Lumipulse-HQ	coefficient	0.747	0.543	0.627	1.20
	R^2	0.868	0.995	0.996	0.984

R^2: coefficient of determination. GT: genotype.

detection limits of these kits is unreliable, and careful interpretations are needed for the clinical use of these kits when measuring specimens with the low HBV DNA titers. In the evaluation using specimens from the HBV-positive panel, inconsistent data of HBV DNA detection was observed in 8 specimens. These discrepancies may reflect the difference in the target region of these kits. Another specimen was determined to be negative by both kits. We presumed that this specimen had no or a very low titer of the HBV genome. When evaluating the quantifiable specimens in the HBV-positive panel by both kits, the results were well

Table 8
Detection of HBsAg in WHO International Standard by ICTs.

International Standard	Theoretical titer (mIU/mL)	Determine	Determine 2	ESPLINE
HBs-IS 1	10,000	+	+	+
HBs-IS 2	3,000	+	+	+
HBs-IS 3	1,000	+	+	+
HBs-IS 4	300	–	+	–
HBs-IS 5	100	–	+	–
HBs-IS 6	30	–	+	–
HBs-IS 7	10	–	–	–
Diluent	0	–	–	–

HBs-IS: WHO International Standard for HBsAg.

Table 9
Detection of HBsAg in HBV reference panel specimens by ICTs.

HBV reference panel	Determined as	Determine	Determine 2	ESPLINE
HBV-negative panel (N = 53)	Negative	53	53	53
HBV-positive panel (N = 80)	Positive	71 (88.8%)	80	71 (88.8%)

correlated and not affected by HBV genotypes that are predominant in Japan.

We evaluated five HBsAg qualitative kits. These kits report in cut off index (COI). Four of the five kits could detect HBs-IS 5 at a concentration of 100 mIU/mL, and 1 kit could detect HBs-IS 6 at a concentration of 30 mIU/mL. The presumed lower detection limits of these kits were comparable to those of the HBsAg quantitative kits. For the evaluation of the HBV reference panel, three of five kits could detect all positive specimens, and the remaining two kits failed to detect a few specimens. Thus, the performance of these HBsAg qualitative kits was not inferior to that of the quantitative kits.

The HBsAg quantitative kits report in IU. We evaluated seven kits, including ARCHITECT that is used worldwide [8,9]. The lower detection limits of five of the seven kits are similar to those of the HBsAg qualitative kits and are 30–50 mIU/mL. The remaining two kits (BLEIA and Lumipulse-HQ) have high sensitivities and the lower detection limits are 5 mIU/mL. In the assessment of the HBV-positive specimen panel, two kits did not detect HBsAg in all specimens and failed in a few specimens. The specimens that were not detected had low HBsAg titers, suggesting that the titers of these specimens were below the lower detection limits of these kits. Notably, the HBsAg quantitative by these kits was not well correlated and was influenced by HBV genotype. The regression equation coefficient was different among genotypes, and the R^2 values were higher when calculated using genotype-matched specimens in comparison with using all specimens. The kit responsible for this inconsistency and genotype dependency was not identified in this study because the evaluations were the results of relative comparisons. The further study will be needed to identify the kit responsible for this discrepancy and clarify the mechanisms.

We also evaluated ICTs for HBsAg. These are known to have the advantages of simplicity, limited need for instrumentation, minimal training, and rapid performance at room temperature. In general, ICTs are considered to have low sensitivity. In fact, in this study, the conventional ICTs exhibited much lower sensitivity compared with HBsAg qualitative and quantitative kits. However, the newly developed ICT proved to have high sensitivity, and the lower detection limit of this ICT was comparable to ARCHITECT. This novel ICT will work as an excellent tool for large-scale screening in global settings, including developing countries or countries with limited electricity status.

In conclusion, by using the International Standard and the established HBV reference panel, we evaluated the performance of HBV DNA quantitative kits, HBsAg qualitative and quantitative kits, and ICTs for HBsAg.. The HBV DNA quantification data correlated with excellent agreement, whereas the HBsAg quantification data were affected by HBV genotypes. Such evaluations will be useful for estimating the quality of currently available and upcoming HBV assay kits, and for the quality control of these kits. When using these diagnostics kits for HBV infection, it is necessary to understand their advantages and disadvantages for the proper use.

Conflicts of interests

All authors have nothing to disclose.

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Author’s contributions

AM, HM, IH and TK designed the study. AM, HM, NY, YH, KI and TK prepared the materials. AM and TK analyzed the data. AM and TK wrote the manuscript. MM, TW, and IH supervised the projects. All authors approved the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2019.05.011.

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