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Establishment of an HBsAg mixed titer performance panel and HBsAg working standard for quality control of HBsAg diagnostic kits in Korea

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ABSTRACT

Background: International Standards or commercial panels used for performance validation of diagnostic kits might not reflect the viral characteristics common in Korea. Also, continuous use of these materials is difficult because of limited quantity and high cost.
Objectives: Establishment of HBsAg reference materials to be used as National Standards for validation of HBsAg diagnostic kits.
Study design: 568 plasma units with OD less than 2.0 on HBsAg EIA were collected. HBsAg testing with 3 EIAs and 1 CIA was performed on all units. HBsAg positive units were subjected to HBV DNA quantification, genotyping and subtyping. Candidates for the mixed titer performance panel and working standard were confirmed for HBsAg by neutralization. A collaborative study was conducted for the candidates of the mixed titer performance panel and the working standard.
Results: Based on the results of the collaborative study, a working standard (KFDA08/024) consisting of a series of four-fold dilutions of 2 materials, one with genotype/subtype C2/adr and the other with C1/adw, was established. A mixed titer performance panel composed of 2 negative and 16 positive samples was also established. A G1896A and a T/I126S mutant are included in the positive samples.
Conclusions: An HBsAg mixed titer performance panel and a working standard reflecting HBV genotypes/subtypes prevalent in Korea have been established as National Standards. This will enable consistent supply of validation materials, improve the validation system of HBsAg diagnostic kits in Korea and lead to quality improvement of diagnostic kits.

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1. Background

Performance validation of HBsAg diagnostic kits used for donor screening is crucial as false-negative results can lead to transfusion-transmitted HBV infection.

The Korea Food and Drug Administration require manufacturers of HBsAg diagnostic kits to validate performance using 4 panels for product approval.[1] The required panels are (1) HBsAg sensitivity panel consisting of dilutions of an HBsAg positive serum, (2) HBsAg seroconversion panel, (3) HBsAg low titer performance panel consisting of undiluted HBsAg positive plasma with an HBsAg concentration of less than 1 IU/mL, and (4) HBsAg mixed titer performance panel consisting of undiluted HBsAg positive plasma with various HBsAg concentrations. To comply with these requirements manufacturers have to rely on International Standards (ISs) or other imported commercial panels, because only two HBsAg National Standards (NSs) have been established so far.[2] However, validation materials made from biological specimens obtained from demographically different populations might not reflect the viral characteristics common in Korea. A sustainable use of these materials can also not be guaranteed because of limited supply and high cost.

Abbreviations: IS, International Standard; NS, National Standard; OD, optical density; EIA, enzyme immunoassay; CIA, cemiluminescence immunoassay; S/CO, specimen absorbance to cutoff ratio; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; COI, cutoff index; NIBSC, National Institute for Biological Standards and Control; ECBS, Expert Committee on Biological Standardization; BRP, biological reference preparation; CTS, common technical specifications.

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2. Objectives

To establish an HBsAg mixed titer performance panel and an HBsAg working standard that reflect the viral characteristics common in Korea to be used as NSs for the validation of HBsAg diagnostic kits.

3. Study design

3.1. Materials

To select candidate materials, 568 plasma units with an optical density (OD) of 2.0 or less on HBsAg enzyme immunoassay (EIA) were collected from Korean Red Cross-blood centers from November 2004 to July 2006. During 2004–2006 GENEDIA HBsAg ELISA 3.0 (GREEN CROSS MS, Kyounggi-do, Korea), Behring Enzygnost HBsAg 5.0 (Dade Behring GmbH, Marburg, Germany), BIO-RAD MONOLISA HBsAg ULTRA (BIO-RAD, Marnes la Coquette, USA) or Murex HBsAg V.3 (Murex Biotec Limited, Dartford, UK) was used for HBsAg screening at blood centers. Six aliquots of 1.5 mL were taken from each plasma unit and stored at −20 °C until testing. Plasma units were stored at −70 °C until further processing.

638 plasma units with an ALT value of 65–100 IU/mL but negative for HBsAg, anti-HCV, anti-HIV 1/2 and non-reactive on HCV and HIV NAT were collected from March to May 2007 to be used as diluents for production of the working standard.

3.2. Serological analysis

568 plasma units were retested for HBsAg using 3 EIAs (GENEDIA HBsAg ELISA 3.0, BIO-RAD MONOLISA HBsAg ULTRA [BIO-RAD, SF, USA], Murex HBsAg V.3) and 1 chemiluminescence immunoassay (CIA) (ARCHITECT HBsAg [ABBOTT, Wiesbaden, Germany]) according to the manufacturers' instructions. Samples with a specimen absorbance to cutoff ratio (S/CO) greater than or equal to 1.0 on EIA were considered reactive. For CIA, samples with results greater than or equal to 0.05 IU/mL were considered reactive.

Candidate materials, that were selected based on the results of HBsAg testing, HBV genotyping and subtyping, were confirmed for HBsAg with ARCHITECT HBsAg Confirmatory V.1 (ABBOTT, Sligo, Ireland). Samples with percent neutralization greater than or equal to 50% were considered confirmed positive for HBsAg.

To select anti-HBs and HBsAg negative plasma to be used as diluents, plasma units were tested for anti-HBs and HBsAg using ARCHITECT Anti-HBs (ABBOTT, Sligo, Ireland) and ARCHITECT HBsAg, respectively.

3.3. Molecular analysis

Viral DNA was extracted using QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) or Magna Pure LC DNA Isolation Kit I (Roche Diagnostics GmbH, Mannheim, Germany).

Genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), type-specific primer PCR, and Inno-LiPA HBV Genotyping kit (Innogenetics, Ghent, Belgium). PCR-RFLP was done using previously described methods with modifications.[3,4] Type-specific primer PCR was modified from Naito et al.[5]

Subtyping and HBV mutant analysis was done using primers adopted from Sato et al.[6] The PCR products were sequenced with the Big Dye terminator ready reaction cycle sequencing kit (Applied Biosystems, Warrington, UK) and loaded onto the ABI 3730xl sequencer (Applied Biosystems, Foster City, CA, USA). The sequences obtained were analyzed with BLAST search in the National Center for Biotechnology Information database (NCBI Genebank).

HBV DNA was quantitated with COBAS AMPLICOR HBV MONITOR Test (Roche Molecular System Inc., Branchburg, USA).

3.4. Filling and labelling

Filling and labelling of candidate materials was performed by LG Life Sciences (Daejon, Korea). Candidate materials for the mixed titer performance panel were processed undiluted and no preservatives were added. Candidate materials for the working standard were heat inactivated (56–60 °C, 30–40 min) and converted to serum by adding $CaCl_2$ to a final concentration of 0.25 M. After filtration and freeze-thawing, candidate materials were diluted with HBsAg and anti-HBs negative serum to an HBsAg content of approximately 50 IU/mL. Thereafter, they were serially diluted four-fold to give a proposed HBsAg unitage of 12.50 IU/mL, 3.13 IU/mL, 0.78 IU/mL, 0.20 IU/mL, and 0.05 IU/mL, respectively. Bronidox (Sigma, St. Louis, USA) was added to a final concentration of 0.05% to prevent growth of contaminants. All candidate materials were filled into 1.5 mL micrewtube with O-ring (SARSTEDT AG & Co., Nürnbrecht, Germany) as 0.5 mL aliquots.

3.5. Collaborative study

A collaborative study with 4 participating laboratories was conducted for the candidate materials of the HBsAg mixed titer performance panel using 8 HBsAg diagnostic kits (GENEDIA HBsAg ELISA 3.0, BIO-RAD MONOLISA HBsAg ULTRA, Murex HBsAg V.3, LG HBsAg ELISA [LG Life Sciences, Daejon, Korea], AxSYM HBsAg V.2 [ABBOTT, Wiesbaden, Germany], ARCHITECT HBsAg, PRISM HBsAg [ABBOTT, Wiesbaden, Germany], Elecsys HBsAg [Roche Diagnostics GmbH, Mannheim, Germany]). Samples were tested in duplicate for 3 days and the mean value was provided as a reference value. For LG HBsAg ELISA, AxSYM HBsAg and PRISM HBsAg an S/CO greater than or equal to 1.0 was considered reactive. For Elecsys HBsAg a cutoff index (COI) greater than or equal to 1.0 was considered reactive.

For the working standard, collaborative study was done with 5 participating laboratories using the ARCHITECT HBsAg assay. The working standard was also validated by the National Institute for Biological Standards and Control (NIBSC, United Kingdom) using Murex HBsAg V.3. Samples were tested in duplicate for 3 days and the results were calibrated against the Second IS for HBsAg, subtype adw2, genotype A (NIBSC 00/588) that has an assigned unitage of 33 IU/vial. The unitage for each member of the working standard was assigned by calculating the mean value of the test results obtained through the collaborative study.

3.6. Real-time stability test

Stability of the proposed standard materials was evaluated by real-time stability testing. The proposed working standard was tested in triplicate using the ARCHITECT HBsAg assay at the time of storage (0 month) and then at 3 months interval.

4. Results

4.1. Serological analysis

Among 568 plasma units retested, only 11.1% showed reactive results with all 4 immunoassays. 50.5% had non-reactive results with all immunoassays and the rest showed discrepant results (Table 1).

Among 638 HBsAg negative plasma units, 250 units (39.2%) tested negative for anti-HBs. These plasma units were used as diluents to prepare the working standard.

Table 1
HBsAg immunoassay results of candidate materials with a reactive result on initial screening ($n = 568$).

Group	No (%)		Architect HBsAg	BIO-RAD MONOLISA HBsAg ULTRA	GENEDIA HBsAg ELISA 3.0	Murex HBsAg V.3
G1	63	(11.1)	R	R	R	R
G2	9	(1.6)	R	R	NR	R
G3	2	(0.4)	R	NR	R	R
G4	4	(0.7)	R	R	NR	NR
G5	2	(0.4)	R	NR	NR	R
G6	4	(0.7)	R	NR	NR	NR
G7	1	(0.2)	NR	R	R	R
G8	3	(0.5)	NR	R	R	NR
G9	7	(1.2)	NR	R	NR	R
G10	3	(0.5)	NR	NR	R	R
G11	108	(19.0)	NR	R	NR	NR
G12	33	(5.8)	NR	NR	R	NR
G13	42	(7.4)	NR	NR	NR	R
G14	287	(50.5)	NR	NR	NR	NR

Abbreviations: R, reactive; NR, non-reactive.
Candidate materials are plasma units that had an OD value of 2.0 or less on initial HBsAg screening performed at Korean Red Cross-blood centers from November 2004 to July 2006.

4.2. Molecular analysis

97 units were subjected to HBV genotyping and subtyping (Table 2). With the exception of 9 samples, that were not amplified, all samples were identified as genotype C. Subtype adr was present in 81.4% and adw in 4.1% of the samples. One sample had subtype ayw.

Two mutants were identified. One mutant had a mutation in the precore region resulting in a G1896A mutant and the other one had a mutation in the S region resulting in a T/I126S mutant.

4.3. Establishment of an HBsAg mixed titer performance panel (BTRI HBV/MP, KFDA 08/025) and an HBsAg Working Standard (KFDA 08/024)

Based on the results of HBsAg assay and molecular analysis, an HBsAg mixed titer performance panel (BTRI HBV/MP, KFDA 08/025) consisting of 18 members was established. The HBsAg content of the positive members ranged from 0.05 IU/mL to >250 IU/mL (Table 3). The genotype/subtype was C1/adr in 11 samples, C2/adr in 5 samples, and C1/adw in 2 samples. Two HBV mutants not detected by some HBsAg kits were also included. BTRI HBV/MP 1 consists of a G1896A mutant and BTRI HBV/MP 18 of a T/I126S mutant. Two samples (BTRI HBV/MP 11 and 17) negative for HBsAg were included as negative controls.

Two HBsAg positive units were selected to constitute the HBsAg working standard (KFDA 08/024). The established working standard A–E contains HBsAg subtype adr, genotype C2 and working standard F–J contains HBsAg subtype adw, genotype C1. The unitage assigned for each standard member the results of the NIBSC are shown in Table 4.

Table 2
HBV genotyping and subtyping results of candidate materials.

Subtype	Genotype			Total	
	C1	C2	NI		
adr	68	9	2	79	(81.4%)
adw	4	–	–	4	(4.1%)
ayr	–	–	–	–	
ayw	–	–	1	1	(1.0%)
NI	7	–	6	13	(13.4%)
Total	79 (81.4%)	9 (9.3%)	9 (9.3%)	97	

Abbreviations: NI, not identified.

4.4. Real-time stability test

The real-time stability test results have been obtained till 12 months after storage of the HBsAg working standard and showed a good correlation with the assigned unitage (Fig. 1).

5. Discussion

The WHO Expert Committee on Biological Standardization (ECBS) is commissioned by the WHO to establish recommendations and guidelines for the manufacturing, licensing, and control of blood products and related diagnostic tests and also for the establishment of the WHO International Biological Reference Preparations (BRPs). Standards for diagnostic kits are used for the assessment and monitoring of performance of kits and also to meet regulatory requirements. Regarding HBsAg kits the ECBS has established the Second IS for HBsAg (NIBSC 00/588) with an assigned value of 33 IU/vial in 2003. It has also established an International Reference Panel for HBsAg, which is a series of four-fold dilutions of the Second IS. Because these materials are available in relatively limited quantities, the ECBS has recommended that regional or national authorities may consider establishing their own secondary reference standards, calibrated against and traceable to the primary WHO materials.[7]

The WHO IS and Reference Panel for HBsAg corresponds to genotype A2. Other genotypes are found more predominantly in some parts of the world and the sensitivity of commercial kits for geno-

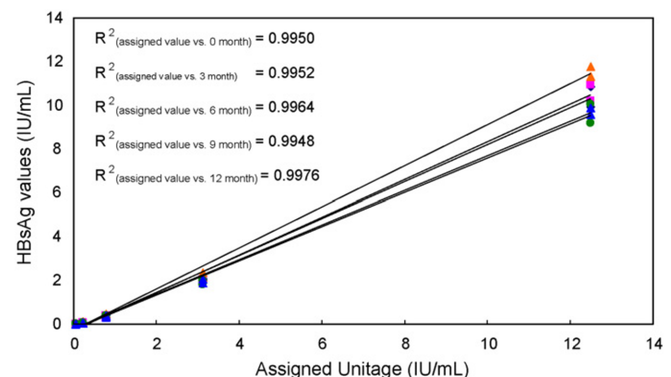

Fig. 1. Results of real-time stability test for the HBsAg Working Standard (KFDA 08/024). Correlation between the assigned HBsAg unitage and the HBsAg results at 0 month (◆) and at 3 (■), 6 (▲), 9 (●), and 12 months (▲) tested in triplicate using the ARCHITECT HBsAg assay.

Table 3
Results of HBsAg immunoassays, HBV genotype/subtype, and HBV DNA quantification for the HBsAg Mixed Titer Performance Panel (BTRI HBV/MP) (KFDA 08/025).

Member ID	HBsAg Neut	EIA[a]				MEIA	CIA		ECL	Genotype		Subtype	HBV DNA quant[d]
		A	B	C	D	E[a]	F[a]	G[b]	H[c]	PCR	I-LiPA		
BTRI HBV/MP 1[e]	100	0.69	0.75	1.22	1.81	0.90	1.76	0.06	1.04	C1	C	adr	1.3×10^4
BTRI HBV/MP 2	99.8	25.31	52.01	13.63	42.95	99.94	33.60	250.00	567.30	C2	C	adr	1.5×10^7
BTRI HBV/MP 3	100	28.60	52.65	13.54	49.61	152.30	72.37	250.00	6248.30	C1	C	adw	5.4×10^3
BTRI HBV/MP 4	82.6	1.80	1.73	2.72	5.19	0.96	4.31	0.14	1.90	C2	C	adr	2.3×10^3
BTRI HBV/MP 5	100	27.80	51.13	13.70	49.61	155.07	45.16	250.00	3455.30	C1	C	adw	1.9×10^3
BTRI HBV/MP 6	100	25.10	48.07	14.83	42.87	105.11	16.32	250.00	750.10	C1	C	adr	4.7×10^7
BTRI HBV/MP 7	99.0	3.95	2.54	4.84	6.17	3.75	9.33	0.34	2.82	C1	C	adr	6.9×10^3
BTRI HBV/MP 8	100	25.34	48.07	14.38	46.42	112.56	11.47	250.00	877.50	C1	C	adr	6.7×10^7
BTRI HBV/MP 9	100	26.10	51.19	13.38	46.49	109.45	63.04	250.00	768.80	C2	C	adr	1.7×10^7
BTRI HBV/MP 10	99.0	6.05	5.64	7.92	16.05	2.68	15.28	0.52	7.65	C1	C	adr	9.2×10^1
BTRI HBV/MP 11	NT	0.32	0.54	0.90	0.71	0.50	0.05	0.02	0.60	C2	C	adr	ND
BTRI HBV/MP 12	100	7.91	6.13	11.32	18.92	6.80	20.85	1.02	16.10	C1	C	adr	6.0×10^1
BTRI HBV/MP 13	99.0	10.58	4.31	17.00	15.79	5.41	29.67	1.03	9.05	C1	C	adr	1.4×10^2
BTRI HBV/MP 14	99.0	4.93	2.47	13.00	8.95	6.46	27.21	1.00	12.89	C1	C	adr	4.5×10^1
BTRI HBV/MP 15	100	25.00	47.00	13.46	43.41	97.19	22.81	250.00	568.80	C2	C	adr	5.4×10^7
BTRI HBV/MP 16	98.0	5.85	4.12	6.90	13.37	2.45	12.67	0.44	5.13	C1	C	adr	1.1×10^1
BTRI HBV/MP 17	NT	0.45	0.31	0.91	0.51	0.43	0.03	0.03	0.40	C1	C	adr	ND
BTRI HBV/MP 18[f]	94.0	0.47	0.64	1.04	0.72	0.64	1.30	0.05	0.57	C1	C	adr	ND

Abbreviations: HBsAg Neut: HBsAg neutralization test; EIA: enzyme immunoassay; MEIA: microparticle enzyme immunoassay; CIA: chemiluminescence immunoassay; ECL: electrochemiluminescence immunoassay; A: GENEDIA HBsAg ELISA 3.0 (GREEN CROSS MS: Kyounggi-do: Korea); B: LG HBsAg ELISA (LG Life Sciences: Daejon: Korea); C: Murex HBsAg V.3 (Murex Biotec Limited: Dartford: UK); D: BIO-RAD MONOLISA HBsAg ULTRA (BIO-RAD: SF: USA); E: AxSYM HBsAg V.2 (ABBOTT: Wiesbaden: Germany); F: PRISM HBsAg (ABBOTT: Wiesbaden: Germany); G: ARCHITECT HBsAg (ABBOTT: Wiesbaden: Germany); H: Elecsys HBsAg (Roche Diagnostics GmbH: Mannheim: Germany); I-LiPA: Inno-LiPA; HBV DNA quant: HBV DNA quantification; NT: not tested; ND: not detected.
Immunoassay results are mean values of testing performed in duplicate for 3 days.

[a] Specimen absorbance to cutoff ratio (S/CO), S/CO $\geq$ 1.0 are considered reactive.
[b] IU/mL, values $\geq$ 0.05 IU/mL are considered reactive.
[c] Cutoff index (COI), COI $\geq$ 1.0 are considered reactive.
[d] Copies/mL.
[e] HBV G1896A.
[f] HBV T/I126S.

types of HBV other than A2 might differ. Because of this, the WHO recommended that regulatory authorities devise panels for kit evaluation that include HBsAg reactive specimens with subtypes and genotypes from their local regions.[8] In 2005 the ECBS has endorsed the proposal of the WHO Collaborating Center Paul Ehrlich Institute to establish an HBV DNA panel and an HBsAg panel covering all HBV genotypes/subtypes identified worldwide.[9] Genotype A (subtypes adw2 and ayw1) is most prevalent in North America and northwestern Europe. Genotypes B (subtypes adw2, adw3 and ayw1) and C (subtypes adw2, adw3, ayw3, adr and ayr) are highly prevalent in East Asia. Genotype D (subtypes adw3, ayw2 and ayw4) is most prevalent in the Mediterranean and the Middle East. Genotype E (subtype ayw4) has been found in West Africa. Genotypes F (subtypes adw4 and ayw4) and H (subtype adw4) are found in Central and South America. Genotype G (subtype adw2) is found in the United States and Europe.[10,11] Studies in Korean patients with chronic HBV infection have shown that genotype C with a prevalence of 95.6–100% predominates in Korea.[12–15] Other genotypes found were genotype A and B. Subtype adr predominates in Korea followed by subtype adw.[16,17] With all samples that could be identified in this study being genotype C and with a predominance of subtype adr, our genotyping/subtyping results were comparable with those of previous reports and thus the established mixed titer performance panel and the working standard reflect the HBV genotype/subtype prevalent in Korea.

The result of the collaborative study for the working standard was used to assign a unitage to each member. To correlate the assigned unitage with the Second IS for HBsAg, the samples were

Table 4
Results of the collaborative study and NIBSC for the HBsAg Working Standard (KFDA 08/024).

Member I.D.	HBV subtype/ genotype	Expected unitage (IU/mL)	HBsAg results of participating laboratories (IU/mL)[a]					Assigned unitage[b] (IU/mL)	HBsAg results of NIBSC (IU/mL)[c]
			PL 1	PL 2	PL 3	PL 4	PL 5		
A	adr/C2	12.50	10.91	11.77	13.26	10.79	10.87	11.52	15.06
B		3.13	2.09	2.15	2.57	2.09	2.23	2.23	2.34
C		0.78	0.38	0.34	0.45	0.35	0.39	0.38	0.33
D		0.22	0.07	0.07	0.10	0.06	0.07	0.07	0.06
E		0.05	0.03	0.02	0.04	0.01	0.02	0.02	0.03
F	adw/C1	12.50	10.94	11.45	13.22	10.80	10.89	11.46	12.89
G		3.13	2.21	2.29	2.83	2.31	2.20	2.37	2.56
H		0.78	0.46	0.43	0.57	0.43	0.42	0.46	0.30
I		0.22	0.10	0.09	0.12	0.08	0.08	0.09	0.09
J		0.05	0.04	0.02	0.04	0.01	0.02	0.03	0.05

Abbreviations: PL 1–5: participating laboratory No. 1–5; NIBSC: National Institute for Biological Standards and Control.

[a] Represents mean values for samples tested in duplicate for 3 days with ARCHITECT HBsAg (ABBOTT, Wiesbaden, Germany).
[b] The assigned unitage for each member of the HBsAg Working Standard represents the mean value of the results of each participating laboratory.
[c] Represents mean values for samples tested in duplicate for 3 days with Murex HBsAg V.3 (Murex Biotec Limited, Dartford, UK).

also validated at the NIBSC, and the results were found to be compatible.

Mutations in the 'a' determinant region of the HBV S gene can result in false-negative results in some HBsAg kits.[18–20] Because evaluation of analytical sensitivity alone is not sufficient to assess the performance of HBsAg kits, it was recommended that a requirement for evaluation of HBsAg mutant samples be added to the European Common Technical Specifications (CTS).[21,22] The revised CTS required for CE marking now require consideration of mutants.[23] The mutation at nt 1896 of the precore gene gives rise to a translational stop codon mutant (G1896A). Although some studies implicated the HBV precore stop codon mutation as a possible virulence marker for severe liver disease and fulminant hepatitis, it has also been found, as in this study, in asymptomatic carriers.[24–26] The second mutant that we have identified is the most frequently observed naturally occurring S gene mutant (T/I126S).[27,28] These 2 mutants were detected by only some of the kits used in the collaborative study. As the G1896A mutant does not affect the 'a' determinant region, low sensitivity might have been the reason why 3 kits failed to give a reactive result. In the case of the T/I126S mutant, only 3 kits used in the collaborative study were able to detect the HBsAg. In this case the mutation might have been the cause for undetectability. However, since the concentration of HBsAg is very low in this panel member, insufficient sensitivity of the kits cannot be ruled out.

Stability studies provide information on the length of time of stability of a preparation under the recommended storage conditions. Although the Arrhenius equation is a satisfactory model for freeze-dried materials, real-time stability studies are needed for liquid stored preparations.[29] We have performed real-time stability studies for the HBsAg working standard (KFDA 08/024) and the results for 12 months after storage at −20 °C showed a very good correlation with the assigned unitage. The real-time stability study needs to be performed until the standard will be replaced by new materials.

As a result of this study an HBsAg mixed titer performance panel (BTRI HBV/MP, KFDA 08/025) and an HBsAg working standard (KFDA 08/024) that reflect the viral characteristics common in Korea have been established as NSs. This will enable the use of validation materials on a long-term basis, improve the validation system for HBsAg diagnostic kits in Korea and lead to quality improvement of diagnostic kits.

Conflict of interest

No conflict of interest is declared.

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