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International collaborative study on the 3rd WHO International Standard for hepatitis B surface antigen

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ABSTRACT

Background: The WHO International Standard (IS) for hepatitis B surface antigen (HBsAg) is used to standardize HBsAg assays. Stocks of the 2nd IS for HBsAg are depleted. The proposal to establish its replacement was endorsed by WHO in 2012.
Objective: Preparation of a freeze-dried candidate 3rd IS (NIBSC 12/226); evaluation of its suitability in a WHO international collaborative study; calibration of its potency in International Units (IU).
Study design: The 3rd IS is based on plasma-derived, purified, inactivated HBsAg from Vietnam. Qualitative and quantitative HBsAg assays were used to evaluate 12/226 alongside the 2nd IS and 1st IS. Blinded study samples included a duplicate of 12/226, a negative control and two diluted plasma samples representing hepatitis B virus (HBV) genotypes A and B.
Results: Twelve laboratories from 9 countries returned 22 data sets from 15 methods. The overall geometric mean potency of 12/226 is 47.3 IU/mL (±13% CV) when compared to the 2nd IS with HBV subgenotype A2. The 3rd IS has HBV subgenotype B4 with a heterogeneous HBsAg subtype population of *ayw1* and *adw2*. Some genotype-dependent effects on the inter-laboratory variability were observed but overall mean potencies were virtually identical irrespective of the IS used for calibration. Stability studies indicate that the candidate is stable for long-term use.
Conclusions: 12/226 was established in October 2014 by the WHO Expert Committee on Biological Standardization as the 3rd IS for HBsAg with a potency of 47.3 IU per ampoule maintaining the continuity in the standardization of HBsAg assays.

1. Background

The WHO Expert Committee on Biological Standardization (ECBS) establishes International Standards (IS) as the highest order of references for biological substances with potencies assigned in International Units (IU) [1]. The WHO IS for HBsAg is used for validation of HBsAg assays by clinical laboratories, blood transfusion laboratories, manufacturers of blood products and manufacturers of in vitro diagnostic kits and to calibrate secondary reference materials for HBsAg. The 2nd IS is now depleted and the ECBS endorsed its replacement in October 2012. This report describes the development and worldwide evaluation of the 3rd WHO IS for HBsAg.

2. Objectives

Assess the suitability of a freeze-dried formulation to serve as 3rd IS; calibrate the candidate relative to the 2nd IS in IU per ampoule; assess the candidate's potency in assays performed in different laboratories; assess the commutability of the candidate to serve as a standard for different sample types; evaluate its stability.

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Table 1
Collaborative study samples.

Sample Code	Sample Name	Description	Presentation
12/226	Candidate 3rd WHO IS for HBsAg	Non-adjuvanted plasma-derived HBsAg vaccine bulk. HBV genotype B4, HBsAg subtypes *ayw1/adw2* formulated to ~50 IU/mL.	Freeze-dried Ampoule
00/588	NIBSC 00/588	2nd WHO IS for HBsAg genotype A2 subtype *adw2* (33 IU/mL) [3,4]	Freeze-dried Vial
80/549	NIBSC 80/549	1st WHO IS for HBsAg subtype group *ad* (100 IU/mL) [5,6]	Freeze-dried Vial
sample A	NIBSC 01/402	Blinded reference panel member inactivated HBsAg genotype A2 subtype *adw2* (~2 IU/mL) [3]	Freeze-dried Vial
sample B	NIBSC 00/616	Blinded negative reference panel member consisting of normal re-calcified plasma [3]	Freeze-dried Vial
sample C	NIBSC 12/226	Blinded duplicate candidate 3rd WHO IS for HBsAg	Freeze-dried Ampoule
sample D	[a]PEI Sample N4222	Blinded HBV genotype B2 HBsAg subtype *adw2* [10] Unitage not assigned Pre-diluted to a target 79 IU/mL	Liquid Screw-capped tube
sample E	[a]PEI sample N4879	Blinded HBV genotype A2 HBsAg subtype *adw2* [10] Unitage not assigned Pre-diluted to a target 74 IU/mL	Liquid Screw-capped tube

[a] The PEI Samples are the unprocessed source materials used in the preparation of members of the 1 st WHO International Reference Panel for HBV genotypes for HBsAg assays (PEI product code 6100/09) [10].

3. Study design

3.1. Candidate material

The source material is a plasma-derived HBsAg vaccine bulk (lot HB-PL01) without adjuvant (VABIOTECH, Vietnam). The plasma was obtained from asymptomatic carriers with high viremia. The HBsAg was purified and inactivated using validated methods for the manufacture of plasma-derived hepatitis B vaccines [2]. HB-PL01 was found positive for HBV DNA and the sequence of the HBV DNA showed two different wildtype strains, both with subgenotype B4 and HBsAg subtype *ayw1* or *adw2* (Seiz et al., doi: http://dx.doi.org/10.1016/j.jcv.2016.05.009).

Based on a preliminary mean potency of 36,450 IU/mL, the bulk was adjusted to ~50 IU/mL in thrombinized and declotted plasma which was negative for HIV, HCV and HBV viral markers. Bronidox was included as preservative at 0.05%. The formulation was prepared and filled into ampoules in 1 mL aliquots and freeze-dried using standard operating procedures. The material is NIBSC product code 12/226.

The target concentration and the detection limit of HBsAg of 12/226 were confirmed by the IVD Test Laboratory at the Paul-Ehrlich-Institut (PEI), Germany.

3.2. Additional study samples

The 2nd IS (NIBSC 00/588) [3,4] and the 1st IS (NIBSC 80/549) [5,6] were included in the study un-blinded. Blinded samples included a duplicate of 12/226, a diluted version of the 2nd IS, a negative control and two diluted non-inactivated plasma samples representing HBV subgenotypes A2 and B2 (Table 1).

3.3. Collaborative study

Participating laboratories are listed in Table 2. For each method, participants were requested to perform 3 assays using fresh samples each time, to test 2 independent dilution series of the study samples and assay all samples concurrently. Codes for the assay methods used are summarized in Table 3. Raw data for each dilution point were reported in Excel spreadsheets.

3.4. Statistical methods

All laboratories are referred to by code number not representing the order of listing in Table 2. Where a laboratory returned data using different assay methods, the results were assessed separately for each method and are referred to according to the lab number and assay code e.g. 3 Eql and 3 Mv3.

Individual assays were analyzed as multiple parallel-line assays using CombiStats [7] and expressing the potency of the study samples relative to the different references. All study samples were included in the analysis of each assay. A log transformation of each response and subtraction of the assay's average blank, if applicable, were used to achieve linearity and parallelism. Where assays were spread over different plates, the data from individual plates were analyzed independently, where the relevant reference was included on the plate. Potency estimates were then combined across plates, to give single estimated potencies for each sample for each assay.

Potency estimates were combined using unweighted geometric means (GM), to give laboratory mean potency estimates for the study samples against the appropriate reference. Overall means for the study were calculated as unweighted geometric means of the individual laboratory means. Variability within (intra-laboratory) and between (inter-laboratory) laboratories was expressed as a geometric coefficient of variation (% GCV) [8].

Differences in potency estimates between different methods were assessed using a multiple comparison Analysis of Variance with Tukey's correction for multiple comparisons [9].

3.5. Stability studies

3.5.1. Accelerated degradation

Ampoules were stored at indicated temperatures, then removed after 343 days and held at −80° C until assayed. Freshly reconstituted ampoules were tested concurrently in 3 independent assays using method Mv3. The potencies of the samples were calculated relative to the sample stored at −70° C. The long-term stability of 12/226 was assessed using the Arrhenius model for accelerated degradation studies using weighted and semi-weighted data [9].

Table 2
Collaborative study participants. (In alphabetical order by country).

Name	Laboratory	Country
T McDonald	National Reference Laborarory, Fitzroy	Australia
V Lievre	L'Agence nationale de sécurité du médicament et des produits de santé – ANSM, Saint-Denis cedex	France
R Bäeuerlein and I Krüger	Roche Diagnostics GmbH, Penzberg	Germany
H Scheiblauer and S Nick	Testing Laboratory for in-vitro Diagnostic Medical Devices at the Paul-Ehrlich-Institut (PEI-IVD), Langen	Germany
R Chhabra	Immuno Diagnostic Kit Laboratory, National Institute of Biologicals, Ministry of Health and Family Welfare, NOIDA	India
L. Pallavicini andM. DeLuca	DiaSorin SpA, Saluggia (VC)	Italy
G Pisani and F Marino	Biologicals Unit, National Center for Immunobiologicals Research and Evaluation (CRIVIB), Istituto Superiore di Sanita, Rome	Italy
K Ishii	National Institute of Infectious Disease, Dept. of Virology II, Tokyo	Japan
C Morris	National Institute for Biological Standards and Control – NIBSC, South Mimms	UK
F Kori	U.S. Food and Drug Administration-FDA/CBER/OCBQ/DBSQC/LACBRP, Kensington	USA
M Kuhns	Abbott Diagnostics Division, Abbott Park	USA
N Thu Van	VABIOTECH, Ha Noi	Vietnam

Table 3
Collaborative study assay methods and codes.

Quantitative automated assays		
Assay Code	Assay	No. of data sets
Aqn	ARCHITECT HBsAg, Abbott Diagnostics	2
Eqn	Elecsys HBsAg II, Roche Diagnostics	2
LxL	LIAISON XL murex HBsAg Quant, Diasorin S.p.A.	1
Qualitative automated assays		
Assay Code	Assay	No. of data sets
ADq	ADVIA Centaur HBsAg, Siemens Healthcare Diagnostics Inc.	1
Aql	ARCHITECT HBsAg Qualitative II, Abbott Diagnostics	1
Eql	Elecsys HBsAg II, Roche Diagnostics	2
LDi	LIAISON HBsAg, Diasorin S.p.A.	1
PRq	PRISM HBsAg, Abbott Diagnostics	3
VBi	VIDAS HBsAg ULTRA ELFA, BioMérieux	1
Qualitative semi-automated/manual assays		
Assay Code	Assay	No. of data sets
En6	Enzygnost HBsAg 6.0,Siemens Healthcare Diagnostics Products GmbH	1 manual1 semi-automated
DPr	HBsAg one, Dia. Pro	1
HeB	Hepanostika HBsAg Ultra, BioMérieux	1
MiS	Microscreen HBsAg ELISA, Span Diagnostics	1
Mv3	Murex HBsAg Version 3, Diasorin Ltd./S.p.A UK Branch	1 manual1 semi-automated
SD3	SD HBsAg ELISA 3.0, Standard Diagnostics	1

Table 4
Thermal degradation assessment of 12/226 stored for ~1 year at the indicated temperatures. Potencies are expressed relative to the material stored at −70 °C (assigned a unitage of 1 for the purpose of this assessment). The % loss per month or year was predicted using the Arrhenius model[a].

Temperature (°C)	Assay	Potency	Combined Potency	Predicted % Monthly Loss	Predicted % Yearly Loss
	1	1.00			
−20	2	1.12	1.05	< 0.01	< 0.01
	3	0.99			
	1	1.02			
+4	2	1.04	1.02	< 0.01	0.03
	3	1.00			
	1	1.00			
+20	2	0.99	0.99	0.07	0.84
	3	0.89			
	1	0.89			
+37	2	0.75	0.78	2.00	21.52
	3	0.73			
	1	0.33			
+45	2	0.38	0.35	NC	NC
	3	0.40			
+56[b]	NT				

Abbreviation: NC = not calculated. NT = not tested.

[a] The predictions are dependent on the estimated potencies at +37 °C and above being reliable, and the apparent drop in potency not being affected by problems of reconstitution. This was seen with +45 °C and the sample cannot be assigned a percentage predicted loss due to the difficulty in reconstituting the material. It is not possible to obtain reliable predictions from the data for +4 °C and +20 °C alone, as insufficient degradation has occurred.

[b] The ampoules stored at 56° C could not be reconstituted.

3.5.2. *Stability after reconstitution*

Ampoules stored as described above for accelerated degradation, were reconstituted and the samples returned to storage at +4 °C for 2 months. Potencies of the stored reconstituted samples were determined relative to a freshly reconstituted sample that had been stored continuously at −70° C.

Table 5
Potencies (IU/mL) relative to 2nd IS.

Study Sample	1st IS sgt A2 (100 IU/mL)			Sample A inactivated sgt A2 (01/402) (~2 IU/mL)			Sample E sgt A2 (PEI N4879) (~74 IU/mL)			Candidate 3rd IS sgt B4 (12/226) (~50 IU/mL)			Sample C duplicate 3rd IS (~50 IU/mL)			Sample D sgt B2 (PEI N4222) (~79 IU/mL)			Sample B (00/616) Negative	
Lab	N	GM	GVC	N	GM	GCV	N	GM	GCV	N	GM	GCV	N	GM	GCV	N	GM	GCV	N	GM
1 VBi	3	168.9	2.4	3	2.25	1.5	3	138.3	0.5	3	56.0	1.7	3	53.5	1.3	3	90.4	2.0	3	ND
2 PRq	3	82.5	7.8	3	1.76	0.9	3	100.5	3.0	3	45.2	5.9	3	45.2	2.6	3	73.5	4.8	3	ND
3 Eql	3	97.2	6.3	3	1.84	8.5	3	96.4	3.0	3	43.9	2.5	3	45.0	1.5	3	73.1	3.3	3	ND
3 Mv3	3	132.7	10.0	3	1.72	11.9	3	105.4	15.7	3	53.4	6.8	3	47.4	14.9	3	60.0	8.7	3	ND
4 LDi	3	132.3	8.4	3	1.80	6.7	3	165.3	2.1	3	42.3	4.7	3	45.5	7.5	3	103.6	6.5	3	ND
4 LxL	3	91.0	2.7	3	2.02	7.6	3	138.0	5.2	3	62.2	6.2	3	62.0	8.1	3	99.1	0.7	3	ND
5 Eqn	9	104.9	4.2	9	1.84	5.2	9	118.1	2.8	9	42.0	2.3	9	41.1	2.8	9	71.2	3.6	9	ND
6 Eql	3	95.5	10.9	2	1.44	21.2	3	89.3	12.3	3	45.3	3.9	3	39.0	17.1	3	75.8	22.9	3	ND
7 Aql	3	74.5	1.8	3	1.77	1.3	3	103.1	9.5	3	63.2	1.8	3	61.7	1.3	3	122.4	1.9	3	ND
7 Aqn	3	86.5	2.8	3	1.72	7.3	3	107.9	4.8	3	48.5	5.0	3	47.0	5.0	3	80.0	2.1	3	ND
7 PRq	3	97.7	4.7	3	1.87	5.1	3	119.7	4.3	3	49.8	3.8	3	49.1	2.6	3	84.6	4.1	3	ND
8 HeB	1	116.2	NA	1	1.45	NA	1	128.8	NA	1	50.6	NA	1	38.1	NA	0	NT	NA	1	ND
8 MiS	0	IV	NA	0	1.90	NA	0	NT	NA	1	47.7	NA	1	47.5	NA	1	127.2	NA	1	ND
8 SD3	0	IV	NA	0	IV	NA	1	[a]	NA	0	IV	NA	1	[a]	NA	0	NT	NA	1	ND
9 Mv3	4	118.6	11.5	4	2.05	10.5	4	66.2	11.2	4	62.6	7.3	4	48.7	6.5	4	70.1	5.3	4	ND
10 ADq	3	66.7	0.2	3	1.88	3.0	3	75.5	3.0	3	43.7	1.3	3	41.4	4.2	3	72.2	2.6	2	ND
10 Aqn	3	91.4	0.7	3	1.84	7.8	3	69.0	4.5	3	49.3	8.4	3	46.3	5.0	3	69.4	2.3	3	ND
10 Eqn	3	93.9	2.3	3	1.90	3.2	3	81.9	4.8	3	43.8	1.9	3	42.0	3.3	3	69.1	3.2	0	NT
10 En6	3	115.5	5.7	2	1.98	4.5	3	90.2	1.4	3	43.4	6.6	3	41.2	6.9	3	62.2	1.3	0	NT
10 PRq	3	88.3	5.9	0	IV	NA	0	NT	NA	3	50.6	3.7	3	42.0	6.5	0	NT	NA	2	ND
11 En6	3	120.4	4.2	3	1.90	2.9	3	120.6	10.0	3	39.5	4.4	3	39.5	5.8	3	69.0	4.2	3	ND
12 DPr	1	57.9	NA	1	1.73	NA	3	145.6	7.4	3	46.1	11.5	1	57.1	NA	1	101.1	NA	3	ND
overall GM	98.7			1.8			105.2			48.6			46.2			80.9			NA	
% GCV	28			11			29			15			15			24			NA	

Abbreviations:: *sgt* = subgenotype; GM = Geometric mean; %GCV = Geometric coefficient of variation; ND = not detected; NA = not applicable; NT = assay not performed; IV = invalid.

[a] Discrepant values from a single assay were not included in calculation of overall GM and overall %GCV.

Table 6
Overall mean estimates (IU/mL) for potency and inter-laboratory variability (%GCV) for samples A, C, D and E relative to the 2nd IS, the candidate 3rd IS or the 1st IS.

Reference				2nd IS (33 IU/mL) sgt A2		Candidate 3rd IS (50 IU/mL) sgt B4		1st IS (100 IU/mL) sgt A2 (plasma)	
Sample			No. ofassays	Overall GM	Overall% GCV	Overall GM	Overall% GCV	Overall GM	Overall% GCV
Code	*sgt*	Type							
A	A2	2nd IS	56	1.8	11	1.9	18	1.9	29
C	B4	3rd IS	63	46.2	15	47.2	10	46.8	37
D	B2	plasma	60	80.9	24	83.1	23	80.2	44
E	A2	plasma	63	105.2	29	107.9	34	106.2	39

Abbreviations: *sgt*: subgenotype; GM = Geometric mean; %GCV = Geometric coefficient of variation.

3.5.3. Stability after freeze-thaw

Ampoules stored at −20° C were reconstituted and then subjected to freeze-thaw cycles. Potencies of the freeze-thawed samples were determined relative to a freshly reconstituted sample that has been stored continuously at −70° C.

4. Results

4.1. Production, validation and stability assessment of 12/226

The product summary for 12/226 is shown in Supplemental Table 1. Validation assessment performed at PEI indicated that the potency (50 IU/mL, 31% GCV) and analytical sensitivity (0.016–0.029 IU/mL) for 12/226 (Supplemental Tables 2 and 3) were acceptable and that the candidate was suitable for evaluation in the international collaborative study.

The long-term stability of 12/226 was predicted using the Arrhenius model (Table 4). The predicted percentage potency loss, at the different temperatures of storage, indicates that 12/226 is adequately stable to serve as IS, and is suitable for ambient transportation as the loss at 37 °C is only 2% after 1 month.

Additional potency estimates were determined for the degradation samples to evaluate the stability of 12/226 after reconstitution and subsequent storage at +4 °C for 2 months (Supplemental Table 4). For temperatures up to 37 °C, there was no significant difference in potency found for any of the temperature points stored in the freeze-dried state for 1 year compared to storage at +4 °C for 2 months after reconstitution. Additional assessment of reconstituted and refrozen samples of 12/226 showed no significant loss of potency for up to 3 freeze-thaw cycles compared to freshly reconstituted samples (Supplemental Table 5).

4.2. Collaborative study

4.2.1. Data received

Twelve laboratories submitted 22 data sets for 15 qualitative and quantitative assay methods. Five participants performed more than one method. The majority of laboratories returned the requested three assays with each method that they used. Laboratory 8 only performed one assay with each of three different methods. The results for method 8SD3 for 12/226, the 1st IS and sample A were invalid due to non-parallelism/non-linearity. For sample C, the result obtained by 8SD3 was much higher than all other laboratories and methods, including results reported by laboratory 8 with different assay methods. The data were only from a single assay and because of this discrepancy; the results from 8SD3 were excluded from the calculations of overall GM and %GCV. Laboratory 12 used multiple plates per assay so not all samples comparisons could be made against the different references for each assay.

4.2.3. Potencies

The individual laboratory GM potencies expressed relative to the 2nd IS along with the intra-laboratory % GCVs are shown in Table 5 for the 1st IS, 12/226 and samples A-E (Supplemental sta-

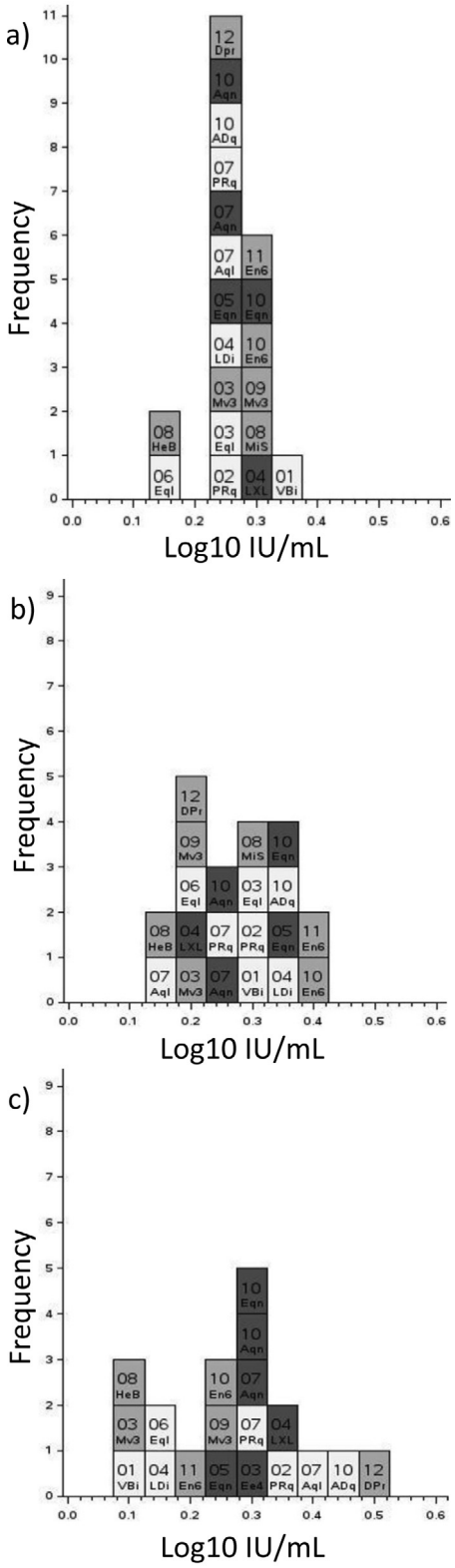

Fig. 1. Relative potencies of sample A (diluted 2nd IS) against the (a) 2nd IS, (b) candidate 3rd IS and (c) 1st IS. Each box in the histogram represents the laboratory geometric mean potency (log10 IU/mL) for a particular assay method, and is labelled with the laboratory and method code. Automated quantitative assays are represented by the dark grey boxes. Manual and automated qualitative assays are represented by the light grey and white boxes, respectively.

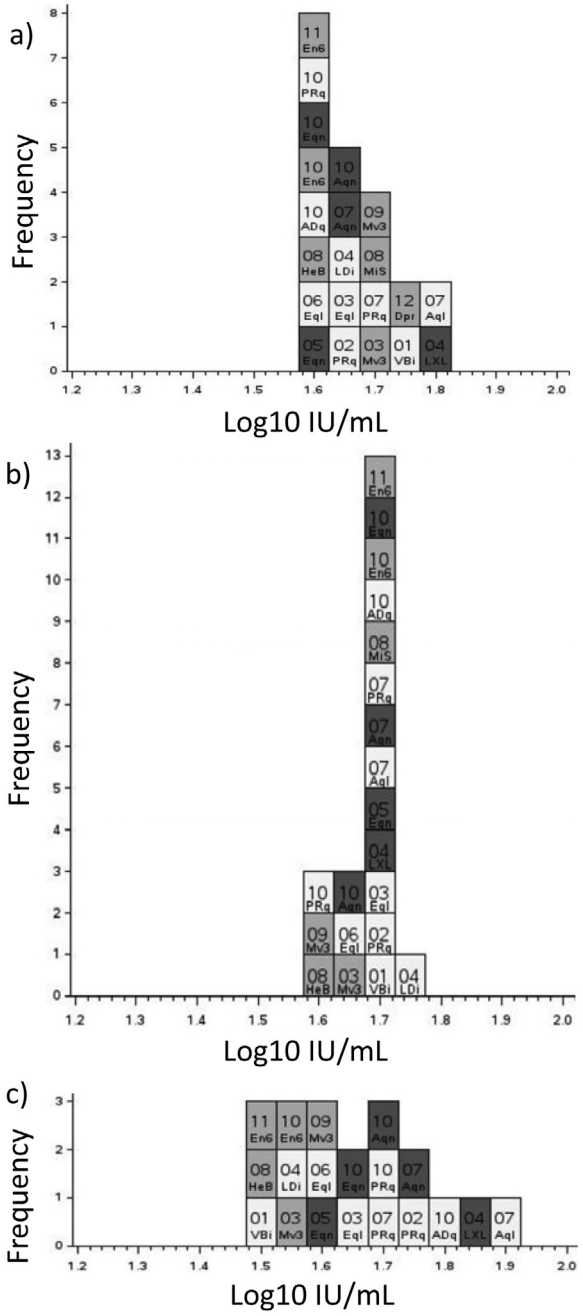

Fig. 2. Relative potencies of sample C (duplicate 3rd IS) against the (a) 2nd IS, (b) candidate 3rd IS and (c) 1st IS. Each box in the histogram represents the laboratory geometric mean potency (log10 IU/mL) for a particular assay method, and is labelled with the laboratory and method code. Automated quantitative assays are represented by the dark grey boxes. Manual and automated qualitative assays are represented by the light grey and white boxes, respectively.

tistical methods). For each sample, the overall GM potency across laboratories is also shown, along with the inter-laboratory % GCV. No false positives were found with the negative control (Sample B). The overall GM potency of the 1st IS (98.7 IU/mL) was in excellent agreement with its assigned potency of 100 IU/mL. The inter-laboratory agreement for the 1 st IS (28% GCV) is close to results determined in the collaborative study that established the 2nd IS (30% GCV) [3]. These results indicate that the continuity of the 2nd IS with respect to the 1st IS had been maintained since its establishment in 2003.

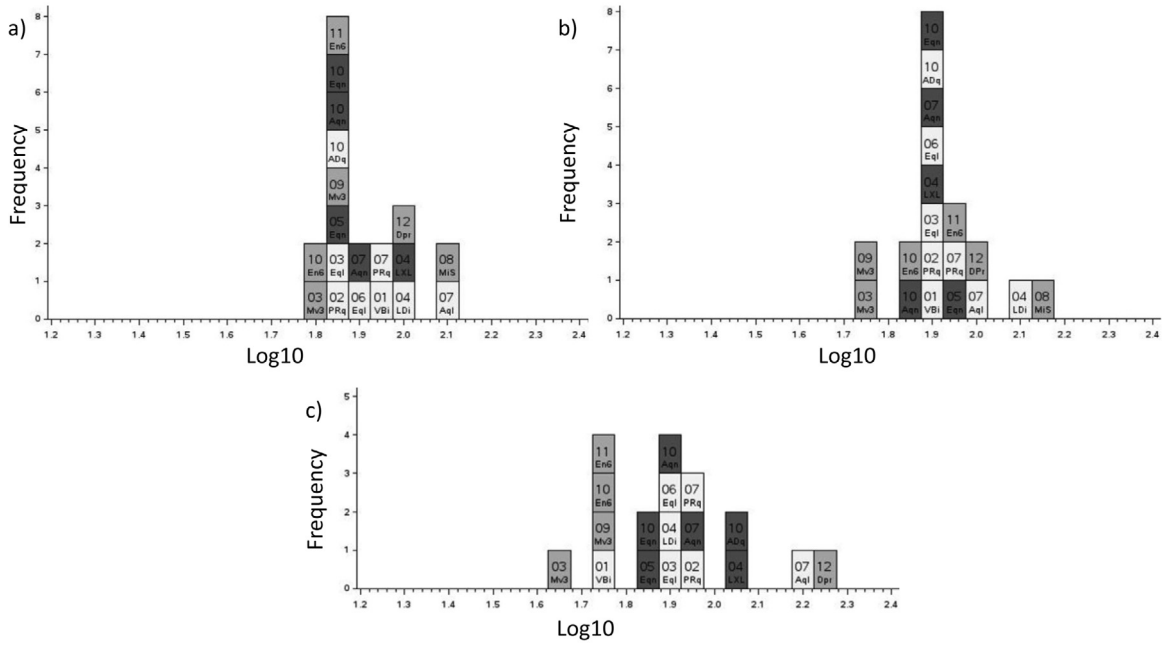

Fig. 3. Relative potencies of sample D (diluted plasma with *sgt* B2) against the (a) 2nd IS, (b) candidate 3rd IS and (c) 1st IS. Each box in the histogram represents the laboratory geometric mean potency (log10 IU/mL) for a particular assay method, and is labelled with the laboratory and method code. Automated quantitative assays are represented by the dark grey boxes. Manual and automated qualitative assays are represented by the light grey and white boxes, respectively.

The potency of 12/226 (48.6 IU/mL) and its duplicate sample C (46.2 IU/mL) is close to the target 50 IU/mL. The difference between the potency of 12/226 and sample C is not statistically significant and combining the data across the two samples gives a mean potency of 47.3 IU/mL (Supplemental statistical methods and Supplemental Table 6).

4.2.3. Intra-laboratory and inter-laboratory variability

The individual laboratory%GCVs for potencies expressed relative to the 2nd IS are shown for the study samples in Table 5 and Supplemental Table 6. Generally, laboratories show a good level of reproducibility for each method. For 12/226 and its duplicate sample C, the majority of laboratories have a%GCV below 8%, with the highest%GCV at 17.1%. Comparing the%GCV values, the inter-laboratory variability generally is greater than the intra-laboratory variability. There is, however, an improvement in inter-laboratory variability for 12/226 (15% GCV) with respect to the 1 st IS which gives a higher%GCV of 28% (Table 5). The candidate plus sample C against the 2nd IS shows similar levels of inter-laboratory variability giving a combined overall%GCV of 13% (Supplemental Table 6).

The overall mean potencies of samples A, C, D and E relative to the 2nd IS, 12/226 and the 1st IS are shown in Table 6 along with the inter-laboratory%GCVs. The results are also shown in histogram form in Figs. 1–4 . Assays of these samples give inter-laboratory%GCVs in the range of 11%–44%, which is consistent with inter-laboratory variability reported for similar collaborative studies [3,10]. There were no significant differences in potency estimates between quantitative and qualitative methods (Figs. 1–4).

The best inter-laboratory agreement is obtained when the potency of a sample is expressed relative to a reference that is most like itself. For example, sample A is a dilution of the same inactivated bulk used to prepare the 2nd IS. The inter-laboratory variability for sample A, is lowest when potency is expressed relative to the 2nd IS, giving a%GCV of 11%. The inter-laboratory variability is somewhat increased (18% GCV), when potencies for sample A are expressed relative to 12/226 (B4 *ayw1*/*adw2*) which, like the 2nd IS, is derived from purified and heat-inactivated HBsAg. However, calibration with the 1st IS, which is derived from diluted native HBsAg-containing plasma, gives a higher variability (29% GCV) even though it has the same HBV subgenotype (Table 6 and Fig. 1).

Similarly, inter-laboratory variability of sample C is lowest when potencies are expressed relative to its duplicate, 12/226 (10% GCV). When expressed against the 2nd IS or the 1st IS, the inter-laboratory%GCV for sample C increases to 15% and 37%, respectively (Table 6 and Fig. 2).

The inter-laboratory%GCV values for Sample D (B2 *adw2*) relative to the 2nd IS, 12/226 or 1st IS are 24%, 23% and 44%, respectively. For sample E (A2 *adw2*), the inter-laboratory%GCVs are 29%, 34% and 39% respectively (Table 6 and Figs. 3–4). While the variability between laboratories is greatest for these samples, the overall%GCVs for samples D and E is typical for current HBsAg assays [10].

5. Discussion

Candidate 3rd WHO IS for HBsAg (NIBSC 12/226) is a batch of 3200 ampoules containing 1.0 mL freeze-dried HBsAg formulated in thrombinized and declotted plasma. The material used to produce 12/226 is a soluble inactivated HBsAg vaccine bulk purified from human plasma obtained from viremic carriers. Biosafety is the rationale for using HBsAg derived from a vaccine in that ideally the IS should not contain infectious HBV. This is the case for 12/226 and the 2nd IS.

The IS should also be representative for a region with high HBV prevalence. The HBV B4 genotype of 12/226 is highly prevalent in Vietnam [11], whereas the HBV A2 genotype of the 1 st and 2nd ISs is prevalent in Europe and the USA. Because multiple donors were used for preparing the bulk, 12/226 is heterogeneous containing HBV strains encoding subtypes *ayw1* and *adw2* (Seiz et al. accepted).

When calibrated against the 2nd IS, 12/226 has an overall geometric mean potency of 47.3 IU/mL, a value close to the intended target of 50 IU/mL (Table 6).

The extent to which a reference is suitable to serve as a standard for the variety of test samples being assayed, i.e. its commutabil-

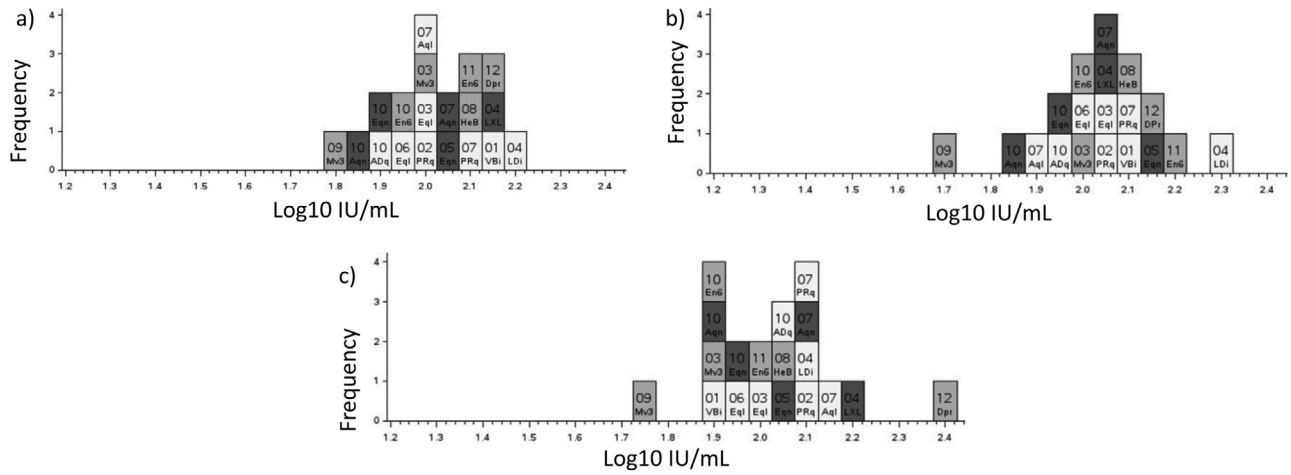

Fig. 4. Relative potencies of sample E (diluted plasma with *sgt* A2) against the (a) 2nd IS, (b) candidate 3rd IS and (c) 1st IS. Each box in the histogram represents the laboratory geometric mean potency (log10 IU/mL) for a particular assay method, and is labelled with the laboratory and method code. Automated quantitative assays are represented by the dark grey boxes. Manual and automated qualitative assays are represented by the light grey and white boxes, respectively.

ity, is determined by many factors including methods of reference preparation such as purification, inactivation and freeze-drying steps; the sample matrix (e.g. native plasma or serum); and molecular and antigenic variants of the analyte (e.g. differences in HBs protein composition, genotype or vaccine escape mutants). An aspect of commutability was addressed in this study by including samples representing different HBV genotypes. The HBV subgenotype is an important factor contributing to the variability of HBsAg assays [3,10] and, some (sub)genotype-dependent effects on the inter-laboratory variability were observed (Table 6 and Figs. 1–4); however, the inter-laboratory%GCV obtained in this collaborative study, indicate that the performance of 12/226 is comparable to that of the 2nd WHO IS in harmonising HBsAg assays. The observed inter-laboratory variability may thus be related to design differences between assay methods. Ideally, assays should be able to detect all known HBV genotypes equally well, however the methods of antigen capture and detection contribute to assay sensitivity and specificity. Fifteen of the 22 data sets represent different assay methods and thus inter-assay variability can be inferred from the inter-laboratory variability. The study on the WHO HBsAg genotype panel identified also some HBsAg assays which performed differently with the inactivated IS and native plasma samples of the same subgenotype [10]. Taken together the data of Tables 5 and 6 and Figs. 1–4 suggest that the nature of the sample – HBsAg positive plasma or purified and inactivated HBsAg – creates larger differences between different methods than the HBV genotype or HBsAg subtype. Irrespective of the detectable differences between different methods, all samples yielded virtually identical overall GM with the three ISs (Table 6). In addition, the overall%GCVs for samples expressed against 12/226 are in line with overall%GCVs observed in similar studies [3,10].

Along with the WHO HBV genotype panel for HBsAg assays (PEI product 6100/09) [10] and the WHO reference panel (NIBSC 03/262) [3], the use of 12/226 as calibrator, will facilitate the characterization and standardization of the factors that contribute to assay sensitivity and variability and assist in the development of uniform management strategies for HBV-associated disease.

Stability studies indicate that 12/226 is stable for long-term storage at −20 °C as well as for short-term storage at +4 °C and shipment at ambient temperatures. After reconstitution 12/226 may be stored at +4 °C for up to 2 months or may undergo up to 3 freeze-thaw cycles with no significant loss of potency.

In October 2014, the WHO ECBS established 12/226 as the 3rd WHO IS for HBsAg (HBV genotype B4, HBsAg subtypes *ayw1*/*adw2*) with a potency of 47.3 IU/mL when reconstituted as directed in the instructions for use. The 3rd IS for HBsAg is available from NIBSC (http://www.nibsc.org/). It is intended to be used by laboratories to calibrate secondary reference materials for HBsAg and in the determination of analytical sensitivity of HBsAg assays.

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Competing interests

None declared.

Ethical approval

Not required.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2016.06.003.

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