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Diagnostic evaluation of the serological detection performance on HBV surface antigens in the Netherlands

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Contents

Abstract

Hepatitis B virus (HBV) poses a substantial global health challenge, with close to 300 million individuals experiencing chronic infections worldwide. Categorized into ten distinct types (A-J), HBV exhibits a high genetic variability. In the Netherlands, genotype A is most prevalent, followed by genotype D. However, since 2019, a notable increase in genotype F is reshaping the epidemiological landscape of HBV infections. The impact of HBV's high genetic variability on serological assay performance, especially in detection efficacy of HBV surface antigen (HBsAg) from different genotypes by assays used in routine diagnostics, remains an understudied area. Here, we report on the results obtained from the study that aimed to investigate the serological detection performance on HBsAg diagnostic assays used in Dutch Medical Microbiology and Clinical Chemical laboratories for different HBV genotypes.

An external quality assessment (EQA) panel containing sixteen well-characterized serological samples with known antigen amounts, representing various HBV genotypes (A, B, C, D, E, F, and H; WHO international HBV reference panel (1)), including a negative serum control, was designed and distributed to twenty-seven participating Dutch laboratories. Each sample contained between 21 and 32 IU/mL of HBsAg. Laboratories were instructed to perform routine diagnostic tests and report their results.

Three quantitative and eight qualitative diagnostic assays were evaluated. The EQA data revealed consistent detection of all genotypes, including genotype F2. Quantitative assays demonstrated variability in antigen detection of the same genotype among laboratories employing the same diagnostic system, e.g., Liaison XL (DiaSorin). Additionally, all quantitative assays exhibited variations in HBsAg detection rates for different HBsAg genotypes, with values ranging from 10 IU/mL (subgenotype A2) to 76 IU/mL (subgenotype E). The lowest measured values (average HBsAg < 29 IU/mL) were observed for genotypes A2, B2, D1, D2, D3, and F2.

Our study shows consistent serological detection of HBsAgs of the evaluated distinct HBV genotypes, indicating good assay performance of the tested immunoassays. However, variations in detection levels among different genotypes, and assay performance discrepancies have been observed and warrant attention with diagnostic interpretation of HBsAg values, in particular for antiviral resistance testing.

1. Introduction

According to the latest available data, Hepatitis B virus (HBV) poses a substantial global health challenge, with an estimated 250 - 260 million individuals experiencing chronic infections worldwide. Annually, approximately 1.1 million people die due to complications associated with HBV (2). The WHO has initiated efforts towards elimination of HBV by 2030 by active case finding, contact training, treatment, and vaccination plans.

Characterized as a small, enveloped, double-stranded DNA reverse-transcribing virus (*Orthohepadnavirus, Hepadnaviridae* family), HBV exhibits a preference for hepatocytes, leading to both acute and chronic hepatic infections. The primary mode of HBV transmission is vertical transmission (3). Other modes of transmission include blood and sexual transmission, as well as intrafamilial spread, which may occur through prolonged contact between children or household members, likely via exposure to fluids from infected individuals (4). HBV is known to exhibit a high genetic variability because of the lack of the proof-reading mechanism (5). Therefore HBV is categorized into 10 distinct types (A-J) with a genome-wide intergenomic sequence divergence of at least 7.5% (6). Additionally, HBV is categorized into four major serotypes (adr, adw, ayr, ayw) based on antigenic epitopes found on its envelope proteins. These serotypes are characterized by a common determinant present in all wild-type variants (a) and two mutually exclusive determinant allele pairs (d/y and w/r) (7). The geographic distribution of HBV genotypes (Table 1) is well-defined and correlates strongly with regional host populations, as well as socio-demographic, ethnic, or migratory factors, in addition to pathogenesis and patient outcomes (8-11). Genotype A is highly prevalent across Southeast Africa, Europe, North America, and India, whereas genotypes B and C are more commonly found in the Asia-Pacific region. Genotype D is the most widespread and is prevalent in North Africa, Europe, the Mediterranean region, North America, and India, while genotype E is primarily restricted to West and Central Africa, as well as Saudi Arabia (12). Genotypes F and H are found in Central and South America and are rare in other parts of the world (13), while the distribution of genotype G is not completely known (14). Genotype I and J are found in Asia and probably result from recombination events with other genotypes (15, 16).

Table 1. Geographical distribution of HBV genotypes and subtypes.

Adapted from Lin et.al, 2017 (10).

Genotypes	Subtypes	Geographic location
A	A1 – A8	Sub-Saharan Africa, Asia, Northern Europe, America, India, Central and Western Africa, Gambia, Nigeria, Haiti
B	B1 – B10	East Asia, Taiwan, China, Indonesia, Vietnam, Philippines, Japan, Alaska, Northern Canada, Greenland
C	C1 – C17	Taiwan, China, Korea, Japan, Southeast Asia, Australia, Philippines, Vietnam, Indonesia, Papua New Guinea
D	D1 – D12	North Africa, Europe, Mediterranean countries, North America, India, Indonesia, Oceania
E		West and Central Africa, Saudi Arabia
F	F1 – F6	Central and South America
G		France, Germany, the United States
H		Central America
I	I1 – I3	Vietnam, Laos, southwestern China, eastern India
J		Japan

HBV genotype F

The S gene, preceded by a preS1 and S2 gene, produces the large, medium, and small forms of the surface antigen (HBsAg). Genotype F displays marked differences from the other genotypes in pre-S/S gene and studies have indicated that detecting genotype F may pose challenges due to the relatively low sensitivity of assays designed for this particular type (1, 17). Genotype F is also the most divergent of the genotypes and is subdivided into six subgenotypes (F1 to F6). Within subgenotypes F1 and F2, distinct clades have been identified. As for subgenotype F1, multiple clades (F1a, F1b, F1c, and F1d) have been described, each representing unique genetic lineages within the broader subgenotype classification. Similarly, within subgenotype F2, distinct clades (F2a and F2b) have been identified. These subclades likely reflect additional levels of genetic diversity and evolutionary divergence within the overall framework of genotype F. (15). The phylogeny of genotype F is unique and shows little intra-subgenotype diversity. However, long evolutionary distances are observed between its six

subgenotypes. This suggests that while the subgenotypes themselves exhibit relatively little genetic variation, they have undergone significant evolutionary divergence from one another over time.

Moreover, there is evidence suggesting that genotype F might have the capacity to evade immunity induced by vaccines (18). Vaccines against HBV primarily target the HBsAg and induce an immune response that produces antibodies against HBsAg. However, one study has shown that HBV genotype F strains may show variations in the HBsAg protein compared to other genotypes (19). These variations could potentially affect the recognition and binding of antibodies produced by vaccinated individuals, reducing the efficacy of vaccination against genotype F strains. However, more data is needed to substantiate this hypothesis.

HBV diagnosis

The accurate detection of HBV infection is essential for early intervention, appropriate patient management, and the prevention of transmission. In the Netherlands, HBV diagnosis is based on detection of HBV antigen and antibodies in serum using serological immunoassays. Key markers, such as HBV surface antigen (HBsAg), HBV envelope antigen (HBeAg), and antibodies to HBV core antigen (anti-HBc), are predominantly tested to determine infection status.

The impact of HBV's high genetic variability on serological assay performance, specifically in detection efficacy of HBsAg from different genotypes by assays used in routine diagnostics, remains an understudied area.

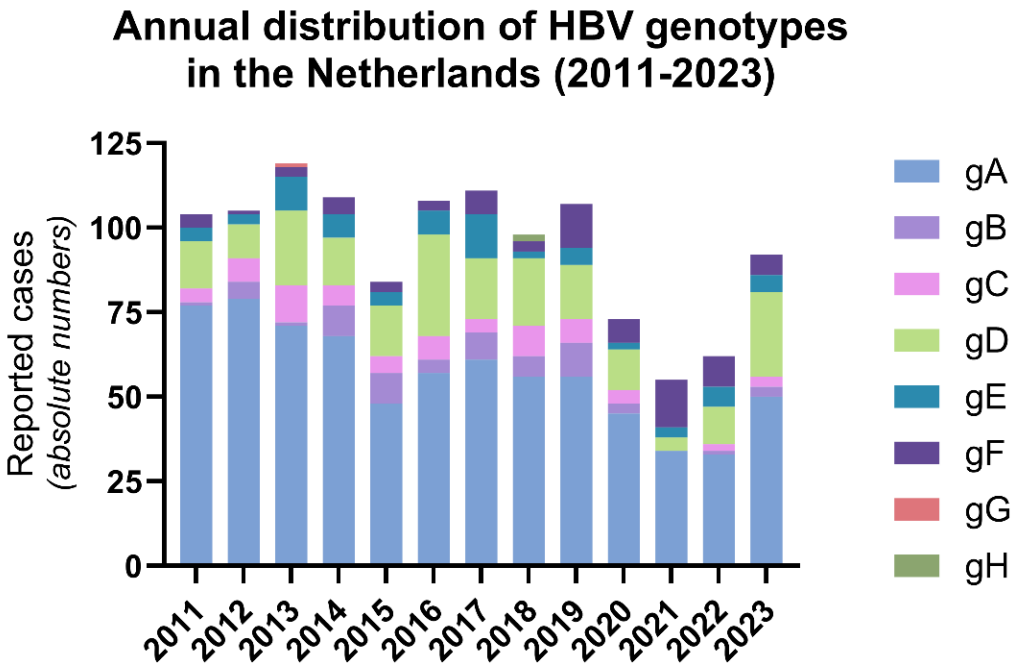
HBV surveillance in the Netherlands

In the Netherlands, all acute HBV infections are reported anonymously by the GGD (Municipal or Community Health Service) to OSIRIS (a public health database used for surveillance purposes). However, clinical, patient, and test data on HBV markers are not reported, limiting the ability to analyze the relationship between different HBV marker levels and substitutions. Acute cases are reported to OSIRIS based on a positive HBsAg test result and/or an anti-HBc IgM result (if available). Chronic cases are notifiable when a positive HBsAg or HBV DNA result is diagnosed for the first time in the Netherlands. To determine risk exposure, trace source research and/or notify partners, interviews are conducted with affected individuals. Since 2004, blood samples have been requested for typing from all acute cases reported to OSIRIS. From 2010 onwards, samples from chronic cases showing risk behavior (i.e., tested in the context of the HBV vaccination program for behavioral risk groups or individuals reporting sexual contact by MSM as a route of transmission), were also requested for typing (20).

In line with patterns observed in most European countries, genotype A is most prevalent in the Netherlands, followed by genotype D (20, 21). Genotype A is consistently the most common genotype each year, but the number of reported cases has generally decreased over the period from 77 cases in 2011 to 50 cases in 2023 (Figure 1). Genotype D shows some fluctuation, but a noticeable decline has also been observed in recent years, peaking at 30 cases in 2016, decreasing to 11 reported cases in 2022, but saw an increase again to 25 cases in 2023. One of the most striking trends in the data is the remarkable increase in the prevalence of genotype F, especially since 2019, even surpassing genotype D as the second dominant type in 2021 (Figure 1). In the first part of the decade (2011-2018), genotype F cases were relatively low and stable, typically ranging from 1 to 7 cases per year. As of 2019, there has been a significant increase in the number of reported cases of genotype F, with 13 cases in 2019, 7 in 2020, 14 in 2021, 9 in 2022, and 6 in 2023. This upward trend suggests that genotype F is becoming increasingly common in the Netherlands.

This shift in genotype prevalence was identified in the Netherlands because of the implementation of standard typing of all reported samples. Since 2004 blood samples from all acute cases are requested for typing based on the S and C gene or complete genome (since 2017), facilitating the identification of genotype variations and contributing to a more comprehensive understanding of the evolving landscape of HBV infections in the region (Figure 1) (20).

Figure 1. Annual distribution of HBV genotypes in the Netherlands over a period of 13 years, between 2011 and 2023.



Role of EQA/SKML

In the framework of promoting the quality of medical laboratory tests in the context of diagnosis and treatment and to raise and maintain those tests at the highest possible level, the routinely designed EQAs are distributed by the Foundation for Quality Assessment in Medical Laboratory Diagnostics (Stichting Kwaliteitsbewaking Medische Laboratoriumdiagnostiek, SKML). EQAs of medical laboratory diagnostics is required by ISO15189 as a means by which laboratories verify whether their methods perform as expected and to what extent their results match those of other users of the same method. The same data also provides insight into the performance of groups of users with the same method and therefore into the performance of those methods. SKML is responsible for providing external quality control distributions for all laboratory specialties, thereby promoting the standardization of diagnostic procedures across laboratories. By participating in EQA programs, laboratories can align their testing procedures with national and international standards, guaranteeing consistent and reliable results.

Given the diversity and complexity of HBV genotypes, the need for a comprehensive HBV genotype EQA panel has become increasingly evident. Traditional EQA panels may not adequately represent the full genetic diversity of HBV, especially for less common genotypes. This can lead to gaps in diagnostic accuracy and reliability, as assays may be less sensitive to these genotypes. The previous HBV genotype panel used by SKML for Hepatitis B focuses on testing the measurement of HBsAg in serum, with the positive/negative cut-off receiving a lot of attention, ensuring that laboratories can accurately distinguish between positive and negative samples. However, the increase in the number of genotype F cases in the Netherlands, has highlighted the possible need for more targeted assessment tools. By incorporating a comprehensive HBV genotype panel, SKML can guarantee that laboratories can accurately detect a wide range of HBV genotypes. This is crucial for effective patient management, especially given the increasing diversity of HBV variants in the Netherlands.

Objectives

The surge in genotype F prevalence is reshaping the epidemiological landscape of HBV infections in the Netherlands. The current diversification of genotypes in the Netherlands raises concerns about potential vaccine evasion, especially with the influx of migrants from regions with a variety of dominant genotypes.

In this study we aimed to investigate the serological detection performance on HBsAg diagnostic assays used in Dutch Medical Microbiology and Clinical Chemical laboratories for different HBsAg genotypes. For this we used the WHO HBV type specific panel (22) as a template for the design of an EQA trial panel. The obtained EQA for different HBsAg genotypes will help in determining whether diagnostic panels uniformly detect all HBV genotypes. In an ideal scenario, the assays should be able to detect all included HBV genotypes equally well.

2. Study design and methods

Selection and characteristics of the EQA subgenotypes

An EQA panel consisting of sixteen well-characterized serological samples (15 HBV positive and 1 negative serum control) with known HBV types and antigen amounts, representing various HBV subgenotypes was designed using WHO reference materials (PEI code 6100/09; version 3; Nov 13, 2017) and published by Chudy et al. (1). The HBsAg concentration was determined by quantitative chemiluminescent immunoassay in IU/mL (CLIA, ARCHITECT HBsAg, Abbott, Germany). Detailed information on the panel is shown in Table 3. In detail, the materials used in the EQA consisted of non-inactivated freeze-dried plasma samples, obtained from the Paul-Ehrlich institute, a WHO collaborating centrum for quality assurance of blood products and *in vitro* diagnostic devices. As part of the blinding procedure, this information was not shared with the participating laboratories.

Table 2. Characterization of the HBV genotype panel members and HBsAg measurements.

Target dilution to ca. 30 IU HBsAg/mL[a]

Sample	Origin	HBsAg (serological) subtype	HBV sub-genotype	HBsAg (IU/mL)[a]
1	Brazil	adw2	A1	29.23
2	Germany	ayw2	D1	27.77
3	Germany	adw2	A2	26.77
4	West Africa	ayw4	E	28.50
5	Japan	adw2	B2	24.90
6	South Africa	adw2	A1	31.80
7	Japan	adr	C2	28.40
8	Russia	adr	C2	30.00
9	South Africa	ayw2	D3	24.13
10	None	/	/	0.00 (NEG CTRL)
11	Japan	adr	C2	29.23
12	Brazil	adw4	F2	31.67
13	Russia	ayw3	D2	29.40

14	Japan	adw2	B2	21.10
15	Brazil	adw4	F2	26.80
16	Germany	adw4	H	29.50

[a]1 in 3 dilutions based on mean CLIA values in IU/mL (ARCHITECT QT, Abbott)(23)

Procedure for participating laboratories

Identification of eligible laboratories

This EQA was set up as a pilot project in collaboration with SKML and Medisch Centrum Alkmaar (MCA). We distributed invitations with forms for collection data on available testing systems; Appendix 1) to laboratories to perform serological testing on samples containing different HBV genotypes. All laboratories expressing interest were included to consider diverse detections systems (both quantitative and qualitative assays). Both the participating laboratories as the reported assays received a specific laboratory and test code.

Distribution of EQA subgenotypes to laboratories and reporting of results

All samples were first allowed to equilibrate to ambient temperature, before being resuspended in 0.4 mL of sterile nuclease-free water. This dilution step ensured that the assays were challenged near their lower detection limits, providing a more rigorous evaluation of their performance. Samples were then left for a minimum of 10 minutes with occasional agitation before being aliquoted into new, relabeled vials (maintaining a new, randomized sample order). All samples were stored and shipped as you would with handling patient samples.

Upon arrival, diagnostic laboratories were requested to check all samples for damage, completeness, and shipping conditions, and to store the samples like patient material until testing. The samples were deemed stable up to 5 days after reception if stored under defined conditions (i.e., refrigerated). Laboratories were advised to freeze samples when processing was expected to start only after five days. MCA was responsible for the distribution of the EQA panel to the participating laboratories.

The distributed panel was accompanied by detailed instructions for testing, consisting of data collection forms (Appendix 2) and a testing protocol (Appendix 3). The diagnostic laboratories participating in the EQA were instructed to assess the panels using each of their routine serological procedures and to report the results obtained for each of the sixteen samples using the provided form (Appendix 3). Results should be reported in IU/mL for quantitative tests and in sample-to-cutoff (S/CO) ratios for qualitative tests. Data about the type of assay utilized, detection methods, instrument and

reagent details, manufacturers, raw detection data, quantitative, and qualitative results of HBsAg were requested as well.

Analysis of the results

The data collection was performed through the RIVM during 2023 up to January 2024 and the data obtained from each diagnostic laboratory were reported as total number of participating laboratories reporting data. The amount of different serological antigenic assays was also reported (Table 3). Per each serological assay the results were analyzed.

Fictional standardization

The results obtained were further analyzed through intra-assay fictional standardization to identify the causes of differences in results. The idea behind fictional standardization is that if each laboratory had included the same calibrator-sample in the measurements, the results could have been converted to that calibrator-sample. If those converted values were then remarkably close to each other, the original difference between the results of the participating laboratories could be explained by difference in standardization. This can also be done fictionally with the obtained results. Let say that we have *n* participating laboratories and each laboratory measures a value m_{ij} (the individual measurement from laboratory i for sample j). If we had made a mixture with equal parts of all tested samples, and measured this mixture by all participating laboratories, the mixture value (MV) found would have been equal to the average of all the measured values. This essentially normalizes the measurements across different laboratories, allowing for a more direct comparison. It is like creating a “composite sample” that represents the average of all samples. In this way, the measurement from each individual laboratory can be directly compared to this “composite sample” measurement.

In this study, the average value of all measured subgenotype samples across all laboratories (a_g) was calculated. From there, the average value of all measured values across all subgenotypes was calculated by dividing a_g by the number of samples (s) in our panel (i.e., 16 – 1 negative control = 15).

$$V = \frac{a_g}{s}$$

This value (V) is then the assigned value of the fictitious calibrator.

Now, to calculate the calibration factor c_i for each laboratory, the fictitious calibrator (V) is divided by a_i, where a_i is the average value measured per each laboratory (i) across all subgenotypes.

$$c_i = \frac{V}{a_i}$$

This calibration factor c_i allows us to standardize the values reported by different laboratories.

As explained above, m_{ij} is the individual measurement from laboratory i for sample j. This measurement is then multiplied by the calculated calibration factor c_i to get the fictitious calibrated value f_{ij} for sample j from laboratory i.

$$f_{ij} = c_i \cdot m_{ij}$$

In this way, all measurements are normalized and can be directly compared across the different participating laboratories.

3. Results

In 2023, the EQA had been tested by 27 laboratories across the Netherlands, including the Caribbean islands Aruba, Curaçao, and Sint-Maarten (Figure 2). The results were evaluated for all these laboratories. The HBV subgenotype panel for HBsAg contains 16 samples representing the most prevalent HBV subgenotypes (Table 1). The HBV DNA concentration in the samples ranged from 21.10 (sample 14) to 31.80 IU/mL (sample 6), with a median of 28.50 IU/mL.

Figure 2. Numbering of participating MMLs returning external quality assessment results, by municipality.

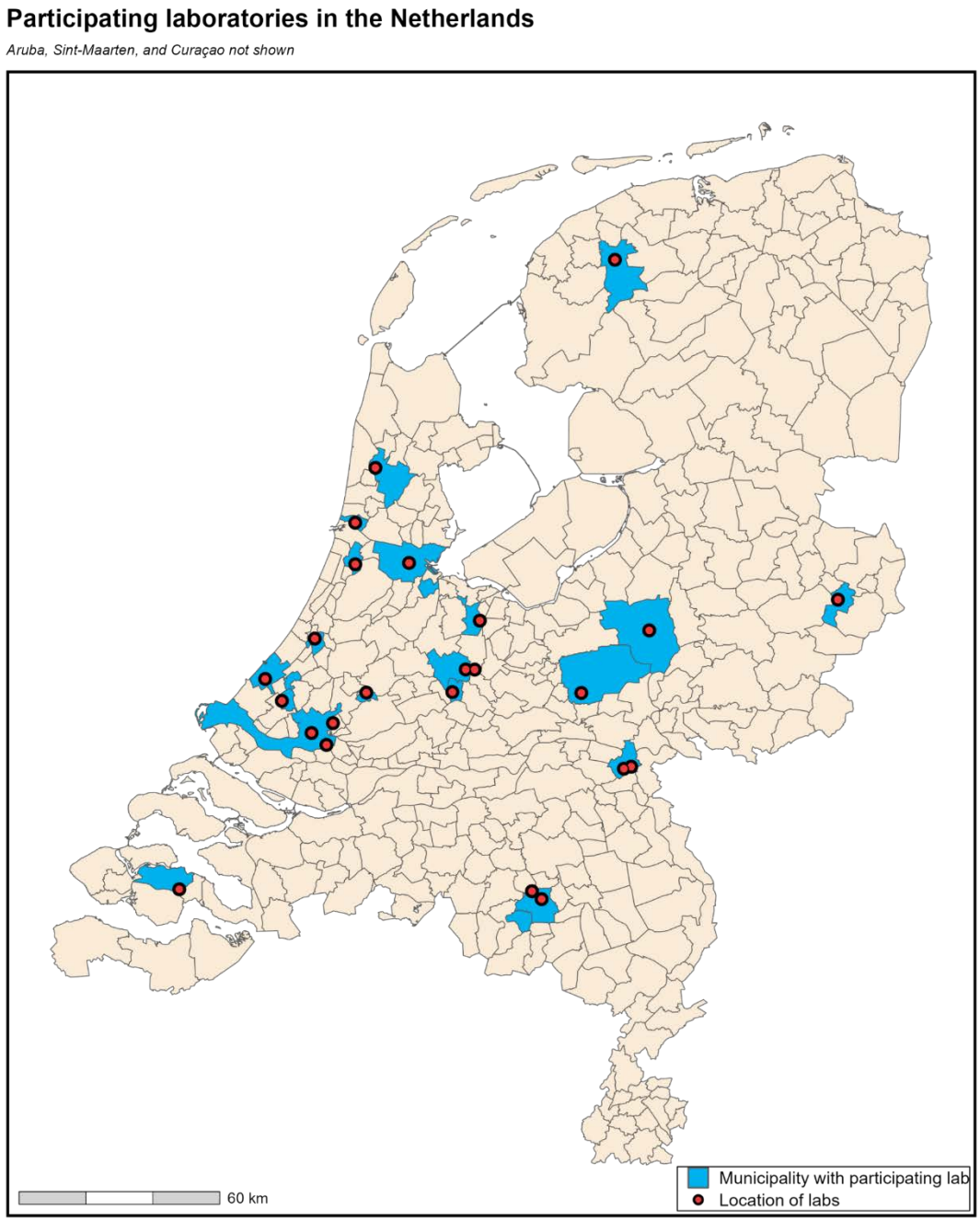


Table 3 and 4 report the number of all the different diagnostic test assays used by the participating laboratories and the units of reported results.

A wide range of assays was used across the participating laboratories (Table 3), with laboratories reporting results in different units, such as S/CO ratio, IU/mL, and reflective lights unit (RLU) (Table 4). In total, 27 laboratories submitted 13 quantitative data sets obtained with 3 different quantitative tests and 21 qualitative data sets from 8 different qualitative tests. One laboratory (HBVg18) performed a quantitative HBsAg test (Liaison-XL Murex HBsAg Quant, test code 2A and 2B) and reported results in both IU/mL and in reflective light units (RLU). Laboratories HBVg06, 11, 14, 17, 19, 22, 23, and 25 reported results in multiple units for the same assay, such as both RLU and S/CO ratio. Four laboratories (HBVg02, 09, 17, and 22) used multiple assays and reported in several units. Dataset from 5 laboratories (HBVg09, 15, 25, 11, and 14) were excluded from analysis as they did not provide S/CO ratio values when a single measurement exceeded 1000 S/CO ratio. Despite this, S/CO ratios ≥ 1000 were considered positive. These datasets were reported from using either the Advia Centaur XPT HBsAg II (test code 6B, 6C) or the Atellica IM analyzer HBsAg II (test code 6A, 6D) assays (both from Siemens Healthcare Diagnostics inc.).

262 **Table 3. List of all assay kits used by the participating MMLs.**

Test no	HBsAg test	Kit manufacturer	Test code
	Quantitative assays		
1	Liaison-XL Murex HBsAg Quant	DiaSorin S.p.A.	2A
2	Elecsys HBsAg II Quant using Cobas 8000 modular analyzer systems	Roche Diagnostics Division	3A
3	Alinity-i HBsAg II QT	Abbott Diagnostics Division	4A
	Qualitative		
4	Advia Centaur XPT HBsAg II	Siemens Healthcare Diagnostics Inc.	6B, 6C
5	Atellica IM Analyzer HBsAg II	Siemens Healthcare Diagnostics Inc.	6A, 6D
6	Vidas HBsAg ULTRA	bioMeriéux	5
7	ARCHITECT HBsAg II Qual	Abbott Diagnostics Division	4B
8	Alinity-i HBsAg II Qual	Abbott Diagnostics Division	4C
9	Liaison-XL Murex HBsAg Qual	DiaSorin S.p.A.	2B
10	UniCel DxI 600 Access 2 HBsAg	Beckman Coulter Diagnostics	1
11	Elecsys HBsAg II Qual using Cobas 6000 or 8000 modular analyzer systems	Roche Diagnostics Division	3B, 3C

263

264

265

266 **Table 4. Reported assays used for HBV genotype testing by all participating laboratories.**

Lab code	Used assay (by test code)	Results reported in
HBVg01	1	S/CO ratio
HBVg02	2A	IU/mL
	1	S/CO ratio
HBVg03	2A	IU/mL
HBVg04	3C	S/CO ratio
HBVg05	2A	IU/mL
HBVg06	5	RLU[a]
		S/CO ratio
HBVg07	4B	S/CO ratio
HBVg08	2A	IU/mL
HBVg09	3C	S/CO ratio
	6A/D	S/CO ratio
HBVg10	2A	IU/mL
HBVg11	6B/C	RLU[a]
		S/CO ratio
HBVg12	2A	IU/mL
HBVg13	2A	IU/mL
HBVg14	6B/C	RLU[a]
		S/CO ratio
HBVg15	6A/D	S/CO ratio
HBVg16	3C	S/CO ratio
HBVg17	4A	IU/mL
	4C	RLU[a]

		S/CO ratio
HBVg18	2A	IU/mL
	2B	RLU[a]
HBVg19	4C	RLU[a] S/CO ratio
HBVg20	3C	S/CO ratio
HBVg21	2A	IU/mL
HBVg22	3A	IU/mL
	3C	S/CO ratio
	4B	RLU[a] S/CO ratio
HBVg23	4C	RLU[a] S/CO ratio
	5	RLU[a] S/CO ratio
HBVg24	2A	IU/mL
HBVg25	6A/D	RLU[a] S/CO ratio
HBVg26	3B	S/CO ratio
HBVg27	2A	IU/mL

[a]RLU: Reflective Lights Unit

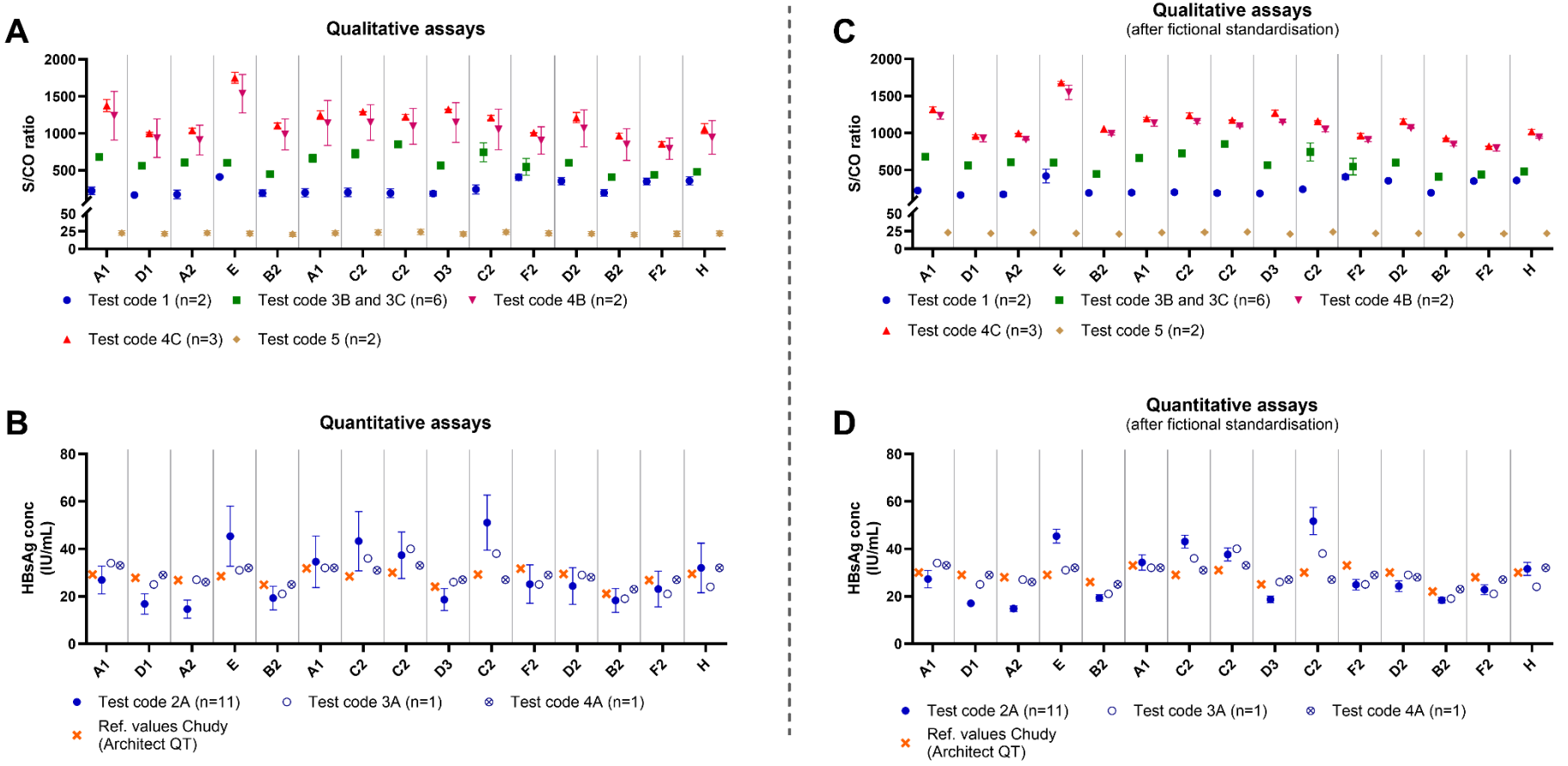
Fifteen qualitative data sets were analyzed (Supplementary Figure 1), with the Elecsys HBsAg II Qual assay (test code 3B, 3C; Roche Diagnostics) being the most frequently used assay and reported by six different laboratories (Table 4). As depicted in Figure 3A, consistent detection of all HBV genotypes, including genotype F2, was demonstrated by all assays. Of interest, the Vidas HBsAg ULTRA assay (test code 5) reported S/CO ratios at a much lower magnitude (average S/CO ratio of 24.70) compared to the other four assays. Excluding the Vidas HBsAg ULTRA assay (test code 5), the lowest S/CO ratio was

obtained when using the UniCel DxI 600 Access assay (test code 1) and was lowest for sample 2 (127.72; HBV subgenotype D1). Additionally, both ARCHITECT HBsAg II (test code 4B) and Alinity-I HBsAg (test code 4C; Abbott) yielded the highest S/CO ratios for the tested samples. Notably, the Alinity-I HBsAg II QL assay (test code 4C) exhibited the highest standard deviation (SD) in reported values.

Three quantitative assays, reporting 13 data sets, were analyzed (Supplementary Figure 2). The Liaison-XL MUREX assay (test code 2A) was by far the most frequently reported assay, with as many as eleven laboratories utilizing this specific assay (Table 4). Conversely, the other two assays were each reported only once. Consistent detection of all HBV genotypes was observed across all assays (Figure 3B). The Liaison-XL Murex quantitative assay (test code 2A) displayed variability in antigen detection for the same subgenotype among laboratories employing this specific diagnostic system. Furthermore, all quantitative assays exhibited variations in HBsAg detection rates, with values ranging from 10 (subgenotype A2) to 76 (subgenotype E) IU/mL. When using the Liaison-XL Murex quantitative assay (test code 2A), the lowest measured values (average HBsAg < 29 IU/mL) were observed for subgenotypes A2, B2, D1, D2, D3, and F2, whereas the highest values were noted for subgenotypes C2 with Japanese origin (average HBsAg of 51 IU/mL) and E with West African origin (average HBsAg of 45 IU/mL).

To distinguish whether the variation between genotypes originated from variations in intra-assay standardization procedures among participating laboratories or from differences in the type of sample measured, we applied the concept of fictional standardization (see methods section for detailed explanation). Fictional standardization was applied to both qualitative and quantitative assays, and as demonstrated in Figure 3 C and D, after fictional standardization, the SD was reduced compared to data presented in Figure 3 A and B, thus reducing the variability across the different assays used. For instance, HBV subgenotype A1 had an average IU/mL value of 26.91 across all laboratories using Liaison-XL Murex (test code 2A), with an initial SD of 5.9. Following fictional standardization, the SD decreased to 3.7.

Figure 3. Comparative analysis of qualitative (A and C) and quantitative (B and C) diagnostic HBsAg serological assays, before (A and B) and after (C and D) fictional standardization. For A and C, HBsAg concentration is expressed as S/CO ratio. For B and D, HBsAg quantity is shown in IU/mL. Mean values ± standard deviation (SD) is shown. In brackets the number (n) indicates the total number of datasets reported per assay. In B and D, the reference HBsAg values (Table 2), adjusted from Chudy et al. (1) provide a benchmark for comparison. Note: Test code 5 (Vidas HBsAg ULTRA assay) displays significantly lower S/CO ratios, appearing near zero despite actual non-zero values.



Observed challenges

Limitations in reporting S/CO ratio values

One of the challenges observed was the limitations in reporting S/CO ratio values. The sample-to-cutoff ratio is a measure used in immunoassays to determine the presence of an analyte. This is calculated by dividing the signal of the test sample by the signal of a defined internal control (cutoff). A ratio above a certain threshold indicates a positive test result.

In our study, five laboratories reported the use of either the Advia Centaur XPT HBsAg II or the Atellica IM analyzer HBsAg II assays (both Siemens Healthcare Diagnostics inc., respective teste code number 6B/6C and 6A/6D). These five data sets were excluded as they did not specify S/CO ratio values when a single measurement exceeded 1000 S/CO ratio. This limitation hindered our ability to evaluate and compare the performance of these assays with others, particularly in instances of high viral load.

Inconsistent assay usage

An inconsistency was noted in the usage of the Liaison-XL Murex HBsAg by one laboratory for qualitative reporting. This test is primarily used as a quantitative assay. Because of this deviation from standard practice, the results from this laboratory using this assay for qualitative reporting were excluded in the analysis.

Communication obstacles

One laboratory became unreachable for further inquiries or comments regarding their submitted report. This communication barrier hindered the clarification of certain aspects of their data.

4. Conclusions

EQA programs play a crucial role in evaluating the performance and status of diagnostic assays in clinical laboratories (24). Ensuring the reproducibility and reliability of diagnostic assays are of particular importance for effective clinical management and public health initiatives (24). Laboratories should conduct verification studies before and continuously monitor reliability through routinely quality management processes (25). Moreover, the inter-laboratory comparison facilitates the assessment of participants' performance, enabling the evaluation of analytical performance, test interpretation, and method performance. By implementing these measures, laboratories can enhance the accuracy and consistency of diagnostic testing.

The results of the EQA presented here was organized in 2023 to evaluate the serological detection performance on HBsAg assays used for different genotypes in routine diagnostics in Dutch Medical Microbiology and Clinical Chemical laboratories. This HBV genotype panel was specifically designed to represent the wild type strains of the most prevalent HBV subgenotypes. The 27 laboratories participating in this EQA program were required to test the panel using their routine procedures and report their qualitative and/or quantitative results. A total of eleven different diagnostic assays were evaluated, three quantitative and eight qualitative assays, covering 34 data sets. The EQA demonstrated consistent and adequate serological detection of Hepatitis B surface antigen from all tested HBV genotypes, including F2, across the wide range of assays used. The results from this study indicate good assay performances of HBsAg immunoassays. However, as demonstrated in Figure 3 C and D, after fictional standardization, the SD was reduced compared to data presented in left panels of the same figure (Figure 3 A and B) . This indicates that the discrepancies observed in the reporting results can be attributed, at least partly, to the utilization of various standardization methods or conventional instrumental-specific calibrators across participating laboratories. Variations in the calibration protocols, reference materials, or calibration intervals used by different laboratories may introduce differences in assay performance. In addition, differences in instrument models and/or manufacturers may require adjustments in calibration procedures, further contributing to these discrepancies in the observed results. It is noteworthy that a larger SD is observed when considering variations between different genotypes. This is more pronounced for subgenotypes such as C2, or subgenotype E, where greater intra- and inter-variability is observed between different test systems compared to other subgenotypes.

5. Recommendations

Addressing the challenges observed is essential for improving the overall quality and consistency of diagnostic practices for HBV in Dutch medical microbiology laboratories. Several strategies can be implemented to achieve this objective. Standardization of reporting is one option. For example, working closely with the assay manufacturers to establish clear guidelines for reporting S/CO ratio values, particularly for measurements exceeding 1000. This may include updating assay protocols or developing additional guidelines to ensure consistent reporting practices across laboratories.

Another option is to invest in capacity-building with harmonized practices. Provide additional training and educational sessions for laboratory personnel to ensure proficiency in the appropriate use and interpretation of diagnostic assays. This would lead to minimizing discrepancies in the reporting of assays.

Having clear communication protocols between medical microbiology and clinical chemical laboratories on the one hand and EQA organizers on the other, would help ensure timely and effective information exchange. One option is to use an online webtool or platform for submitting results, supplemented with an electronic feedback survey for participating laboratories, instead of using a preformatted Excel sheet.

Another recommendation is the development of follow-up procedures for laboratories that become unreachable or fail to provide adequate information during the EQA process. Providing assistance to laboratories experiencing difficulties is one option. This could include technical support, guidance on what to do, on how to submit required information, or addressing other obstacles they may face. This would enable proactive resolution of encountered barriers. By implementing these strategies, the EQA program can improve the proficiency and consistency of diagnostic practices among participating laboratories.

Consistent HBV diagnostics is especially important when considering antiviral testing. While HBsAg serology levels provide valuable information, the decision to initiate antiviral therapy or assess the efficacy of antiviral treatments primarily depends on accurate measurements of HBV DNA levels (26, 27). HBV DNA quantification is the key marker used to assess viral replication and determine the need for antiviral therapy. Discrepancies in assay results underscore the need for improved standardization or calibration methods across different laboratories and assay systems. Accurate calibration ensures that all laboratories report consistent and comparable results, which is crucial for making informed treatment decisions. Using data from these and similar EQAs to inform and enhance HBV diagnostics

will improve the reliability of antiviral testing schemes and assist in making reliable decisions about initiating antiviral therapy.

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Appendices

Appendix 1. Invitation letter.

Call for interest – Serologic hepatitis B EQA: assessment of diagnostic capacity for distinct genotypes

Dear colleagues,

Since 2019, an uprise of HBV genotype F has been noted in the Netherlands (and no data from other countries is available to our knowledge). Most recently, this genotype detection surpassed the most common genotype D, giving place to a new epidemiological picture of HBV infections which we would like to better understand.

The aim is to study the detection capacity of distinct HBV genotypes and subgenotypes by using different diagnostic systems. At the same time, this EQA will allow the collection of valuable information of ongoing monitoring/surveillance activities and foster preparedness (capacity to detect uncommon genotypes).

We would like to ask for your interest to collaborate in testing your HBsAg detection assay with an HBV serological panel.

Unfortunately, as funds are limited, we will only be able to distribute the panel to 12-15 laboratories expressing interest. In case more than 15 laboratories have expressed interest we will have to make a selection, taking into account diverse detection systems (quantitative assays, preferential). The panel (maximum of 18 samples) will be sent out by the end of July with a testing period of 3 weeks.

We would appreciate if you could complete the attached short survey to indicate whether you would like to participate or not. Please return the survey to us **before the 23rd June 2023**, to kim.benschop@rivm.nl and maggie.pires.simoes@rivm.nl.

A final decision will be shared among those willing to contribute by end of June.

This study is performed in collaboration with SKML. Results will be collected, analysed, and reported via RIVM.

Thank you in advance for your collaboration.

Yours sincerely,

Kim Benschop, Maggie Simões

Laboratory details

Name of the laboratory: _____

City / Province: _____

Contact name: _____

E-mail, phone number: _____

Interested in participating? **Please check your choice**: Yes___, No___

If not interest to participate in this Ring Trial, no further answers are needed.

Diagnostic method	Diagnostic reagents/assay	System (Manufacturer and Model)	Min. sample volume input required (in µL)	Detection limits (IU; ng/mL)	Quantitative assay?	Lowest reliable detection limit	Assay cut-off to define positive and negative result	HBV Ag sample matrix validated (manufacturer, own validation)
					Yes / No			

E-mail this form to: kim.benshop@rivm.nl & maggie.pires.simoes@rivm.nl

Thank you for your collaboration

Appendix 2. Reporting form.

Results of Ring Trial / Laboratory Proficiency Testing

Hepatitis B subgenotype serologic detection

Laboratory code		
Lab name		
Responsible name		
Responsible email		
Diagnostic System/Method		
Diagnostic reagents/ assay (kit)		
Diagnostic system (Manufacturer and Model)		
Type of assay please select from drop down list		If Qualitative assessment, further reporting in row 17 If Quantitative assessment, further reporting in row 40
Panel received (date: dd-mm-yyyy)		
Panel condition		**If issues,** please specify here:
Testing period (date: dd-mm-yyyy to dd-mm-yyyy)		

Qualitative with cutt-off							
Sample number	OD sample	OD cut-off	S/CO ratio	Results		Result/Interpretation	Remark if applicable. Clarify if technical issues
				HBsAg concentration (value)	Units		
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							
16							

NOTES (if needed)	
Disclaim any differences to the manufacturer's instructions	
General observations	

NB: Columns in yellow consisted of drop-down choices to be selected from.

Quantitative					
Sample number	**Results**		**Result / Interpretation**	Remark if applicable	Clarify if technical issues
	HBsAg concentration (value)	Units			
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					

NOTES (if needed)	
Disclaim any differences to the manufacturer's instructions	
General observations	

NB: Columns in yellow consisted of drop-down choices to be selected from.

Appendix 3. Instructions for use of the trial EQA panel.

Please find enclosed the samples for the Pilot serologic HBV EQA panel

Contact Information:
For all questions please contact: Kim Benschop and Maggie Simoes
kim.benschop@rivm.nl; maggie.pires.simoes@rivm.nl; +31 629637711

Control:
Please check all samples for damage, completeness, and shipping conditions.
☒ These samples were sent without specified shipping conditions.

Special Comments:
An email will be sent with your assigned laboratory code and reporting template.
All communications should have your unique identifier (Lab Code) in the subject.

Closing date results:
Results are to be reported until the 15th.September.2023 (end of working day).

Responsible coordinator:

Dr. Kimberley S.M. Benschop
Centre for infectious Disease Control (Cib) | Centre for Infectious Disease Research, Diagnostics, and laboratory Surveillance (IDS).
Dutch National Institute for Public Health and the Environment
PO Box 1 | 3720 BA Bilthoven
Antonie van Leeuwenhoeklaan 9 | 3721 MA Bilthoven

Contents of this shipment:
One box labeled “serologic HBV EQA panel, Samples 1 to 8 ” and one box “serologic HBV EQA panel, Samples 9 to 16 ”.
Each box contains 8 vials with each 0.4 mL of sample material.

Storage:
The samples must be stored like patient material.
The shelf life of the samples equals patient material.

Expiry date samples:
The samples are stable up to 5 days of reception if stored under defined conditions.

Preparation for use:
a. Mix carefully.
b. Process as patient material.

Measurement of samples:
The samples should be treated in the same manner as regular patient samples and in accordance with the regular used method(s).

Submission of results:
Results should be sent to : kim.benschop@rivm.nl; maggie.pires.simoes@rivm.nl until the 15th.September.2023 (end of working day)

Risks:
Like patient specimens the material should be regarded as potentially infectious and be treated as such.

Appendix 4. Steps followed for fictional standardization

1. **Collect Data**:

   Gather measurement results from each laboratory for the samples of interest.

2. **Calculate Averages**:

   Compute the average value for each sample within each laboratory, and then calculate the average of these values across all labs. This gives you the assigned value of the fictitious calibrator.

3. **Apply Calibration Factor**:

   Divide each laboratory's average value by the assigned value of the fictitious calibrator. This gives you a calibration factor for each laboratory.

4. **Apply Calibration Factor to Individual Measurements**:

   Multiply each individual measurement from each laboratory by the corresponding calibration factor obtained in step 3.

5. **Assess Variability**:

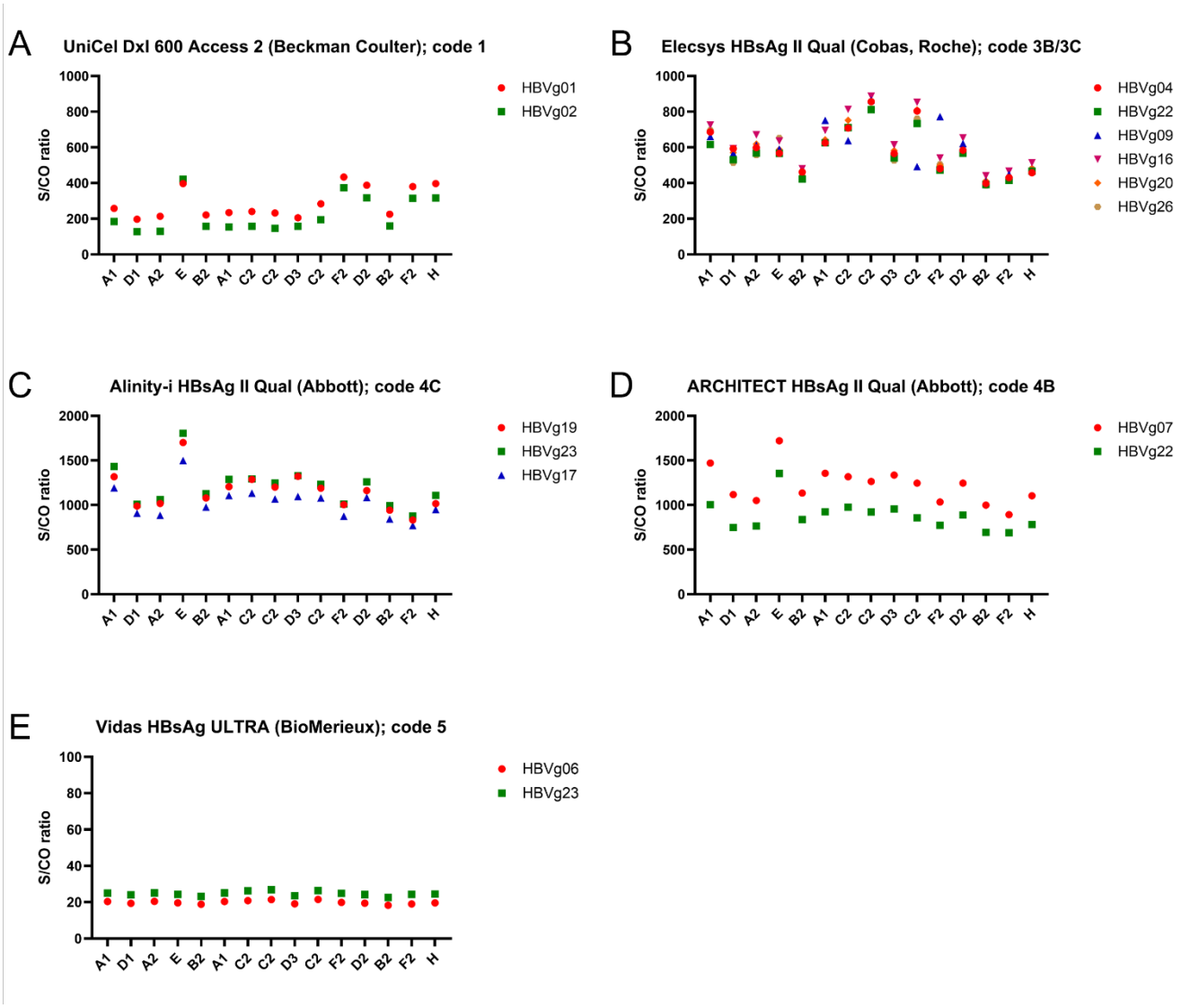
   Calculate the standard deviation (SD) of the measurements before and after calibration. Compare the SDs to assess the impact of standardization differences on the variability of the measurements.

6. **Analyze Results**:

   Interpret the changes in variability and assess the extent to which differences in standardization contribute to the variability in the measurements.

Supplementary Figure 1.

Individual graphs of the 5 qualitative HBsAg assays used to test the 15 serological HBV genotype samples (excluding the negative control). HBsAg concentration is expressed as S/CO ratio.



Supplementary Figure 2.

Individual graphs of the 3 quantitative HBsAg assays used to test the 15 serological HBV genotype samples (excluding the negative control). HBsAg quantity is shown in IU/mL.