

assays have been equally reliable in collaborative studies in the past.<sup>11</sup>

## 2. Objectives

During the 'WHO Consultation on Global Measurement Standards and their use in the in vitro Biological Diagnostic Field' in June 2004 concern was raised that NAT test kits for the detection of HBV DNA or test systems for the detection of HBsAg might be less efficient for some HBV genotypes other than genotype A2.<sup>12</sup> The Paul-Ehrlich-Institut (PEI) in Germany, as one of the three WHO Collaborating Centres involved in the Biological Standardization Programme for in vitro diagnostics (IVDs), proposed in collaboration with the German Reference Laboratory for Hepatitis B and D in Giessen projects to establish WHO International Biological Reference Panels for HBV DNA and for HBsAg representing different subgenotypes of HBV.

## 3. Study design

### 3.1. Human plasma samples

HBsAg and HBV DNA high titre plasma units were collected worldwide. In total, 215 potential candidate materials were kindly received from: H. Yoshizawa and J. Tanaka, Hiroshima University, Japan; E. Zhiburt, Federal Blood Center, Moscow, Russia; E. Sabino and M. Otani, Fundação Pró-Sangue Hemocentro de São Paulo, Brazil; W. Gerlich, Institute of Medical Virology, University Giessen, Germany; M. Schmidt, German Red Cross Frankfurt/Main, Germany; M. Cheraghali and H. Abolghasemi, Iranian Blood Transfusion Organization, Tehran, Iran; and W. Sykes and D. Watts, South African National Blood Service, Durban, South Africa.

### 3.2. NAT assays for characterization of HBV plasma samples

HBV DNA in the plasma specimens was initially quantitated using four different CE-marked NAT assays: Cobas AmpliPrep/Cobas TaqMan HBV Test, Cobas Amplicor HBV Test (Roche Diagnostics GmbH, Mannheim, Germany), Abbott RealTime HBV assay (Abbott GmbH & Co. KG, Wiesbaden, Germany), and *artus* HBV LC PCR Kit (Qiagen GmbH, Hilden, Germany). The arithmetic mean value was taken for further calculations. The plasma samples were checked for the absence of HCV RNA and HIV-1 RNA using the Procleix HIV-1/HCV Assay (GenProbe, San Diego, USA). HBV genotyping was performed by using the INNO-LiPA HBV Genotyping kit (Innogenetics N.V., Gent, Belgium) and checked by sequence analysis (see below).

### 3.3. Serological assays for characterization of HBV plasma samples

Quantitative HBsAg determination was performed using Architect HBsAg L/N 6C36 (Abbott GmbH & Co. KG, Wiesbaden, Germany). The status of anti-HBc, anti-HBe and anti-HBs was determined using the Architect system (Architect Anti-HBc II L/N 8L44, ARCHITECT Anti-HBe, L/N 6C34 and ARCHITECT Anti-HBs, L/N 7C18), and HBeAg was determined with Elecsys HBeAg (Roche Diagnostics GmbH, Mannheim, Germany). Potential coinfection with HDV was determined by using the Murex anti-Delta (total) (Abbott GmbH & Co. KG, Wiesbaden, Germany).

### 3.4. HBV sequence analysis

For sequence analysis primers PreS1 sense (2816–2835; 5' GTCACCATATTCTTGGGAAC 3') and S6 antisense (997–973; 5'

**Table 1**

Participants in the international collaborative study.

Scientist	Affiliation
J. Chen/L. Wong	Siemens Clinical Laboratory, Berkeley, CA, USA
M. Chudy/C.M. Nübling	Paul-Ehrlich-Institut, Langen, Germany
T. Grewing/M. Rudolph	Qiagen GmbH, Hamburg, Germany
O. Hsu/Y. Shih-Chieh	General Biologicals Corp., HsinChu, Taiwan, ROC
B. Jansson/I. Bokliden	Cepheid AB, Bromma, Sweden
R. Jardi	Universitary Hospital Vall Hebron, Barcelona, Spain
S. Kerby	Center for Biologics Evaluation and Research/Food and Drug Administration, Bethesda, MD, USA
A. Kramvis/C.-Y. Chen	University of the Witwatersrand, Johannesburg, South Africa
H. Leying/F. Boehl	Roche Diagnostics AG, Rotkreuz, Switzerland
J.M. Linnen	Gen-Probe Incorporated, San Diego, CA, USA
S. Mizusawa/F. Ban	National Institute of Infectious Diseases, Tokyo, Japan/BML, Inc., Saitama, Japan
C.R. Mullen	AbbottMolecular, Des Plaines, IL, USA
M. Rapicetta	Istituto Superiore di Sanita, Rome, Italy
E. Sabino/M. Otani	Fundação Pró-Sangue, Hemocentro de São Paulo, Sao Paulo, Brazil
W.R. Willems	Justus-Liebig-University Giessen, Giessen, Germany
H.-S. Yim	Biosewoom, Inc., Seoul, Korea

CKTTGACADACTTTCCAATCAATAG 3') were used. Amplicons were directly sequenced by the company GATC Biotech AG (Konstanz, Germany). Sequences determined for the entire S open reading frame were used for initial subgenotyping and HBsAg subtyping.

The sequence data were checked by a modified approach to construct the phylogenetic tree (Fig. 1): sequences were determined for concatenated 1128 bp belonging to the preS region (nucleotide positions 2762–177) and the S gene (nucleotide positions 248–738) of HBV reference sequences (retrieved from GenBank) reflecting genotypes A–H (subgenotypes are indicated at the end of reference sequence accession numbers). HBV sequences from members of the WHO International Reference Panel are indicated as S1–S15, the WHO International Standard (97/750) as SD.

Woolly-Monkey HBV sequences were used as outgroups.

### 3.5. Design and manufacture of the HBV genotype reference panel

Each panel member was diluted to a target HBV DNA concentration of 10<sup>6</sup> IU/ml in a volume of 1.2 l, with the exception of panel samples 6 (B4; 10<sup>4</sup> IU/ml), 14 (F2; 10<sup>5</sup> IU/ml), and 15 (G; 10<sup>4</sup> IU/ml) because of limited volumes of the source samples. Dilutions were performed with a plasma pool consisting of donations which had been tested negative for the following markers: HIV-1 RNA, HCV RNA, HBV DNA, HBsAg, anti-HBs, anti-HBc (IgG and IgM), anti-HIV-1/2, and anti-HCV.

Filling and lyophilization of the panel members was performed by an ISO EN 13485 certified company. The coefficient of variation of the fill volume is described as being within an acceptable range (0.7–1.1%). In total, 2000 vials were produced for each of the 15 panel members. Additional 144 vials filled with 0.5 ml of negative diluent plasma pool were used for "non-infectious" residual moisture determination based on an European Pharmacopeia method.<sup>13</sup> Stability testing of the panel was initiated using the Cobas AmpliPrep/Cobas TaqMan HBV Test.

### 3.6. Collaborative study

16 laboratories from 12 countries participated in the characterization of the panel (Table 1). They were requested to analyse the coded 15 panel samples concurrently with the 2nd WHO IS for HBV DNA (97/750) and to perform 3 separate assay runs when



Fig. 1. Phylogenetic tree of HBV genotypes. Bootstrap values for genotype or subgenotype clusters are indicated. \*This bootstrap support increased to 95 when bootstrapping was performed by Neighbour Joining method (1000 replicates).

**Table 2**  
Assays used by participants.

Assay type (quantitative or qualitative)	Extraction/assay method	Region of HBV genome amplified
Quantitative	Cobas Amplicor HBV Monitor Test	pre-C/C
Quantitative	Real-Q HBV Quantification Kit	S
Quantitative	Cobas TaqMan HBV Test for Use with the High Pure System	pre-C/C
Quantitative	Cobas AmpliPrep/Cobas TaqMan HBV Test	pre-C/C
Quantitative	Abbott RealTime HBV	S
Quantitative	QIAamp MinElute Virus spin/HBV RealQuant PCR	X
Quantitative	affigene HBV trender	n.a.
Quantitative	Smart HBV	
Quantitative	In house TaqMan PCR 1	S
Quantitative	In house TaqMan PCR 2	S
Quantitative	Versant HBV DNA 3.0 Assay (bdNA)	n.a.
Quantitative	QIAamp DSP Virus Kit/artus HBV RG PCR Kit	C
Qualitative	cobas TaqScreen MPX Test	pre-C/C
Qualitative	Procleix Ultrio Plus assay	S/pol

n.a.: information not available.

performing quantitative tests. For qualitative assays, participants were requested to assay each panel member by a series of one  $\log_{10}$  dilution steps, followed by 3 subsequent assays with half- $\log_{10}$  dilutions around the determined end-point estimation. The types of assays used are recorded in Table 2.

### 3.7. Statistical methods

#### 3.7.1. Quantitative assays

For comparison of laboratories the replicate results of each laboratory, corrected for the dilution factor, were combined as arithmetic mean of  $\log_{10}$  IU/ml values. These estimates were then combined across assays to obtain an overall laboratory mean value of  $\log_{10}$  IU/ml. Results outside a range of the overall mean of  $\pm 0.5 \log_{10}$  IU/ml were removed for a second evaluation excluding outliers. The box-and-whisker-plot shows the distribution of the data. The box itself contains the middle 50% of the results (interquartile range, IQR) and the median as horizontal line. Between the whiskers lie 100% of the observations. The statistical analysis was performed with SAS<sup>®</sup>/STAT software, version 9.2, SAS System for Windows. Estimation of end-point dilution was done with CombiStats Software, version 4.0, from EDQM/Council of Europe.

#### 3.7.2. Qualitative assays

The results from the 4 assay runs were pooled to give a series of number positive out of number tested at each dilution. The pooled results of the single assays were evaluated by using probit analysis to estimate the concentration at which 63% of the samples tested were positive (i.e. the dilution at which on average one single copy per sample tested could be expected under the assumption of an underlying Poisson distribution). The calculated end-point gives estimated NAT detectable units/ml after correcting for an equivalent volume of the test sample.

## 4. Results

The 15 member HBV genotype panel for use with NAT assays was designed to contain the HBV (sub)genotypes A1, A2, B2, B4, C2, D1, D3, E, F2 and G. Selection criteria were availability of the HBV (sub)genotype, mean HBV DNA concentration  $> 10^7$  IU/ml as determined by four quantitative NAT assays and an HBsAg content preferably  $> 15,000$  IU/ml, in order to potentially use common source materials for a differently manufactured WHO HBV genotype panel for HBsAg assays designed in parallel (Chudy et al., manuscript in preparation). All source specimens used for the panel members were anti-HBc positive, and most of them HBeAg positive and anti-HBe negative (except panel members 14 and 15),

indicating highly replicative HBV infection with low immune pressure. It was intended to provide samples typical for the corresponding (sub)genotype with a minimum of individual mutations (e.g. exclusion of known HBsAg escape variants) or quasi-species-like heterogeneity. There was no evidence for any HDV coinfection. The individual panel candidates were also checked for the absence of HIV RNA and HCV RNA in order to facilitate future use of the panel for validation of the HBV component in multiplex assays (e.g. HBV/HCV/HIV multiplex blood screening NATs). Detailed information on the characterization of the source materials is summarized in Table 3.

The preS/S Gene region of all panel members was sequenced and the obtained sequence confirmed the HBV genotype as determined by the INNO-LiPA HBV Genotyping test. Furthermore, the sequence analysis identified HBsAg subtypes (Table 3). These sequences are available on the PEI homepage ([www.pei.de](http://www.pei.de)). It will also provide users with sequence information of the genome region targeted by some NAT assays. Fig. 1 shows a phylogenetic tree constructed on HBV reference sequences (retrieved from GenBank) reflecting genotypes A–H and from HBV sequences of the reference panel members (S1–S15) and the 2nd WHO IS HBV DNA (97/750).<sup>14,15</sup> The subgenotypes are included at the end of the names of the reference sequences.

The residual moisture content was determined as 0.82% (standard deviation  $\pm 0.03\%$ ) which satisfies the requirements for biological reference preparations.<sup>16</sup> A stability testing programme of the panel members was introduced. Interim results demonstrate that the panel members are now stable for more than two years under recommended storage conditions, i.e. at  $-20^\circ\text{C}$  or below.

Laboratory mean estimates for quantitative (IU/ml) and qualitative assays (NAT detectable units) are summarized (Table 4). In the collaborative study, the highest accuracy of test results was obtained with the 2nd WHO IS HBV DNA (97/750) with a mean value of  $6.01 \log_{10}$  IU/ml virtually identical to designated content and the smallest SD of 0.17. There was also a narrow range of the quantitative test results for samples 1–3 (genotype A) and samples 10–12 (genotype D) (Table 4, Fig. 2) with a difference factor of 0.6 (sample 12) to  $0.95 \log_{10}$  (sample 2) between the highest and lowest value. In contrast, all other samples exhibited difference factors in reported results in a range between 1.0 and  $2.2 \log_{10}$ . Some of the quantitative assays clearly underestimated some genotypes or provided false-negative test results. These kinds of outliers are marked in bold (Table 4). These outliers were found with genotypes B, C, E, F and G but not with A or D. The mean estimates using all assays were also calculated for the individual panel members. The distribution of the quantitative results has been summarized using box plot (Fig. 2). The distribution of test results between different NAT

**Table 3**  
Characterization of human plasma source materials used for HBV genotype panel.

Sample no.	Origin	HBsAg subtype	HBV genotype <sup>a</sup>	HBV subgenotype <sup>b</sup>	HBV DNA (IU/mL)	HBsAg (kIU/mL)
1	South Africa	adw2	A	A1	6.08E+08	131.9
2	Brazil	adw2	A	A1	6.53E+08	94.0
3	Germany	adw2	A	A2	6.87E+08	74.3
4	Japan	adw2	B	B2	1.48E+08	51.4
5	Japan	adw2	B	B2	2.84E+08	95.3
6	Viet Nam	ayw1	B	B4	6.29E+06	4.6
7	Japan	adr	C	C2 (Ce)	3.99E+08	70.2
8	Japan	adr	C	C2 (Ce)	1.25E+08	47.0
9	Russia	adr	C	C2 (Ce)	2.92E+08	54.4
10	Germany	ayw2	D	D1	1.17E+09	130.4
11	South Africa	ayw2	D	D3	1.04E+08	63.8
12	Iran	ayw2	D	D1	1.00E+08	17.7
13	West Africa	ayw4	E	E	9.45E+08	82.6
14	Brazil	adw4	F	F2	1.10E+07	32.2
15	Germany	adw2	G	G	1.40E+07	0.9

All panel members were tested anti-HBc positive, HBeAg positive (except no. 14, 15), anti-HBe negative (except no. 14), anti-HDV negative, HIV-1 RNA negative, HCV RNA negative.

<sup>a</sup> INNO-LiPA.

<sup>b</sup> Sequencing.

**Table 4**

Laboratory mean estimates for quantitative NAT assays ( $\log_{10}$  IU/ml) and for qualitative NAT assays ( $\log_{10}$  NAT detectable units/ml; greyed boxes).

Lab no.	Assay type	Panel member/HBV subgenotype															IS A2
		1 A1	2 A1	3 A2	4 B2	5 B2	6 B4	7 C2	8 C2	9 C2	10 D1	11 D3	12 D1	13 E	14 F2	15 G	
1	Quantitative	5.73	5.41	5.80	5.99	5.84	4.00	6.03	6.15	5.94	5.97	6.01	5.92	5.93	5.11	3.72	5.99
2	Quantitative	6.57	6.35	6.16	6.31	6.02	4.41	6.41	6.42	6.16	6.30	6.31	6.37	5.91	<b>3.54</b>	4.30	6.30
3A	Quantitative	5.85	5.63	5.42	5.88	5.74	3.74	5.88	5.95	5.88	5.94	5.90	5.79	5.70	4.29	3.62	5.66
3B	Quantitative	6.32	6.06	5.99	6.34	6.03	4.15	6.28	6.38	6.28	6.12	6.38	6.35	6.28	4.69	4.10	6.01
4	Quantitative	6.30	6.03	5.96	6.05	5.79	4.19	6.09	6.04	5.99	6.22	5.89	6.12	5.95	4.54	<b>5.01</b>	5.92
5	Quantitative	6.07	5.89	5.71	6.13	5.81	4.13	5.98	6.03	5.87	5.84	5.88	5.84	5.81	5.00	3.78	5.87
6	Quantitative	6.09	5.88	5.70	5.96	5.98	3.57	5.80	6.07	5.91	5.97	5.87	5.90	5.81	–	<b>2.81</b>	5.90
7A	Quantitative	6.29	6.08	6.01	5.76	5.57	4.05	6.09	6.23	6.17	5.87	6.29	6.23	6.02	5.12	3.63	6.21
7B	Quantitative	6.39	6.15	6.02	5.68	5.69	4.20	6.08	6.25	6.20	6.10	6.33	6.33	6.03	5.28	3.52	6.26
8	Quantitative	6.13	5.86	5.81	<b>5.27</b>	<b>5.03</b>	<b>2.58</b>	<b>5.34</b>	<b>5.25</b>	5.70	5.68	5.78	5.94	5.68	–	<b>2.94</b>	6.09
9	Quantitative	5.71	5.50	5.35	5.45	5.40	3.87	5.61	5.96	5.60	5.63	5.80	5.76	5.57	4.97	3.75	5.96
10	Quantitative	6.17	5.97	5.81	6.29	6.05	4.13	6.07	6.20	5.98	5.99	5.94	5.87	5.82	4.86	4.07	6.05
11	Quantitative	6.01	5.80	5.82	5.71	5.49	<b>3.41</b>	5.82	5.99	<b>5.28</b>	5.95	5.92	5.87	<b>5.31</b>	5.04	3.56	6.01
12	Quantitative	6.26	6.09	6.02	6.35	6.13	4.27	6.22	6.39	6.25	6.27	6.28	6.30	6.22	4.75	4.17	6.20
13	Quantitative	6.05	5.86	5.73	6.06	5.89	4.19	6.02	6.05	5.96	5.99	5.99	5.96	5.88	4.78	3.99	5.91
14	Quantitative	5.98	5.75	5.64	6.00	5.59	4.14	5.89	5.98	5.80	5.74	5.81	5.86	5.79	4.97	3.85	5.84
15	Qualitative	<b>6.31</b>	<b>5.97</b>	<b>6.27</b>	<b>6.31</b>	<b>6.80</b>	<b>3.88</b>	<b>6.31</b>	<b>6.61</b>	<b>6.31</b>	<b>6.95</b>	<b>6.61</b>	<b>6.61</b>	<b>6.31</b>	<b>4.95</b>	<b>3.88</b>	<b>6.31</b>
16	Qualitative	<b>6.34</b>	<b>6.00</b>	<b>5.95</b>	<b>5.68</b>	<b>5.95</b>	<b>4.20</b>	<b>6.07</b>	<b>6.37</b>	<b>6.40</b>	<b>6.03</b>	<b>5.86</b>	<b>6.20</b>	<b>6.19</b>	<b>5.06</b>	<b>4.00</b>	<b>6.26</b>
Overall mean ( $\log_{10}$ IU/ml) <sup>a</sup>		6.12	5.89	5.81	6.00	5.80	4.07	6.02	6.14	5.98	5.97	6.02	6.03	5.89	4.88	3.85	6.01
95%-confidence interval ( $\log_{10}$ IU/ml) <sup>a</sup>		6.00	5.76	5.69	5.85	5.68	3.95	5.91	6.05	5.87	5.87	5.91	5.91	5.79	4.71	3.70	5.92
Standard deviation <sup>a</sup>		0.24	0.24	0.22	0.27	0.22	0.22	0.20	0.16	0.20	0.20	0.21	0.22	0.19	0.27	0.25	0.17

–, not detected; bold, outliers.

<sup>a</sup>Quantitative assays; calculated without outliers.

**Table 5**

Overall mean estimates ( $\log_{10}$  IU/ml) relative to concurrently tested WHO IS (97/750) (combined from quantitative and qualitative assays, with outlier exclusion).

Sample	HBV Sub GT	Number of NAT assays	Overall mean ( $\log_{10}$ IU/ml)	95%-confidence intervals ( $\log_{10}$ IU/ml)	Standard deviation	Min	Max	Range
1	A1	18	6.10	6.02 6.18	0.16	5.74	6.38	0.64
2	A1	18	5.86	5.77 5.95	0.18	5.42	6.11	0.69
3	A2	18	5.80	5.73 5.87	0.14	5.39	6.04	0.65
4	B2	17	5.96	5.80 6.12	0.31	5.42	6.33	0.91
5	B2	17	5.83	5.68 5.98	0.29	5.36	6.49	1.13
6	B4	16	4.03	3.92 4.14	0.21	3.57	4.30	0.73
7	C2	17	6.00	5.91 6.08	0.17	5.65	6.27	0.62
8	C2	17	6.14	6.09 6.20	0.11	5.98	6.37	0.39
9	C2	17	5.98	5.89 6.07	0.17	5.61	6.27	0.66
10	D1	18	5.99	5.86 6.12	0.25	5.59	6.64	1.05
11	D3	18	6.01	5.91 6.10	0.19	5.60	6.37	0.77
12	D1	18	6.03	5.95 6.10	0.15	5.80	6.34	0.54
13	E	17	5.89	5.80 5.98	0.18	5.59	6.27	0.68
14	F2	15	4.86	4.75 4.98	0.20	4.55	5.13	0.58
15	G	14	3.85	3.72 3.97	0.21	3.42	4.09	0.67

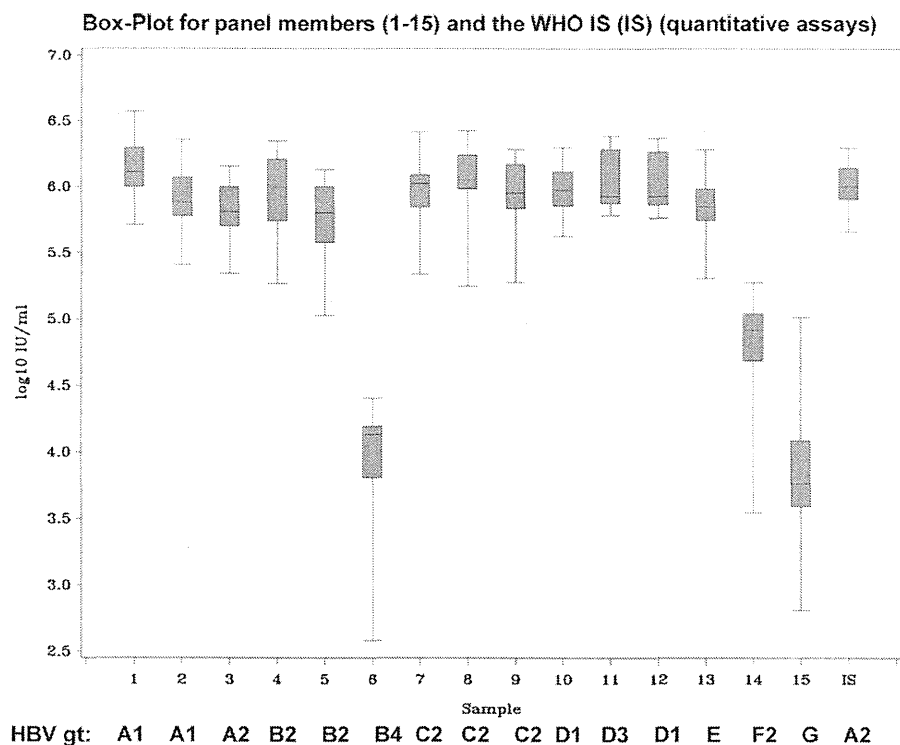


Fig. 2. Box plot for panel members (1–15) and the WHO IS (IS) (quantitative assays).

assays becomes closer if outlier results are excluded and mean estimates are calculated relative to the co-analysed 2nd WHO IS which has a defined HBV DNA content of  $10^6$  IU/ml (Table 5). Two assays (laboratories 8 and 11) showed lower potencies for more than one sample from the panel (for genotypes B, C, E and G). Additionally, the genotype F sample could not be detected by laboratory 8. The assay used by laboratory 6 was also not able to detect genotype F and in addition underquantitated the genotype G panel member. Laboratory 4 represented a significant higher result for sample 15/G compared to the overall mean potencies. One further NAT assay (laboratory 2) significantly underquantitated the genotype F sample.

## 5. Discussion

The overall results of the collaborative study demonstrate that the range of the results for the samples 1–15 are very close to the HBV DNA concentrations which were chosen for the final preparation of the panel samples: approximately  $10^6$  IU/ml (samples 1–5, 7–13), approximately  $10^5$  IU/ml (sample 14), and approximately  $10^4$  IU/ml (samples 6 and 15). The results of this study confirm the validity of the assigned value for 97/750, the 2nd IS for HBV DNA, i.e.  $10^6$  IU/ml and successful calibration of all assay methods to determine this value since the range of reported results is close around the assigned concentration (Fig. 2). Some HBV NAT assays showed deficiencies in the detection of certain HBV genotypes other than genotype A2 (IS), thus confirming the need for such a genotype reference panel. However, the results of the collaborative study demonstrate that the majority of the quantitative assays and both qualitative assays are able to detect HBV genotypes A–G quite consistently.

Unfortunately we were not in the situation to include HBV genotype H into this reference panel because of lack of material. Genotype H shows a variation in nucleotide sequence equivalent to genotype F. The prevalence of genotype H is restricted to Central America, mainly in Nicaragua and Mexico, and few cases in

California. More recently new HBV strains were identified in Laos and Vietnam which phylogenetically clustered in a new group and were proposed as new genotype I.<sup>17</sup> Currently it is still under discussion whether this HBV strain reflects a recombinant of several genotypes or a separate genotype.<sup>18</sup>

Based on the availability of improved molecular diagnostic tools and performance of epidemiological studies worldwide new subgenotypes and recombinant forms may be identified in the future. The global distribution of genotypes may also change in the future and therefore updating the HBV genotype reference panel, to reflect the changing global epidemiological trend of HBV genotypes, may be necessary.

## 6. Conclusion

The global availability of a well characterized WHO HBV genotype panel is an essential prerequisite for the evaluation and validation of HBV NAT assays. This reference panel will allow comparison of different NAT assays on a standardized basis and may be used for the quality control of NAT tests by kit manufacturers, national control laboratories and diagnostic laboratories. The panel will be available from the PEI with ordering details announced on the PEI homepage.

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## Competing interests

The authors have no conflict of interest.

## Ethical approval

Not required.

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