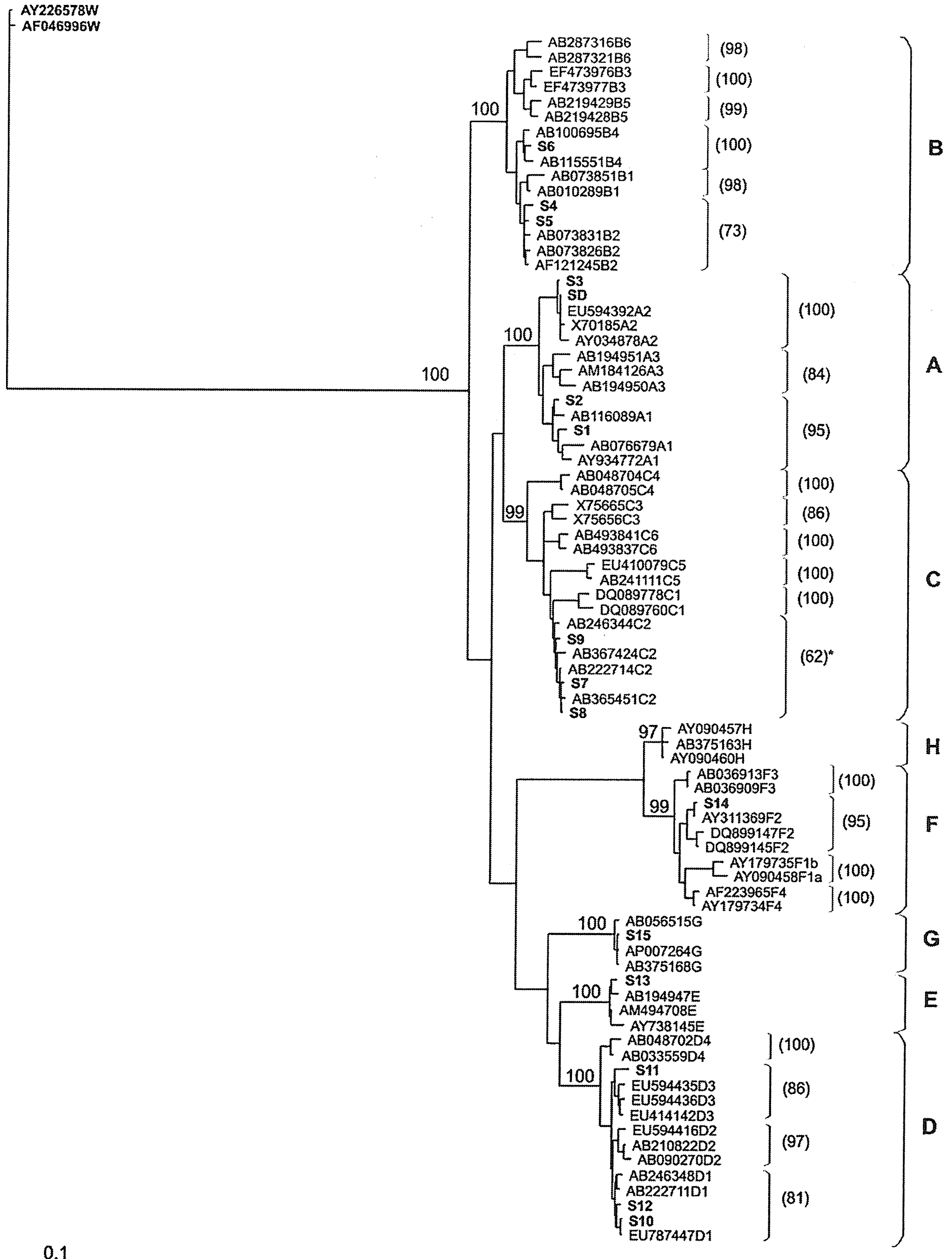

Fig. 1. Phylogenetic tree of HBV genotypes. Bootstrap values for genotype or subgenotype clusters are indicated. *This bootstrap support increased to 95 when bootstrapping was performed by Neighbour Joining method (1000 replicates).

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 $\pm0.5 \log_{10}$ 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®/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). 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).[14,15] 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 ±0.03%) which satisfies the requirements for biological reference preparations.[16] 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 °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[a]	HBV subgenotype[b]	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.

[a] INNO-LiPA.

[b] 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 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	IS A2
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	**3.54**	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	**5.01**	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	–	**2.81**	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	**5.27**	**5.03**	**2.58**	**5.34**	**5.25**	5.70	5.68	5.78	5.94	5.68	–	**2.94**	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	**3.41**	5.82	5.99	**5.28**	5.95	5.92	5.87	**5.31**	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	6.31	5.97	6.27	6.31	6.80	3.88	6.31	6.61	6.31	6.95	6.61	6.61	6.31	4.95	3.88	6.31
16	Qualitative	6.34	6.00	5.95	5.68	5.95	4.20	6.07	6.37	6.40	6.03	5.86	6.20	6.19	5.06	4.00	6.26
Overall mean (log_{10} IU/ml)[a]		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		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
interval (log_{10} IU/ml)[a]		6.25	6.03	5.93	6.14	5.92	4.20	6.13	6.23	6.09	6.08	6.14	6.14	6.00	5.04	4.01	6.10
Standard deviation[a]		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.

[a]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

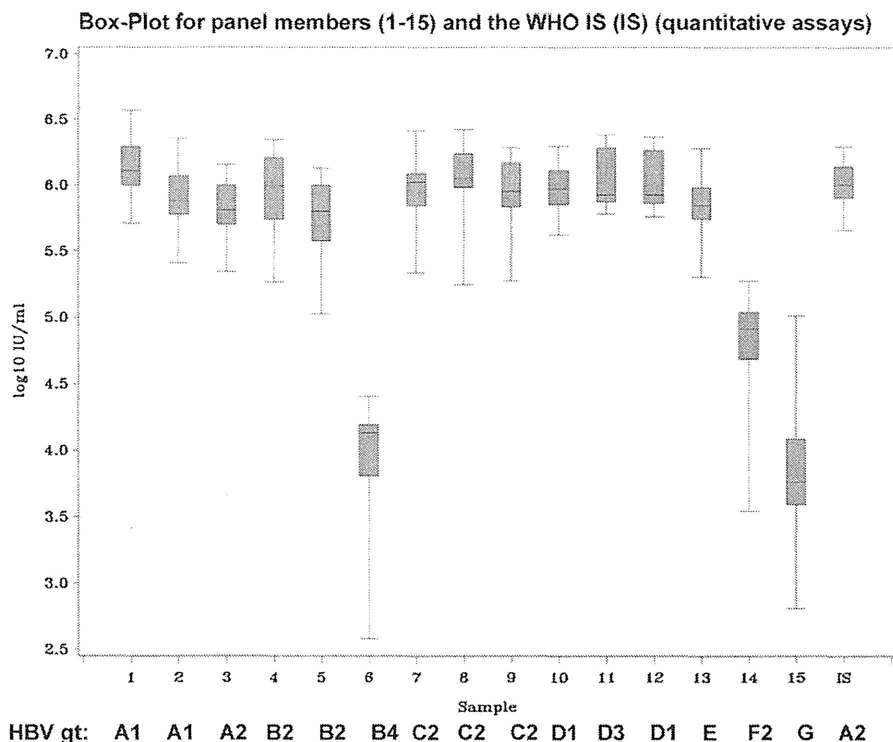

Fig. 2. Box plot for panel members (1–15) and the WHO IS (IS) (quantitative assays).

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.[17] Currently it is still under discussion whether this HBV strain reflects a recombinant of several genotypes or a separate genotype.[18]

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.

Funding

All funding was provided by the Paul-Ehrlich-Institut, Langen.

Competing interests

The authors have no conflict of interest.

Ethical approval

Not required.

References

1. Stramer SL, Zou S, Notari EP, Foster GA, Krysztof DE, Musavi F, et al. Blood donation screening for hepatitis B virus markers in the era of nucleic acid testing: are all tests of value? *Transfusion* 2012;**52**:440–6.
2. Linauts S, Saldanha J, Strong DM. PRISM hepatitis B surface antigen detection of hepatitis B virus minipool nucleic acid testing yield samples. *Transfusion* 2008;**48**:1376–82.
3. Roth WK, Busch MP, Schuller A, Ismay S, Cheng A, Seed CR, et al. International survey on NAT testing of blood donations: expanding implementation and yield from 1999 to 2009. *Vox Sang* 2012;**102**:82–90.
4. Raimondo G, Allain JP, Brunetto MR, Buendia MA, Chen DS, Colombo M, et al. Statements from the Taormina expert meeting on occult hepatitis B virus infection. *J Hepatol* 2008;**49**:652–7.
5. Blaich A, Manz M, Dumoulin AC, Schuttler CG, Hirsch HH, Gerlich WH, et al. Reactivation of hepatitis B virus with mutated hepatitis B surface antigen in a liver transplant recipient receiving a graft from an antibody to hepatitis B surface antigen- and antibody to hepatitis B core antigen-positive donor. *Transfusion* 2012, http://dx.doi.org/10.1111/j.1537-2995.2011.03537.x.
6. Cornberg M, Protzer U, Petersen J, Wedemeyer H, Berg T, Jilg W, et al. Prophylaxis, diagnosis and therapy of hepatitis B virus infection—the German guideline. *Z Gastroenterol* 2011;**49**:871–930.
7. Gunson RN, Shouval D, Roggendorf M, Zaaijer H, Nicholas H, Holzmann H, et al. Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections in health care workers (HCWs): guidelines for prevention of transmission of HBV and HCV from HCW to patients. *J Clin Virol* 2003;**27**:213–30.
8. Saldanha J, Gerlich W, Lelie N, Dawson P, Heermann K, Heath A, et al. An international collaborative study to establish a World Health Organization international standard for hepatitis B virus DNA nucleic acid amplification techniques. *Vox Sang* 2001;**80**:63–71.
9. Baylis SA, Heath AB, Chudy M, Pisani G, Klotz A, Kerby S, et al. An international collaborative study to establish the 2nd World Health Organization International Standard for hepatitis B virus DNA nucleic acid amplification technology-based assays. *Vox Sang* 2008;**94**:358–62.
10. Norder H, Courouce AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, et al. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology* 2004;**47**: 289–309.
11. Heermann KH, Gerlich WH, Chudy M, Schaefer S, Thomssen R. Quantitative detection of hepatitis B virus DNA in two international reference plasma preparations. *J Clin Microbiol* 1999;**37**:68–73.
12. *WHO Consultation on Global Measurement Standards and their use in the in vitro Biological Diagnostic Field*. Geneva, Switzerland; June 2004. http://www.who.int/bloodproducts/publications/en/Minutes-220804.pdf.
13. Water: micro determination. Methods of analysis EP 2.5.32. In: *European pharmacopoeia*. 6th ed. Strasbourg, France: Council of Europe; 2008.
14. Swofford DL. *PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4.0b10*. Sunderland, MA, USA: Sinauer Associates; 2003.
15. Guindon S, Gascuel O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 2003;**52**:696–704.
16. Recommendations for the preparation, characterization and establishment of international and other biological reference standards. WHO Expert Committee on Biological Standardization. Fifty-fifth report; 2004 (WHO Technical Report Series No. 932).
17. Hannoun C, Norder H, Lindh M. An aberrant genotype revealed in recombinant hepatitis B virus strains from Vietnam. *J Gen Virol* 2000;**81**: 2267–72.
18. Kurbanov F, Tanaka Y, Kramvis A, Simmonds P, Mizokami M. When should "I" consider a new hepatitis B virus genotype? *J Virol* 2008;**82**: 8241–2.