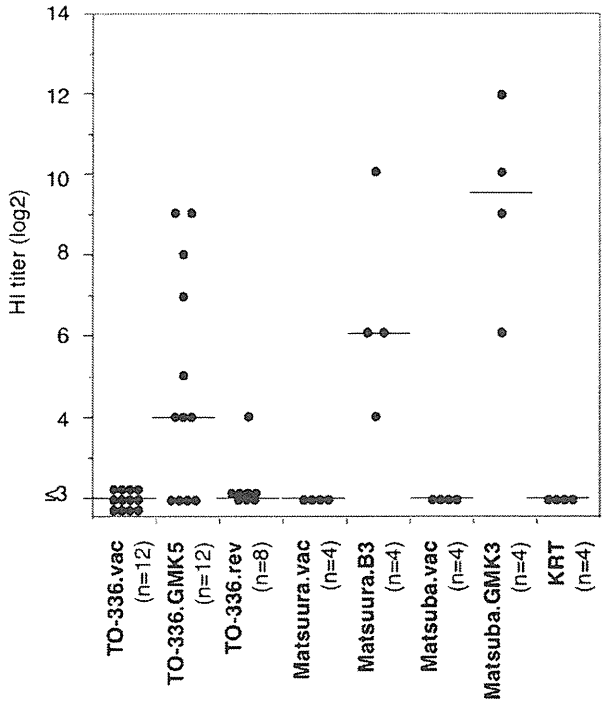

Fig. 7. Production of HI antibodies in guinea pigs inoculated with RuV strains. Guinea pigs (4, 8, or 12 animals per RuV strain) were subcutaneously inoculated with 5000 PFU of RuV. The serum HI antibody titers were measured at 5 weeks p.i. The horizontal bars indicate the median values of the HI antibody titers of the animals inoculated with each RuV strain.

suggest that both of the mutations contribute to the phenotypic reversion of TO-336.rev.

The Y1042H substitution in P150 is known to be responsible for the ts phenotype of KRT [12]. The $NSP_{994-1548}$ peptide of KRT (KRT-$NSP_{994-1548}$) was also subjected to the expression analysis. A strong signal for KRT-$NSP_{994-1301}$ was detected in cells incubated at 35 °C, while only a faint signal was detected in cells incubated at 39 °C (Figs. 6F and G, KRT). A histidine residue at amino acid position 1042 of NSP was replaced with tyrosine, the wt residue. This mutation (H1042Y) made the KRT-$NSP_{994-1301}$ stable at 39 °C (Figs. 7F and G, H1042Y). Therefore, the amino acid residues at 1042, 1126, 1159, and 1277, which are located in the protease domain, seem to be individually involved in the thermal stability of $NSP_{994-1301}$.

Collectively, the data suggest that substitutions in the protease domain may be predisposed to cause thermal lability of the NSPs, conferring the ts phenotype on some rubella vaccine strains.

3.8. A high growth capacity of RuV in cultured cells at a high temperature is neither essential nor sufficient to elicit antibody responses in guinea pigs

Japanese rubella vaccines lack the ability to elicit anti-RuV antibodies in experimentally infected guinea pigs and rabbits [6]. This is used as an *in vivo* marker phenotype of Japanese rubella vaccine strains [6]. According to the protocol for a marker test of rubella vaccines documented in the MRBP [8,9], 5000 PFU of each wt or vaccine strain was inoculated subcutaneously into guinea pigs, and their HI antibody titers were analyzed at 5 weeks after the inoculation. HI antibody titers were undetectable in the sera of all animals inoculated with vaccine strains (TO-336.vac, Matsuura.vac, Matsuba.vac, and KRT) (Fig. 7). The TO-336.GMK5 and Matsuura.B3 strains induced anti-RuV antibody responses in 66.7% and 100% of the inoculated animals, respectively (Fig. 7). It was noteworthy that all of the animals inoculated with Matsuba.GMK3, which showed a moderate ts phenotype (Fig. 1B), produced high HI titers (Fig. 7). The median value of the HI titers induced by Matsuba.GMK3 was even higher than those induced by TO-336.GMK5 and Matsuura.B3 (Fig. 7). The reversion mutant, TO-336.rev, which was able to replicate at a high temperature (Fig. 5B), hardly induced any antibody responses in the animals (Fig. 7). Most of the animals were seronegative, and only one of eight animals showed a low HI titer (Fig. 7). These findings demonstrate that the ability of RuV to grow at a high temperature was not necessarily correlated with the potency to elicit humoral immune responses in guinea pigs.

4. Discussion

Many vaccine strains for live attenuated vaccines have been successfully generated by adaptation of clinical isolates through numerous passages in various cultured cells [6,25–28]. During this process, the viruses have often been propagated at low temperatures (29–35 °C), and have acquired the ts phenotype [6,26,28]. Although these adaptations often reduce viral virulence, the molecular mechanisms of the attenuation have been poorly elucidated. Comparisons of nucleotide and amino acid sequences between vaccine strains and their progenitors provide basic and solid information toward understanding of the molecular bases that underlie the attenuation and the acquisition of other unique phenotypes of vaccine strains. In the present study, we determined the entire nucleotide sequences of the progenitors of currently used rubella vaccine strains. Unfortunately, the detailed records of the old isolates were unavailable. The passage histories of two viruses, however, could be predicted from their strain names, since the names of vaccine progenitors are usually designated on the basis of their passage history [6]. TO-336.GMK5 and Matsuba.GMK3 seemed to have been isolated in GMK cells and passaged in these cells five and three times, respectively. However, the history of Matsuura.B3 was unclear. In addition to these viruses, the entire genome nucleotide sequences of three Japanese rubella vaccines (Matsuba.vac, TCRB19, and Matsuura.vac) were determined. Phylogenic analyses confirmed that TO-336.GMK5 and Matsuura.B3 were progenitors or closely related progenitors of the currently used TO-336.vac and Matsuura.vac strains, respectively, whereas Matsuba.GMK3 was apparently unrelated to the currently used Matsuba.vac strain. However, it could be the progenitor of a vaccine candidate that has not been licensed. Comparative analyses of these strains and other RuV strains provided full lists of the mutations introduced into the genomes of TO-336.vac and Matsuura.vac during their passages under laboratory conditions. Matsuura.vac had acquired greater number of amino acid substitutions than TO-336.vac. This may be caused by differences in the host cell types used to produce these vaccine strains and/or the numbers of passages in these cells. TO-336.vac was generated after seven passages in GMK cells, followed by 20 passages in guinea pig kidney cells and three passages in rabbit kidney cells at 29–32 °C [6,19]. Matsuura.vac was generated after 14 passages in GMK cells, 65 passages in chick embryo amniotic cavities, and 11 passages in Japanese quail embryo fibroblasts at 32–35 °C [6,19].

A single amino acid substitution, Y1042H, has been demonstrated to be responsible for the ts phenotype of the KRT vaccine strain [12]. This mutation is located in the protease domain of P150 [20]. TO-336.vac became able to grow at a high temperature by acquiring second-site mutations in the protease domain. Therefore, we focused on the mutations in the protease domain for determining the ts phenotype. The protease domain possesses a cysteine-rich Ca^{2+} and Zn^{2+}-binding domain, which is essential for the protease activity and virus replication [29,30]. This domain contains a CaMBD with an alpha-helical structure, which also plays important roles in the protease activity and virus

replication [24]. Mutations in this domain have been shown to reduce its conformational stability at a high temperature [29]. The ts phenotype-determining mutation, Y1042H, found in the KRT vaccine strain rendered the protease domain-containing peptide, $NSP_{994-1301}$, unstable at a high temperature. In contrast, the N1126T and A1277V mutations found in the reversion mutant, TO-336.rev, rendered the TO-336.vac-derived $NSP_{994-1301}$ thermostable. Thus, the present data suggest that reduced stability of the conformation of the protease domain of P150 at a high temperature is a cause of the ts phenotype of some rubella vaccine strains. RuV with any mutations that have similar effects on the protease domain may exhibit a ts phenotype. It is of interest that other vaccine strains also possessed unique mutations in the protease domain.

The most important properties of vaccines are their safety and efficacy. For attenuated live vaccines, avirulence is critical for safety. Therefore, understanding of the molecular bases of the attenuation is crucial for quality control of vaccines. However, no reliable animal models for analyzing RuV virulence have been established. Humans are the only natural host for RuV, and it exhibits poor infection and replication in experimentally infected animals. Nonetheless, infections with clinical isolates of RuV induce considerable levels of humoral immune responses in animals, and the lack of these responses in the majority (>80%) of infected guinea pigs has been used as an *in vivo* marker of licensed rubella vaccines in Japan [6]. This phenotype is documented in the MRBP [8,9]. Although the low potency to induce antibody responses may be correlated with the attenuated phenotype of vaccine strains, no scientific evidence has been provided. A marker test that checks the *in vivo* marker phenotype of vaccine strains has been performed to verify the constancy of the vaccine quality, but not the avirulence [8,9]. It is difficult to determine the safety or avirulence of vaccines using cell culture systems. However, it is generally accepted that a ts phenotype, which can be analyzed in cultured cells, may play a role in virus attenuation [31–37]. Mutations in various genes can cause the ts phenotypes of viruses [33–35,38–43]. Since the body temperatures of guinea pigs and rabbits range from 37.5 to 39.5 °C, the inability of rubella vaccine viruses to elicit humoral immune responses in these animals may be partly and reasonably explained by the ts phenotype [5]. Surprisingly, however, Matsuba.GMK3 with a partial ts phenotype was highly potent in eliciting humoral immune responses in animals. On the other hand, the reversion mutant, TO-336.rev, was able to replicate better than Matsuba.GMK3, but was still unable to elicit these responses. These data demonstrate that a high growth capacity at a high temperature is not necessarily critical for eliciting humoral immune responses in animals. In the view of the care and use of laboratory animals, it is desirable to replace the marker test by a test involving cultured cells. However, our data show that a test for the ts phenotype using cultured cells cannot be a substitute for the marker test using animals. The present data showed that a phenotypic reversion of the virus, by which TO-336.vac became able to grow at a high temperature, was insufficient to elicit humoral immune responses. These data suggest that TO-336.vac has one or more mutations that specifically abolish the potency to elicit these immune responses in animals. It is of interest that TO-336.vac has mutations in the E1 surface glycoprotein, because it is known to be involved in cell entry and induction of neutralizing and HI antibodies. Functional or antigenic changes to this surface glycoprotein may play a role in determining the potency of viruses to elicit humoral immune responses. Analyses of these mutations are in progress in our laboratory.

In summary, the entire nucleotide sequences of all the Japanese rubella vaccines became available with the data obtained in the present study. Nucleotide sequence analyses of progenitor RuV strains and their resulting vaccines revealed mutations that were introduced into the genomes of TO-336.vac and Matsuura.vac during their passages in laboratories. Among these, the N1159S mutation in the protease domain of P150 seems to affect the thermal stability of the protein. The data further suggested that a reduction in the thermal stability of the protease domain is a cause of the ts phenotype of some rubella vaccines. Finally, our data showed that the ability of RuV to grow at a high temperature was not necessarily correlated with the potency to elicit humoral immune responses in animals. These findings indicate that the molecular mechanisms underlying the inability of vaccines to elicit humoral responses in animals are more complicated than the hitherto considered mechanism involving the ts phenotype as the major cause.

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REPORT

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World Health Organization collaborative study to calibrate the 3rd International Standard for Hepatitis C virus RNA nucleic acid amplification technology (NAT)-based assays

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Background and Objectives A collaborative study was undertaken to evaluate a replacement World Health Organization International Standard for hepatitis C virus (HCV) RNA for nucleic acid amplification technology (NAT)-based assays. The candidate preparations were calibrated in International Units (IUs).

Materials and Methods Three new candidate preparations were produced from a single bulk containing anti-HCV-negative, genotype 1a HCV RNA–positive plasma. Two samples were lyophilized (coded Sample 2 and Sample 3), whilst a third (Sample 4) contained liquid/frozen material. The samples were distributed together with the 2nd International Standard (Sample 1, NIBSC code 96/798) for evaluation by thirty-three laboratories, from fourteen countries. The panel of samples were assayed on four separate occasions. Stability studies were performed for the lyophilized samples by accelerated thermal degradation.

Results Participants returned data from a wide range of commercial and in-house quantitative and qualitative assays. Twenty-five data sets were returned for quantitative assays and fourteen for qualitative assays. Excellent agreement was observed between laboratories and assay methods. The mean relative potencies of Samples 2–4 were 5·19, 5·41 and 5·70 $\log_{10}$ IU/ml, respectively, when compared against the 2nd International Standard. Samples 2 and 3 demonstrated stability of a similar order to the previous standards.

Conclusions Based upon the results of the collaborative study, Sample 2 (code number 06/100) was established as the 3rd International Standard for HCV RNA with an assigned unitage of 5·19 $\log_{10}$ IU/ml. Each vial contains the equivalent of 0·5 ml of material; each vial contains 4·89 $\log_{10}$ IU of HCV RNA.

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Introduction

The safety of plasma-derived medicinal products and blood for transfusion has been greatly enhanced by the introduction of nucleic acid amplification technology (NAT)-based assays for the detection of the major blood-borne viruses. The success of this strategy and the implementation of regulations for testing for viral nucleic acids have been underpinned by activities of the World Health Organization (WHO). The WHO has established International Standards (ISs) for a variety of blood-borne virus for NAT-based assays [reviewed in 1], the first of which was for hepatitis C virus (HCV) RNA. The 1st IS for HCV RNA was established by the WHO Expert Committee on Biological Standardization (ECBS) in 1997 [2] and was replaced by the 2nd IS in 2003 [3]. The two preparations were lyophilized from the

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same bulk material in consecutive freeze-drying runs. The availability of the standards has enabled laboratories to ensure that assays are of sufficient sensitivity and comply with regulatory requirements for limits of detection of HCV RNA, both in plasma fractionation organizations and in blood banks, where local rules dictate assay sensitivities based upon specific pooling strategies. The standards have been used extensively for validation purposes and in the calibration of secondary standards and working reagents [4–6].

The use of the standards has extended to clinical laboratories and kit manufacturers, where HCV RNA loads are expressed in International Units (IU) per ml of plasma and form a basis for monitoring antiviral therapy in patients infected with HCV [7]. Because of the importance of the IU for HCV RNA in the plasma and blood safety field, as well as its widespread acceptance as a reporting unit in the clinical setting, a study was undertaken to calibrate replacement preparations against the 2nd IS for HCV RNA to ensure continuity of supply of calibrated reference material for HCV RNA. A study to replace the standard was endorsed by the WHO and was discussed at the eighteenth meeting of SoGAT held at the NIH in Bethesda, USA (24th–25th May 2005). It was proposed that like the 1st and 2nd IS, HCV genotype 1a would be used, which should be anti-HCV negative (a 'window period'). The genotype 1a replacement was proposed because of concerns that the IS based upon a different (sub)-genotype might not be suitable for calibration. The HCV RNA–positive plasma donation(s) were to be diluted in pooled human plasma negative for both HCV RNA and anti-HCV antibodies [8]. The study was designed to calibrate the candidate replacement preparations against the 2nd IS.

Materials and methods

Samples evaluated in the collaborative study

Four materials were included in the study. Sample 1 was the 2nd IS for HCV RNA (NIBSC code 96/798). Genotype 1a, anti-HCV-negative, HCV RNA–positive plasma was diluted in human plasma to create a bulk preparation which was lyophilized in two batches (NIBSC code numbers 06/100 and 06/102; Sample 2 and Sample 3, respectively, in this study). The bulk material was included in the study as a liquid/frozen preparation (Sample 4).

Preparation of bulk material

To produce the bulk preparation, three vials of genotype 1a HCV RNA–positive plasma samples were pooled and diluted 1:20 in pooled citrated plasma that tested negative for the following markers: anti-HIV-1, anti-HCV, HBsAg, anti-syphilis, anti-HTLV, HIV-1 RNA, HCV RNA. The plasma diluent was filtered to remove coagulated matter. The respective concentrations, as determined by the COBAS® Amplicor HCV Monitor Test, v2.0 (Roche Molecular Systems, Pleasanton, CA, USA), and volumes of the three HCV-positive plasma samples were as follows: (i) $6{\cdot}2 \times 10^6$ IU/ml (41 ml), $6{\cdot}1 \times 10^5$ IU/ml (73 ml) and $1{\cdot}7 \times 10^7$ IU/ml (42 ml). The virus stock material for Samples 2, 3 and 4 was negative for HIV-1 RNA, HBV DNA, HAV RNA and parvovirus B19 DNA. The bulk was mixed for 40 min at room temperature, divided into three aliquots and stored at −70 °C prior to processing. Two of the aliquots were lyophilized (Sample 2 and Sample 3 in the study). The third aliquot was thawed and dispensed into 0·5 ml volumes in Sarstedt vials and coded as Sample 4 and stored at −70 °C until dispatch on dry ice.

The HCV genotype of the plasma samples used to prepare Samples 2, 3 and 4 was determined using DNA sequence analysis and reverse hybridization using the Bayer Versant HCV genotype 2.0 assay (LiPA2.0). The genotyping assay was performed in accordance with the manufacturer's instructions except for the amplification step, which was carried out using the COBAS Amplicor HCV test, version 2.0 (Roche Molecular Systems).

Lyophilization of Samples 2 and 3

Samples 2 and 3 were filled and lyophilized in two separate processing runs. The material was processed as follows: on the day of filling, the bulk sample was thawed in a water bath at 37 °C with constant agitation until the sample had just thawed out. During filling, the bulk material was kept at 0 °C. Adelphi vials were filled with 0·5 ml of material and rubber seals were placed on the vials before loading into the freeze-dryer. The shelves of the freeze-dryer were precooled to 4 °C followed by loading of the vials. The shelf temperature was then ramped down to −40 °C over a period of 4·5 h then maintained at this temperature for 7·5 h in the absence of a vacuum. After this initial period, a vacuum of 200 μb was applied for 2 h and then further pulled to 100 μb over a period of 1 h with the shelf maintained at −40 °C. The temperature was ramped up to −35 °C over a period of 75 min and held at this temperature for 48 h with a vacuum of 100 μb. The shelf temperature was then ramped from −35 to 0 °C over a period of 2 h and 55 min and held at this temperature for 2 h with vacuum of 100 μb. The shelf temperature was further ramped to 25 °C over a period of 2 h and 5 min at 100 μb. The temperature was maintained at 25 °C for 2 h with the vacuum pulling to 30 μb. The shelf temperature was then maintained at 25 °C for 39 h at 30 μb. Upon completion of freeze-drying, the vials were back filled with nitrogen and sealed within the freeze-dryer. The vials were crimp-sealed with

aluminium over-seals and stored at −20 °C with constant temperature monitoring at the National Institute for Biological Standards and Control (NIBSC).

The coefficient of variation (CV) of the fill volume was 0·62% for Sample 2 (06/100), determined by measuring every thirtieth vial filled. Measurements were made for a total of 70 vials. In the case of Sample 3 (06/102), the CV of the fill volume was 1·82%, determined by measuring every thirtieth vial filled. Measurements were made for a total of 74 vials.

Study protocol

The collaborative study participants were sent four vials of each reagent coded Sample 1, Sample 2, Sample 3 and Sample 4. Participants were instructed to store Samples 1–3 at −20 °C and Sample 4 at or below −60 °C. The three lyophilized preparations (Samples 1–3) were to be reconstituted at the start of each assay in 0·5 ml of nuclease-free water. Participants are requested to perform testing (and as appropriate, dilutions prepared in the diluent normally used in the participating laboratories) of these reagents in four independent assays using a fresh vial of each reagent in each assay. Participants were requested to use quantitative methods in preference to qualitative ones and to report results in IU/ml; however, the use of qualitative assays was also acceptable and participants were requested to perform end-point dilution assays as previously described [2].

Statistical methods

All participating laboratories were assigned a code number, allocated at random, for the duration of the study and are referred to in this report by their code number.

Qualitative assays

For each laboratory and assay method, data from all assays were pooled to give a number positive out of number tested at each dilution step. A single 'end-point' for each dilution series was calculated, to give an estimate of 'PCR detectable units/ml', as described in previous studies [2]. It should be noted that these estimates are not necessarily directly equivalent to a genuine genome equivalent number/ml.

Quantitative assays

Analysis was based on the results supplied by the participants from kits and in-house assays calibrated in IU/ml. For each assay run, a single estimate of $\log_{10}$ IU/ml was obtained for each sample, by taking the mean of the $\log_{10}$ estimates of IU/ml across replicates, after correcting for any dilution factor. A single estimate for the laboratory and assay method was then calculated as the mean of the $\log_{10}$ estimates of IU/ml across assay runs.

Analysis

All analysis was based on the $\log_{10}$ estimates of IU/ml or PCR detectable units/ml. Mean estimates for each assay method were calculated as the mean of the individual laboratory means. Overall means and confidence intervals were calculated as the means of all individual laboratories (not the mean of the methods). Confidence intervals were not calculated for assay method means where there were only three or fewer laboratories using that method. Variation between laboratories was expressed as standard deviations (SD) and %CV. Differences between methods were assessed using an analysis of variance, comparing method differences to variation between laboratory mean estimates. An analysis of variance using the variation between individual assay runs within laboratories was used to assess the significance of differences between laboratories, and with the Duncan multiple comparison test, to investigate any 'anomalous' outlying laboratory results. The significance of differences in potency between the study samples was assessed using paired *t*-tests.

Stability studies

Accelerated thermal degradation studies were performed for Sample 2 (06/100) and Sample 3 (06/102). Vials of the respective preparations that had been stored at +4 °C for 10·5 months and +20 °C for 4 weeks were tested by real-time RT-PCR for HCV RNA. These were assayed concurrently with vials Samples 2 and 3 that had been stored at −20 °C, on three separate occasions at NIBSC. Vials had also been stored at higher temperatures (+37 °C and +45 °C) or for longer periods at +20 °C (8 months) but it was not possible to reconstitute the contents and they are not included in the stability study. Samples were analysed in triplicate on three separate occasions.

For analysis, 1000-µl volumes of the reconstituted Samples 2 and 3 were extracted using the MagNA Pure LC instrument with software version 3.0 (Roche Applied Science, Mannheim, Germany) using the Total Nucleic Acid Isolation Kit (Roche Applied Science) according to the manufacturer's instructions. Elution was performed with 50 µl of elution buffer. Real-time RT-PCRs were performed on the LightCycler 2.0 instrument (Roche Applied Science) using a previously published real-time PCR assay [9]. Amplification reactions were performed using the LightCycler FastStart RNA Master Hybprobe kit (Roche Applied Science). The concentration of each primer in the reaction was 0·6 µM, and the probe was used at a final concentration of 0·2 µM. The amplification conditions were as follows: 95 °C for 10 min, then 45 cycles of the following sequential steps: 95 °C for 15 s, 60 °C for 1 min. Fluorescence data were collected during the combined annealing/extension step and detected at 530 nm.

Results

Collaborative study materials were distributed to 33 laboratories in 14 countries. Results were returned from all participating laboratories.

Data received

Data were received from a total of 33 laboratories (listed in Appendix S1 in the supporting information accessible in the online version of this article), performing a variety of different assay methods. Some laboratories performed more than one assay method. All laboratories are referred to by a code number, allocated at random, not necessarily representing the order of listing in Appendix S1 in the supporting information accessible in the online version of this article. In total, data sets were received from 25 quantitative assays and 14 qualitative assays. The breakdown of the different assay methods used is shown in Table 1.

One laboratory performing the Procleix qualitative assay only returned data from a single assay run for each sample, and it was therefore not possible to obtain reliable estimates of 'PCR detectable units/ml'. This data is not included in the results or tables. The laboratory using the TS (COBAS TaqScreen MPX) assay did not provide details of the volume of sample amplified. It was therefore not possible to estimate a figure for 'PCR detectable units/ml' from this assay. The data could be used to estimate potencies for the study samples relative to the current IS however. Laboratory 1 returned additional data on diluted samples that had undergone a freeze/thaw cycle. There was some evidence of a drop in activity for these samples, and these data were not included. Only the results for the freshly reconstituted study samples were used. Laboratory 29 indicated on their results sheets that run 1 was 'invalid'. Also, the samples tested in their run 2 had been stored overnight at +4 °C, and the results appeared low. Only the data from runs 3 and 4 were analysed. Apart from the cases noted above, there were no exclusions of data.

Table 1 Types of assay used by participants in the collaborative study

Assay code	Assay	Number of data sets
Quantitative		
AB	Abbott RealTime HCV	7
BA	Bayer Versant HCV RNA 3.0 (bDNA)	4
CTM	COBAS AmpliPrep/COBAS TaqMan	6
CTMH	HPS/COBAS TaqMan	1
IHRT	In-house real-time	3
MO	COBAS Amplicor HCV Monitor	4
Qualitative		
A	Amplicor HCV 2.0	2
AC	COBAS Amplicor HCV 2.0	1
ASC	COBAS AmpliScreen HCV 2.0	3
IHQ	In-house qualitative	2
IHRTQ	In-house real-time qualitative	2
P	Procleix	1[a]
PU	Procleix Ultrio	1
PUT	Procleix Ultrio (TIGRIS)	1
TS	COBAS TaqScreen MPX	1

The type of assay and assay code are indicated together with the number of data sets received for each type of assay.

[a]One laboratory (26) also used the Procleix test but, in a single assay run, giving single positive or negative results for 10-fold (1 $\log_{10}$) dilution series for all samples. Any estimate of PCR detectable units on such limited data was considered to be too unreliable for inclusion and the results are therefore not included in the totals.

Analysis of results

Quantitative assays

The mean estimates of IU/ml ($\log_{10}$) from the quantitative assays are shown in Tables S1 and Table 2 for the different laboratories and different assay methods. Table S1 is available in the Supporting Information accessible in the online version of this article. The individual laboratory mean estimates are plotted in histogram form in Fig. 1a–d. Each box represents the mean estimate from one laboratory, and the boxes are labelled with the laboratory code number and the assay code. There is generally good agreement between laboratories and assay methods. Although there appear to be some small differences in the means from the different assay methods, the differences between methods were not statistically significant when compared to the between laboratory, within method, variation. Analysis of variance indicated that the within laboratory variability, across the four requested assays, was significantly lower than the between laboratory variability, which is not surprising. No individual laboratory results appeared anomalous with the overall distribution of estimates, with the exception of laboratory 27 for Sample 2, which was low. The consistency of assay methods across laboratories was good, with all %CV of $\log_{10}$ IU/ml values being below 5%, again with the exception of the Roche Monitor assay for Sample 2, because of the low result from laboratory 27.

The assays used in this study have been calibrated against the IS and using this calibration, the 2nd IS – Sample 1, has an overall estimate (overall mean of individual laboratory means) of 5·10 $\log_{10}$ IU/ml, whereas the IS has an assigned unitage of 5·0 $\log_{10}$ IU/ml. The 95% confidence interval, based on the between laboratory variation, was 5·02–5·17. The bDNA assay, which has the best

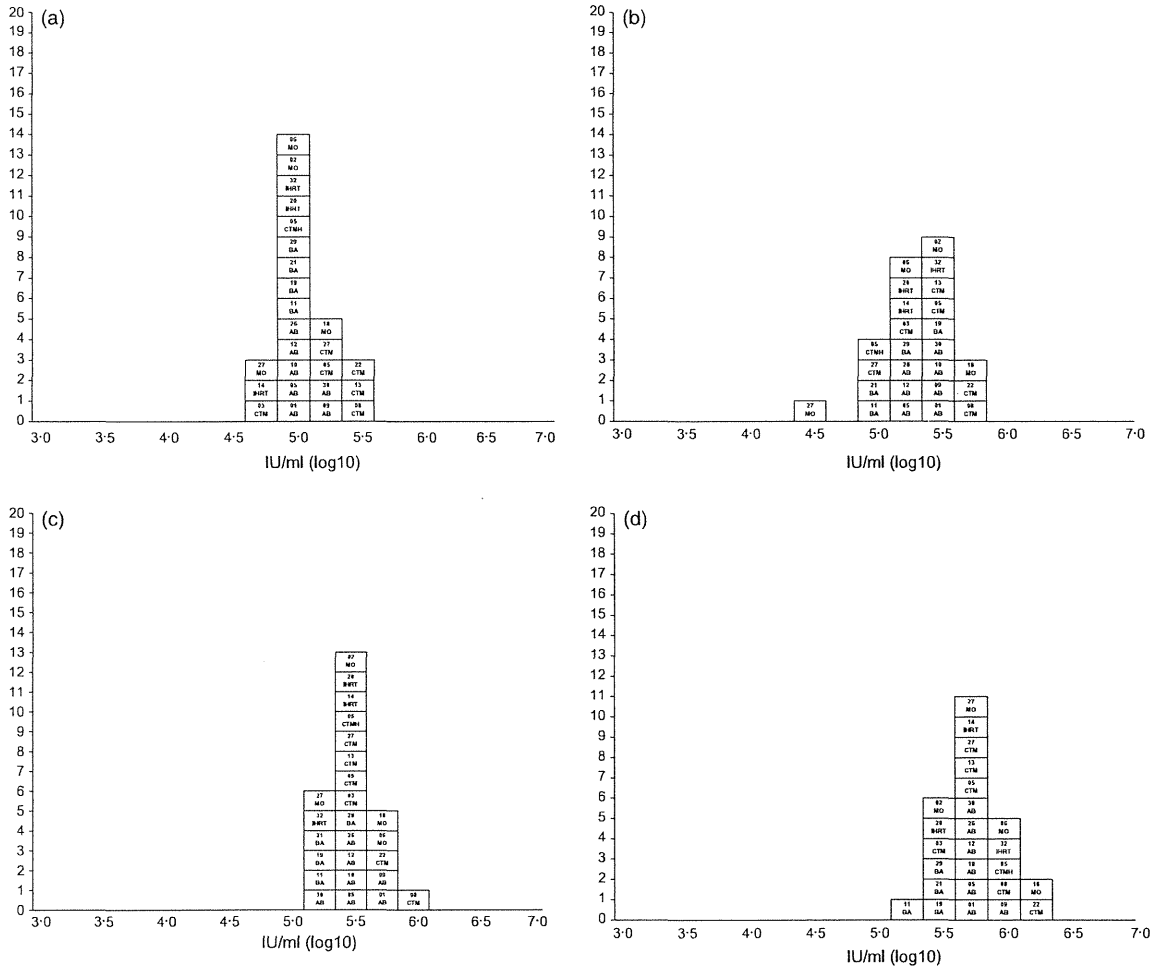

Fig. 1 (a) Histograms of the mean laboratory estimates in IU/ml for Samples 1–4 (Fig. 1a–d, respectively), from the different quantitative assay methods. The estimated IU/ml values are shown on the horizontal axis and the number of laboratories is indicated on the vertical axis. The laboratory code number together with the type of assay is indicated in each box. The assay abbreviations are indicated in Table 1.

consistency across laboratories, gives a mean estimate of 4·98 $\log_{10}$ IU/ml however.

Qualitative assays

The estimates of PCR detectable units/ml from the qualitative assays are shown in Table S2 (available in the Supporting Information accessible in the online version of this article) and Table 3, for the different laboratories and different assay methods. The laboratory using the TS (COBAS TaqScreen MPX) assay did not provide details of the volume of sample amplified. It was therefore not possible to estimate a figure for 'PCR detectable units/ml' from this assay. There is a wide range of different assay methods, and the variability between them is greater than for the quantitative assays, as expected. The increase in estimates across the four samples reflects the same pattern as for the quantitative assays.

Potency estimates

The potency estimates relative to Sample 1, the 2nd IS, for the individual laboratories and assay methods are shown in Tables S3 and S4, for the quantitative and the qualitative assays, respectively. Tables S3 and S4 are available in the Supporting Information accessible in the online version of this article. The mean potencies relative to the 2nd IS (Sample 1) are shown in Table 4a–c and are plotted in histogram form in Fig. 2a–c. From the figures, there is good agreement between the different assay methods. The result from laboratory 27 for Sample 2 no longer appears anomalous, as this laboratory had results for all samples that were consistently lower than for other laboratories using the same method, and expressing results as relative potencies has brought them more closely into line with the other laboratories. The %CV between individual laboratories is <5% for all samples. The overall potencies, along with 95%

Table 2 Estimated IU/ml (log_{10}) from quantitative assays for Samples 1–4

Method	No. of assays	Geometric mean	95% CI	SD	%CV
Sample 1 (2nd IS, 96/798)					
AB	7	5·11	4·98–5·24	0·14	2·68
BA	4	4·98	4·88–5·09	0·06	1·29
CTM	6	5·26	5·01–5·50	0·23	4·44
CTMH	1	5·01	–	–	–
IHRT	3	4·96		0·11	2·17
MO	4	5·07	4·69–5·46	0·24	4·77
Overall	25	**5·10**	**5·02–5·17**	**0·19**	**3·73**
Sample 2					
AB	7	5·37	5·27–5·47	0·11	2·00
BA	4	5·19	4·84–5·54	0·22	4·23
CTM	6	5·44	5·18–5·70	0·25	4·61
CTMH	1	5·10		–	–
IHRT	3	5·32		0·11	1·99
MO	4	5·25	4·55–5·95	0·44	8·42
Overall	25	**5·32**	**5·22–5·42**	**0·24**	**4·51**
Sample 3					
AB	7	5·56	5·45–5·68	0·12	2·20
BA	4	5·32	5·13–5·51	0·12	2·24
CTM	6	5·62	5·44–5·81	0·18	3·18
CTMH	1	5·58		–	–
IHRT	3	5·39		0·11	2·04
MO	4	5·51	5·23–5·79	0·18	3·18
Overall	25	**5·51**	**5·44–5·58**	**0·17**	**3·10**
Sample 4					
AB	7	5·77	5·69–5·85	0·09	1·54
BA	4	5·46	5·30–5·61	0·10	1·75
CTM	6	5·82	5·54–6·09	0·26	4·49
CTMH	1	5·89	–	–	–
IHRT	3	5·72		0·21	3·70
MO	4	5·86	5·43–6·29	0·27	4·63
Overall	25	**5·74**	**5·65–5·83**	**0·22**	**3·84**

CV, coefficient of variation.

confidence intervals, are shown in the Table 4a–c. Samples 2, 3 and 4 have mean relative potencies in log_{10} IU/ml of 5·17, 5·41 and 5·70, respectively. The differences in potency between all four samples are statistically significant ($P < 0·0001$) in all cases.

Stability studies

It was not possible to reconstitute samples stored at +20 °C for 8 months, or samples stored at +37 °C or +45 °C. The estimated drop in potency (log_{10}) is shown in Table 5. The differences between Sample 2 (06/100) and Sample 3 (06/102) were not statistically significant. Given the difficulties with reconstitution of the higher temperature

Table 3 Estimated PCR-detectable units/ml (log_{10}) from qualitative assays for Samples 1–4

Method	No. of assays	Geometric mean	95% CI
Sample 1 (2nd IS, 96/798)			
A	2	4·89	
AC	1	4·92	
ASC	3	5·40	
IHQ	2	5·25	
IHRTQ	2	5·37	
P	1	5·48	
PU	1	5·28	
PUT	1	5·19	
TS	1	(a)	
Overall	13	**5·24**	**5·04–5·43**
Sample 2			
A	2	5·07	
AC	1	5·27	
ASC	3	5·84	
IHQ	2	4·92	
IHRTQ	2	5·41	
P	0	–	
PU	1	5·40	
PUT	1	5·24	
TS	1	(a)	
Overall	12	**5·35**	**5·09–5·62**
Sample 3			
A	2	5·63	
AC	1	5·59	
ASC	3	5·88	
IHQ	2	5·50	
IHRTQ	2	5·63	
P	1	6·01	
PU	1	5·14	
PUT	1	5·64	
TS	1	(a)	
Overall	13	**5·65**	**5·45–5·86**
Sample 4			
A	2	5·88	
AC	1	5·24	
ASC	3	6·35	
IHQ	2	5·85	
IHRTQ	2	6·20	
P	1	6·55	
PU	1	6·16	
PUT	1	5·72	
TS	1	(a)	
Overall	13	**6·04**	**5·76–6·33**

(a) Details of the volume of sample amplified were not supplied by the laboratory using the TS assay (COBAS TaqScreen MPX). It was therefore not possible to estimate a figure for 'PCR detectable units/ml' from this assay. The data were used to generate relative potencies in Table 4 a–c however.

Table 4 Mean potencies (log_{10} IU/ml) relative to the 2nd IS (Sample 1, = 5·0 IU/ml) for (a) Sample 2, (b) Sample 3, (c) Sample 4

Assay method	*n*	Mean	95% CI	SD	%CV
(a)					
Quantitative					
AB	7	5·26	5·19–5·33	0·08	1·49
BA	4	5·20	4·93–5·47	0·17	3·27
CTM	6	5·18	4·95–5·42	0·22	4·34
CTMH	1	5·08	–	–	–
IHRT	3	5·36	–	0·11	1·96
MO	4	5·18	4·86–5·50	0·20	3·90
Qualitative					
A	2	5·18		0·13	2·54
AC	1	5·35		–	–
ASC	3	5·44		0·37	6·88
IHQ	2	4·67		0·18	3·80
IHRTQ	2	5·04		0·48	9·44
P	0	–		–	–
PU	1	5·12		–	–
PUT	1	5·05		–	–
TS	1	4·91		–	–
Overall	**38**	**5·19**	**5·11–5·27**	**0·24**	**4·62**
(b)					
Quantitative					
AB	7	5·46	5·27–5·64	0·20	3·62
BA	4	5·34	5·19–5·48	0·09	1·73
CTM	6	5·37	5·15–5·58	0·21	3·82
CTMH	1	5·57		–	–
IHRT	3	5·43		0·12	2·24
MO	4	5·44	5·28–5·61	0·10	1·89
Qualitative					
A	2	5·74		0·35	6·19
AC	1	5·67		–	–
ASC	3	5·48		0·16	2·97
IHQ	2	5·24		0·24	4·59
IHRTQ	2	5·26		0·04	0·82
P	1	5·53		–	–
PU	1	4·86		–	–
PUT	1	5·45		–	–
TS	1	5·16		–	–
Overall	**39**	**5·41**	**5·34–5·48**	**0·21**	**3·82**
(c)					
Quantitative					
AB	7	5·66	5·58–5·73	0·08	1·40
BA	4	5·47	5·41–5·53	0·04	0·65
CTM	6	5·56	5·34–5·78	0·21	3·82
CTMH	1	5·88		–	–
IHRT	3	5·76		0·28	4·88
MO	4	5·79		0·22	3·72
Qualitative					
A	2	5·99		0·06	0·99
AC	1	5·32		–	–
ASC	3	5·95		0·19	3·21
IHQ	2	5·59		0·13	2·35
IHRTQ	2	5·83		0·66	11·31
P	1	6·06		–	–
PU	1	5·88		–	–
PUT	1	5·53		–	–
TS	1	5·54		–	–
Overall	**39**	**5·70**	**5·62–5·78**	**0·25**	**4·31**

CV, coefficient of variation.

samples, the results for the +20 °C samples at 4 weeks shown in Table 5 may not be reliable. Although there appears to be some loss of potency on prolonged storage at +4 °C, it is difficult to relate this predicted stability at −20 °C. The small collaborative study carried out when the 1st IS was replaced by the 2nd IS, looked at samples of both the 1st and the 2nd ISs that had been stored at +4 °C for 5 years and 9 months [3]. Assays by three laboratories indicated a mean drop of 0·9 log_{10} (relative to the −20 °C samples) over this period (Table 6), with no evidence of any drop at −20 °C, based upon assays calibrated in IU/ml. If there were a constant degradation over time, this would equate to a drop of 0·0132 log_{10} per month. The figures for Sample 2 (06/100) and Sample 3 (06/102) are a little higher than this, but of a similar magnitude. Experience suggests that rather than constant degradation over time, the initial degradation may be higher, and then stabilize.

It should be further noted that in this study, the overall estimate for the 2nd IS was 5·10 log_{10} IU/ml. There is no evidence that this preparation has lost potency since it was manufactured in 1996. Since manufacture it has been stored at −20 °C for 9·5 years prior to analysis in the present collaborative study and supports the long-term stability of such preparations. Studies on the 1st IS (96/790) reported no loss of potency in vials stored at −20 °C after 6 years [3].

A small study was also carried out in 2010, testing the 1st, 2nd and 3rd IS in parallel, by six laboratories, using either the Roche COBAS AmpliPrep/COBAS TaqMan assay, or the Abbott Real-Time assay (R. Anderson and A. Heath, unpublished data). The results represent over 3 years real-time storage at −20 °C for the 3rd IS and over 12 years for the 1st and 2nd IS. The mean estimates of log_{10} IU/ml from the six laboratories were 5·05, 5·08 and 5·12 for the 1st, 2nd and 3rd IS, respectively. A comparison of the results from the six laboratories, and their results in the 2007 study described in this paper, did not indicate any statistically significant loss of potency for the 3rd IS. Further accelerated degradation studies, and investigations using different assay formats, are planned.

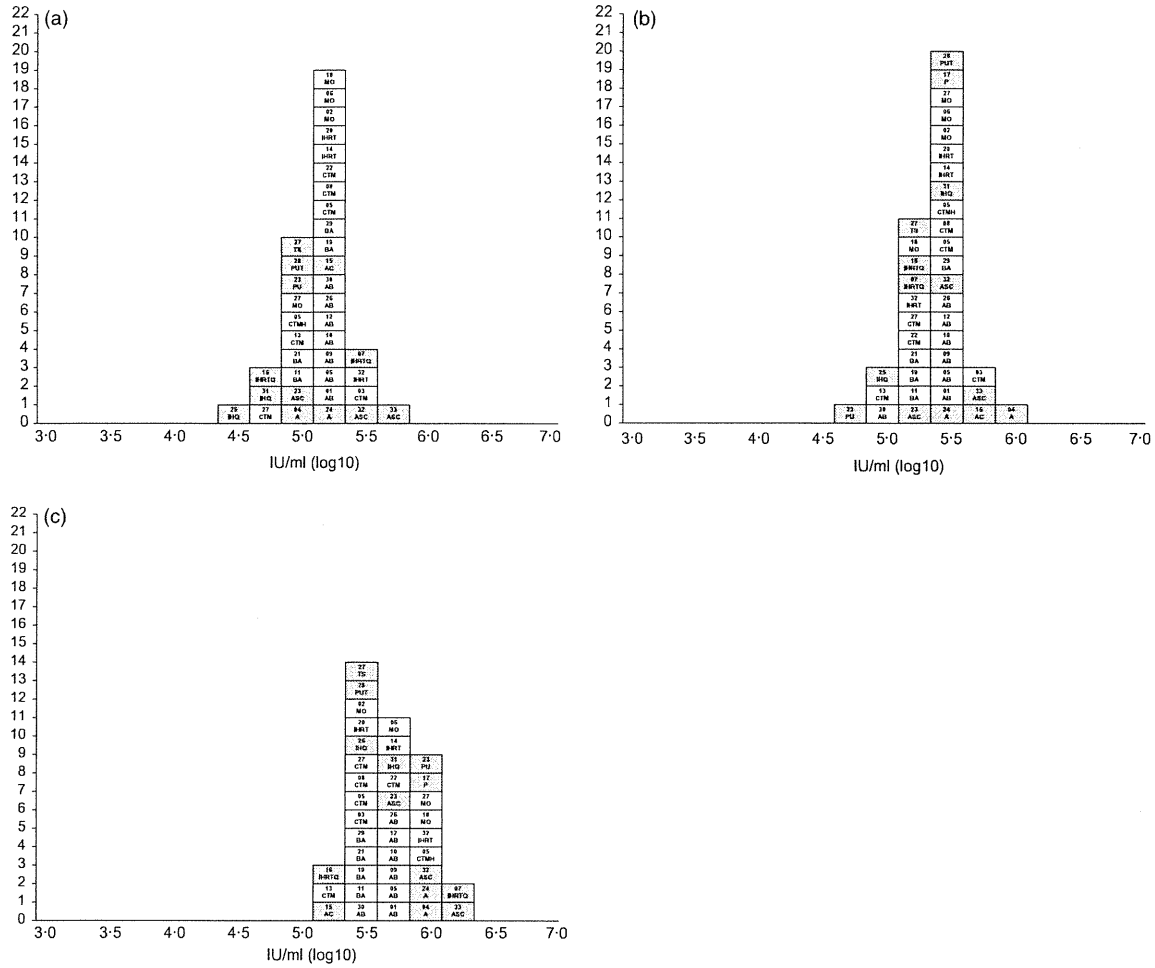

Fig. 2 (a) The mean potencies of Samples 2, 3 and 4 (Fig. 2a–c, respectively) compared to the 2nd IS (Sample 1). Potencies determined from for both quantitative (un-shaded) and qualitative (shaded) assays are indicated on the horizontal axis and the number of laboratories is indicated on the vertical axis. The laboratory code number together with the type of assay is indicated in each box. The assay abbreviations are indicated in Table 1. In the case of Sample 2, no data are available from laboratory 17.

Conclusions

In this study, a wide range of commercial and in-house quantitative and qualitative assays were used to evaluate candidate replacements for the 2nd IS for HCV RNA (96/798). The study aimed to calibrate the replacement preparations. Unlike in the original study [2] to establish the 1st IS, the majority of assays were quantitative, particularly real-time RT-PCR assays, although some qualitative assays utilizing limiting dilution were performed.

The data presented in this study confirm the validity of the assigned value of 96/798 and also demonstrate its stability under normal conditions of storage (i.e. −20 °C). The overall estimate for 96/798 was 5·10 $\log_{10}$ IU/ml, slightly higher than the assigned value of 5·0 $\log_{10}$ IU/ml, despite the assays being calibrated against the 2nd IS. There was good agreement observed between laboratories and assay methods with no outlying estimates. In this study, the confidence intervals around the determination of the 2nd IS for HCV RNA [3] are narrower than for previous studies, despite a larger number of participating laboratories. This effect is likely to be due in part to improvements in available assays and operator performance. The mean relative potencies of Samples 2–4 were 5·19, 5·41 and 5·70 $\log_{10}$ IU/ml when compared against the 2nd IS for HCV RNA (Sample 1, 96/798). These differences in relative potencies were statistically significant. Samples 2 and 3 were lyophilized from the same bulk material that is represented by Sample 4. It is clear that there has been a loss of potency of Samples 2 and 3 following processing when

Table 5 Stability of Sample 2 (06/100) and Sample 3 (06/102) in accelerated thermal degradation studies shown as an estimated drop in potency (log_{10})

Sample	+4 °C (10·5 months)	+20 °C (4 weeks)
06/100	0·22	0·2
06/102	0·34	0·24

Table 6 Stability of 1st IS (96/790) and 2nd IS (96/798) after 5·8 years accelerated thermal degradation studies from the study to establish the 2nd IS [3]. Mean log_{10} IU/ml based on three laboratories

Sample	−20 °C	+4 °C	+20 °C
96/790	5·11	4·18	3·07
96/798	5·09	4·20	3·31

compared to the unprocessed bulk (Sample 4). The loss of titre is slightly different between the two batches, but generally consistent with the effects of lyophilization observed during the preparation of the 1st and 2nd ISs for HCV RNA. Levels of HCV core antigen were determined for Sample 2 and Sample 4 using an Abbott Architect i2000SR analyzer and the HCV Ag assay kit (Abbott GmbH & Co KG, Wiesbaden, Germany). Lyophilization resulted in a 0·4-log_{10} drop in the levels of HCV core antigen (S. Nick, Paul-Ehrlich-Institut, personal communication), which is very similar to the drop in RNA titre determined in the collaborative study. Predictions of stability indicate that 06/100 is stable and suitable for long-term use.

The calculated mean relative potency for Sample 2 is a consensus value based on the overall mean of all participating laboratories, irrespective of assay method used. The 95% confidence interval of (5·11–5·27) around the mean of 5·19 log_{10} IU/ml reflects the uncertainty in this calculation. Based upon the results of the collaborative study, Sample 2 (NIBSC code number 06/100) was established as the 3rd IS for HCV RNA and has been assigned a unitage of 5·19 log_{10} IU/ml. Each vial contains the equivalent of 0·5 ml of material, and the content of each vial is 4·89 log_{10} IU of HCV RNA. Vials of the 3rd IS are available from NIBSC (http://www.nibsc.ac.uk).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Estimates from quantitative assays log_{10} (IU/ml) for Samples 1–4.

Table S2 Estimates from qualitative assays – PCR-detectable units/ml (log_{10}).

Table S3 Estimates of potency relative to the 2nd IS (Sample 1) for quantitative assays (log_{10} IU/ml).

Table S4 Estimates of potency relative to the 2nd IS (Sample 1) for qualitative assays (log_{10} IU/ml).

Appendix S1 Collaborative study participants.

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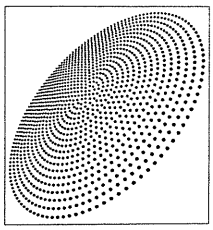

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肝炎ウイルスの核酸増幅試験法のための標準品

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はじめに

ウイルスの核酸増幅検査（NAT）のために最初に作られたWHO国際標準品は，1997年に制定されたC型肝炎ウイルスRNA NATのための第1次国際標準品（HCV RNA第1次国際標準品）である．供血者のHCV抗体スクリーニングが実施されると輸血後肝炎は激減した．しかし，ヨーロッパやアメリカ合衆国では抗体陰性の血漿をプールして製造した免疫グロブリン製剤によるHCV感染が報告された[1]．これは，感染初期のウィンドウ期のHCV陽性血漿が原料血漿プールに混入していたためと考えられた．そこで，1997年に欧州医薬品委員会（CPMP：Committee for Proprietary Medicinal Products）は適切なランコントロールを用いて原料血漿プールのHCV RNA検査を1999年7月1日から実施することを決めた．欧米諸国は自国で実施するNATのために標準品を作製したが，HCV RNA量の単位表示が異なっていたので，標準品のHCV RNA量やNAT法の感度を相互に比較することができなかった．そこで，WHOコラボレイティングセンターラボラトリーであるイギリスのThe National Institute for Biological Standards and Control（NIBSC）が中心となってWHO国際共同研究を実施し，力価100,000国際単位（IU）/mLの世界共通の標準品として1997年に制定されたのがHCV RNA第1次国際標準品である．その後，B型肝炎ウイルス（HBV）DNA，A型肝炎ウイルス（HAV）RNAおよびE型肝炎ウイルス（HEV）RNAの国際標準品が制定された．これらの国際標準品は血液製剤の原料血漿プールや輸血用血液のウイルスのスクリーニングとして実施するNATの標準品として使用することを目的とする一方，NATを用いた体外診断薬の評価・標準化のために使用することも目的としている．本稿では，HCV RNA国際標準品の制定を例にして，肝炎ウイルスのNATのためのWHO国際標準品の特徴と整備状況について述べる．また，日本の国内標準品をはじめとする入手可能な二次標準品について紹介する．

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1. C型肝炎ウイルスRNAの国際標準品

1.1 国際標準品の制定

NIBSCが中心となって実施した国際共同研究において，世界中の実績のある22の研究室が参加して濃度約10^5 genome equivalents/mLの3つの候補品（同一のHCV遺伝子型1の陽性血漿を陰性の脱クリオ血漿で希釈し，アンプルに0.5 mLずつ分注，凍結乾燥したロットの異なるAAとBB，凍結した液体のCC）を測定した．測定には，nested PCR法をはじめとする種々

の自家開発の試験法や市販の測定試薬が用いられた．当時は希釈した検体を定性法で測定するエンドポイント法による測定が主であった．定量法の測定結果も少数ながら報告されたが，もともと定量法は臨床検体のウイルス濃度の定量を目的とした測定法であるので低濃度検体の測定には不向きであった．定性法の測定結果に基づく候補品AAとBBの力価はともに5.0 $\log_{10}$ PCR detectable units/mLであった．この結果に基づいて，候補品AAを力価10^5 IU/mLのHCV RNA第1次国際標準品（96/790）として1997年に制定した[2]．翌年，国際共同研究を実施して，イギリス，ドイツ，イタリア，アメリカ，オランダの5ヵ国が自国で使用しているワーキング試薬の力価を国際標準品を用いて値付けし，IUで表示した．その結果，それぞれのワーキング試薬を使用して血液製剤の原料血漿プールや輸血用血液で実施しているNATスクリーニング試験法の標準化とバリデーションが可能になった[3,4]．

1.2 国際標準品の更新

その後，第1次国際標準品の更新が必要になった．主だった研究機関による小規模な共同研究を実施して，最初の共同研究で第1次国際標準品と同時に力価を決定した候補品BBをHCV RNA第2次国際標準品（96/798）に制定した（2003年）[5]．次の更新の時は，新たに製造した3つの候補品（HCV抗体陰性，HCV遺伝子型1a陽性血漿を血漿で希釈し，ガラス瓶に0.5 mLずつ分注，凍結乾燥したロットの異なるSample 2とSample 3，凍結した液体のSample 4）の力価を第2次国際標準品を用いて決定するための国際共同研究が実施され，14ヵ国33研究室が参加した．Real Time PCR法の普及により，定量法25組，定性法14組の測定結果が報告された．全体の測定結果がよく一致したので，定性法と定量法の両方の測定結果に基づいて候補品（Sample 2，Sample 3）の第2次国際標準品に対する相対力価を各々，5.19，5.41 $\log_{10}$ IU/mLと決定した．結論として，Sample 2を力価5.19 $\log_{10}$ IU/mLのHCV RNA第3次国際標準品（06/100）に制定した[6]．Sample 3は，次の更新時に主だった研究機関による小規模な共同研究によって，2011年に力価260,000 IU/mL（~5.41 $\log_{10}$ IU/mL）のHCV RNA第4次国際標準品（06/102）に制定された[7]．ところが，共同研究中に，第3次国際標準品（06/100）と第4次国際標準品（06/102）は常温輸送中に力価が低下することが判明したので，輸送にドライアイスを使用している．現在，第5次標準品と置き換えるべきか検討中である．

2. 肝炎ウイルス等遺伝子の国際標準品の制定

2.1 WHO ECBSとSoGAT会議

血液製剤のウイルス安全性にかかわる国際標準品の制定はWHO生物学的製剤標準化専門家会委員会（ECBS：Expert Committee on Biological Standardization）が行っており，肝炎ウイルスのNATのための国際標準品もこれに含まれる．1995年，WHOがスポンサーとなってNIBSCが第1回遺伝子増幅試験法の標準化に関する国際ワーキンググループ（SoGAT：The International Working Group on the Standardisation of Genomic Amplification Techniques）会議を開催した．会議には公的機関，血液製剤メーカー，血液センター，試薬メーカー，ウイルスの専門家等が参加し，血液製剤のウイルス学的安全性のために実施するNATの標準化について討議した[8]．以後，SoGATでは，WHO文書「国際標準品及びその他の生物学的参照品の作製と制定のための留意事項」[9]に基づいて国際標準品や国際パネルの必要性，候補品の性状，国際共同研究について科学的な討議を行い，ECBSに対して国際標準品の制定に関する報告や提案をしている．1997年のHCV RNA第1次国際標準品（96/790）の制定に引き続き，1999年にHBV DNA第1次国際標準品（97/746）[10]，2004年にHAV RNA第1次国際標準品（00/560）[11]，2011年にHEV RNA第1次国際標準品（6329/10）[12]が制定された．また，この間にHCV RNAとHBV DNA[13,14]の国際標準品が順次更新された（**表1**）．

2.2 国際標準品の性状

国際標準品には“Commutability”が求められている[9]．“Commutability”とは，種々の異

表1　肝炎ウイルス核酸増幅試験法のための WHO 国際標準品と WHO 遺伝子型パネルの制定

名称	制定年	力価 (IU/mL)	共同研究による測定値	交付機関	文献
WHO 国際標準品					
HCV RNA 第1次国際標準品（96/790）	1997	100,000	5.0 $\log_{10}$ PCR detectable units/mL	NIBSC	2
HBV DNA 第1次国際標準品（97/746）	1999	1,000,000	6.42 $\log_{10}$ 'equivalents' /mL	NIBSC	10
HCV RNA 第2次国際標準品（96/798）	2003	100,000	5.0 $\log_{10}$ PCR detectable units/mL	NIBSC	5
HAV RNA 第1次国際標準品（00/560）	2003	100,000	5.29 $\log_{10}$ PCR detectable units/mL or genome equivalents/mL	NIBSC	11
HBV DNA 第2次国際標準品（97/750）	2006	1,000,000	6.30 $\log_{10}$ 'equivalents' /mL	NIBSC	13
HCV RNA 第3次国際標準品（06/100）	2007	5.19log10	5.19 $\log_{10}$ IU/mL	NIBSC	6
HCV RNA 第4次国際標準品（06/102）	2011	5.41log10	5.41 $\log_{10}$ IU/mL	NIBSC	7
HBV DNA 第3次国際標準品（10/264）	2011	850,000	5.93 $\log_{10}$ IU/mL	NIBSC	14
HEV RNA 第1次国際標準品（6329/10）	2011	250,000	5.39 $\log_{10}$ NAT detectable units/mL or 'copies' /mL	ポールエーリッヒ研究所	12
WHO 遺伝子型国際パネル					
HCV RNA 遺伝子型国際パネル	2002	3.81 ～ 4.65	遺伝子型 1，2，3，4，5，6	NIBSC	15
HBV DNA 遺伝子型国際パネル（5086/08）	2009	1.10 ～ 6.87	遺伝子型 A，B，C，D，E，F，G	ポールエーリッヒ研究所	16

なる測定法を用いて測定しても国際標準品が実際の試験検体と同様の挙動を示すことを意味する．血液中のウイルスの遺伝子の NAT は核酸の抽出と検出の2つの過程からなっている．両方の過程を反映するにはウイルス粒子を含む血漿（ウイルス陽性血漿）が標準品に適しているとの考えから，肝炎ウイルスの NAT のための国際標準品は陽性血漿を陰性血漿で希釈した凍結乾燥品である．NAT の対象となるウイルス遺伝子の物理的な量は微量であるので，測定結果は血液中の他の成分の影響を受けやすい．よって，これらの標準品は陰性血漿で希釈してから核酸の抽出，検出を行うべきである．緩衝液で希釈したり，標準品原液から抽出した核酸を希釈して使用すると，実際の臨床検体よりも核酸の抽出効率は高く，検出反応液中の阻害物質濃度は低くなる可能性があるため，試験法の性能の過大評価になり得るので注意が必要である．

2.3　国際標準品の表示単位

前述の WHO の文書には生物学的な国際標準品の表示単位は国際単位系（SI）でも任意の IU でもよいと明記してある．これは，肝炎ウイルス遺伝子の NAT 標準品のような陽性血漿を原料とする標準品に含まれるウイルス遺伝子量を SI で表すのは困難だからである．HCV RNA 第1次国際標準品の制定の目的は原料血漿に混入する微量の HCV 遺伝子を検出するための NAT の検出限界の評価にあったことから，高感度の定性法を用いたエンドポイント法の測定結果（PCR detectable unit）に基づいて力価を決定し，表示単位を IU とした．ところで，HCV RNA 第2次国際標準品のエンドポイント法による測定値は，1997 年の共同研究においては 10^5 PCR detectable units/mL であったが，2007 年の共同研究においては $10^{5.24}$ PCR detectable units/mL であった．10 年間に試験法の感度が向上したことによって PCR detectable unit で表示する測定値は高くなったが，HCV RNA 第1次国際標準品に対する相対力価は 10^5 IU/mL であることに変わりなく，国際標準品を更新しても 1IU が表す HCV RNA 量は一定である．

2.4　遺伝子型国際パネル

ウイルスの NAT 試験法のバリデーションにおいて，特異性として主な遺伝子型を検出できることが求められている．WHO はウイルス毎に世界各地から様々な遺伝子型の陽性血漿を収集し，標準品と同様に国際共同研究を実施して WHO 国際パネルを制定している．2002 年に HCV RNA 遺伝子型国際パネル[15]が，2009 年に HBV DNA 遺伝子型国際パネル[16]が制定された．力価（IU/mL）を定めた標準物質は国際標準品のみであるとの考えから，HBV パネルの個々のメンバーの力価は定めないこととした．国際共同研究におけるパネルメンバーの測定値は公表

表2　国際標準品に基づくNAT国内標準品の整備

ウイルス	国内標準品			国際標準品		
	制定年	力価 IU/mL	遺伝子型	制定年	力価 IU/mL	遺伝子型
HCV	1999	1.0×10^5	1b	1997	1.0×10^5	1a
HBV	2002	4.3×10^5	C	1999	1.0×10^6	A
HIV*	2002	1.8×10^5	B	1999	1.0×10^5	B
HEV	2012	2.5×10^5	3b	2011	2.5×10^5	3a

＊国際標準品の名称はHIV-1

されているので参考にすることは可能である．HCV遺伝子型パネルの更新と，HAVおよびHEVの遺伝子型パネルを新規に作製することが決まっている．

3. WHO国際標準品の二次標準品

WHO国際標準品は配布する数量に限りがあり，国や地域で流行しているウイルス株が異なることから，二次標準品を作製することが推奨されている．

3.1　日本の国内標準品

わが国において，血液を介して感染するNATの国内標準品の制定は薬事・食品審議会血液事業部会安全技術調査会血液製剤の安全性確保対策に関する検討小委員会（NAT小委員会）が行っている．**表2**に国内標準品と国際標準品の対応を示す．国立感染症研究所が中心となって国内標準品作製のための国内共同研究を実施し，WHO国際標準品に準拠したHCV RNA国内標準品を1999年に[17]，HBV DNAおよびHIV RNA国内標準品を2002年に整備した．国内標準品はウイルス陽性の国内献血血漿を陰性血漿で希釈，分注した液体の凍結品である．2010～2011年にポールエーリッヒ研究所が中心となってHEV RNA国際標準品候補品を評価するための国際共同研究を実施した．わが国においても国内標準品の作製を計画中であったので，国立感染症研究所が共同研究者として参画し，日本からは6施設が参加して，国際標準品候補品（遺伝子型3a）と日本の国内標準品候補品（同3b）を同時に評価した．2つの候補品は日本の献血由来のHEV陽性血漿を原料とし，ISO13485：2003を取得したスイスの同一の会社に委託して分注，凍結乾燥した製品である．ほとんどの参加施設が自家開発のReal Time PCR法を用いて測定を行った．20組の定量法，14組の定性法の測定結果が報告され，両方の測定結果に基づいてHEV RNA第1次国際標準品（6329/10）の力価を250,000 IU/mLと決定した．2つの候補品の力価の差は無視できるほどわずかであったので，日本の国内標準品候補品が国際標準品と同等の品質，同じ力価（250,000 IU/mL）であることが示された．2012年第1回NAT小委員会においてHEV RNA国内標準品の制定と，パルボウイルスB19 DNA国内標準品を作製することが決定された．国内標準品の交付は国立感染症研究所が有償で行っている．

3.2　海外の参照品

HCV RNA第1次国際標準品が制定されると欧米諸国のワーキング試薬の力価をIUで表示したことは1.1で述べたが，HCV RNAやHBV DNAの国際標準品の二次標準品が順次整備されていった．アジアにおいては日本，台湾，韓国，中国が国内標準品を作製した．現在，交付あるいは販売されている肝炎ウイルス遺伝子NATのための主な二次標準品を**表3**に示した．

おわりに

現在の国際標準品は，血液製剤の原料血漿プールに混入する微量のウイルス遺伝子を検出する試験法の標準品として制定され，性状は血漿の凍結乾燥品，力価は様々な試験法を用いた測定結果に基づいて決定され，IUで表示される．WHOは2007年「血液のウイルス学的安全性にかかわるウイルスの体外診断薬のための国際標準品整備の5か年戦略」[18]に基づいて血清学的

表3　海外の参照品とパネル

NIBSC	
	HCV RNA ワーキング試薬
	HCV RNA 遺伝子型パネル
	マルチプレックスワーキング試薬 (HCV, HBV, HAV, HIV, B19)
ポールエーリッヒ研究所 (PEI)	
	HBV-DNA PEI 参照品
	HCV-RNA PEI 参照品
欧州医薬品品質部門 (EDQM)	
	PH. Eur.BRP　HBV-DNA
	PH. Eur.BRP　HCV-RNA
SeraCare ライフサイエンス株式会社	
	ACCURUN® コントロール HCV
	ACCURUN® コントロール HBV
	HCV リニアリティーパネル
	HBV リニアリティーパネル

検査や NAT のための標準品の整備を進めている．しかし，近年，種々のウイルス遺伝子を定量するための体外診断薬が開発されるに従い，合成核酸を標準物質とする SI 表示の国際標準品を求める声がある[19]．SoGAT において議題にされてきたが，合成核酸は核酸抽出の過程を反映することができないことから現段階では血漿中のウイルスの NAT の国際標準品には使用されていない．合成核酸を国際的な標準物質にする場合の“Commutability”の問題をどうすれば解決できるか，科学的な議論をすすめる必要があるだろう．

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Standards for hepatitis viruses for nucleic acid amplification technology (NAT)-based assays

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First WHO International Reference Panel containing hepatitis B virus genotypes A–G for assays of the viral DNA

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ABSTRACT

Background: WHO International Standards (IS) are provided for the calibration and validation of diagnostic and screening assays, e.g. for hepatitis B virus (HBV). HBV forms numerous subgenotypes and the current IS for HBV DNA reflects subgenotype A2.
Objective: A reference panel with the most prevalent subgenotypes should facilitate evaluation of genotype-specific detection efficiencies.
Study design: 215 HBV positive plasma samples collected worldwide were characterized for HBV markers and sequenced. Fifteen subgenotype A1, A2, B2, B4, C2, D1, D3, E, F2 and G samples were selected for the panel. The lyophilized samples were tested in parallel with the IS in an international collaborative study with 16 laboratories using 13 different nucleic acid amplification techniques (NATs).
Results: Eight of 13 NAT had a HBV DNA detection efficiency which was independent of the genotype and consistent with the IS, while with five assays, certain deviations were noted, particularly with genotype F which was under quantitated or even missed by three assays. The panel was accepted by the WHO as the "1st WHO International Reference Panel for HBV Genotypes for HBV NAT-Based Assays".
Conclusions: The evaluation of HBV DNA assays should include many different genotypes. The WHO Reference Panel is universally available for manufacturers of HBV DNA assays, diagnostic laboratories and control authorities to facilitate standardized validation of HBV genotype specific detection efficiency of both diagnostic (quantitative and qualitative) and screening NAT assays.

1. Background

Sensitive screening and accurate diagnostic assays play a crucial role in the prevention and management of HBV associated diseases. Sensitive HBV DNA assays by nucleic acid amplification techniques (NATs) detect more infected subjects in the early and in the low level chronic infection phase when compared to highly sensitive immune assays for HBsAg.[1,2] Over the past years HBV NAT has been increasingly introduced in different countries into the screening programmes for blood donors.[3] Highly sensitive detection of HBV DNA by NAT is also important for the detection of occult HBV infection, for the early detection of re-activating HBV infection under immune suppression, and for the decision on a potential discontinuation of long-term antiviral therapy.[4–6] Quantitative HBV DNA assays are required for the estimation of infectivity of an HBV infected subject, and for the decision on whether an antiviral therapy is indicated according to HBV treatment guidelines.[6,7]

Due to the importance of HBV DNA as a screening and diagnostic marker, the first WHO International Standard (IS) for HBV DNA (97/746) was established in 1999 followed by the subsequent replacement standards, the 2nd WHO IS for HBV DNA (97/750) and the 3rd WHO IS (10/264). All these were derived from the same plasma of one highly viremic HBV carrier.[8,9] HBV strains from different regions of the world differ in their genomic sequence. Eight HBV genotypes (A–H) have been defined so far with >8% intergenotype nucleotide divergence over the entire viral genome.[10] Some of the HBV genotypes are further differentiated into subgenotypes characterized by >4% intra-genotype divergence. The IS preparations are subgenotype A2 which is mainly prevalent in Western Europe and North America. However, on a global basis, this genotype represents only 1% of the HBV infected population. Currently available NAT assays target different regions of the HBV genome, e.g. the preS-, S-, core- or X-gene region. Despite the choice of conserved sequences for primers and probes, not all

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assays have been equally reliable in collaborative studies in the past.[11]

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.[12] 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 Homocentro 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′ 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.

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

3.5. Design and manufacture of the HBV genotype reference panel

Each panel member was diluted to a target HBV DNA concentration of 10^6 IU/ml in a volume of 1.2 l, with the exception of panel samples 6 (B4; 10^4 IU/ml), 14 (F2; 10^5 IU/ml), and 15 (G; 10^4 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.[13] 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