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Quantitative Detection of Hepatitis B Virus DNA in Two International Reference Plasma Preparations

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Quantitative detection of hepatitis B virus (HBV) in serum or plasma is of significance for monitoring of therapy and establishment of the prognosis of the disease, as well as for infectivity assessment and quality control of the diagnosis. Unfortunately, various commercially available test kits for HBV DNA yielded conflicting quantitative results, with differences of up to a factor of 120. The Eurohep Pathobiology Group has established two reference samples of plasma from HBV carriers and determined as accurately as possible the number of HBV DNA molecules in these samples. Plasma donations from two single highly viremic carriers of HBV genotype A (HBV surface antigen subtype *adw2*) and genotype D (*ayw2*/*3*), respectively, were collected, and coded dilutions of these samples were analyzed by members of the Eurohep Pathobiology Group. Quantitative results from the seven laboratories reporting consistent results were initially divergent. Limiting dilution and nested PCR assays suffered from incomplete DNA extraction. Hybridization assays used inaccurately quantitated cloned DNA as a reference. Two hybridization assays could not be calibrated directly with cloned HBV DNA, because virion-derived DNA reacted much less efficiently. After identification and elimination of these problems, limiting-dilution assays from three laboratories and hybridization assays from two producers generated consistent and concordant results: 2.7×10^9 HBV DNA molecules/ml (range, 2.1×10^9 to 3.4×10^9 HBV DNA molecules/ml) in the plasma from the carrier of genotype A and 2.6×10^9 HBV DNA molecules/ml (range, 2.1×10^9 to 3.0×10^9 HBV DNA molecules/ml in the plasma from the carrier of genotype D. The two Eurohep reference plasma samples have already been used for the standardization of test kits and in quality control trials, and the plasma from the carrier of genotype A will probably be the basis of a World Health Organization reference sample.

Quantitative detection of hepatitis B virus (HBV) DNA in serum or plasma provides a means of measuring the viral load in the blood of patients before, during, and after antiviral therapy studies (16). The reduction of the amount of HBV DNA to undetectable levels is a favorable prognostic sign of acute or chronic hepatitis B (25). Furthermore, the level of HBV DNA makes it possible to estimate the potential infectivity of a hepatitis B patient or an HBV carrier (1, 21). A highly sensitive means of detection of HBV DNA is useful for the early detection of blood donors who were recently infected with HBV and for the detection of HBV in therapeutic plasma protein preparations (5).

For quantitation of high levels of HBV DNA, various nucleic acid hybridization techniques have been used, and several of them are commercially available as test kits. Unfortunately, these test kits generated highly divergent results which differed by factors of up to 120 (3, 13, 24; this study). Determination of the accurate number of HBV DNA molecules (*N*) in a sample is particularly important in determining whether HBV-infected medical staff may participate in operations that place the patients at risk of exposure (4). Furthermore, reference samples with a well-defined number of HBV DNA molecules would allow an objective and reproducible standardization of assays for the detection of HBV DNA. This is particularly relevant for the internal and external quality control of PCR assays for the detection of HBV DNA (17). In view of these problems, the Eurohep Pathobiology Group decided to generate two reference plasma samples for HBV DNA and to determine as accurately as possible in an international trial *N* in these samples (8). The plasma samples have already proven to be useful in a quality control trial (17) and have provided a basis for the comparison of test kits for the detection of HBV DNA (24). During the evaluation in phase 1 of the Eurohep study, some of reasons for the divergent results between different quantitative assays became apparent. It was recognized that cloned and purified HBV DNA may in certain assays behave very differently from virion-derived HBV DNA and that even quantitation of cloned HBV DNA itself was not always accurate and comparable between assays. After resolution of these problems, in phase 2, the accurate determination of *N* in the two Eurohep reference plasma samples was possible.

MATERIALS AND METHODS

Samples. The two Eurohep reference samples, reference samples 1 (R1) and 2 (R2), were collected by plasmapheresis from two HBV carriers who were persistently hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) positive. Five hundred milliliters of plasma was distributed into 1,000 aliquots, stored at −80°C, and supplied by request on dry ice. The genotypes of the virus in R1 and R2 were determined by enzyme-linked immunosorbent assay with a monoclonal anti-d antibody (7a) or by immunodiffusion with a polyvalent adsorbed guinea pig anti-y serum (2), by sequencing of R1 (19b), and by multiplex PCR of R2 (19). Dilutions A (1:64) and B (1:64,000) from R1 or C (1:32) and D (1:32,000) from R2 were made in human plasma negative for HBV,

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TABLE 1. Primers and conditions of nested PCR used for limiting-dilution assays by the three laboratories participating in phase 2 of the study

PCR and laboratory	Gene region	Position		Annealing temp (°C)	No. of cycles	Sequence reference no.
		Forward primer[a]	Reverse primer			
PCR 1						
1a	S	392–416	750–726	65–57.4[b]	30	14
1b	X	1275–1302	1577–1551	67.9–60.4[b]	30	14
2	pre-S and S	2718–2738	1305–1285	55	35	8
8	S	194–213	706–687	55	40	27
PCR 2						
1a		427–449	694–672	60.1/58.1[b]	20/20	
1b		1305–1328	1542–1522	63.5/61.5[b]	20/20	
2		2821–2843	191–168	60	35	
8		305–324	433–414	55	40	

[a] Numbering starts at the unique *Eco*RI site at position 3221/0.
[b] Laboratory 1 used a touchdown program for annealing temperatures from 5°C above the melting temperature of the primer down to −2.5°C for 15 cycles and then 15 further cycles at the lower temperature.

human immunodeficiency virus, and hepatitis C virus serological markers and were stored at −80°C or were transported on dry ice. Aliquots are available on written request from K. H. Heermann (Department for Medical Microbiology, Kreuzbergring 57, D 37075 Goettingen, Germany; Fax, 49-551-395860). The samples are not available as control for routine diagnosis but are used for the calibration of test kits, trials, and research purposes only.

Laboratories 4 to 7 used their own cloned and purified HBV DNAs as in-house references in their assays for the Eurohep trial. In phase 2 of this study, a well-characterized sample of cloned HBV DNA at 2 μg/ml was provided by P. D. Neuwald (Chiron Corporation, Emeryville, Calif.) and was used by laboratories 4, 5, and 9 for calibration. Later, J. C. Wilber from Chiron Corp. reported that the concentration of HBV DNA had to be corrected by multiplying by a factor of 0.65. The results presented in this report were corrected accordingly, whereas a previous preliminary report (8) did not consider this correction.

Performance of the trial. Members of the Eurohep Pathobiology Group (see the footnote to the article title) and some laboratories that produce HBV DNA test kits each received four vials of samples R1, R2, and A to D, instructions, suggestions for testing, and protocol forms for the recording of results. They were asked to determine the dilution factors of A or B with respect to R1 and C or D with respect to R2. The dilution factors were unknown to the investigators in phase 1. It was recommended that they use a highly sensitive nested PCR which is able to detect one copy of HBV DNA and to test at least six replicates of 10-fold dilutions in order to determine the limiting dilution at which 50% of the PCR assays would be positive. Furthermore, the absolute values for the HBV DNA concentration (in picograms per milliliter or *N* per milliliter) should be determined. The results, including all experimental details, were reported under code to the coordinator of the Eurohep Pathobiology Group, S. Schalm (Rotterdam, The Netherlands). In phase 1, seven laboratories provided data sets; laboratories 1, 2, 4, and 5 also participated in phase 2. Laboratories 8 to 10 provided data only for phase 2. Results from a laboratory were considered to be consistent if the dilution factors for samples A to D were correctly determined within a factor of 4. Results from different laboratories were considered to be concordant if the geometric mean values (GMVs) were within the 95% confidence interval (CI), i.e., ±2 standard deviations (SDs) of the log GMV obtained by the other methods.

Extraction of the DNA. In phase 1, laboratory 1 treated 200 μl of the plasma samples with 300 μl of lysis buffer (2.5 mg of proteinase K per ml, 1% sodium dodecyl sulfate in 0.13 M NaCl, 0.015 Tris-HCl [pH 8.0]) for 4 h at 56°C, extracted this lysate with phenol-chloroform, and precipitated the DNA with ethanol. The dried DNA extract was dissolved and diluted in one series of experiments. In another series the plasma samples were diluted first in negative plasma and extracted afterward. For phase 2, laboratory 1 diluted the plasma samples in 0.1% bovine serum albumin–10 mM Tris-HCl (pH 7.4)–0.13 M NaCl–0.1 mM EDTA and subjected the 10-fold dilution series to alkali lysis with 1 volume of 0.2 N NaOH for 1 h at 37°C and reneutralization with 3 volumes of 0.2 M Tris-HCl (pH 7.4) (method 1.1 in Table 3). Alternatively, 200 μl of the 10^{-4} dilutions were extracted with the QIAamp Blood Kit (Qiagen, Hilden, Germany) and the extracts were diluted further (method 1.2). Laboratory 2 used a similar proteinase K lysis (at 65°C) and extraction procedure but prediluted the plasma samples 1:100 in water prior to lysis. The dried extract was dissolved and diluted further in 0.1 mg of bovine serum albumin per ml–10 mM Tris-HCl (pH 8.4)–0.1 mM EDTA–0.1% Triton X-100. Laboratory 8 extracted DNA from 100 μl of plasma with NaI as described by Wang et al. (23) and thereafter used the same diluent as laboratory 1 in phase 2.

Limiting-dilution assays with nested PCR. Tenfold dilution series were made by careful mixing of samples and diluent for 15 min per step up to a dilution of 10^{-10}, and between 7 and 16 replicates of the respective dilutions were tested in parallel or in different runs. All runs were controlled with numerous negative controls and dilution series of cloned HBV DNA covering the limiting-dilution range. All precautions used to avoid contaminations were observed, including the use of different rooms for sample extraction, amplification, and amplicon detection. Laboratories 1, 2, and 8 used different optimized nested PCR protocols (5, 7, 10), as listed in Table 1, and ethidium bromide staining after agarose gel electrophoresis. The titer of the sample theoretically yielding positive results for 50% of the samples by the nested PCR (PCR positive unit 50 [PPU_{50}]) was calculated in phase 1 by the formula of Reed and Muench (18) as described by Davies et al. (6) and in phase 2 by the maximum-likelihood method. The PPU_{50} titer was multiplied by ln 2 (0.7), and this result was divided by the sample volume of the aliquot in the PCR assay to give *N* per milliliter in the original sample. The estimated SD of the log PPU_{50} was calculated by the formula $0.79/n$, where n is the number of replicates per dilution if the method of Reed and Muench (18) was used as described in reference 6. Log $PPU_{50} \pm 2$ SDs gave the 95% CI of a titration. The maximum likelihood and the 95% CI were calculated with a computer program (available previously from M. Chudy).

Hybridization assays. Laboratory 4 was at Orion Pharmaceutica (Helsinki, Finland), which used its quantitative test kit, Affiprobe Hep B (12). The bound amount of an ^{35}S-labeled probe bound was converted to *N* per milliliter with the aid of a calibration curve based on the results obtained with purified cloned HBV DNA. Laboratory 9 was at Chiron Corporation and used cloned HBV DNA as the "gold standard" and its branched-DNA (bDNA) assay for the detection of HBV DNA (11). This reference DNA was distributed to laboratories 1, 2, 4, and 8 in phase 2. Laboratory 10 used the Genostics liquid hybridization assay from Abbott Laboratories, North Chicago, Ill. (14). Picogram results for HBV DNA were converted to *N* by using a factor of 2.85×10^5, assuming a molecular weight of 2.1×10^6.

RESULTS

Design of the reference samples. From the divergent data in the literature and from our own previous experience (25), it was suspected that problems associated with the extraction of HBV DNA from virions caused unsatisfactory results. Thus, the Eurohep Pathobiology Group decided that HBV particles should be used as reference materials. Since some assays did not react equally well with different genotypes (24), two reference samples, samples R1 and R2, containing HBVs of different genotypes were generated. To measure naturally occurring concentrations up to the nanogram-per-milliliter range (16), we selected plasma samples from among those with the highest concentrations of HBV available to us.

Although a very large volume of reference material would be desirable (e.g., several liters), we preferred to use one plasma donation from one well-characterized donor infected with

TABLE 2. Phase 1 results from seven laboratories for *N* in R1, R2, and dilutions A to D[a]

Sample (dilution)	*N*							
	Laboratory 1a	Laboratory 1b	Laboratory 2	Laboratory 3	Laboratory 4	Laboratory 5	Laboratory 6[b]	Endpoint titer laboratory 7[c]
R1	5.5×10^8	5.5×10^8	2.2×10^9	4.3×10^8	1.6×10^9	4.0×10^8	1.2×10^7	1:1,000
A (1:64)	2.4×10^7 (1:23)[d]	3.2×10^7 (1:17)	1.1×10^8 (1:20)	4.3×10^5[b] (1:1,000)	3.0×10^7 (1:53)	1.1×10^7 (1:35)	8.2×10^5 (1:14)	1:128
B (1:64,000)	1.1×10^4 (1:50,000)	2.1×10^4 (1:26,000)	4.8×10^4 (1:46,000)	4.3×10^3[e] (1:100,000)	$<5 \times 10^6$	$<2.9 \times 10^6$	6.6×10^2 (1:17,900)	Negative
R2	8.6×10^8	5.2×10^8	4.7×10^9	4.3×10^8	2.0×10^9	3.4×10^8	2.7×10^6	1:>100
C (1:32)	5.5×10^7 (1:16)	1.3×10^7 (1:39)	1.3×10^8 (1:36)	4.3×10^7[e] (1:10)	5.2×10^7 (1:38)	1.1×10^7 (1:30)	3.4×10^5 (1:8)	1:32
D (1:32,000)	3.0×10^4 (1:28,700)	9.3×10^4[b] (1:5,600)	1.7×10^5 (1:28,000)	1.4×10^4[e] (1:30,000)	$<5 \times 10^6$	$<2.9 \times 10^6$	1.2×10^3 (1:2,390)	Negative
No. of replicates per dilution step	7	7	11–16	2				

[a] Laboratories 1 to 3 used nested PCR and limiting dilution; laboratories 4 to 7 used reference samples.
[b] Data not considered for further evaluation because of fourfold or greater deviation from the true dilution factor.
[c] Endpoint titer of a hybridization assay.
[d] Values in parentheses are dilution factors determined by the laboratories.
[e] Due to low numbers of replicates, these data were not considered for calculation of the GMV.

HBV genotype A as reference 1 (R1) and a donation from a carrier infected with HBV genotype D as reference 2 (R2). The genotypes of the HBVs in these two reference samples are predominant in Europe and North America. The corresponding HBsAg subtypes are *adw2* in R1 and *ayw* in R2 (15). We avoided the pooling of donations from different HBV carriers, because many carriers contain antibodies against epitopes of heterologous HBV genotypes (2). This could cause aggregation of HBV and problems in the testing of dilutions made from the reference samples. According to the principles of the World Health Organization, it would also be desirable to freeze-dry official reference samples or even to inactivate the infectivity of HBV. This was avoided because the effects of these procedures on HBV DNA quantitation by various techniques were unknown.

Results of phase 1. The main goal of the trial was to measure *N* for single HBV genomes by limiting-dilution assays. The ability of the PCR to detect single molecules was indicated if a strong signal from the nested PCR appeared for only some of the replicates of the limiting-dilution assay. Such data were reported by laboratories 1a, 1b, 2, and 3. Hybridization assays with a quantitative signal and calibration of this assay with a highly purified cloned HBV DNA with a known concentration were used by laboratories 4, 5, 6, and 7. The results are listed in Table 2. The determination of the dilution factors in samples A to D derived from R1 and R2 was sufficiently precise for six sets of data from five laboratories. Laboratories 4 and 5 were unable to detect HBV DNA in highly diluted samples B and D, as was expected for hybridization assays. The data from laboratory 6 were not precise enough. Furthermore, the results from laboratory 6 were lower than the next lowest results by a factor of 30 or more. Laboratory 7 provided only the endpoint titer of a semiquantitative hybridization assay but no value for *N* or picograms of HBV DNA.

The results from laboratories 1 to 5 for dilutions A to D were multiplied by the corresponding true dilution factors and used together with the results for R1 and R2 to calculate the geometric mean value of *N* per milliliter: 1.04×10^9 (95% CI, 6.0×10^8 to 1.5×10^9) for R1 and 1.13×10^9 (95% CI, 6.3×10^8 to 2.0×10^9) for R2.

From the data in Table 2, it is apparent that laboratory 2 found consistently more HBV DNA in all samples than laboratory 1, although both laboratories used the limiting dilution assay and nested PCR for quantitation. This technique inherently has a large SD, but the observed differences were significant. The results obtained by laboratories 4 and 5, which used different hybridization assays and different cloned HBV DNA samples for reference, were also significantly different.

The Eurohep Pathobiology Group decided after this evaluation to consider this result for *N* to be preliminary. Because, however, international reference samples were urgently needed, we decided to assign 10^9 arbitrary Eurohep Units/ml of HBV DNA to the two reference samples (8) and to consider 1 Eurohep Unit as a preliminary estimate for 1 HBV DNA molecule.

Evaluation by additional assays in phase 2. In order to find the reasons for the divergent results and to obtain a more accurate determination of *N*, the participants in phase 1, including some of those who had not yet provided data, were asked to repeat their determinations by improved methods. Laboratory 1 compared its original extraction procedure with phenol-chloroform with simple lysis of virions with 0.1 N NaOH and with the Qiagen procedure with silica columns. The last two methods yielded five times higher values of *N* than the previous one. For phase 2, laboratory 1 used two different primer sets covering the X and the S gene regions as described

TABLE 3. *N* in R1 and R2 measured in phase 2 by improved or reevaluated limiting-dilution assays

Sample and laboratory	Sample	*N*/ml (10^9)
R1[a]		
2	A	5.8
1b	R1	4.9
8	A	3.2
1a	R1	2.1
2	B	2.0
2	R1	1.7
8	R1	0.96
8	B	0.93
R2[b]		
8	R2	3.9
2	D	3.5
2	C	3.4
2	R2	3.1
1a	R2	2.5
8	C	2.1
8	D	1.2
1b	R2	0.96

[a] GMV, 2.2×10^9/ml; 95% CI, 1.3×10^9 to 3.7×10^9/ml.
[b] GMV, 2.3×10^9/ml; 95% CI, 1.6×10^9 to 3.5×10^9/ml.

in Table 1. Identical results were obtained with both primer sets, and the results are not shown separately in Table 3. Laboratory 2 reanalyzed R1 by the limiting-dilution assay with NaOH lysis of virions and another PCR technique and confirmed its previous results within the expected CI, as described elsewhere (7). An additional laboratory (laboratory 8) tested all samples from the Eurohep trial by PCR after lysis with NaI and provided consistent results. With the highly purified cloned HBV DNA sample from Chiron Corp, which contained 3.8×10^{11} copies of HBV DNA/ml, laboratory 8 found by limiting dilution 2.3×10^{11} copies of HBV DNA/ml, which is within the statistical variation of this assay. Furthermore, laboratories 1, 2, and 8 used the maximum-likelihood method to calculate *N* and obtained results slightly different from those obtained by the method of Reed and Muench method (18) that they used initially. The results of the limiting-dilution assays performed in phase 2 are summarized in Table 3.

Laboratory 4 repeated its hybridization assay also by using the reference sample from Chiron. Now, the *N* values were 2.8×10^9 for R1 and 2.4×10^9 for R2, which were higher by a factor of 1.6. Chiron Corporation found *N* values of 3.1×10^9 for R1 and 2.9×10^9 for R2 by using their reference DNA and their bDNA hybridization assay. Laboratory 5 repeated its hybridization assay with the Chiron reference sample and also obtained values that were higher than those obtained in phase 1 by a factor of 1.5. However, even after this correction, all the values were lower by a factor of 3 to 7, depending on the genotype, than those found by limiting-dilution assays. Thus, according to the predefined criteria, these data were not concordant and were not considered for further evaluation. Laboratory 10 provided data for samples R1, A, R2, and C obtained by the liquid hybridization assay (Genostics) from Abbott Laboratories. By this method the dilution factors in samples A and C were determined very precisely, with very low SDs, but the absolute values of 0.08×10^9/ml for R1 and 0.12×10^9/ml for R2 were lower than those obtained by the limiting-dilution assay by factors of 26 and 19, and these data were also not considered in further evaluations.

The two hybridization assays used by laboratories 4 and 9 are much more precise than the limiting-dilution assays, with coefficients of variation of 17 and 8.5%, respectively. The results of the hybridization assays agreed with those of the limiting-dilution assays within the 95% CI. It appeared to be appropriate to give the two determinations by the hybridization assays as much weight as the GMVs of the eight determinations obtained by the limiting-dilution assay. The GMVs of these three assays were 2.7×10^9 genomes/ml (95% CI, 2.1×10^9 to 3.4×10^9 genomes/ml) for R1 and 2.6×10^9 genomes/ml (95% CI, 2.1×10^9 to 3.0×10^9 genomes/ml) for R2.

DISCUSSION

The results of this study indicate that the accurate determination of *N* in serum or plasma samples containing HBV particles is not a trivial task. We confirm previous reports (3, 13, 24) that different hybridization assays generate highly divergent results. Butterworth et al. (3) described an in-house dot blot assay by which the values obtained for an HBV DNA-positive serum sample were 120 times lower than those obtained by the bDNA assay. The values obtained by the widely used Genostics liquid hybridization assay were also 100 times lower than those obtained in that particular study. The results of our own (K.-H.H. and W.H.G.) in-house dot blot hybridization assay for R1 and R2 (25) had to be corrected by use of a factor of 8 in light of our new data. This assay had a design similar to that of the dot blot assay from laboratory 5, which was not included for determination of the final result in phase 2. Virion-derived DNA does not behave, in most hybridization assays, like the cloned HBV DNA which is used as a reference sample. The attempt to use cloned HBV DNA encapsidated within a bacteriophage as a more realistic reference sample for the Genostics assay (14) also failed. Virion-derived HBV DNA contains a covalently linked protein. This protein remains bound to the DNA if it is not removed by digestion with protease. It significantly enhances the binding of HBV DNA to glass fibers in low-salt buffers and causes incomplete elution from purification columns. Furthermore, it causes extraction of HBV DNA in the phenol phase unless it is completely removed (9). Plasmid DNA into which HBV DNA is inserted is protein-free. Thus, calibration of assays of virion-derived DNA extracts with cloned DNA or consideration cloned DNA as a gold standard for the determination of virion-derived DNA is not justified unless the validity of the assumption has been proven. To overcome this problem, four participants in our study determined *N* in extracts from virions by limiting-dilution assays which were able to recognize one molecule. For this type of assay no reference sample is required. Laboratory 1 initially had difficulties completely recovering virion-derived HBV DNA from the samples, probably because undiluted plasma exhausted the capacity of proteinase K digestion under the conditions that were used. Incomplete recovery of HBV DNA from virions also seems to have occurred in the Eurohep quality control trial of the HBV PCR (17). The 39 participants in that trial received coded dilution series made from R1 or from purified cloned DNA and seven positive serum samples. Virus in one of these serum samples was missed by nine participants, who reported otherwise faultless results. These were considered "moderate performers." Six of the 10 "good performers" who detected all seven positive serum samples were able to detect 300 Eurohep Units/ml, but none of the moderate performers was able to do so. Even 30,000 Eurohep Units/ml was not detected by six of the nine moderate performers. In contrast, 300 and 30,000 copies of cloned DNA were detected with equal success rates by the good and the moderate performers. In theory, detection of HBV DNA in dilution of R1

should have been easier than detection of DNA in the samples with cloned HBV DNA because, as shown in this report, they contained 2.7 times more HBV DNA than anticipated by the data from phase 1. The incomplete extraction could not be recognized because no virion-based reference sample with known *N* was available to the participants at that time.

The consistent PCR results for R1 and R2 obtained by three laboratories that used four different methods of lysis or extraction and four different primer sets for PCR suggest that, within the statistical confidence intervals, the GMVs of *N* for R1 and R2 are close to the true value of *N*, although the possibility that minor amounts of HBV DNA were lost before PCR cannot be excluded. The fact that two quite different commercially available hybridization assays yielded concordant values which were well within the 95% CI of the limiting dilution assays suggests that these two assays detect cloned and virion-derived DNA equally well, at least within the accuracies of the limiting-dilution assays.

The numbers of HBV genomes in R1 and R2 are typical for low-level or asymptomatic HBV carriers with high concentrations of HBsAg and HBeAg. By immune electron microscopy, similar plasma samples were found to contain 2×10^{10} HBV-like particles per ml which were coimmunoprecipitated with the known number of 20-nm HBsAg particles in these samples (20a). The higher value for particle numbers than for genome numbers found in this study may be explained by the similar but nonidentical plasma samples, the potential overrepresentation of HBV particles in HBsAg immune complexes, and the existence of a variable proportion of empty HBV particles which may account for more than half of the particles (23a). Although not directly comparable to this study, our previous studies on particle numbers indicate that the virion-associated genome numbers found in this study are not unexpectedly high. For standardization of HBV DNA assays, the use of particle counts is, however, not technically feasible. Infectivity titers in HBeAg-positive samples from chimpanzee virus carriers are about 10^8 infectious doses/ml or greater (20). This implies, in agreement with previous studies (1, 21), that in such HBV carriers almost every 10th virion is infectious, although variations in this ratio are likely to occur in individual carriers. Our data also imply that virions are not heavily aggregated in such samples because *N* was found to be the same irrespective of whether the limiting-dilution assay was done with plasma dilutions or dilutions of the DNA extract (10a).

This study shows that the results of the widely used Genostics assay are very precise, but they must be multiplied by a factor of 28 if the genotype of HBV DNA is not known. The correction factor suggests that the total viral release in a carrier with a high level of viremia is not $\simeq 10^{11}$ virions/day, as determined by Genostics, but is closer to 10^{13}/day (16). It appears that the Genostics assay reacted better with genotype D than with genotype A by a factor of 1.5. A genotype effect was also found by laboratory 5, but in laboratory 5 genotype A was better detected than genotype D by a factor of 2. In light of these observations it is highly advisable to evaluate a new method for the quantitative determination of HBV DNA first with R1 and R2 or similarly designed reference samples infected with different genotypes. Only if samples with virions of different genotypes yield consistent results that are comparable to those obtained with cloned DNA may these less practical reference samples be replaced by samples containing cloned HBV DNA of one genotype. Assays showing significant differences by genotype are not suitable for use for routine diagnosis in the laboratory because genotyping will usually not be done. If such assays are used, the calibrations obtained with R1 and R2 may not be valid for genotypes (15) which are prevalent in East Asia (genotypes B and C), Africa (genotype E), and South America (genotype F). Depending on the genome region of the PCR amplification or hybridization probe, different results may be obtained if the HBV carrier contains genomes with large deletions. This was, however, not the case for R1 or R2 because PCRs covering either the X or S gene region yielded identical results.

The use of reference samples R1 and R2 has been essential for clarifying the divergences between various assays and for quality control trials. They do not satisfy the requirements of a World Health Organization-approved international standard concerning volume and lyophilization. It is planned that such a standard be derived by diluting R1 in HBV-negative cryoglobulin supernatant and freeze-drying it for that purpose (19a). R1 and R2 remain available for special studies.

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REFERENCES

1. **Berninger, M., M. Hammer, B. Hoyer, and J. L. Gerin.** 1982. An assay for the detection of the DNA genome of hepatitis B virus in serum. J. Med. Virol. **9:**57–68.
2. **Biswas, R., W. H. Gerlich, A. Schober, and R. Thomssen.** 1976. Determination of antibodies against subtypes of hepatitis B surface antigens (HBsAg) proceedings. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **235:**310–315.
3. **Butterworth, L. A., S. L. Prior, P. J. Buda, J. L. Faogali, and W. G. Cooksley.** 1996. Comparison of four methods for quantitative measurement of hepatitis B viral DNA. J. Hepatol. **64:**686–691.
4. **Caspari, G., and W. H. Gerlich.** 1998. Mandatory hepatitis B virus testing for doctors. Lancet **1352:**991.
5. **Chudy, M., C. M. Nübling, H. Scheiblauer, H. Willkommen, R. Kurth, and J. Löwer.** 1995. Virusübertragungen durch Blutprodukte: Mögliche Ursachen und Konsequenzen. Hämostaseologie **15:**215–219.
6. **Davies, B. D., R. Dulbecco, H. N. Eisen, and H. S. Ginsberg.** 1980. Microbiology, 3rd ed., p. 880–883. Harper & Row, Publishers, Inc., New York, N.Y.
7. **Erhardt, W., S. Schaefer, N. Athanassiou, M. Kann, and W. H. Gerlich.** 1996. Quantitative assay of PCR-amplified hepatitis B virus DNA using a peroxidase-labelled DNA probe and enhanced chemiluminescence. J. Clin. Microbiol. **34:**1885–1891.

7a. **Gerlich, W. H.** Unpublished data.

8. **Gerlich, W. H., K.-H. Heermann, R. Thomssen, and The Eurohep Group.** 1995. Quantitative assays for hepatitis B virus DNA: standardization and quality control. Viral Hep. Rev. **1:**53–57.
9. **Gerlich, W. H., and W. S. Robinson.** 1980. Hepatitis B virus contains protein attached to the 5′ terminus of its complete DNA strand. Cell **21:**801–809.
10. **Heermann, K. H., Y. Hagos, and R. Thomssen.** 1994. Liquid-phase hybridization and capture of hepatitis B virus DNA with magnetic beads and fluorescence detection of PCR product. J. Virol. Methods **50:**43–57.

10a. **Heermann, K. H., and R. Thomssen.** Data not shown.

11. **Hendricks, D. A., B. J. Stowe, B. S. Hoo, J. Kolberg, B. D. Irvine, P. D. Neuwald, M. S. Urdea, and R. P. Perillo.** 1995. Quantitation of HBV DNA in human serum using a branched DNA (bDNA) signal amplification assay. Am. J. Clin. Pathol. **104:**537–546.
12. **Jalava, T., M. Ranki, M. Bengtstrom, P. Pohjanpelto, and A. Kallio.** 1992. A rapid and quantitative solution hybridization method for detection of HBV DNA in serum. J. Virol. Methods **36:**171–180.
13. **Janssen, H. L., Y. A. Schoenmaker-Weber, H. Kruining, S. W. Schalm, and R. A. Heijtink.** 1993. Quantitative assessment of hepatitis B virus DNA in chronic hepatitis B comparison of two solution hybridization assays. J. Med. Virol. **40:**307–312.
14. **Kuhns, M. C., A. L. McNamara, R. P. Perillo, C. M. Cabal, and C. R. Campbel.** 1989. Quantitation of hepatitis B viral DNA by solution hybridization: comparison with DNA polymerase and hepatitis B antigen during antiviral therapy. J. Med. Virol. **27:**274–281.
15. **Magnius, L. O., and H. Norder.** 1995. Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. Intervirology **38:**24–34.

16. **Nowak, M. A., S. Bonhoeffer, A. M. Hill, R. Boehme, H. C. Thomas, and H. McDade.** 1996. Viral dynamics in hepatitis B virus infection. Proc. Natl. Acad. Sci. USA **93:**4398–4402.
17. **Quint, W. G., R. A. Heijtink, J. Schirm, W. H. Gerlich, and H. G. Niesters.** 1995. Reliability of methods for hepatitis B virus DNA detection. J. Clin. Microbiol. **33:**225–228.
18. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27:**493–497.
19. **Repp, R., S. Rhiel, K. H. Heermann, S. Schaefer, C. Keller, P. Ndumbe, F. Lampert, and W. H. Gerlich.** 1993. Genotyping by multiplex polymerase chain reaction for detection of endemic hepatitis B virus transmission. J. Clin. Microbiol. **31:**1095–1102.
19a. **Saldanha, J.** Personal communication.
19b. **Schaefer, S., and H. Naumann.** EMBL accession no. AJ012207.
20. **Shikata, T., T. Karasawa, K. Abe, T. Uzawa, T. Oda, M. Imai, M. Mayumi, and Y. Moritsugu.** 1977. Hepatitis B e antigen and infectivity of hepatitis B virus. J. Infect. Dis. **136:**571–576.
20a. **Tralle, S., and R. Thomssen.** Unpublished data.
21. **Ulrich, P. P., R. A. Bhat, B. Seto, D. Mack, J. Sninsky, and G. N. Vyas.** 1989. Enzymatic amplification of hepatitis B virus DNA in serum compared with infectivity testing in chimpanzees. J. Infect. Dis. **160:**37–43.
22. **Valenzuela, P., M. Quiroga, J. Zalvidar, P. Gray, and W. J. Rutter.** 1980. The nucleotide sequence of the hepatitis B viral genome and the identification of the major viral genes, p. 57–70. *In* B. N. Fields et al. (ed.), Animal virus genetics. Academic Press, Inc., New York, N.Y.
23. **Wang, L., K. Hirayasu, M. Ishizawa, and Y. Kobayashi.** 1994. Purification of genomic DNA from human whole blood by isopropanol-fractionation with concentrated NaI and SDS. Nucleic Acids Res. **22:**1774–1775.
23a. **Welzbacher, W., and W. Gerlich.** Unpublished data.
24. **Zaaijer, H. L., F. ter Borg, H. T. Cuypers, M. C. Hermus, and P. N. Lelie.** 1994. Comparison of methods for detection of hepatitis B virus DNA. J. Clin. Microbiol. **32:**2088–2091.
25. **Zyzik, E., W. H. Gerlich, A. Uy, H. Köchel, and R. Thomssen.** 1986. Assay of hepatitis B virus genome titers in sera of infected subjects. Eur. J. Clin. Microbiol. **5:**330–335.