Quantitative PCR for Hepatitis B Virus with Colorimetric Detection

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A novel, sensitive colorimetric test is described for quantification of the initial number of hepatitis B virus (HBV) genomes amplified in PCR. The viral genomes are amplified together with a synthetic internal standard (IS) to correct for the variability of the efficiency factor. One of the two primers is biotinylated, and the amplified mixtures of HBV and IS DNAs are bound to streptavidin-coated microtiter plates for quantitative detection. The ratio of HBV to IS DNA is determined for each sample by hybridization with DNP-containing probes and immunoenzymatic detection. The colorimetric detection is quantitative, rapid, and accurate with a dynamic range from $\sim 10^8$ to $>10^{11}$ DNA molecules. The initial number of HBV genomes in a clinical sample is interpreted from the signal ratio HBV/IS by using a standard curve, obtained from coamplification of known quantities of synthetic HBV templates with IS. The assay quantified 15 viral genomes from 10 μ l of serum, and its dynamic range was up to five orders of magnitude. After the amplification step, the assay takes <2 hr, and the method is applicable to automation.

The *in vitro* DNA amplification methods are restricted primarily to qualitative analyses, including the "semiquantitative PCR assays."⁽¹⁾ There would be need for validated quantitative assays, especially if they were simple enough for routine diagnostic use. We describe here a practical, rapid, and sensitive nonradioactive method for quantification of the initial number of hepatitis B virus (HBV) templates from clinical samples. The method is principally applicable to any DNA.

In PCR the amount of amplified material is $N = N_0 (1 + \text{eff})^n$, where N_0 is the initial amount of template, *eff* is the efficiency, and *n* is the number of cycles. The efficiency of amplification varies from sample to sample and also decreases during cycling. The estimation of the initial quantity of template has been considered difficult because the efficiency factor may be decreased, especially in clinical samples, depending on their quality. Some PCR inhibitors from serum may not be removed completely, and reagents used in pretreatment can be inhibitory. False-negative results may occur without proper standardization. Moreover, the reproducibility of releasing the DNA from the clinical specimen is an important factor affecting the result. In the present test, quantification is based on coamplification with an internal standard, which has been applied to eliminate sample-dependent variation in amplification efficiency.^(2–8) We have described earlier the principle of coamplification in the form a prototype test.⁽⁹⁾

Qualitative PCR for HBV DNA has been described frequently,^(10–13) and the sensitivity of PCR tests has been shown to be at least as good as that of infectivity

testings with chimpanzees.⁽¹⁴⁾ A quantitative PCR assay, capable of monitoring HBV DNA levels at higher sensitivity than present tests, would be required for assessing the efficacy of antiviral therapy.^(12,15–17) A sensitive and quantitative test would also be useful in assessing potential infectivity of problematic cases of donated blood units⁽¹⁸⁾ and for assessing whether asymptomatic chronic carriers are contagious.

MATERIALS AND METHODS

DNA Standards, Respective Primers, and Probes

The HBV DNA standard template was synthesized using an Applied Biosystems 381 A Synthesizer. It contained the 104-bp long sequence 1864–1967 from the pre-C and C gene region of HBV genome of subtype ayw.⁽¹⁹⁾ The synthetic internal standard (IS) DNA was a mutant version of the former, in that the 21-mer internal sequence was replaced by an unrelated sequence. The molecular concentrations of the purified DNAs were determined by spectrophotometry. The primers used were 5'-biotin-GTT CAI GCC TCC AAG CTG TG and 5'-TCA GAA GGC AAA AAI GAG AGT AAC T (I is inosine). These primers were amplifying DNA from the C and pre-C region of the HBV genome⁽¹⁹⁾ and were obtained from MedProbe A.S. (Oslo, Norway).

In the reference amplification system 2, longer and nonhomologous competing DNAs were amplified.⁽⁹⁾ The HBV and IS sequences in this system were amplified with common primers and were recognized by the probes used in the test. The standard plasmid DNAs were linearized. The primers for the reference amplification system were 5'-B₂₋₄-TTC

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AAG CCT CCA AGC TGT GCC T (which is overlapping with the biotinylated primer above), and 5'-GTT AGC GGC GCA GCG TCT.⁽²⁰⁾ Here, B was a biotinylated C added to the oligonucleotide in an Applied Biosystems A 381 synthesizer using the respective phosphoramidite reagent (M. Bengtström, L. Paulin, and B. Sproat, unpubl.).

The detection probe for HBV DNA (5'-CTT TAT AAG GAT CAA TGT CCA TGC) was modified with a dinitrophenyl (DNP) group at the 5' and 3' ends. Also a probe with three DNP groups at the 5' end was used (British Biotechnology, Abingdon, Oxon, UK). The detection probe for IS DNA was similarly labeled.

Clinical Samples

Patient sera were obtained from the Department of Virology, University of Helsinki, Finland. Negative control serum was from the Finnish Red Cross. All samples were also tested with the commercial quantitative AffiProbe HepB test for HBV DNA (Sangtec Medical, Bromma, Sweden). A HBV-positive reference plasma (see ref. 1, genotype A) analyzed in the Eurohep standardization project⁽²¹⁾ was obtained from W.H. Gerlich (University of Giessen, Germany).

For the quantitative PCR, serum was pretrated by precipitation with polyethylene glycol followed by lysis with NaOH, neutralization, boiling, and centrifugation. An aliquot of the supernatant corresponding to 10–40 μ l of original serum was subjected to amplification by PCR (M. Uusi-Oukari, A. Kähärä-Uppgård, M. Ranki, and P. Lehtovaara, unpubl.).

Amplification in Competitive PCR

In addition to HBV DNA, the PCR reaction contained 50 mM Tris-HCl (pH 8.8), 1.5 mM MgCl₂, 15 mM (NH₄)₂SO₄, 0.1% Tween 20, 0.2 mM dNTPs, 0.2 μ M amplification primers, 10³ molecules of IS DNA, and 1 unit of *Taq* polymerase (Promega Corp., Madison, WI) or thermostable DNA polymerase (DynaZyme, Finnzymes, Finland) in a total volume of 100 μ l. The samples were overlaid with two drops of mineral oil. Thirty cycles of amplification (DNA Thermal Cycler, Perkin-Elmer Cetus or PTC-100-60, MJ Research Inc., Watertown, MA) consisted of 30 sec of denaturation at 96°C, 1 min

of primer annealing at 55°C, and 1 min of primer extension at 72°C, except that in the first cycle the denaturation step at 96°C was extended to 6 min and the last extension step to 11 min.

Detection on Microtiter Plates

Each sample was to be hybridized with two different probes (HBV and IS specific, respectively); therefore, 50- μ l aliquots from each PCR sample, first diluted 1 : 4, were pipetted to microtiter plate wells coated with streptavidin (see

Fig. 1; HepB sensitive test kit, Sangtec Medical, Bromma, Sweden). Two parallel hybridizations were usually performed. The plate was shaken for 15 min at room temperature in a plate shaker. Fifty microliters of 100 mM NaOH and 300 mM NaCl were added to the wells and, after shaking for 1 min, the plates were washed three times with 25 mM Tris-HCl (pH 7.5), 125 mM NaCl, 2 mM MgCl₂, and 0.3% Tween in a microtiter plate washer. The HBV- or IS-specific DNP probe was added at a concentration of 10¹² molecules per well, and hybridization oc-

FIGURE 1 Principle of the quantification assay for HBV DNA. The assay includes (1) Competitive amplification of 104-bp-long HBV DNA and IS DNA sequences using common primers (one of which is biotinylated). The IS sequence is designed to differ from the amplified HBV sequence only in the 21-mer region shown in black. (2) Binding of the biotinylated DNAs to the streptavidin-coated microtiter plate. Each sample is to be detected with two different probes. (3) Removal of the nonbiotinylated strands and hybridization with DNP-labeled HBV and IS-specific oligonucleotides, respectively. (4) Colorimetric immunoassay detection of the hybridized probes, giving numerical quantitative results for HBV DNA and IS DNA content in each sample. The signal ratio HBV/IS is calculated.

curred at room temperature for 15 min with shaking.

The plate was washed six times and anti-DNP-alkaline phosphatase conjugate was added [50 μ l of 25 mM HEPES (pH 7.5), 125 mM NaCl, 2 mM $MgCl_2$, 1% BSA, and 0.3% Tween]. After the plate was shaken at room temperature for 15 min, it was washed six times, and the substrate *p*-nitrophenyl phosphate (pNPP) was added as 4 mg/ml of solution in standard diethanolamine- $MgCl_2$ buffer in a volume of 100 μ l. The reaction was allowed to proceed at room temperature with shaking, and the plate was read spectrophotometrically at 405 nm (Multiscan Plus plate reader, Lab-systems, Helsinki, Finland) after suitable time intervals (e.g., 4, 10, 30 min).

RESULTS

Outline of the Assay

The substance of the assay is illustrated in Figure 1. The clinical sample is pre-treated to release the viral genome quantitatively, and an aliquot corresponding to 10 μ l (up to 50 μ l) of serum is subjected to PCR amplification, together with 10^3 molecules of a synthetic (IS). The IS sequence is identical to the amplified 104-mer sequence of the HBV genome except that it contains an internal 21-mer replacement. Coamplification is continued for 30 cycles.

The amplified HBV and IS DNA, both of which are biotinylated at one 5' end, are quantified by binding to streptavidin-coated microtiter plates, removing the nonbiotinylated strands, and hybridizing with an HBV-specific and an IS-specific probe, respectively. The hybridized DNP label is quantified in an immunoenzymatic reaction, and the ratio of the HBV- and IS-specific signals is determined. The initial quantity of the HBV genome is interpreted from a standard curve.

The Standard Curve

To obtain the standard curve (Fig. 2), known numbers of molecules of the synthetic HBV standard template (usually 10^2 – 10^6 molecules) were coamplified with 10^3 molecules of IS. The signal ratios of HBV/IS were determined and plotted against the initial number of HBV DNA molecules in a logarithmic scale. If the two competing templates were am-

FIGURE 2 Competitive amplification of HBV DNA and IS DNA: the standard curve for the assay with 104-bp long synthetic templates with 80% homology (●) and from the reference amplification system 2 (■). The initial number of IS DNA molecules was always 10^3 . The two standard curves were each a mean of three to four individual experiments. The third linear curve is theoretical, assuming an identical amplification efficiency for HBV and IS DNA at all combinations.

plified with an identical amplification efficiency, and the initial ratio of the templates would not affect the efficiency, it would be possible to calculate the results directly (see theoretical curve, Fig. 2). The ratio of HBV/IS after amplification would be multiplied by the known initial quantity of IS, as the ratio would remain constant. However, the slope of the experimental curve in Figure 2, obtained by coamplifying the synthetic 104-bp standards, was less steep than that of the theoretical slope. Also dilution series of the authentic viral genome showed results similar to those from the synthetic template. Thus, the initial ratio of the two templates had some effect on the amplification efficiency, which had to be corrected by using a standard curve. The experimental standard curve established the correlation between the signal ratio HBV/IS and the initial quantity of HBV DNA over four to five orders of magnitude.

The synthetic 104-bp-long HBV and IS DNA standards were designed to have a high degree of homology (80% of the sequences were identical), because it has been generally assumed that the sequence of a competing IS ought to be as homologous as possible to the target DNA.^(4,22) We also studied the performance of the assay with an IS template totally unrelated to the HBV template except at the common primer regions (amplification system 2) (Fig. 2). Interest-

ingly, the competition among highly homologous DNAs and the competition of unrelated longer sequences gave almost indistinguishable results for the standard curves. The only minor differences were that the linear range was reproducibly larger and the efficiency was better with the homologous standards, which were amplified as shorter fragments. Both amplification systems can, in principle, be used in the quantitative test.

Validation of Quantitative Nonradioactive Detection

The amplified biotinylated products were quantitatively bound to a streptavidin-coated microtiter plate well in 15 min with shaking. The bound DNA was made single stranded with brief alkali treatment before hybridization with single-stranded, DNP-labeled oligonucleotide probe. Quantitative hybridization occurred rapidly, within 5–15 min at room temperature, depending on the probe concentration (data not shown).

The DNP-labeled hybrids were detected by an anti-DNP-alkaline phosphatase conjugate, which produced a spectrophotometrically measurable product from the pNPP substrate. Because the kinetics of this reaction was linear over 4 hr (Fig. 3), the dynamic range for the colorimetric detection could be expanded by taking advantage

FIGURE 3 Linearity of the enzyme reaction in colorimetric quantification of biotinylated HBV DNA on microtiter plates. Various amounts of purified PCR product (from amplification system 2) were bound to the plate and quantified. Hybridization was with DNP-labeled oligonucleotide containing an average of 1.5 DNP groups, and the hybrids were detected with anti-DNP-alkaline phosphatase conjugate. Formation of the reaction product from the pNPP substrate was followed at 405 nm at time intervals. The results were a mean of four parallel determinations.

of repeated measurements and extrapolation, for example, routinely measuring the enzyme reaction after 4, 10, and 30 min. It was possible to cover more than three orders of magnitude in this colorimetric measurement: The lowest detectable amount was $\sim 10^8$ DNA molecules

(in a 30-min reaction time) and the highest was $>10^{11}$ molecules (4 min) as shown in Figure 4. A cutoff value defined as a signal-to-noise ratio of 2, corresponded to $\sim 2 \times 10^8$ molecules of DNA, and defined as the mean of blank values + 3 s.d. to $\sim 10^8$ molecules (Fig. 4).

FIGURE 4 The dynamic range of the colorimetric quantification of biotinylated HBV DNA. The amplified 104-bp-long biotinylated HBV DNA was purified, and quantified spectrophotometrically, and different amounts were bound to the microtiter plate in a volume of 50 μ l. The detection probe contained three successive DNP groups at the 5' end. The cutoff limits 1 and 2 denote the mean of blank readings plus three standard deviations and a signal-to-noise ratio of 2, respectively.

The detection assay was quantitative and highly reproducible. The coefficient of variation (CV) for the detection step was 3.2%, measured in the experiment shown in Figure 3 with four parallel samples at each point.

Accuracy of Quantification of HBV DNA from Serum Samples

An important prerequisite for a quantitative PCR assay for HBV DNA is that the specimen pretreatment results in quantitative and reproducible DNA yields from different sera, irrespective of the virus concentration or the quality of the sample. We have developed a pretreatment method that gives quantitative and reproducible yields for viral DNA (M. Uusi-Oukari, A. Kähärä-Uppgård, M. Ranki, and P. Lehtovaara, in prep.).

A reference test for quantification was used to demonstrate the reliability of the quantitative PCR test. The AffiProbe HepB test⁽²³⁾ is a radioactive hybridization assay for nonamplified HBV DNA covering the dynamic range of 5×10^6 –

TABLE 1 Accuracy of Quantification of HBV DNA in Clinical Specimens

Serum sample	Number of HBV genomes (in 10 μ l)	
	expected ^a	detected in the PCR test
1	1.2×10^5	4.8×10^5
2	1.7×10^5	7.0×10^5
3	$<5 \times 10^4$	2.7×10^3
4	2.3×10^5	4.1×10^5
5	2.3×10^5	1.4×10^5
6	1.2×10^4	1.2×10^4
7	6.2×10^4	1.9×10^5
8	5.4×10^4	3.5×10^4
9	1.0×10^4	2.7×10^4
10	3.6×10^4	9.0×10^4
11	1.5×10^4	2.1×10^4
12	2.3×10^4	4.2×10^4
13	2.0×10^4	4.0×10^4
14	1.5×10^4	1.5×10^4
15 ^b	1.4×10^4	2.3×10^4

^aThe expected number of molecules is based on the AffiProbe results. The samples (except AffiProbe-negative sample 3) were diluted 1:100 with negative control serum before quantification with the PCR test.

^bThe undiluted sample 15 was pretreated with the method used for the PCR test before AffiProbe detection. After pretreatment, it was diluted with buffer and quantified with the PCR test using the reference amplification system.

5×10^8 viruses/ml and starting with 100 μ l of serum. The AffiProbe pretreatment inhibited the PCR test, but other data obtained (see sample 15, Table 1) suggest that both pretreatment methods were quantitative, and thus allowed the comparison.

Table 1 shows quantification of HBV genomes from 15 HBsAg- and HBeAg-positive clinical serum samples. The undiluted sera were quantified with the reference test, and the positive samples were diluted 1 : 100 with HBV-negative serum before the PCR test. The quantitative results from the two tests were in good agreement for all samples, demonstrating that the PCR test is reliable and accurate. The consistent, slightly higher titers obtained with the PCR method (on the average of 2.1-fold) presumably were caused by differences in standardization. The results also indicated that the test is not sensitive to strain differences (the viral strains were not known, but the sera were obtained from patients who were from Asia, Africa, and Western and Eastern Europe).

Dynamic Range and Reproducibility of Quantification from Serum Samples

Clinical samples were serially diluted

with normal human serum to assess the dependence of the quantitative result on the amount of virus in serum and to determine the sensitivity and dynamic range for quantification. As shown by an example in Figure 5A, the quantitative results were correctly dependent on virus concentration over up to five orders of magnitude. On the basis of the titer determined by the AffiProbe HepB test,⁽²³⁾ the dynamic range shown in Figure 5A was from 15 to 1.5×10^6 viral genome templates in 10 μ l of serum. Other clinical samples also gave similar curves. Thus, it can be concluded that the test is quantitative in a range of five orders of magnitudes.

The most sensitive range of the method was studied in more detail using the Eurohep reference specimen at our disposal. Its HBV DNA content had been determined to be 10^9 Eurohep U/ml, with 1 Eurohep unit being 1–5 HBV DNA molecules.⁽²¹⁾ By our quantitative AffiProbe HepB test,⁽²³⁾ its HBV DNA titer was 10^9 molecules/ml. The specimen was serially diluted with HBV-negative serum and these dilutions were subjected to the quantitative PCR test. Figure 5B shows that the test was capable of quantifying 16 viral genomes/10 μ l of serum. The titer of the undiluted Eurohep sample, determined from the dilution

(1 : 2×10^6) by using the standard curve was 1.4×10^9 /ml (Fig. 5B).

The reproducibility of the quantitative PCR test was good, taking into account the dynamic range. When four clinical samples with titers 10^4 – 10^6 HBV/ml were pretreated and quantified four times, the titers obtained had a CV percent of ~20% and maximally threefold differences could be detected (data not shown). Variation caused by the amplification reaction gave rise to no more than twofold differences in the titer, showing that a single amplification reaction from each sample was sufficient. The reproducibility of the test in the most sensitive region of the assay is shown in Table 2, where clinical samples containing very low quantities of virus (2.8×10^3 – 9×10^4 HBV/ml) were analyzed. Control serum gave extremely low signals irrespective of sample quantity, whereas the signals from viral samples responded to the quantity applied to PCR. Thus, volumes corresponding to at least 40 μ l of serum could be subjected to PCR to increase the sensitivity and reliability of the assay. The quantitative results obtained were highly reproducible even at the lowest end of the dynamic range.

DISCUSSION

The PCR test enables accurate quantifi-

FIGURE 5 The dynamic range and sensitivity of the quantitative test for clinical samples. (A) A clinical serum sample was serially diluted to negative control serum. The titer of the undiluted sample was 1.5×10^9 HBV DNA/ml in the AffiProbe assay, and the volume applied to the PCR reaction corresponded to 10 μ l. (B) Sensitivity of the PCR quantification as analyzed with the Eurohep standard plasma ($\blacktriangle$), serially diluted to negative control serum. The number of synthetic HBV and IS standard molecules ($\bullet$) applied to PCR is based on concentration determination of the stock solutions. The number of molecules shown for the dilution series of the Eurohep sample are calculated from the AffiProbe titer (1×10^9 HBV DNA/ml) of undiluted sample. (The cutoff for the lowest positive measurement was determined as follows: Both the HBV signal and HBV/IS signal ratio of the sample were at least twofold as compared with those from control serum.)

TABLE 2 Reproducibility of the Quantification from Clinical Specimens: Response to Sample Quantity

Serum sample ^a	$A_{405} \times 10^3$		Ratio (HBV/IS)	Quantity ^b	Titer (molecules/ml)
	HBV	IS			
0-10	7	675	0.01	—	—
0-20	6	813	0.01	—	—
0-40	8	634	0.01	—	—
I-10	40	596	0.07	70	7×10^3
I-20	124	972	0.13	130	6.5×10^3
I-40	187	735	0.25	260	6.5×10^3
II-10	28	512	0.05	40	4×10^3
II-20	46	801	0.06	60	3×10^3
II-40	85	750	0.11	110	2.8×10^3
III-10	68	574	0.12	100	1×10^4
III-20	197	716	0.28	290	1.5×10^4
III-40	413	628	0.66	700	1.8×10^4
IV-20	1070	698	1.53	1.8×10^3	9×10^4
IV-40	1380	640	2.2	2.9×10^3	7.3×10^4

^aTen microliters (first experiment) or 20 and 40 μ l (second experiment) of the clinical samples I-IV and the HBV-negative 0 control serum sample were pretreated and subjected to amplification.

^bThe ratio of the measured absorbance values was calculated and the quantity interpreted from the respective standard curve.

cation of HBV DNA from samples containing amounts of virus too low for conventional quantitative assays. The test is easy to perform and is well suited for research use and routine clinical diagnostics. The post-PCR steps are carried out on microtiter plates rapidly, with low coefficient of variation, and with enlarged dynamic range of colorimetric measurement. Amplified DNAs can be quantified in <2 hr.

The quantitative analysis of competitive PCR reactions typically has been tedious so far, with separation of the two products by electrophoretic techniques or HPLC.^(1,6,8,20) In most cases, quantification has been performed with isotopic label. Moreover, the competitive amplification is tedious when multiple PCR reactions are performed to titrate one sample with IS.^(2,4-8,22) In the present test, a single amplification is sufficient; therefore, several samples can be analyzed in one experiment. No tedious separation is necessary to quantify the amplified products. The nonradioactive detection system permits adequate and sensitive quantification, whereas the nonisotopic detection systems described previously for HBV DNA were used for qualitative assays only.^(24,25)

The IS template of the present assay was synthetic, 104-bp long, and 80% identical to the HBV DNA amplified. The assay was also completely functional,

however, with a nonhomologous IS (amplification system 2), indicating that there are several choices for a suitable IS DNA.

When different quantitative tests are compared, it is of interest to analyze their standardization and evaluate how close the results are to the absolute titers. The Eurohep reference plasma (1×10^9 to 5×10^9 HBV DNA/ml) gave the result 1.4×10^9 HBV DNA/ml in the PCR test and 1×10^9 HBV DNA/ml in AffiProbe HepB. The other clinical specimens that could be tested by both assays showed on the average of 2.1-fold (s.d. 1.2) higher titers in the PCR assay as compared with the AffiProbe HepB. In the PCR test, during the very first cycles, mispriming was more possible theoretically from the 3.2-kb genomic template than from the 104-bp-long HBV or IS templates. This could slightly overestimate the sample in the PCR test. The reference test AffiProbe, on the other hand, has a slightly underestimating standardization (vector sequences present both in standards and nick-translated probes). The conclusion is that all of the results were in good agreement and thus reliably quantified HBV DNA.

As in all PCR tests, contamination of unamplified samples with amplified material has to be prevented by standard precautions. The limitations caused by strain differences have been minimized

by amplifying a well-conserved region and using the universal base inosine in primers and probes. The AffiProbe test with long probes and no amplification is not sensitive to strain differences, and none of the samples tested so far with the two tests have shown clearly test-dependent results.

With each step optimized and validated, the test offers a convenient new possibility for accurate monitoring of HBV DNA levels in serum and plasma samples in a large dynamic range. The present sensitivity is likely to be sufficient for quantitative monitoring of the virus in most clinical applications.

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