

METHODOLOGY

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Development of a new ultra sensitive real-time PCR assay (ultra sensitive RTQ-PCR) for the quantification of HBV-DNA

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Abstract

Background: Improved sensitivity of HBV-DNA tests is of critical importance for the management of HBV infection. Our aim was to develop and assess a new ultra sensitive in-house real-time PCR assay for HBV-DNA quantification (ultra sensitive RTQ-PCR).

Results: Previously used HBV-DNA standards were calibrated against the WHO 1st International Standard for HBV-DNA (OptiQuant[®] HBV-DNA Quantification Panel, Accrometrix Europe B.V.). The 95% and 50% HBV-DNA detection end-point of the assay were 22.2 and 8.4 IU/mL. According to the calibration results, 1 IU/mL equals 2.8 copies/mL. Importantly the clinical performance of the ultra sensitive real-time PCR was tested similar (67%) to the Procleix Ultrio discriminatory HBV test (dHBV) (70%) in low-titer samples from patients with occult Hepatitis B. Finally, in the comparison of ultra sensitive RTQ-PCR with the commercially available COBAS TaqMan HBV Test, the in-house assay identified 94.7% of the 94 specimens as positive versus 90.4% identified by TaqMan, while the quantitative results that were positive by both assay were strongly correlated ($r = 0.979$).

Conclusions: We report a new ultra sensitive real time PCR molecular beacon based assay with remarkable analytical and clinical sensitivity, calibrated against the WHO 1st International standard.

Background

Chronic hepatitis B virus (HBV) infection can be assessed by evaluating clinical features and biochemical, virologic, and histologic parameters. Knowledge of HBV-DNA viral load is useful for predicting disease prognosis, determining infectivity, evaluating indications for treatment, assessing response to treatment identifying emergence of resistance, and diagnosing occult HBV infection [1-5].

There are several commercially available HBV-DNA tests. The oldest versions suffered from poor sensitivity (approximately $5 \cdot 10^5$ copies/mL) and a narrow dynamic range of HBV-DNA quantification ($\sim 3-4 \log_{10}$), while the current limit of detection of the newer assays is lower than 50 IU/mL with a dynamic range of approximately $8 \log_{10}$ [6-13]. Additionally several in-house

quantitative assays for HBV-DNA have been developed, based mostly on real-time PCR methodology, showing a remarkable sensitivity and a wide linear range for quantification [6,7,11-14].

Importantly, improved sensitivity of HBV-DNA tests is of critical importance for the management of HBV infection as well as for the diagnosis of occult HBV infection [HBsAg(-), HBV-DNA(+)] [15].

The objective of this study was to develop a new ultra sensitive real-time PCR assay based on the knowhow of the existing real-time PCR assay for the quantification of the HBV-DNA [12] and, also, to assess its performance characteristics.

Methods

DNA extraction

The HBV-DNA was extracted from 0.5 mL of serum/plasma using the QIAamp UltraSens Virus Kit (QIAGEN Inc., Valencia CA) and the DNA was eluted in 60 μ l of elution buffer. For each ultra sensitive RTQ-PCR

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HBV-DNA quantification measurement, a positive HBV-DNA sample with known HBV-DNA titer ($\sim 10^4$ IU/mL) and one negative control (negative human plasma) (Procleix® Negative Calibrator, Chiron/GenProbe, CA) were extracted additional to the unknown samples.

Ultra Sensitive RTQ-PCR

The assay was carried out into a LightCycler® 2.0 apparatus. The reaction mixture contained 1× reaction buffer (LightCycler DNA Master HybProbe; Roche Applied Science, Germany), 5 mM MgCl₂, 0.5 μM primer hbv305 (sense) 5'-GCCAAAATTCGCAGTCCC-3', 0.5 μM primer hbv460 (antisense) 5'-GATAGTCCAGAAGAACAACAAGAAG-3', 0.4 μM molecular beacon 5'-CGCGGATGAGGCATAGCAGCAGGATGAAGAACGCGG-3' labelled with FAM and Dabcyl at the 5' and 3' ends, respectively, Taq DNA polymerase, dNTP mix (with dUTP instead of dTTP), and 1 U of uracil-DNA glycosylase (Roche Applied Science, Germany). The amplification profile was as follows: One cycle of initial denaturation (95°C for 10 min) followed by 40 cycles of amplification at 95°C for 0 sec, 55°C for 10 sec, and at 72°C for 8 sec. The final reaction volume was 60 μl containing 30 μl of extracted viral DNA.

Analytical Sensitivity of the ultra sensitive RTQ-PCR

The sensitivity together with the 95% and 50% HBV-DNA detection limits of the assay were estimated by using the WHO 1st International Standard for HBV-DNA (OptiQuant® HBV-DNA Quantification Panel, Accrometrix Europe B.V.), tested in 8 replicas. We performed serial dilutions with human negative plasma (Procleix® Negative Calibrator, Chiron/GenProbe, CA) of the WHO standard at final concentrations of 100, 50, 25, 10 and 5 IU/mL. The 95% and 50% HBV-DNA detection limits were calculated by probit analysis (8 replicas).

Assessment in a low titer HBV-DNA panel

The clinical performance of the assay was tested on 27 samples drawn from 22 individuals with occult Hepatitis B (HBs Ag negative, consistently reactive by multiplex Procleix Ultrio HIV-1/HCV/HBV and Procleix Ultrio discriminatory HIV and HCV negative) screened with the discriminatory HBV assay (dHBV) (Chiron/GenProbe, Emeryville/San Diego, CA).

Calibration of the HBV-DNA values copies/ml vs IU/ml

The calibration of the HBV-DNA values was performed against the WHO 1st International Standard for HBV-DNA (OptiQuant® HBV-DNA Quantification Panel, Accrometrix Europe B.V.). Specifically, serial dilutions of the WHO standard ranging from $2 \cdot 10^2$ to $2 \cdot 10^6$ IU/mL in 5 individual sample preparations were tested by the

ultra sensitive RTQ-PCR method. Based on a linear regression, a conversion formula was calculated for the in-house measurements (copies/mL) to the international standard units (IU/mL).

Comparison of the ultra sensitive RTQ-PCR vs the previous real-time PCR assay

To compare the ultra sensitive RTQ-PCR assay with the previously reported test, HBV-DNA derived from 25 randomly selected samples was quantified using both methods. The correlation of the HBV-DNA quantifications by using the current and the previous in-house assay was also estimated.

Comparison of the ultra sensitive RTQ-PCR vs COBAS TaqMan HBV Test

To compare the ultra sensitive RTQ-PCR assay with the commercially available COBAS TaqMan HBV Test, plasma samples collected from 94 different HBV-infected patients were tested. The correlation of the HBV-DNA quantifications by using the in-house and the commercial test was also estimated.

Statistical analysis

Pearson's correlation coefficient was used to assess the strength of the linear association between the \log_{10} -transformed values of the estimated (copies/mL) versus the expected values (IU/mL) and also between HBV-DNA quantifications using the two in house real-time PCR assays and COBAS TaqMan as well. To compare the quantitative results obtained for the samples found positive by the two different methods (ultra sensitive RTQ-PCR vs COBAS TaqMan HBV Test) the fitted regression line was compared to the line of equality by testing the two-tailed hypothesis of slope = 1 and the intercept = 0.

Results

The ultra sensitive RTQ-PCR was performed in a LightCycler® 2.0 apparatus using a plasmid HBV-DNA standard as described previously. We should note that the specificity of the assay was 100% as shown previously [12]. The differences between the newly developed method (ultra sensitive RTQ-PCR) and the previous one [12] were: 1) the utilization of a molecular beacon instead of 2 hybridization probes, 2) extraction of HBV-DNA from a larger volume of serum/plasma (0.5 versus 0.2 mL), 3) use of 30 μl instead of 10 μl extracted DNA, 4) modified RTQ-PCR conditions, and 5) all experiments were performed in a LightCycler® 2.0 apparatus because the latter shows improved performance characteristics compared to LightCycler® 1.0. Moreover reaction volume in LightCycler® 2.0 can be as large as 100 μL compared to 20 μL in the LightCycler® 1.0.

Analytical sensitivity of ultra sensitive RTQ-PCR

To determine the analytical sensitivity of the ultra sensitive RTQ-PCR, 5 dilutions of the WHO 1st International Standard for HBV-DNA were tested (OptiQuant® HBV-DNA Quantification Panel, Accrometrix Europe B.V.). DNA concentrations above 25 IU/mL were detected on all 8 occasions; concentrations of 10 IU/mL were tested on 4 of 8 occasions (50%), while 5 IU/mL were detected on 2 out of 8 occasions (25%). The 95% and 50% detection end-point of the assay were 22.2 and 8.4 IU/mL respectively.

Performance of ultra sensitivity RTQ-PCR assay in low titer HBV-DNA clinical samples

The performance of the assay was tested on 27 samples collected from 22 individuals with occult Hepatitis B (HBsAg negative, HBV-DNA consistently reactive by Ultrio, Ultrio HIV and HCV negative). This analysis was part of another study concerning the molecular typing of occult Hepatitis B in blood donors across Greece

[15]. The characteristics of these patients are shown in Table 1. These samples were selected because of the low HBV-DNA titer. Among the 27 samples tested with the dHBV test, 19 (70%) were found to be above the threshold of detection, while a similar sensitivity was observed for the ultra sensitive RTQ-PCR method (18 samples tested positive out of 27, 67%). The mean HBV-DNA was 59.1 IU/mL (range, 10.2 to 346.7 IU/mL). We should note, however, that the dHBV assay was applied multiple times to 18 samples (a sample was considered positive, if it was tested above the threshold of detection at least once), while ultra sensitive RTQ-PCR was performed only once. As shown in table 2, 14 samples were detectable by both methods, while 4 and 5 samples were tested positive only by ultra sensitive RTQ-PCR and the dHBV test, respectively. These qualitative findings suggest that the in-house assay performed equally well as the commercial test, although the samples were tested multiple times by the latter method.

Table 1 Epidemiological, molecular and serological features of 27 samples obtained from 22 Blood Donors with Occult Hepatitis B Infection*

Patient ID	Sample	dHBV ¹	In-house Assay	IU/mL	aanti-HBc (IgM)	anti-Hbs	anti-HBe
Blood donor 1	1	+	+	69.2	-	-	-
	2	+	+	89.1	-	+	-
Blood donor 2	3	+	-		-	-	-
Blood donor 3	4	+	-		-	-	-
Blood donor 4	5	+	+	10.2	-	+	-
	6	+	+	40.7	-	+	-
Blood donor 5	7	+	+	25.1			
	8	-	+	97.7	-	+	-
Blood donor 6	9	+	+	46.8	-	-	+
Blood donor 7	10	+	+	25.1	-	+	+
Blood donor 8	11	+	-		-	-	+
Blood donor 9	12	+	+	47.9	-	-	+
Blood donor 10	13	+	+	346.7	-	-	+
Blood donor 11	14	+	+	26.9	-	+	-
Blood donor 12	15	-	-		-	-	+
Blood donor 13	16	+	-		-	+	-
Blood donor 14	17	+	+	13.5	-	+	-
Blood donor 15	18	+	+	27.5	-	+	-
Blood donor 16	19	-	-		-	-	+
Blood donor 17	20	-	+	15.8	-	+	+
	21	-	-		-	+	+
Blood donor 18	22	+	+	41.7	-	-	+
	23	-	-		-	-	+
Blood donor 19	24	-	+	25.1	-	+	+
Blood donor 20	25	+	+	91.2	-	-	-
Blood donor 21	26	+	-		-	-	-
Blood donor 22	27	-	+	24.0	+	+	+

¹dHBV: Procleix Ultrio discriminatory HBV test

*All subjects were HBsAg(-)/HBeAg(-)/IgG-anti-HBc(+)

Table 2 Efficiency of the ultra sensitive RTQ-PCR and HBV Procleix Ultrio discriminatory HBV tests (dHBV) on 27 samples collected from 22 individuals with occult Hepatitis B

Ultra sensitive RTQ-PCR	dHBV		Total
	+ (n. %)	- (n. %)	
+	14 (77.8%)	4 (22.2%)	18 (66.7%)
-	5 (55.6%)	4 (44.4%)	9 (33.3%)
Total	19 (70.4%)	8 (29.3%)	27 (100%)

Calibration of the HBV-DNA values obtained with the ultra sensitive RTQ-PCR against the WHO 1st International Standard for HBV-DNA

The calibration of the in-house assay was done against the WHO 1st International Standard for HBV-DNA (OptiQuant[®] HBV-DNA Quantification Panel, Accrometrix Europe B.V.) DNA was extracted and HBV-DNA was tested in 5 replicates by using the plasmid DNA as a standard. The correlation plot of the expected (\log_{10} HBV-DNA IU/mL) versus the estimated values by ultra sensitive RTQ-PCR (\log_{10} HBV-DNA copies/mL) is shown in Fig. 1. The fitted regression lines between IU/mL and copies/mL was given by the following equation: \log_{10} (IU/mL) = $-0.4475 + 1.0096 \cdot \log_{10}$ (ultra sensitive RTQ-PCR copies/mL), suggesting that in our setting, 1 IU/mL = 2.8 copies/mL. The 95% CI for the estimated intercept was -0.69, -0.21. The 95% CI for the estimated slope was: 0.96, 1.06. The correlation coefficient between

the expected and the estimated values was very good $r = 0.9937$ ($p < .001$) (Fig. 1).

Comparison of the ultra sensitive RTQ-PCR with the previous HBV-DNA test

To assess the correlation between the previous and the new assay, we re-tested 25 previously quantified samples. The average \log_{10} HBV-DNA was 5.38 copies/mL (range, 2.31 to 10.51 \log_{10} copies/mL). Importantly, the linearity was very good between the HBV-DNA values quantified by the two assays ($r = 0.985$, $p < .001$) over the whole range of quantification. The fitted regression line between the ultra sensitive RTQ-PCR and the previous test was: \log_{10} HBV-DNA (copies/mL) with the ultra sensitive RTQ-PCR = $-0.531 + 1.015x \log_{10}$ (copies/mL) with the previous real-time PCR test. The 95% CI for the estimated intercept was -0.92, -0.14. The 95% CI for the estimated slope was: 0.95, 1.08 (data not shown).

Comparison of the ultra sensitive RTQ-PCR with TaqMan

Among the 94 samples tested with the TaqMan test, 85 (90.4%) were found to be above the threshold of detection, while a higher sensitivity was observed for the ultra sensitive RTQ-PCR method (89 samples tested positive out of 94, 94.7%). As shown in Table 3, 83 (88.3%) samples were detectable by both methods, while 6 (6.4%) and 2 (2.1%) samples were tested positive only by the ultra sensitive RTQ-PCR and the TaqMan test,

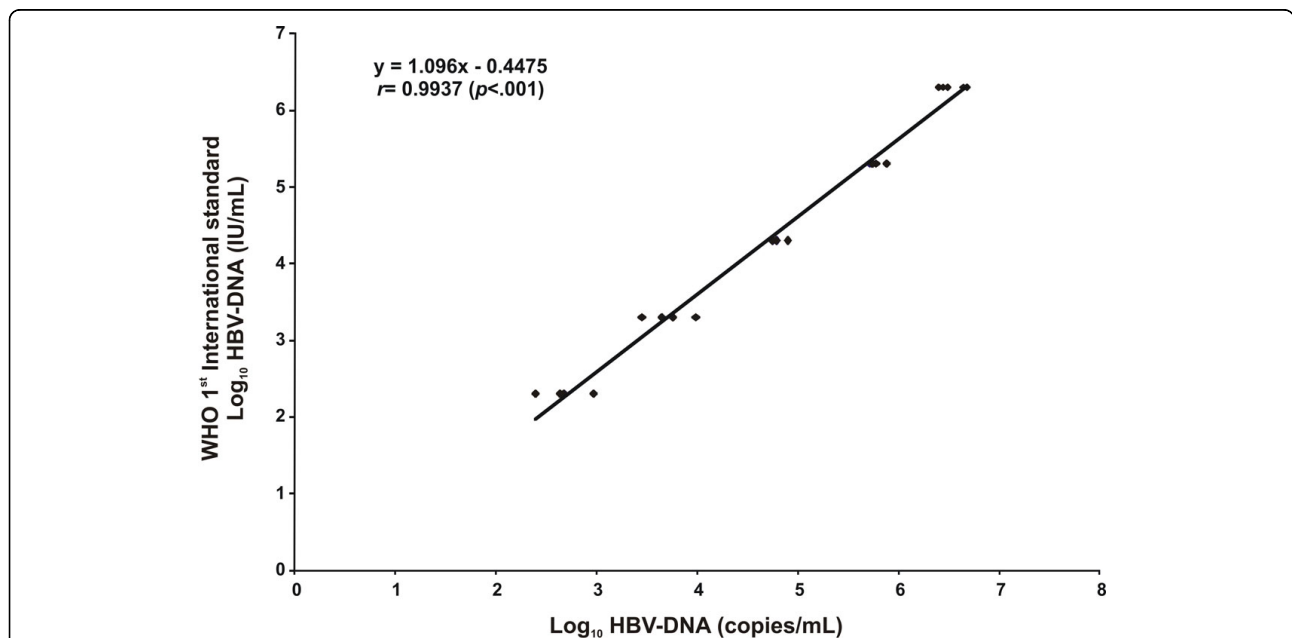


Figure 1 Calibration of the HBV-DNA values using the ultra-sensitive RTQ-PCR assay versus the WHO International Units. Correlation plot of the expected (\log_{10} HBV-DNA IU/mL) versus the estimated values by ultra sensitive RTQ-PCR (\log_{10} HBV-DNA copies/mL) tested on 5 serial concentrations of the WHO International standard ranging between 2×10^2 - 2×10^6 IU/mL by using the plasmid DNA as a standard.

Table 3 Efficiency of the ultra sensitive RTQ-PCR and COBAS TaqMan Test on 94 randomly selected samples with Hepatitis B

Ultra sensitive RTQ-PCR	COBAS TaqMan Test		Total
	+(n. %)	-(n. %)	
+	83 (88.3%)	6 (6.4%)	89 (94.7%)
-	2 (2.1%)	3 (3.2%)	5 (5.3%)
Total	85 (90.4%)	9 (9.6)	94 (100%)

respectively. These qualitative findings suggest that the new version of the in-house assay performed equally well as the commercially available TaqMan Test (McNemar's $p = 0.157$). The in-house assay found positive six samples (ranging from 1.37 to 1.83 \log_{10} IU/mL) with undetectable viral load levels with COBAS TaqMan HBV Test, while it did not quantify two samples (with viral load levels 1.08 and 1.77 \log_{10} IU/mL, respectively).

Fig. 2 shows the scatter plot of \log_{10} HBV-DNA IU/mL determined by the ultra sensitive RTQ-PCR and the TaqMan assays, using specimens with detectable HBV-DNA by both assays ($N = 83$). The results of the two assays were linearly correlated ($r = 0.979$, $p < 0.001$). The fitted regression line differs significantly from the line of equality and was described by the equation: \log_{10} (TaqMan, IU/mL) = $-0.457 + 0.987 \log_{10}$ (ultra sensitive RTQ-PCR, IU/mL) (joint test of intercept = 0 and slope = 1: $p < 0.001$).

Discussion

Sensitive detection and quantification of HBV-DNA is essential for monitoring response to therapy and, also,

for assessment of occult HBV infection. Therefore, ultra-sensitive assays for HBV-DNA quantification are needed in clinical practice.

In the current study, we describe the characteristics of a new ultra sensitive in-house RTQ-PCR performed in a LightCycler® 2.0 instrument (Roche, Molecular Biochemicals, Mannheim, Germany). The new assay showed improved sensitivity of 22 and 8 IU/mL as 95% and 50% detection end-points, respectively, versus the 94 IU/mL (250 copies/mL) of the previously reported quantitative test [12]. The sensitivity of the assay was improved mainly because of a larger volume of HBV-DNA used in ultra sensitive RTQ-PCR, extracted also from a larger volume of serum/plasma. Additionally, the new assay was carried out in the LightCycler® 2.0 that shows improved performance than the LightCycler® 1.0. We should note that the sensitivity of the assay can be potentially improved by using a larger than 0.5 mL volume of plasma, after a concentration step performed before the standard procedure. Moreover, the plasmid standard calibrated against the WHO 1st International standard, thus suggesting that the HBV-DNA values are reported in the international format (IU/mL).

Importantly, the ultra sensitive RTQ-PCR performed equally well as the qualitative dHBV assay tested on low-titer HBV-DNA samples collected from individuals with occult Hepatitis B. These findings are in accordance with the previously reported 95% detection limit of the dHBV, 19 IU/mL [16], which is almost identical to the 95% detection limit (22 IU/mL) of the ultra sensitive RTQ-PCR assay. These findings suggest that the ultra sensitive RTQ-PCR assay is equally reliable as the

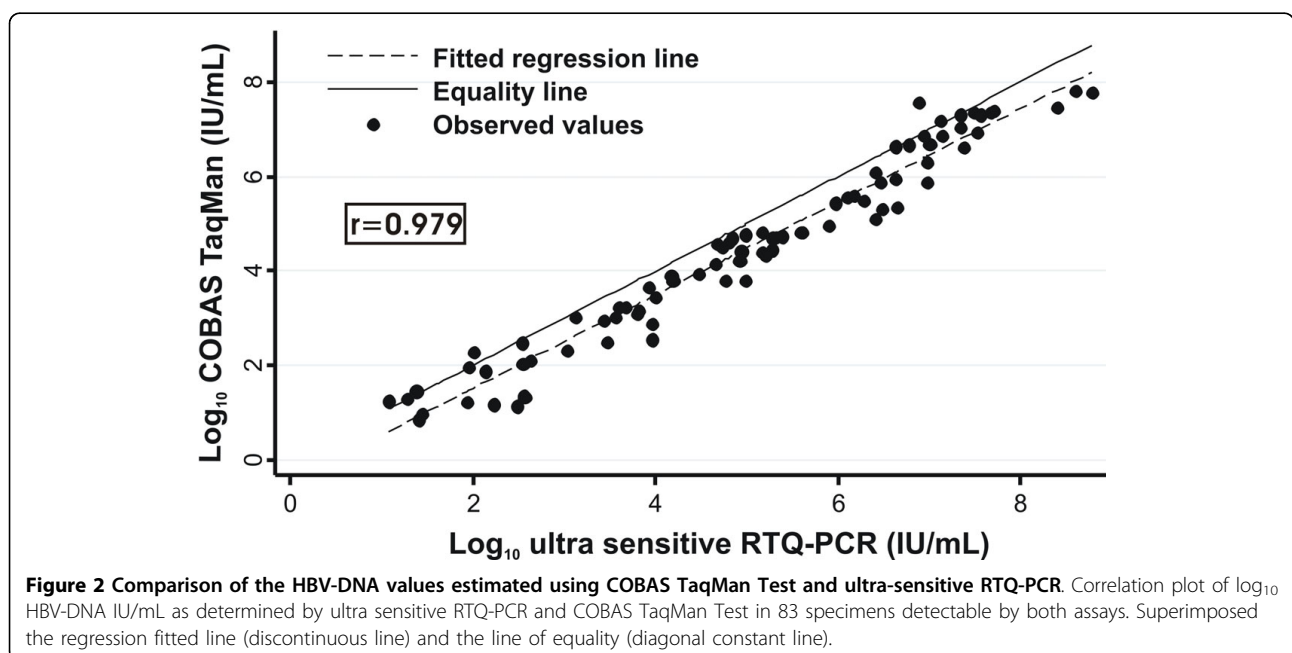


Figure 2 Comparison of the HBV-DNA values estimated using COBAS TaqMan Test and ultra-sensitive RTQ-PCR. Correlation plot of \log_{10} HBV-DNA IU/mL as determined by ultra sensitive RTQ-PCR and COBAS TaqMan Test in 83 specimens detectable by both assays. Superimposed the regression fitted line (discontinuous line) and the line of equality (diagonal constant line).

dHBV for detecting HBV-DNA in low-titer samples as those collected from patients with occult Hepatitis B. We should note however that the ultra sensitive RTQ-PCR is a quantitative test and therefore it shouldn't be used for diagnostic purposes.

The HBV-DNA quantified values between the ultra sensitive RTQ-PCR and the previously reported HBV-DNA test correlated very well, suggesting that the new version of the test can be utilized in occasions in which the original test was used. Furthermore, the ultra sensitive RTQ-PCR was found to be highly correlated with commercial available COBAS TaqMan HBV Test ($r = 0.979$, $p < 0.001$).

Conclusion

In the era of highly potent antivirals (e.g. tenofovir, entecavir) approved for the treatment of chronic Hepatitis B, it is of crucial importance to utilize highly sensitive assays for detecting and quantifying HBV-DNA. We report the new ultra sensitive assay, using molecular beacon as a detection system, with remarkable analytical and clinical sensitivity, calibrated against the WHO 1st International standard.

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Authors' contributions

DP participated to the study design and coordination, he prepared the manuscript and the reply to the reviewer's comments; AB and CH carried out the experiments; AK, ZM, HH, AV were responsible for the part of the study concerning the occult Hepatitis B infection; VS did the statistical analysis; AH was the study coordinator and participated to the writing and editing of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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