Short communication

Detection and quantitation of HBV DNA in miniaturized samples: Multi centre study to evaluate the performance of the COBAS® AmpliPrep/COBAS® TaqMan® hepatitis B virus (HBV) test v2.0 by the use of plasma or serum specimens

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ABSTRACT

Laboratory analysis of blood specimens is an increasingly important tool for rapid diagnosis and control of therapy. So, miniaturization of test systems is needed, but reduced specimens might impair test quality. For rapid detection and quantitation of HBV DNA, the COBAS® AmpliPrep/COBAS® TaqMan® HBV test has proved a robust instrument in routine diagnostic services. The test system has been modified recently for application of reduced samples of blood plasma and for blood serum, too.

The performance of this modified COBAS® AmpliPrep/COBAS® TaqMan® HBV v2.0 (HBV v2.0 (this test is currently not available in the USA)) test was evaluated by comparison with the former COBAS® AmpliPrep/COBAS® TaqMan® HBV v1.0 (HBV v1.0) test. In this study a platform correlation of both assay versions was done including 275 HBV DNA positive EDTA plasma samples. Comparable results were obtained ($R^2 = 0.97$, mean difference $-0.03 \log_{10}$ IU/ml). The verification of equivalency of the sample matrix (plasma vs. serum samples tested in HBV v2.0 in the same run) showed comparable results for all 278 samples with a $R^2 = 0.99$ and a mean difference of $0.06 \log_{10}$ IU/ml.

In conclusion, the new test version HBV v2.0 is highly specific and reproducible and quantifies accurately HBV DNA in EDTA plasma and serum samples from patients with chronic HBV infection.

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Detection of hepatitis B Virus (HBV) derived DNA in blood specimens is an excellent surrogate marker of virus infectivity. Quantitative viral DNA or RNA determination (viral load) is the best marker for evaluating early response to antiviral therapy in patients with chronic hepatitis B and C (Berger et al., 2001). Commercial assays for detecting and quantifying HBV DNA in clinical practice have been available for several years and automation of nucleic acid extraction has also become practical (Berger et al., 2002; Stelzl et al., 2004). More recently, real-time PCR-based assays are replacing other methods for quantitation of HBV DNA in routine diagnostic services. Many modifications of HBV DNA tests have been developed to meet the requirements of both high sensitivity and a broad linear range (Hochberger et al., 2006; Schumacher et al., 2007; Stürmer et al., 2009). Close tube format, internal positive controls, automated DNA extraction and validation of analytical assay properties are critical features for such assays (Panning et al., 2007). The COBAS® AmpliPrep/COBAS® TaqMan® (Roche Diagnostics, Basel, Switzerland) instrument for automated nucleic acid extraction and real-time PCR has been developed for quantitation of HBV, HIV-1 and HCV in human plasma (Hochberger et al., 2006; Schumacher et al., 2007; Sizmann et al., 2007). Recently, the COBAS® AmpliPrep/COBAS TaqMan® HBV Test (HBV v1.0, Roche Diagnostics, Basel, Switzerland) has been optimized to improve the utility of the test in terms of clinical samples. For example, serum specimens can be used in smaller amounts (i.e. 650 μl instead of 1050 μl are needed). In order to achieve the nearly same previous sensitivity and to use serum and plasma samples equally the sample lysis buffer was modified by adding more detergent and lowering the pH value. The linearity of HBV DNA quantitation by the COBAS® AmpliPrep/COBAS® TaqMan® HBV Test, v2.0 in EDTA plasma and serum matrices was approved previously by serial dilutions of highly concentrated HBV positive certified clinical specimens of genotype A. To verify the correct concentration assignment, one of the concentration levels was determined according to the Calibrator Bracketing Method with the HBV Secondary Standard as calibrator traceable to the WHO International Standard for Hepatitis B virus DNA (NIBSC Code 97/746 and NIBSC 97/750), genotype A (Goedel et al., 2009).

The experience with this new test system in the setting of routine diagnostic services is described. The objective of this study was

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(i) to demonstrate diagnostic specificity of the new test version HBV v2.0 using plasma and serum samples of HBV negative patients, (ii) to analyze the platform correlation of HBV v1.0 to HBV v2.0 and (iii) to investigate the matrix equivalency of HBV v2.0 using HBV positive EDTA plasma and serum samples.

Tests were carried out at three different test sites, i.e. site 1: Institute for Medical Virology, University Hospital Frankfurt/Main, Germany, site 2: Bioscientia GmbH Ingelheim, Germany, site 3: Roche Molecular Diagnostics AG, Rotkreuz, Switzerland.

The samples obtained from HBV positive and negative patients were collected and stored at −80 °C until use. All specimens were completely anonymized prior to testing. The study protocol was approved by the Ethics Committee of the University Hospital of Frankfurt.

All samples were processed and amplified using the docked version of the COBAS® AmpliPrep/COBAS® TaqMan® instrument (Roche Diagnostics, Basel, Switzerland). Test procedures of both test versions (COBAS® AmpliPrep/COBAS TaqMan® HBV Test 1.0 and 2.0 further referred to as HBV v1.0 and HBV) were carried out according to the instructions of the manufacturer and were performed as described previously (Allice et al., 2007; Chevaliez et al., 2008; Goedel et al., 2009). Each batch of 24 samples contained a high and low positive control, as well as a negative control. Furthermore, additional in-house internal-run controls (IRC-Ffm, IRC-Ing) were included in each test run of the particular test sites 1 and 2 as assay- and batch-independent positive controls.

The IRCs were prepared from HBV DNA positive plasma samples diluted in negative plasma to about 500 IU/ml (IRC-Ffm, test site 1) and approx. 7000 IU/ml (IRC-Ing, test site 2) as determined by HBV v1.0. The IRCs were divided into aliquots and stored at −80 °C until use.

Intra-assay reproducibility of the new test version was assessed by 20-fold testing of the IRC-Ffm in one experiment. Inter-assay reproducibility was estimated by analyzing the test kit control values (high and low positive controls, HPC and LPC) as well as the IRC-Ffm and IRC-Ing values from each test run.

Specimens from 743 HBV DNA negative patients (HBV surface antigen negative) were tested by HBV v1.0 (plasma only) and HBV v2.0 (matched serum and plasma samples) at three different test sites (test site 1 $n = 50$, test site 2 = 46, test site 3 $n = 647$ samples).

A total of 275 HBV positive plasma samples with HBV DNA concentrations ≥54 IU/ml and <110,000,000 IU/ml (determined by HBV v1.0) were tested by HBV v1.0 and HBV v2.0.

A total of 278 matched serum and EDTA plasma samples from patients infected with HBV and HBV DNA positive were evaluated for matrix equivalency with HBV v2.0 in the same run.

In specimens of 57 patients infected with HBV, the HBV genotype was determined by analyzing a specific part of the surface gene region using primers as described elsewhere (Tenney et al., 2004; Thibault et al., 1999). The HBV genotype was estimated by submitting the consensus sequence to both the HIV-GRADE HBV website (http://www.hiv-grade.de/hbv_grade) and the NCBI virus genotyping page http://www.ncbi.nlm.nih.gov/projects/genotyping).

To compare the performance of the different assay versions, Deming regression and Bland–Altman plots were generated. In a Bland–Altman plot the measurements for the same samples obtained on two different platforms were compared by plotting the pairwise means of the $\log_{10}$-transformed concentration values against the pairwise differences (significance level of 95%) (Bland and Altman, 2004).

All of the 743 HBV DNA free serum and EDTA plasma samples were negative (expressed as "target not detected") in the HBV 2.0 assay. This corresponds to an estimated specificity of 100%.

Intra-assay variability of the HBV v2.0 assay was determined by 20-fold testing of the IRC-Ffm in one test run. The calculated

mean value was 418 ± 145 IU/ml resulting in a coefficient of variation (CV) of 34.7%. Inter-assay variability of both HBV v1.0 and v2.0 was assessed by analyzing the results of the IRC-Ffm (test site 1) and IRC-Ing (test site 2) from several independent test runs. Additionally, values obtained of the high and low positive test kit controls (HPC and LPC) of all runs performed at test site 1 were analyzed. The mean values, standard deviations and CVs of the controls were calculated for the results obtained from 10 independent HBV v1.0 runs and HBV 2.0 runs (all site 1) and are compiled in Table 1. Both test versions had only a low inter-assay variation ranging from 8.8–18.7% in the HPCs and LPCs, respectively. In contrast, the IRC-Ffm demonstrated a relatively high CV of 43.4% in HBV v2.0. For the IRC-Ing (test site 2) CVs were found in a range of 11.5% (HBV v1.0) and 16.4% (HBV v2.0).

A total of 275 plasma samples from patients infected with HBV (HBsAg and HBV DNA positive) were tested in both test versions. Samples were selected according to their HBV DNA concentration within a range of 54–110,000,000 IU/ml (HBV v1.0).

As shown in Fig. 1, a significant correlation between the two test versions was obtained ($R^2 = 0.97$). Bland–Altman plotting of the $\log_{10}$-transformed test results of the 275 samples demonstrated that the mean difference between the two tests (i.e. the $\log_{10}$ IU/ml HBV v1.0 − the $\log_{10}$ IU/ml HBV v2.0) was $-0.03 \log_{10}$ (95% confidence interval: −0.50 to 0.44, Fig. 2). Three samples left showed differences within the range from 0.7 to 0.9 $\log_{10}$ IU/mL. None of the HBV v2.0 results was significantly different (differences >0.9 $\log_{10}$) from the corresponding version HBV v1.0 result.

HBV genotype (A to D) was determined in 57 patient specimens. Eleven patients were infected with genotype A, seven with genotype B, four with genotype C and 35 with genotype D, respectively. As shown in Fig. 3, no significant genotype-dependent difference was observed in the quantitation of the viral load using HBV v1.0 or HBV v2.0.

The analysis of the matrix equivalency (plasma vs. serum samples tested by HBV v2.0 in the same run) showed highly concordant results for all 278 samples, as shown by regression analysis (Fig. 4, $R^2 = 0.99$).

Bland–Altman analysis (plasma vs. serum, HBV v2.0) demonstrated a mean difference between the two samples matrices of 0.06 $\log_{10}$ IU/ml (95% confidence interval −0.23 to 0.34, Fig. 5).

Viral load quantitation assays must be highly sensitive, specific, precise, reproducible, and ideally calibrated by the use of World Health Organization (WHO) international standards. In addition, they should be rapid and automated with a minimum of hands-on time (Chevaliez et al., 2008). The COBAS® AmpliPrep/COBAS® TaqMan® instrument, a combination of real-time PCR with

Table 1
Inter-assay coefficient of variation (CV %) of the internal-run control and test kit controls (HBV v1.0 and HBV v2.0).

Sample		HBV v1.0 (IU/ml)	HBV v2.0 (IU/ml)
HPC site 1	Mean value	1,745,714 ($n = 10$)	1,504,800 ($n = 20$)
	±SD	324,544	257,146
	CV (%)	18.6	17.1
LPC site 1	Mean value	400 ($n = 10$)	556 ($n = 20$)
	±SD	35	104
	CV (%)	8.8	18.7
IRC-Ffm site1	Mean value	541 ($n = 10$)	493 ($n = 20$)
	±SD	117	212
	CV (%)	21.6	43.4
IRC-Ing site 2	Mean value	6807 ($n = 20$)	7731 ($n = 12$)
	±SD	783	1268
	CV (%)	11.5	16.4

HPC: high positive control; LPC: low positive control; IRC: in-house internal-run control; SD: standard deviation.
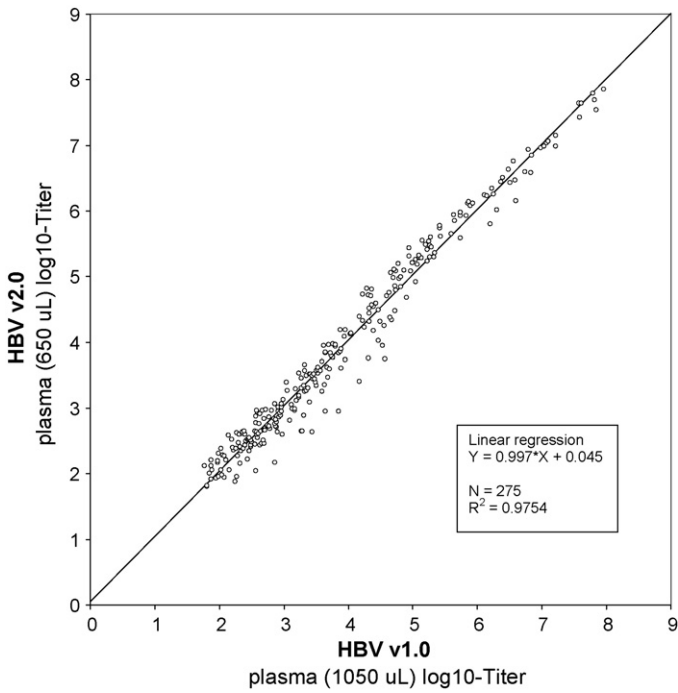
Fig. 1. Correlation between HBV determinations using the new test version HBV v2.0 in comparison to the former version HBV v1.0 by parallel testing of 275 plasma samples. Platform comparison by Deming regression analysis.

automated Nucleic Acid extraction for large routine diagnostic laboratories, has met these requirements (Allice et al., 2007; Hochberger et al., 2006; Ronsin et al., 2006). However, for quantitation of HBV load in human blood specimens, a relatively large amount of EDTA plasma was needed. Serum samples could not be processed. In the new version HBV v2.0 test, the sample input is reduced to almost half of the initial volume with a marginally lower limit of detection. Furthermore, not only plasma samples, but serum specimens can be applied with this version. In this multicentre study, the performance of the HBV v2.0 test was evaluated by comparison with the former HBV v1.0 test by analyzing clinical samples under the settings of a routine laboratory.

Since 743 HBV negative samples showed negative results by the HBV v2.0, the intrinsic performance of the new assay version revealed an excellent specificity. Platform correlation of both assay versions including 275 HBV DNA positive EDTA plasma samples revealed concordant results ($R^2 = 0.97$). Nevertheless, three plasma samples showed different test values between both versions (0.7–0.9 $\log_{10}$ IU/ml) demonstrating underquantitation of
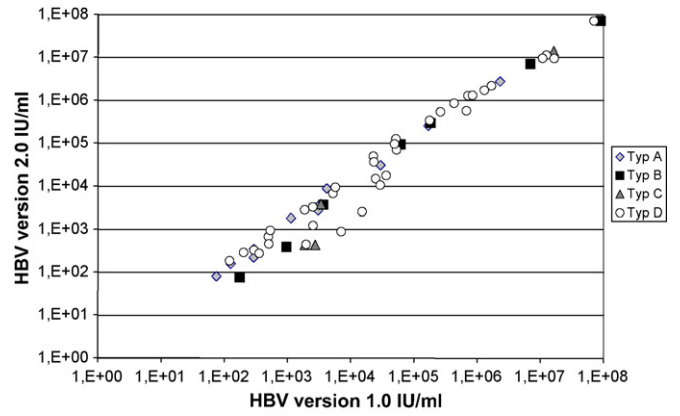

Fig. 3. Linear regression of plasma samples of 57 patients infected with four different HBV genotypes (A–D).
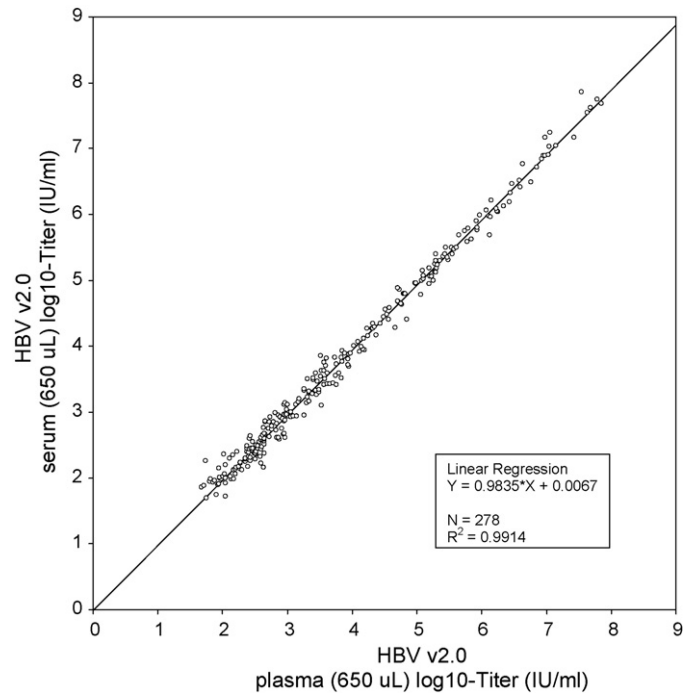

Fig. 4. Correlation of HBV DNA quantitation results using the new version 2.0 assessed on 278 parallel tested plasma and serum samples. Matrix comparison was done by Deming regression.
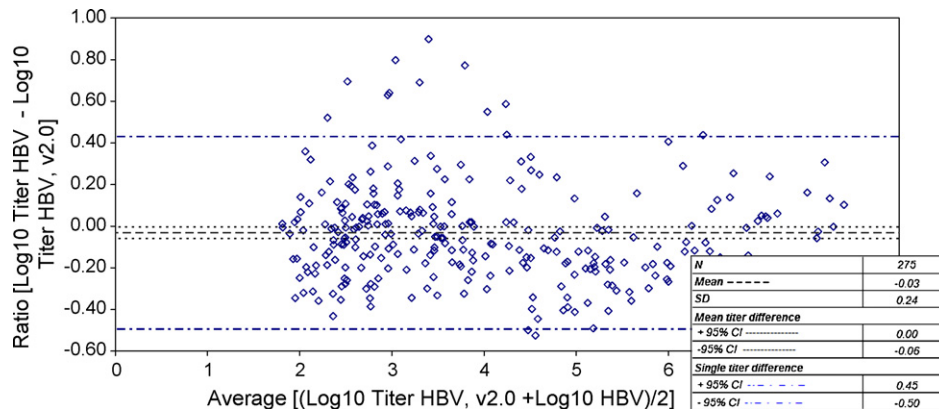

N	275
Mean -----	-0.03
SD	0.24
Mean titer difference	
+ 95% CI -------------	0.00
-95% CI ---------------	-0.06
Single titer difference	
+ 95% CI -- --- -- ---	0.45
- 95% CI -- --- -- ---	-0.50

Fig. 2. Bland–Altman plot analysis of the differences in HBV DNA quantitation between the version HBV v1.0 and HBVv2.0 as assessed on 275 plasma samples.
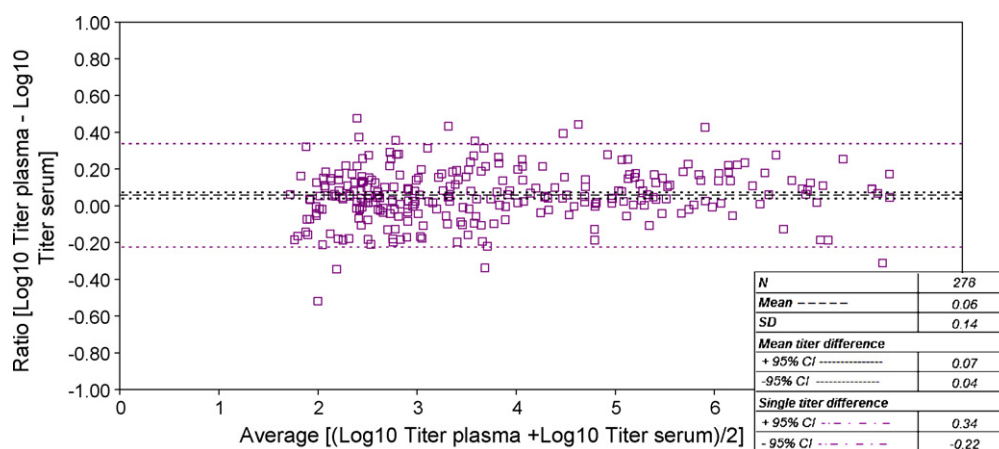
Fig. 5. Bland–Altman plot analysis of the differences in HBV DNA quantitation to determine the matrix equivalency of plasma and serum in the new version (HBV 2.0) as assessed on 278 patient serum and plasma samples tested parallel in the same run.

these sample when using the HBV v2.0 test. Serum samples taken simultaneously from these three patients were also tested using the HBV v2.0. The serum samples showed nearly the same results, confirming underquantitation of the HBV v2.0 in these individual patients (Table 2). Nevertheless, the vast majority of samples were quantified accurately independently from the type of storage (tested immediately or after freezing and thawing).

During the past years it has become evident that HBV genotype has an impact on the severity and progression of chronic hepatitis as well as on the efficacy of antiviral therapy, in particular of interferon treatment (Liu and Kao, 2008; Taylor et al., 2009; Zeuzem et al., 2009). Therefore, viral load should be quantified independent of HBV genotype. The reliability of both the HBV v1.0 and v2.0 systems for specimens from patients infected with HBV genotypes A–G was already demonstrated by other studies (Chevaliez et al., 2008; Goedel et al., 2009; Liu et al., 2007). This study confirms the findings of these investigations and shows an exact quantitation of HBV viral load in EDTA plasma independent of HBV assay version and genotypes A–D (Fig. 3).

Precision and reproducibility of the HBV v2.0 was comparable to the HBV v1.0, when assay LPCs and HPCs were used. Interestingly, a higher variance of the IRC-Ffm results was observed. An explanation could be that the IRC is less stable or less homogeneous than the test kit controls. In comparison to the IRC-Ing the mean viral load of the IRC-Ffm was determined to be more than 1 $\log_{10}$ lower. This could be another explanation for the higher variation of the IRC-Ffm in comparison to the IRC-Ing. In HBV v1.0 the same IRC delivered a mean value of 460 IU/ml with the same high CV of 47% ($n = 65$ runs performed previously, data not shown). The results of EDTA plasma samples obtained by HBV v2.0 and by HBV v1.0 tests revealed a mean difference of 0.03 $\log_{10}$ IU/ml. In addition, a mean difference of 0.06 $\log_{10}$ IU/ml was obtained by the HBV v2.0 when matched plasma and serum samples were analyzed. These results

could be an explanation for the slightly lower mean value of the IRC-Ffm when tested with the HBV v2.0. Despite the higher variation of these "in-house" controls, such controls are useful, because they are test kit and, above all, batch independent (Rabenau et al., 2007).

In conclusion, the results of the study shows that the COBAS® AmpliPrep/COBAS® TaqMan® HBV v2.0 assay yields highly specific and reproducible results and quantifies accurately HBV DNA both in EDTA plasma and serum samples from patients with chronic HBV infection. There are no significant differences of the test results between the HBV v2.0 to HBV v1.0. It is also demonstrated that the new test version works well with smaller sample input volumes of either serum or plasma without a relevant loss of sensitivity independent of the HBV genotype.

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Table 2
Plasma and serum viral loads measured with HBV vs. 1.0 and 2.0 of three samples with discordant results >0.7 $\log_{10}$ IU/ml.

Study number	HBV genotype	Sample matrix	HBV vs. 1.0 (IU/ml)	HBV vs. 2.0 (IU/ml)
G5	n.d.	Serum		328
		Plasma	2740	437
G22	C	Serum		3240
		Plasma	15,100	2550
F55	D	Serum		60,600
		Plasma	45,300	115,000

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