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Comparison of the Amplisure HBV Quantitative Kit with the Qiagen Artus HBV QS-RGQ Assay for Quantifying Viral DNA in Plasma Samples of Monitoring Cases

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Keywords
Hepatitis B virus · Chronic hepatitis B · Viral load · Treatment monitoring

Abstract
Background: Monitoring of hepatitis B virus (HBV) viral load has become an essential phase in the treatment of HBV. There are many commercial assays available for HBV viral load quantification. In this study, we have evaluated the performance characteristics of Amplisure® HBV Kit in comparison with the Qiagen artus HBV QS-RGQ kit for HBV DNA quantitation. ***Methods:*** Comparison of 2 methods was carried out on 200 clinical samples, 150 HBV DNA positive and 50 HBV DNA negative, by a reference method. Results obtained with Amplisure® HBV Kit (Amplisure HBV) were compared using the Qiagen artus HBV QS-RGQ assay results as the comparator method. ***Result:*** The overall performance of the Amplisure HBV compared with the comparator method shows positive and negative clinical agreement of 100 and 76%, respectively. Among the 12 qualitative discrepant samples, all positive with Amplisure HBV were sequenced and 10 were below comparator method's LOD. For 5 weak positives (−0.22 to 0.98 log IU/mL), the sequencing failed. The 7 other positives (0.48 to 1.89 log IU/mL) were confirmed positive by sequencing. Quantitative comparison gave an r^2 of 0.967 with a mean log difference of 0.09 $\log_{10}$ IU/mL. ***Conclusion:*** This study shows that Amplisure® HBV Quantitative Kit shows comparable performance with artus HBV QS-RGQ assays and can be useful in management and therapeutic monitoring of HBV in a clinical practice.

Introduction

Hepatitis B virus (HBV) infection is still a major health concern all over the world. Globally, an estimated 257 million people are infected with HBV, defined as HBsAg positive. In 2015, an estimated 8,87,000 deaths have occurred because of chronic HBV mainly due to complications such as cirrhosis and hepatocellular carcinoma [1]. Hepatitis B (Hep B) prevalence is the highest in the Western Pacific region and the African region, 6.2 and 6.1%, respectively. Also, in adult population, it is estimated 3.3, 2.0, and 1.6% in eastern Mediterranean region, Southeast Asia region, and European region, respectively.

In India, prevalence of HBsAg is 3–4.2% with over 40 million HBV carriers, and every year over 1,15,000 Indians die of HBV-related complications. There are 10

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known HBV genotypes, classified from A to J, all found in India. The most common genotype in India is D, followed by A and C [2–4]. Molecular testing using real-time PCR (RT-PCR) has made a big impact on viral HBV quantification. HBV DNA measurements play a critical role in determining the phase of infection, deciding the treatment, and detecting responses to the antiviral therapy [5, 6]. According to the guidelines for the prevention, care, and treatment for persons with chronic hepatitis B (CHB) infection from the World Health Organization and China, HBV DNA quantification is recommended in the treatment of CHB infections. [6].

The application of RT-PCR for viral diagnostics has been stated to reveal high sensitivity, a broad dynamic range, and short turnaround time, considered as the standard method for quantification [7, 8]. It is important to implement a highly reliable PCR-based assay to quantify HBV DNA in order to enable appropriate clinical management of Hep B infection. To date, many HBV DNA assays are commercially available [9–11]. In our study, we have verified the performance of the Amplisure® HBV Quantitative Kit in comparison with the Qiagen artus HBV QS-RGQ assay for HBV DNA quantification.

Materials and Methods

Flow of the study of Amplisure® HBV Quantitative Kit as compared to the comparator method is shown in Figure 1.

Settings

Sample collection was done by a Thyrocare service provider; DNA extraction and PCR assay was performed at Thyrocare Technologies Ltd., Turbhe, Navi Mumbai; and discrepant results were sequenced by RAS Lifesciences Pvt., Ltd., Hyderabad, through outsourcing to a contract research service provider.

Study Population and Specimens

This was a single-site, blinded, validation study to evaluate the performance of the Amplisure HBV Quantitative Kit considering Qiagen artus HBV QS-RGQ assay results as the comparator method. A total of 200 EDTA plasma samples were included in the study, which were received for routine testing for HBV viral load monitoring by the Thyrocare Laboratory. After processing and reporting the results by the laboratory, the leftover plasma samples that were adequate in quantity were selected for processing further with blinded numbers. The samples used for this study were selected from April 2018 to May 2018.

Artus HBV QS-RGQ Assay (Method 1 – Comparator)

Nucleic acid (60 µL HBV DNA) was extracted from 200 µL plasma by using the QIAsymphony SP and PCR assay and the DSP Virus Mini Pathogen Kit. Setup was performed on QIAgility using artus HBV QS-RGQ assay manufactured by Qiagen, Germany. QIAgility is a liquid dispenser; it mixes the DNA and master mix.

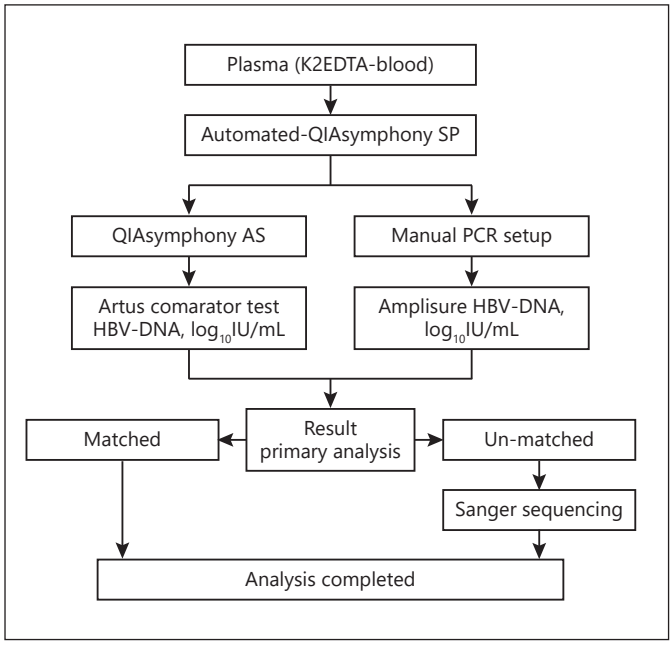

Fig. 1. Study design for evaluation of Amplisure HBV Quantitative Kit.

The assay contains reagents and enzymes for the specific amplification of a 134-bp region of the HBV genome. Five quantitative standards and NTC were incorporated in each batch for accurate quantification of HBV DNA viral load in samples as per manufacturer's recommendations. Rotor Gene Q was used for RT-PCR. The thermal cycling condition was followed as per manufacturer's instruction. The results were analyzed using RGQ software version 2.3.1 by setting the threshold between 0.03 and 0.05. The laboratory verified the limit of detection (LOD) of the assay by using the 4th WHO international standard for HBV DNA for NAT (NIBSC code: 10/266) procured from NIBSC, and the standard was serially diluted (stock 955000, 19100, 1910, 191, 95.5, and 47.75) and tested in duplicates as per protocol. Laboratory-derived LOD of the assay is 40 IU/mL, and the linear range of the assay is from 4×10^1 to 2×10^7 IU/mL.

Amplisure® HBV Quantitative Kit (Method 2 – Amplisure HBV)

DNA (60 µL HBV DNA) extracted for the reference test by using the QIAsymphony SP instrument with DSP Virus Mini Pathogen Kit manufactured by Qiagen, Germany, was used. The assay contains reagents and enzymes for the amplification of a specific region (S gene) of the pathogen genome. The PCR assay setup was done manually with a reaction volume of 5 µL DNA and 20 µL master mix by using Amplisure® HBV Quantitative Kit [12] manufactured by RAS Lifesciences, India. RT-PCR was performed on the Rotor Gene Q instrument as per the assay procedure recommended by the manufacturer. Four quantification standards and NTC were run in each batch for accurate quantification of HBV DNA viral load in samples. The results were analyzed using RGQ Software V2.3.1 by setting the threshold at 0.06. LOD of the assay was verified by using the 4th WHO international standard for

Table 1. Primer sequences used for HBV genotyping by nested PCR

Primer	Sequence
First PCR	
P1[b]	5′-TCA CCA TAT TCT TGG GAA CAA GA-3′ (nt 2,823–2,845, universal, sense)
S1-2	5′-CGA ACC ACT GAA CAA ATG GC-3′ (nt 685–704, universal, antisense)
Second PCR	
Mix A	
B2	5′-GGC TCM AGT TCM GGA ACA GT-3′ (nt 67–86, types A to E specific, sense)
BA1R	5′-CTC GCG GAG ATT GAC GAG ATG T-3′ (nt 113–134, type A specific, antisense)
BB1R	5′-CAG GTT GGT GAG TGA CTG GAG A-3′ (nt 324–345, type B specific, antisense)
BC1R	5′-GGT CCT AGG AAT CCT GAT GTT G-3′ (nt 165–186, type C specific, antisense)
Mix B	
BD1	5′-GCC AAC AAG GTA GGA GCT-3′ (nt 2,979–2,996, type D specific, sense)
BE1	5′-CAC CAG AAA TCC AGA TTG GGA CCA-3′ (nt 2,955–2,978, type E specific, sense)
BF1	5′-GYT ACG GTC CAG GGT TAC CA-3′ (nt 3,032–3,051, type F specific, sense)
B2R	5′-GGA GGC GGA TYT GCT GGC AA-3′ (nt 3,078–3,097, types D to F specific, antisense)

HBV DNA for NAT (NIBSC code: 10/266) procured from NIBSC, and a single concentration of 18 IU/mL was prepared from the standard and was extracted 20 times with High Pure Viral Nucleic Acid Kit. Two hundred microliters of the sample + 10 μL of IC2 were coextracted and eluted in 50 μL of elution buffer. All the extracted samples were amplified in a single amplification run on Bio-Rad CFX 96 with the Amplisure® HBV Quantitative Kit, LOD of the assay is determined as 18 IU/mL (CI: 11–37 IU/mL), and the linear range of the assay is from 25 to 1.25×10^{10} IU/mL.

Nested PCR and Sequencing

Discrepant DNA samples were reamplified by nested PCR by using type-specific primers (Table 1). We amplified the HBV genome by nested PCR using the universal primers (P1 and S1-2) for the outer primers, followed by 2 different mixtures containing type-specific inner primers. The first PCR was carried out with the following thermal cycling conditions: first incubating the samples for 10 min at 95°C, followed by 40 cycles consisting of 94°C for 20 s, 55°C for 20 s, and 72°C for 1 min; 2 second-round PCRs were performed for each sample, with the common universal sense primer (B2) and mix A for types A through C and the common universal antisense primer (B2R) and mix B for types D through F. A 1-μL aliquot of the first PCR product was added to 2 tubes containing the second sets of each of the inner primer pairs and each of the deoxynucleotides, AmpliTaq Gold DNA polymerase, and PCR buffer, as in the first reaction. These were amplified for 40 cycles with the following parameters: preheating at 95°C for 10 min, 20 cycles of amplification at 94°C for 20 s, 58°C for 20 s, and 72°C for 30 s, and an additional 20 cycles of 94°C for 20 s, 60°C for 20 s, and 72°C for 30 s [13]. The PCR products were purified using ExoSAP treatment according to the manufacturer's instructions [14]. The concentration and quality of PCR products was checked by agarose gel electrophoresis. The samples were sequenced by both forward and reverse primers. After ethanol precipitation, the purified PCR products were subjected to sequencing by using Applied Biosystems 3730xl DNA Analyzer (ABI), and results were generated after data analysis.

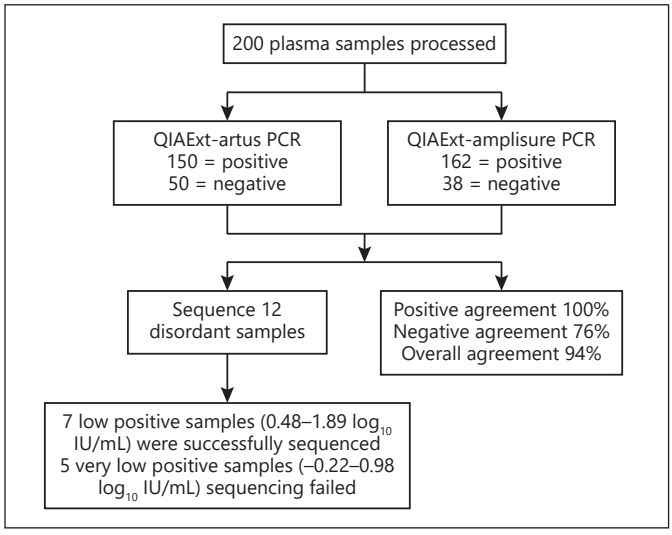

Fig. 2. Enlistment and outcome of the study.

Statistical Analysis

For this validation study, positive agreement and negative agreement for qualitative results values were calculated using Medcalc's diagnostic test evaluation calculator [15, 16]. The differences between 2 methods were presented in a Bland-Altman plot, and linear regression analysis was used for r^2 determination [17].

Results

The results of performance characteristic of Amplisure® HBV Quantitative Kit as compared to the comparator method are shown in Figure 2. The overall perfor-

Table 2. Amplisure HBV in comparison with the comparator method

		Artus HBV DNA		
		+	–	total
Amplisure HBV	+	150	12	162
	–	0	38	38
Total		150	50	200

Table 3. Agreement between the 2 methods

	Amplisure versus artus as a comparator test
Positive agreement	100 (97.57–100.00)
Negative agreement	76 (61.83–86.94)
Overall agreement	94 (89.75–96.86)

Data are presented as %. Figures in the brackets indicate 95% confidence interval.

Table 4. Comparison of artus QS-RGQ assay (IU/mL) and Amplisure® HBV Quantitative Kit (IU/mL) assay by means of SD and % CV as per log values

Log value	Artus comparator test, log_{10} IU/mL				Amplisure HBV, log_{10} IU/mL				Difference (*n* and *n*/*N*) unmatched logs (samples, *n*)
	N	mean	SD	% CV	*n*/*N*	mean	SD	% CV	
<2	3	1.35	0.62	45.5	1/3	2.05	0.16	8	<3 log (2)
2	42	2.52	0.30	11.80	3,242	2.35	0.46	20	<2 log (9), <4 log (1)
3	33	3.36	0.28	8.32	22/33	3.20	0.38	12	<3 log (10), <5 log (1)
4	27	4.43	0.30	6.80	21/26	4.29	0.43	10	<4 log (4); <6 log (1)
5	12	5.42	0.33	6.05	6/12	5.09	0.46	9	<5 log (6)
6	17	6.60	0.23	3.50	14/17	6.28	0.26	4	<6 log (3)
>7	17	7.85	0.59	7.57	15/17	7.78	0.60	8	<7 log (2)

N, number of samples/log by artus assay QS-RGQ assay; *n*/*N*, number of samples/log by Amplisure® HBV Quantitative Kit. Data are presented as mean plus standard deviation; IU, international unit; mL, milliliter.

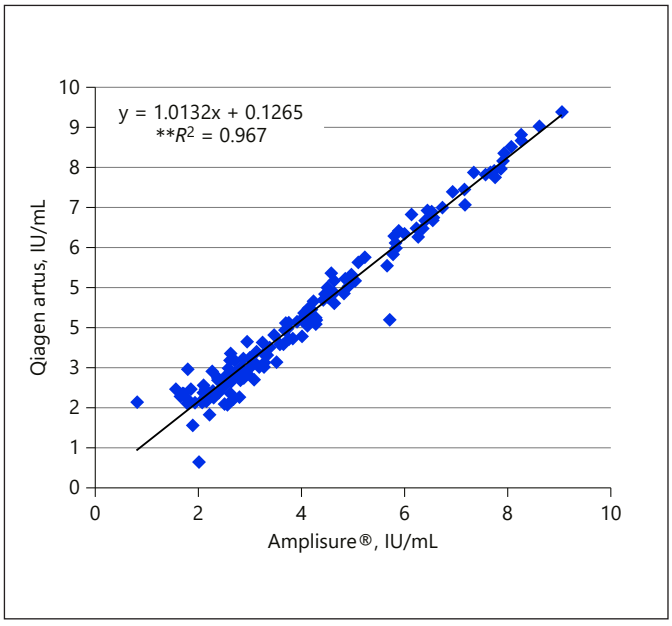

Fig. 3. Linear regression analysis shows the comparison of the comparator method and Amplisure HBV DNA.

mance of the test method, Amplisure HBV, as compared with the comparator method shows that out of 200 samples, 188 samples were concordant and 12 samples showed discordant results (Table 2). These 12 (6%) discordant results have low viral load ranging from –0.22 to 1.89 log_{10} IU/mL according to Amplisure HBV quantification. The positive and negative agreements were 100% (CI: 97.57–100%) and 76% (CI: 61.83–86.94%), respectively (Table 3).

Among the 200 plasma samples tested, HBV DNA was quantified in 150 samples (75%) by both the assays; of these 150 samples, 39 samples show log differences while comparing both results (Table 4). There were 12 (6%) samples not detected by the artus QS-RGQ assay but quantified by Amplisure® HBV Quantitative Kit. Ten of the discordant samples present viral load below the artus QS-RGQ assay LOD and the remaining two are below the 2x artus QS-RGQ assay LOD.

The viral loads quantified by Amplisure® HBV Quantitative Kit were insignificantly lower than those quantified by the artus QS-RGQ assay (mean difference of 0.09 log IU/mL). A total of 4 (2.7%) samples showed significant difference of ≥1 log_{10} IU/mL.

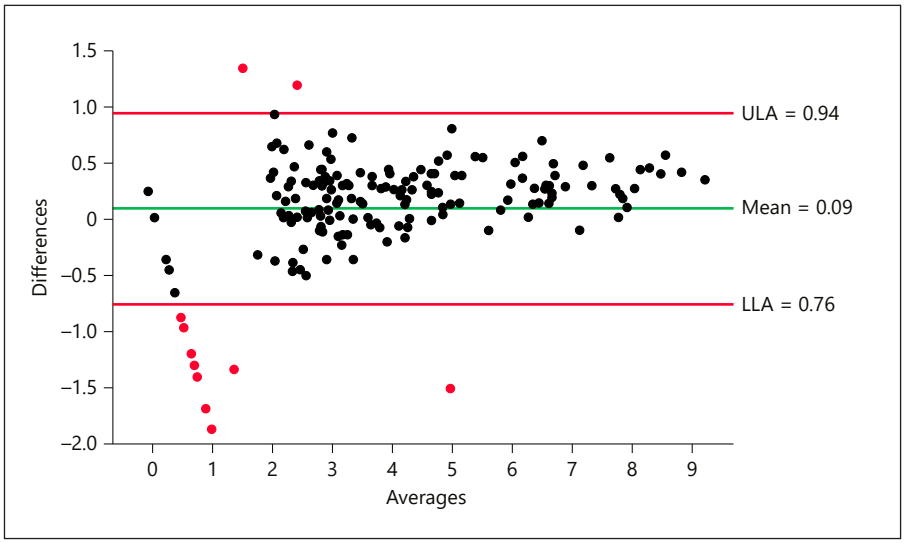

Fig. 4. Bland-Altman analysis was performed to compare the comparator method and Amplisure HBV DNA.

Table 5. Bland-Altman analysis result showing the mean log difference and LOA–ULA

Description	IU/mL
Mean $\log_{10}$ IU/mL difference	0.09
LOA, $\log_{10}$ IU/mL	–0.76
ULA, $\log_{10}$ IU/mL	0.94

IU, international unit; mL, milliliter; LOA, lower limit of agreement; ULA, upper limit of agreement.

Nested PCR and Sequencing Analysis

The 12 discrepant qualitative results, all positive with Amplisure® HBV Quantitative Kit and negative with artus QS-RGQ assay, were taken for nested PCR and then sequenced. As most of the samples are of low viral load, we have selected the nested PCR approach. In spite of the nested PCR approach, concentration of the amplified products of 5 samples was less than the requirement for sequencing. All these 5 samples were below the detection limit of the comparator method, that is, below 40 IU/mL. But, all those 5 samples were positive at the nested PCR level which was confirmed by agarose gel. Of these 12 discrepant samples, 7 were sequenced as genotype A (1), C (1), D (4), and E (1). For the remaining 5 samples, sequencing failed.

Agreement and Correlation between the Assays

As shown in Figure 3, linear regression analysis was done comparing Amplisure HBV and the comparator method. The correlation of $r^2 = 0.967$ shows good correlation of the techniques. The plot was performed to measure the agreement between the assays (Fig. 4). The difference between the HBV viral load $\log_{10}$ IU/mL values of the 2 methods was plotted against the average of the $\log_{10}$ IU/mL results of the 2 methods. As shown in Table 5, the mean difference between Amplisure HBV and the comparator method is 0.09 $\log_{10}$ IU/mL. The lower limit of agreement (LOA) is –0.76, and the upper limit of agreement (ULA) is 0.94.

Discussion

One of the important outcomes of HBV DNA testing has been the identification of patients who are HBsAg, anti-HBe positive but positive for HBV DNA. Such a clinical situation is associated with variations in ongoing chronic disease, fulminant acute hepatitis, acute nonfulminant hepatitis, and poor response to interferon therapy [18]; as this study was focused on active infection and the study was done on the leftover samples, we have not considered serological status of samples selected for comparison or for analysis.

HBV DNA quantification is essential for monitoring disease status and treatment response along with drug resistance detection in CHB patients. Until now, numerous commercially available RT-PCR assays have been developed for this. CHB is a foremost cause of hepatocellular carcinoma and cirrhosis in Southeast Asia, China, and Africa [19]. Newly launched Amplisure® HBV Quantitative Kit assay suggested an equivalent LOD and equivalent LOA to artus QS-RGQ assay by the manufacturer. This new assay has not been researched enough about its performance, so it is unable to compare findings with previous studies.

In our study, there were differences between the artus QS-RGQ assay and the Amplisure® HBV Quantitative Kit assay in the detection rate and viral load when quantifying HBV DNA levels in clinical plasma samples. Correlation between the Qiagen artus assay with Amplisure® HBV Quantitative Kit assay, that is, Amplisure HBV, showed good positive agreement of 100% (CI: 97.57–100) with an overall agreement of 94% (89.75–96.86), and good correlation was observed (r^2 = 0.967); it shows a comparable result with Qiagen artus assay.

However, there were only few samples which had significant log differences in the viral load quantification rate of Qiagen artus assay and Amplisure HBV Quantitative Kit assay. The HBV DNA levels determined by the Amplisure HBV were slightly lower than the results of the comparator method (0.09 $\log_{10}$ IU/mL in mean). Overall, 4 samples showed >±1 log difference ranging from 0.65 to 4.19 $\log_{10}$ IU/mL, and 23 samples showed >±0.5 $\log_{10}$ IU/mL difference viral loads ranging from 2.08 to 8.82 $\log_{10}$ IU/mL. Bland-Altman shows a mean $\log_{10}$ IU/mL difference of 0.09 $\log_{10}$ IU/mL and the LOA of −0.76 with a ULA of 0.94.

The correlation between the results of the 2 assays in low viral load samples (<log 2 and 3) was not as strong as the correlation for all samples (r = 0.967). It is a known characteristic of PCR amplification that weak samples may present higher variability than strong ones. According to the manufacturer's protocol, the LOD of the Amplisure® HBV Quantitative Kit assay is 18 IU/mL which is lower than that of the QS-RGQ assay at a laboratory, generated 40 IU/mL. Consequently, QS-RGQ assay results showed a lower positive rate than the Amplisure® HBV Quantitative Kit in the samples for 1 and 2 log. There were 12 samples which showed qualitative discrepancy. All of the 12 samples were negative from the comparator method. Amplisure HBV results indicated that 7 samples have low viral load log ranging from 0.65 to 1.86 $\log_{10}$ IU/mL, and all those were confirmed positive by sequencing. For the other 5 samples, sequencing failed probably due to even lower viral load (ranging from −0.22 to 0.98 $\log_{10}$ IU/mL). Ten of these 12 qualitative discrepant samples are below the Qiagen artus LOD and the two other are below twice the LOD of Qiagen artus.

Furthermore, 3 of the highest quantitative discrepancies between the assays were observed on low viral load (0.65–2.96 log IU/mL). Only 1 sample presents >1 log IU/mL difference and a high viral load 4.19 log IU/mL for artus QS-RGQ assay versus 6.59 log IU/mL for Amplisure® HBV Quantitative Kit. This sample was sequenced and gave a genotype D result. The good quantitative correlation in medium/high load would lead to a similar classification of inactive HBsAg carriers and active CHB patients and may even change the CHB management strategy.

One more possibility for the sample discrepancies may be mutations in the core region of the 134-bp genome which may have led to the negative results of the comparator method, as all the samples were positive in the nested PCR which had type-specific primers as targets with BA1R (type A 68 bp), BB1R (type B 281 bp), BC1R (type C 122 bp), BD1 (type D 119 bp), BE1 (type E 167 bp), and BF1 (type F 97 bp), while mutations in the core region would have not affected the Amplisure kit result as it targets the S gene, so difference in the target regions of both the kits may have resulted in the sample discrepancies.

As studied before, there are several possible factors that may lead to assay discrepancies [10, 20, 21]. It has been reported that the HBV genotype B and low HBV viral load were 2 factors that contribute to significant differences in HBV DNA viral load detection, that is, ≥1/2 $\log_{10}$ IU/mL, when compared to the real-time HBV assay (Abbott Laboratories, Abbott Park, IL, USA) with CAP/CTM v2.0 assay [9]. Also, identical results were observed previously in comparison with the Abbott real-time HBV assay and artus QS-RGQ assays on 2 different platforms. The difference was marginal, especially in samples with low viral load (<2–3 log) [22]. These observations are also applicable to our study for low viral load samples.

Another aspect for these discrepancies may be mutations in precore and core promoter regions. Mutations generally occur as CHB progresses and may affect the DNA quantification [23–25]. Also, few of the studies shed light on the detection difference between other assays in low viral load samples that may be influenced by the tyrosine-methionine-aspartate-aspartate mutation, which confers lamivudine resistance [26, 27]. Thus, detection sensitivity for HBV mutants in the common target region may differ depending on the assay. We have performed genotyping to identify HBV genotypes. The data represented genotype D followed by genotype A and genotype E.

In conclusion, the Amplisure® HBV Quantitative Kit assay shows good correlation with the Qiagen artus QS-RGQ assay. This assay showed a slightly increased detection rate and similar viral load compared with results of the comparator method in plasma samples. Consequent to these results, it can be concluded that Amplisure® HBV Quantitative Kit assay showed comparable performance with Qiagen artus HBV QS-RGQ assay and can be useful in management and therapeutic monitoring of HBV infection.

Acknowledgement

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Statement of Ethics

The study is exempt from ethics committee approval. We obtained the waiver of consent as the study was carried out on leftover samples identified by a laboratory-generated number with no traceability to the patients. All patients' details were thus kept confidential. The Amplisure® HBV Quantitative Kit results were not used in clinical decision-making.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

P.K.G.: performing testing and writing the manuscript; C.N.: study design and verification of data and manuscript; C.S.: verification of data and manuscript; A.V.M.: reviewing the manuscript; Venkat and R.A.: study performers.

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