Performance evaluation of TRUPCR[®] HBV Real-time PCR assay for Hepatitis B virus DNA quantification in clinical samples: report from a tertiary care liver centre

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Abstract Quantitative Real-time PCR (qPCR) based Hepatitis B virus (HBV) DNA load estimation is crucial for the initiation of treatment and serves as a strong predictor of liver disease progression in HBV infected individuals. HBV DNA quantification has been ever evolving with the addition of new qPCR based kits on a regular basis. The study was carried with an objective to evaluate the performance characteristics of a commercially available qPCR kit (TRUPCR[®], 3B Black Bio Biotech, India Ltd.) and compare with CE approved qPCR kit (Artus HBV Real-time PCR, Qiagen, Germany). 121 HBV infected patients were prospectively enrolled from July to December 2016. Aliquots of serum samples were tested in parallel by TRUPCR[®] and Artus for HBV DNA levels. Genotype D was most predominant genotype in 36.9% (38/121) of patients followed by genotype A in 14.6% (15/121) patients. Median viral load as seen by Artus was log₁₀IU/ml 3.37 (interquartile range log₁₀IU/ml 2.10–10.89) as compared to TRUPCR[®] where it was log₁₀IU/ml 3.54 (interquartile range log₁₀IU/ml 2.67–11.52). A very good correlation was seen between the two assays ($R^2 = 0.964$) with a concordance rate of 92.6% (112/121). The TRUPCR[®] qPCR HBV kit is capable of providing reliable and rapid HBV DNA quantitation and together with its much lower costs, presents itself as a good alternative.

Keywords HBV DNA quantification · TRUPCR[®] · Real-time PCR

Introduction

Hepatitis B virus (HBV) infection is a life-threatening liver infection with estimated 350 million individuals being chronically infected worldwide. HBV belongs to genus *Orthohepadnavirus* and *Hepadnaviridae* family of viruses. In India, the prevalence of HBV is in the range of 3–4.2% with around 40 million HBV carriers which has a heavy economic and social burden for both patients and society in general (<http://www.who.int/mediacentre/factsheets/fs204/en/>). HBV infection can cause chronic hepatitis, cirrhosis and hepatocellular carcinoma [7]. Among patients with active viral replication, cirrhosis will develop in 15–20% cases within 5 years while 70–90% of cirrhotic cases develop hepatocellular carcinoma [2, 8].

Quantitation of HBV DNA in serum or plasma has been an important tool in identifying individuals with active hepatitis B infection to monitor the efficiency of antiviral treatment, and to predict whether the treatment will be successful or not [3, 9, 10, 14, 15]. Several commercial molecular kits have been developed for quantitation of HBV DNA in serum or plasma samples keeping cost-effectiveness in consideration. TRUPCR[®] HBV Viral Load Kit is one such kit which is designed and developed in India (in collaboration with M/S. Bio tools B and M labs Spain) to quantitate HBV DNA viral load.

qPCR based quantitative assays have higher sensitivity, broader dynamic range, and an accurate quantitation of HBV DNA compared to conventional PCR techniques [1, 11–13]. Most of these assays which are available commercially imply cost per test of around 3000–4000

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INR and hence, their utility is being limited to health care centres with high end infrastructure and resources. The search for cheaper, easier to handle and easily available qPCR has become the need of the hour, so that access to testing is enhanced and patients from all strata benefit from rapidly evolving molecular technology.

The present study was done to evaluate the performance characteristics of a commercially available qPCR kit (TRUPCR®, 3B Black Bio Biotech, India Ltd.) and compare its performance with European Conformity (CE) approved qPCR kit (Artus HBV Real time PCR, Qiagen, Germany).

Materials and methods

Study design

This was a prospective study conducted in the Department of Clinical Virology, Institute of Liver and Biliary Sciences New Delhi, a tertiary liver care centre. Overall, 121 Hepatitis B infected patients based on Hepatitis B surface antigen (HBsAg) positivity in blood were recruited in the study from July 2016 to December 2016. Hepatitis C virus (HCV), HIV and other causes of viral hepatitis were ruled out by appropriate serological testing. Patients with co-infection with HCV or HIV were not included in the study. Plasma samples were aliquoted in 2 separate vials and stored at -80°C until further testing. Artus qPCR assay (hereafter, Artus) was used as the routine assay for HBV viral load estimation and reporting to the patients. Parallel plasma samples were re-tested on the TRUPCR® HBV kit (hereafter, TRUPCR®). The HBV serological markers namely hepatitis e antigen (HBeAg), antibody to HBeAg (anti-Hbe), antibody to HB core antigen (HBctotal) were determined by Chemiluminescence immunoassay method (Architect i1000SR, Abbott Diagnostics, IL, USA). Liver enzymes namely, Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) were measured as a part of the routine monitoring. The HBV genotype was determined by sequencing the HBs gene region using *in-house* designed pan genotypic primers. Approval for the study was obtained from the Institutional Review Board and Ethics Committee.

HBV standards

Third WHO international standard for HBV (NIBSC code: 10/264) comprising of genotype A2, HBsAg subtype adw2 was used to evaluate the linearity of TRUPCR®. The lyophilized standard was reconstituted with 500 μl of deionized, nuclease-free molecular grade water. This material was assigned a unit of $5.93 \log_{10}\text{IU/ml}$ in the final concentration as per published literature. Serial five tenfold

dilutions of this were made in human plasma tested negative for all markers of HBV, HCV and HIV.

HBV DNA extraction

Nucleic acid extraction was done using QIAamp DSP virus Purification system using automated extraction system (QIA Symphony SP, Qiagen) as per the manufacturer's instruction with a starting plasma volume of 1000 μl .

ARTUS & TRUPCR® Real-Time HBV quantitation

qPCR by Artus & TRUPCR® kits were performed on Rotor-Gene Q instrument as per the manufacturer's instruction. Both method targets a fragment size of 134 base pairs (bp) in the HBV core gene. Both kits require similar temperature profile and dye acquisition setting. While the reaction volume and template amount for Artus were 50 μl and 20 μl respectively, TRUPCR® required lesser amounts for both i.e., 30 μl and 15 μl respectively. The analytical sensitivity of this assay is 3.8 IU/mL for Artus & 2.5 IU/ml for TRUPCR® with 95% detection limit with a linear range of $1.1\text{--}4 \times 10^9$ IU/ml & $2.5\text{--}1 \times 10^8$ IU/ml respectively for Artus & TRUPCR® kits respectively.

HBV genotyping

HBV DNA was subjected to PCR using *in-house* designed primers for HBV surface gene (forward primer 5'-CAT-CAGGATTCCTAGGACCCCT-3', reverse primer 5'-AGGACAAACGGGCAACATAC-3'). PCR was performed using Phusion High Fidelity DNA Polymerase (Thermo scientific Inc., Waltham, MA). Amplified products were gel-excised & further purified using QIAquick Gel Extraction Kit (QIAGEN, GmbH, Mannheim, Germany) to remove unincorporated dNTPs and primers. This was followed by bidirectional sequencing using ABI Big Dye chemistry on the ABI 3500Dx series genetic analyzer (Life Technologies, Waltham, MA). Forward and reverse sequence reads were aligned and assembled using DNA Baser v3.5.1 (Heracle BioSoft SRL, Romania). Genotype assignment was done by comparing the obtained sequences with the reported sequences on the Basic Local Alignment Search Tool (BLAST) database of NCBI.

Statistical analysis

For analysis, DNA values were $\log_{10}$ transformed. Linear regression analysis was done and correlation coefficients were calculated using SPSS version 22. The agreement between the two assays was evaluated with Bland–Altman plots by using Med Calc software (Med Calc Software

bvba, Ostend, Belgium). A p value of < 0.05 was accepted as statistically significant.

Results

Concordance between the assays

Of the total 121 subjects recruited in the study during routine monitoring by Artus, 85.12% (103/121) had quantifiable viral load. Out of these, HBV DNA below the lower limit of detection (LLOD) of Artus Assay was found in six (5.8%) samples and in three (2.9%) samples, it was found to be below the LLOD of TRUPCR[®] assay. Genotype D was the most common genotype 70.6% (60/85) followed by genotype A in 29.4% (25/85).

Overall concordance between both the assays was seen in 92.6% (112/121) samples. Median viral load as seen by Artus was $\log_{10}$ IU/ml 3.37 (Interquartile Range: $\log_{10}$ IU/ml 2.10–10.89) as compared to TRUPCR[®] where it was $\log_{10}$ IU/ml 3.54 (Interquartile range $\log_{10}$ IU/ml 2.67–11.52). Amongst the 9 discrepant results, 3 samples which were negative by TRUPCR[®] were below the quantification limit in Artus. This is explainable as sometimes such low viral load may not be detected by PCR. However in 6 samples, Artus assay was negative but they were positive by TRUPCR[®]. Repeat sample of these patients during OPD follow up were repeated with both the assays and eventually they all were found to be negative. The negative results in the follow up samples could be explained by the individuals clearing the infection, though their initial ARTUS results produced for routine care were reported as negative. To prevent any contamination, a very strict protocol of decontamination of surfaces were carried out, together with the use of separate hoods for determining the performance of the PCRs were done. Quality control was checked not only by kit controls but also using molecular grade water as negative control in between the samples during the performance of various PCR reactions.

Correlation between the assays

A linear regression analysis was performed with the help of a scatter plot having $\log_{10}$ transformed values of quantifiable viral loads by TRUPCR[®] on Y-axis and viral loads by Artus on X-axis as shown in Fig. 1a. A very good correlation was seen between the two assays ($R^2 = 0.964$) as evidently displayed by the straight line obtained in the scatter plot. A strong correlation was seen for both genotypes D (Fig. 1b) and A (Fig. 1c) with R^2 values of 0.94 and 0.936 respectively. Pearson's coefficient was also evaluated which further measures the strength and direction of correlation of linear relationship between two

variables on a scatter plot. A value of 0.98 was obtained between the two assays & the association was found to be significant with a p value of < 0.001 .

Agreement between the two assays

To determine the level of agreement between the two assays, a Bland–Altman plot was used wherein the difference of HBV viral loads by the two assays were plotted against the mean of the techniques. Horizontal lines were drawn at the mean difference, and at the limits of agreement, which were defined as the mean difference plus and minus 1.96 times the standard deviation of the differences. The mean difference between TRUPCR[®] and Artus kit was -0.096 with limits of agreement ranging from -1.026 to 0.835 . The two systems showed a good agreement with an intra-class correlation coefficient (ICC) of 0.984 (95% CI 0.972–0.991, $p < 0.001$). Genotype D was most predominant genotype in 36.9% (38/121) of patients followed by genotype A in 14.6% (15/121) patients. Though the manufacturers claim that the performance of the assay is pan genotypic, assay validation could only be done on genotype A and genotype D samples as these were the two genotypes obtained in our study. This was also a limitation for our study. There was good agreement for genotype A (ICC = 0.985, 95% CI 0.954–0.995, $p < 0.001$) and genotype D (ICC = 0.984, 95% CI 0.969–0.992) as shown in Fig. 2.

Concordance of assays on the basis of HBeAg status

A good concordance was also found for HBeAg reactive status in both the assays of HBV viral load of patients with ICC of 0.99 (95% CI 0.977–0.996, $p < 0.001$) and HBeAg non-reactive patients with ICC of 0.987 (95% CI 0.979–0.992, $p < 0.001$) shown in Fig. 3a, b.

Evaluation of TRUPCR[®] linear range using reference standards

The linear range of TRUPCR[®] HBV Kit was determined by analyzing a logarithmic dilution series of the plasmid calibrated with 3rd WHO International Standard for Hepatitis B Virus for Nucleic acid amplification techniques (NIBSC code: 10/264) with concentrations ranging from 1 IU/ml to 1×10^8 IU/ml. Each concentration was tested in triplicates for 5 times. On linear regression analysis, good correlation was seen between the measured HBV DNA concentrations and the expected concentrations ($R^2 = 0.99$) and the mean value 4.86 ± 0.06 was comparable ($p = 0.16$) by student's t test. The linear range has been determined to cover concentrations from 2.5 IU/ml to 1×10^8 IU/ml (Fig. 4).

Fig. 1 Scatter plot diagram showing linear regression analysis. **a** Between log₁₀IU/ml HBV DNA values of TRUPCR[®] (Y-axis) and Artus (X-axis), **b** between log₁₀IU/ml HBV DNA values of

TRUPCR[®] (Y-axis) and Artus (X-axis) for Genotype D, and **c** Between log₁₀IU/ml HBV DNA values of TRUPCR[®] (Y-axis) and Artus (X-axis) for Genotype A. Correlation is denoted with R^2

Fig. 2 Bland–Altman analysis to determine the number of cases within agreement limits for HBV DNA quantification by plotting a scatter diagram of differences of HBV DNA viral load between the two assays against their averages with an intra-class correlation coefficient (ICC) of 0.984 (95% CI 0.972–0.991, $p < 0.001$). Horizontal lines are drawn at the mean difference, and at the limits of agreement. The limits of agreement (LoA) are defined as the mean difference $\pm$ 1.96 SD of differences

The analytical sensitivity and limit of detection for TRUPCR[®] HBV Kit was determined by analyzing dilution series of 3rd WHO International Standard for Hepatitis B virus for Nucleic acid amplification techniques (NIBSC code: 10/264) and confirmed by WHO multiplex material (NIBSC Code: 14/198-XXX). The limit of detection for

TRUPCR[®] HBV kit was determined to be 2.5 IU/ml over repeated number of runs which was in concordance with the manufacturer's claim. All the quantitative results were tabulated in Table 1.

Fig. 3 Scatter plot diagram showing linear regression analysis. **a** Between $\log_{10}$ IU/ml HBV DNA viral loads values of TRUPCR® (X-axis) and Artus (Y-axis) in patients with HBeAg (non-reactive

status) and **b** between $\log_{10}$ IU/ml HBV DNA viral loads values of TRUPCR® (X-axis) and Artus (Y-axis) in patients with HBeAg (reactive status). Correlation is denoted with R^2

Fig. 4 Linear regression analysis and boot strapping plot. To determine the linear range of the TRUPCR® HBV Viral Load Kit with concentrations ranging from 2.5 IU/ml to 1×10^8 IU/ml

Table 1 Limit of detection for TRUPCR® HBV assay

IU/mL	Number tested	Number detected	Percent detected
50	30	30	100
5	30	30	100
2.5	30	30	100
1.25	30	10	33
0.625	30	1	3

Limit of detection for TRUPCR® HBV assay evaluated as 2.5 IU/ml determined by analyzing dilution series of 3rd WHO International Standard for Hepatitis B Virus for Nucleic acid amplification techniques

Inter-assay variation and accuracy of TRUPCR® using WHO Standards

To determine the dynamics and accuracy of the TRUPCR® qPCR kit, WHO standard dilutions (IU/mL) were selected and run 3 different times with kits of different lot numbers. TRUPCR® real-time HBV kit detected all five dilutions, and all the quantitative results are shown in Table 2. The inter-assay variation, CV % < 5 clearly is suggestive of low-variance in data.

Intra-assay variation and reproducibility of TRUPCR®

For evaluation of the intra-assay variation of the TRUPCR® qPCR kits, two samples with high and low HBV titers were tested. Each sample was tested 10 times in single run. Table 3 shows the mean $\log_{10}$ (copies/mL) viral loads, SD and coefficient of variation (CV) of the samples. The viral load values from the TRUPCR® qPCR assay were highly reproducible over the various HBV titers as CV % < 5.

Inter-day precision

To determine the precision and variability, a sample was diluted in a concentration of 25 and 2.5 IU/ml. These samples were run in triplicates for three times using the kits of three different lots. Table 4 shows average Ct (Threshold values), SD and CV of the samples. There was no inter day variability as reflected by CV < 5%.

Table 2 Inter-assay variation and accuracy detection

WHO Dilutions	Results (IU/mL)			Mean log ₁₀ IU/mL	SD log ₁₀ IU/mL	CV %
	Run1	Run2	Run3			
Dilution 1	83,232	88,383.6	89,773.2	87,129.6	3446.188	3.955,244
Dilution 2	7668	8132.4	8461.2	8087.2	398.5271	4.927,875
Dilution 3	718.8	750.6	774	747.8	27.70,632	3.705,044
Dilution 4	131.4	135	141	135.8	4.849,742	3.571,239
Dilution 5	13.5	12.6	12.3	12.8	0.6245	4.878,905

Inter-assay variation and accuracy detection of TRUPCR® determined by 5 WHO standard dilutions (IU/mL) run 3 different times with kits of different lot numbers

Table 3 Intra-assay variation and reproducibility calculation

IU/mL	Mean Log ₁₀ IU/mL	SD Log ₁₀ IU/mL	CV %
2.5	2.441	0.117,042	4.794,849
25	25.146	0.736707	2.92972

Intra-assay variation and reproducibility of TRUPCR® calculated by repeated run of low and high titer value HBV samples

Table 4 Inter-day precision

Target	HBV DNA	
IU/ml	2.5	25
Average(Ct)	37.31	34.44
Standard Deviation	0.22	0.25
CV (%)	0.58	0.72

Inter-day precision of TRUPCR® assay calculated by using a sample diluted in a concentration of 25 and 2.5 IU/ml. The samples were run in triplicates during three independent times using kits of three different lots

Assay time and cost

The assay times and costs of both tests were compared. Artus qPCR required 3 h while TRUPCR® test required 2 h for completion of test. The cost of TRUPCR® qPCR per test including consumables and DNA extraction reagents was around 1200 INR as compared to 2000–3000 INR for other PCR kits.

Discussion

The study describes the evaluation and comparison of a qPCR assay for detection and quantitation of HBV DNA (all genotypes) in human plasma specimens. The indigenous TRUPCR® assay for HBV DNA quantification holds an excellent agreement with the CE—marked IVD-

licensed Artus assay in clinical samples when compared using qPCR technology. The target fragment length by TRUPCR® is 130 bp in the core region of HBV gene as compared to 134 bp in Artus. The clinical sensitivity determination showed TRUPCR® and Artus to detect HBV DNA at < 10 IU/ml which meets the recommended guidelines for HBV infection management [5].

There was a 92.6% of concordance between both the assays while performing test using clinical samples. However, TRUPCR® assay used a lesser reaction volume of 30 µL as opposed to 50 µL in Artus thereby, decreasing the requirement of sample volume and making the balance quantity of eluted DNA available for repeat test or for other tests. It is of immense help in cases of neonates and debilitating patients where repeated sample collection through venipuncture is cumbersome. Quantitative HBV DNA measurements can vary widely with the quantification method used and among different laboratories performing the testing hence, 9 out of 121 samples tested did not correlate initially between the two assays. All of these 9 samples were of low viral load DNA. To eliminate any discrepancies in results due to chances of cross contamination during extraction or PCR setup, well-defined strict protocols were followed in samples during extraction and in qPCR along with appropriate positive and negative controls.

To enable result reproducibility, re-evaluation was done from the routine OPD follow-up samples from 6 patients. Both the assays showed no amplification of HBV DNA. We compared the TRUPCR® qPCR assay with the Artus assay and found excellent correlation for results within the linear range ($R^2 = 0.96$). Interestingly, the apparent homogeneous distribution of HBV genotypes with respect to the regression line (see above figures) suggests there is no bias in detection of one genotype over another (genotype A- $R^2 = 0.94$ and genotype D $R^2 = 0.936$) by either qPCR based TRUPCR® assay or Artus assay. The agreement of TRUPCR® assay with Artus assay holds true both for reactive and non-reactive HBeAg patients. This clearly defines that the target region detected by this assay is not

influenced by mutations in the precore and core region of the virus that would have affected the Hepatitis B e antigen expression. Thus, this assay may be especially useful for detection of patients who carry the HBV virus particle but are HBeAg negative either due to mutation or chronic HBV inactive carrier stage.

Most of the literature available is about commercial kits that are manufactured outside India, but the present study is done on kit which is indigenous in nature and is easily available in our country [4, 6, 11]. Chronic Hepatitis B patients have to undergo a battery of tests monthly for follow up, monitoring the staging of disease and for assessing response to the treatment. In a developing country like India, it can pose as a major economic burden on the patients. Hence, exploring newer indigenous, open and cost-effective assays is the need of the hour in country like India, which lacks national health program for HBV to provide free testing to the patients. TRUPCR[®] validated by our study can provide a promising, open and cost-effective alternative for better patient care with cost of around 1200 INR per test as compared to 2000–3000 INR for other PCR kits. This novel qPCR assay can be further used for follow up testing of these patients and monitoring the response to the treatment given and thus, can be started in various referral centers of this country.

The TRUPCR[®] real-time HBV DNA assay fulfills the requisite criterion to become an excellent detection and quantitation kit for the molecular diagnosis of HBV with good sensitivity and genotype inclusivity for low load as well as with a better cost effectivity.

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Compliance with ethical standards

Conflict of interest None declared.

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