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COMPARATIVE DETECTION OF HEPATITIS B VIRAL LOAD USING CHIP BASED QUANTSTUDIO 3D DIGITAL PCR AND COBAS TAQMAN REAL-TIME PCR SYSTEM

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ABSTRACT: Viral load monitoring of hepatitis B virus infection is decisive among the patients for proper management and treatment. Various techniques are being employed for the quantification of HBV DNA in the clinical setting. The chip-based Quantstudio 3D Digital PCR was compared with FDA-approved COBAS Taqman Real-time PCR for the HBV viral load quantitation in 52 samples. Of these samples, six samples were non-detectable by both technologies, 7 samples detected with a viral load of <6 IU/ml by COBAS Taqman method out of which 6 samples were positive by Digital PCR also; while the rest 39 samples were quantifiable viral loads by both. There was a good correlation (r=0.92) between two assays and the most agreement was found for the samples with 2-6Log_{10}IU/mL viral load using the Bland-Altman plot. The average viral loads measured by COBAS Taqman and Digital PCR were 3.34 $\pm$ 1.73 and 3.52 $\pm$ 1.19 respectively. In conclusion, the study demonstrates that chip-based Quantstudio 3D digital PCR can be utilized in assessing HBV monitoring in the patients who have cleared the viral load or possible recurrence. Furthermore, digital PCR also serves as cost-effective technology with similar sensitivity and specificity as FDA-approved COBAS Taqman in future.

Key words: HBV; Digital PCR; COBAS Taqman; Viral Load; Comparison

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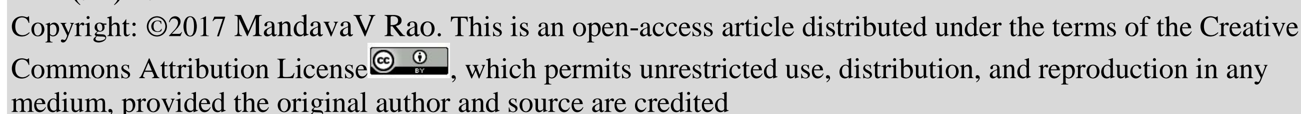
Copyright: ©2017 MandavaV Rao. This is an open-access article distributed under the terms of the Creative Commons Attribution License , which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

INTRODUCTION

Hepatitis B virus (HBV) is a small, partially double-stranded enveloped DNA type that consists of 3.2-kb relaxed circular DNA(Ganem and Prince, 2004), which is converted into a stable covalently closed circular DNA (cccDNA), serves as the template for viral replication and plays an important role in HBV persistence in the nuclei of infected hepatocytes (Lucifora et al., 2014).Worldwide, more than 350 million people have persistent and chronic infection while nearly 2 billion people are infected by HBV (Lee, 1997; Yim and Lok, 2006).Currently, the diagnosis of HBV infection is routinely performed with serological tests and quantitative real-time PCR (qPCR)(Ide et al., 2003). However, false-negative results are major issues due to the presence of PCR inhibitors or low viral load in the specimen as well as HBsAg variation.

At present, several molecular techniques have been developed and validated for the diagnosis and management of HBV infection in the clinical diagnosis setting. Real-time PCR technology based on Taqman Chemistry(Mackay et al., 2002) is one which has been evaluated to quantitate HBV DNA with wider dynamic range, higher sensitivity and accurate quantification(Shyamala et al., 2004; Sum et al., 2004) and is also increasingly used as a powerful diagnostic tool for infectious disease identification. The COBAS TaqMan HBV test is a Taqman based real-time PCR chemistry(Weiss et al., 2004) for HBV DNA quantification with a wider and dynamic linear range(Gordillo et al., 2005; Lindh and Hannoun, 2005)and thereby quantification can be done even without diluting the sample(Gordillo et al., 2005).Digital PCR (dPCR) has the potential to highly accurately quantify the concentration of nucleic acids in a sample, to a much greater extent than traditional quantitative PCR, through counting individual DNA molecules(Vogelstein and Kinzler, 1999).Besides its research abilities, dPCR could serve as a standard tool in nucleic acid quantification especially in the field of viral diagnostics(Sedlak and Jerome, 2013).
HBV DNA viral load detection is required for the proper management of the chronic Hepatitis B patients for which, efficient, reliable and sensitive molecular tests are required to detect the HBV viral load. In this study, we used a new dPCR system to measure the HBV copy number in the clinical samples, and also investigated comparison of Quantstudio 3D digital PCR and COBAS Taqman 48 Analyzer for HBV viral DNA assessment. This is one of its first kindsfor comparing these two methodologies to the best of our knowledge.

MATERIALS AND METHODS

Patient Cohort:

A total of 52 blood/plasma samples of referral cases to SupratechMicropath Laboratory and Research Institute, Ahmedabad, were utilized in the study after filling up consent forms. These were extracted in parallel for an FDA approved real time PCR method and the digital PCR workflow. In order to ensure that the clinical samples were appropriately managed, extraction for the digital PCR workflow was performed only after the FDA real time PCR run was completed. Samples were selected so as to represent a wide spectrum of HBV concentration based on the clinical reports. This project is approved by Human Ethical Committee of Gujarat University, Ahmedabad.

Roche COBAS Taqman 48 Analyzer

The HBV DNA for COBAS Taqman was isolated by a manual FDA approved kit for viral DNA extraction which is a part of the Roche HBV DNA kit (High pure system viral nucleic acid kit) in accordance with the manufacturer’s instructions. This test is based on automatic PCR amplification of target DNA using HBV specific complementary primers, and detection of cleaved dual fluorescent dye-labelled oligonucleotide detection probes that permit quantification of HBV target amplified product (amplicon) and HBV quantification standard DNA which is processed, amplified and detected simultaneously with the specimen. The COBAS Taqman 48 analyzer calculates the HBV DNA titer in the test specimen by comparing the HBV signal to the HBV Quantification standard signal for each specimen and control and is expressed in IU/ml. The conversion between HBV copies to International Units (IU) is made by dividing the result of the HBV copies by 5.82 as per manufacturers instruction manual.

DNA extraction for Digital PCR

Samples which had greater than 500ul of residual plasma and met inclusion criteria for a wide distribution of log ranges, were separated and DNA was extracted with QIAampminelute virus spin kit (Qiagen) according to manufacturer’s instruction. Input volume of the sample was 400ul. The samples were eluted in 40ul and stored in -80°C till further use.

Quantstudio 3D Digital PCR

The viral DNA samples were loaded onto the chips using the Quantstudio 3D Digital PCR chip loader in a mixture consisting of 2X Quantstudio 3D Digital PCR master mix and 20X HBV Taqman assay (Pa_03453405) as mentioned in the digital PCR protocol by Thermofisher. Concentration of the target sequence in the final reaction was set in the Digital PCR range between 200 and 2,000 copies/µlby performing appropriate dilutions. The chips were sealed and loaded onto a Proflex 2x flat PCR system (ThermoFisher Scientific) and cycled according to following instructions: 96°C for 10 min, followed by 39 cycles of 60°C for 2 min and 98°C for 30 sec and a final extension at 60°C for 2 min. The chip images were determined on Quantstudio 3D PCR instrument and further analyzed using Quantstudio 3D analysissuite cloud software. The HBV viral load was measured as the copy numbers per microliter of DNA sample. The final results were expressed in IU/ml as discussed in the above section.

Data analysis

The validity of each chip run on digital PCR was determined by preset metrics set by the manufacture in the Quantstudio 3D analysis suite. For accurate quantification using Quantstudio, threshold was defined at 4000 units of fluorescence intensityfor all the samples.Negative control chips were also run on every day that experimental runs were performed to ensure no contamination was present during these experiments. The background fluorescence of the negative control chips were used to determine the threshold of 4000 units of fluorescence.

Statistical Analysis

Linear regression analysis and Pearson correlation was performed using MedCalc Software. Pearson correlation test was used to measure overall correlation between the two methods. Bland Altman was performed to assess the agreement between the digital PCR and COBAS Taqman method. Statistical significance was set at $p<0.05$.

RESULTS

A total of 52 plasma samples were tested by both the methods i.e. Digital PCR and COBAS Taqman PCR. Among them, 6 samples were 'not detected' by COBAS Taqman method while 46 samples were detected by it. Further a total of 7 samples out of these 46 samples were detected with a viral load of <6 IU/ml by COBAS Taqman method while the rest 39 samples were having the quantifiable viral loads (Table-1).Similarly, 7 samples out of 52 were 'not detected' by Digital PCR while the rest 45 samples were detected by the same. The one sample which was 'not detected' by the digital PCR was 'detected' with COBAS Taqman method, showing the viral load of <6 IU/ml.Thus, out of 7 samples which were detected with a viral load of <6 IU/ml by COBAS Taqman,while 6 samples were positive by Digital PCR method also (Table-1). Among the samples with quantifiable viral loads, a strong correlation between the two techniques appeared based on the linear regression analysis (Figure 1; $r=0.92$; 95%CI = 0.84 to 0.95; $p<0.0001$).

Table 1: HBV viral load detected by both the methods

		COBAS Taqman		
		Detected	Not Detected	Total
Digital PCR	Detected	45	0	45
	Not Detected	1	6	7
	Total	46	6	52

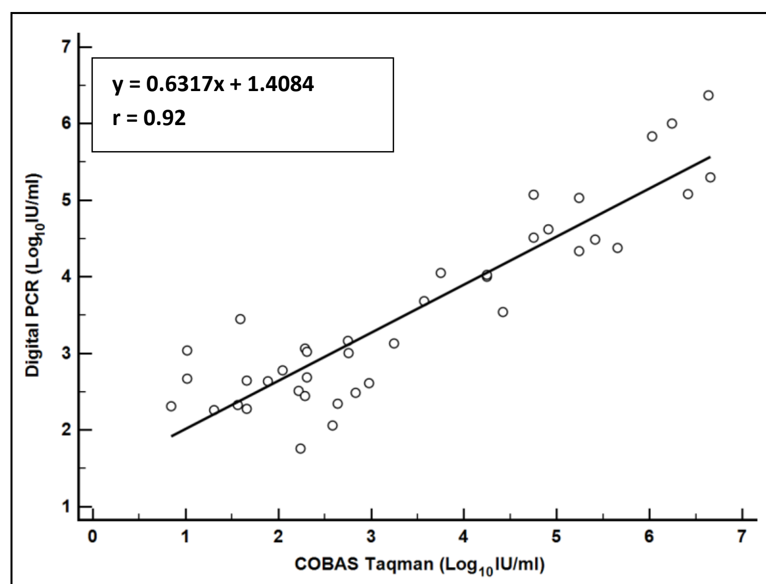


Figure 1: Linear Regression and Correlation of HBV DNA levels in clinical samples measured by Quantstudio 3D Digital PCR system and Roche COBAS Taqman analyzer in Log_{10}IU/ml)

Comparison of HBV DNA viral load was done between the two methods using the Bland Altman plot. Bland Altman analysis was performed in three different ways: All the samples with quantifiable viral loads; the samples with more than 2Log IU/ml and less than 6Log IU/ml, and the samples with less than 2Log IU/ml and more than 6Log IU/ml (Figs: 2a,b,c). The log difference in all the samples were between $-Log_{10}1.74$ and $Log_{10}1.39$ (-0.17 ± 0.79 Log_{10}IU/ml). Further, the log difference in the samples with 2-6Log viral load were between $-Log_{10}0.92$ and $Log_{10}0.99$ (0.03 ± 0.48 Log_{10}IU/ml) while the sample other than 2-6 Log viral load were between $-Log_{10}2.70$ and $Log_{10}1.60$ (-0.55 ± 1.08 Log_{10}IU/ml). The most agreement between the two methods was found 2-6Log viral load for the samples.

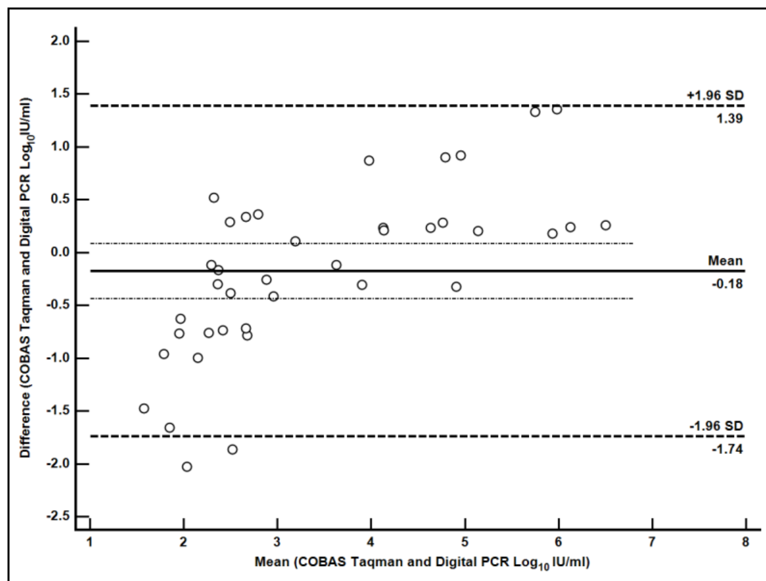


Figure 2a: Bland-Altman analysis showing the differences in quantification between Roche COBAS taqman and Digital PCR for the HBV DNA viral load of all the samples.

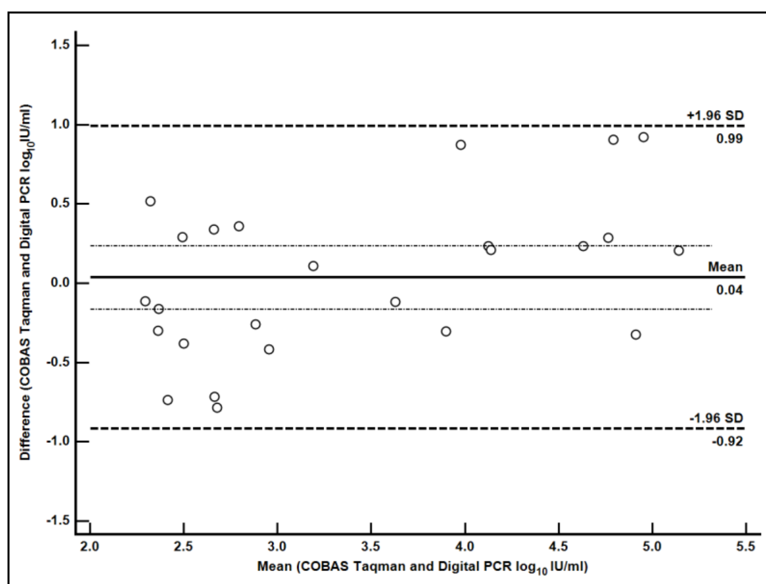


Figure 2b: Bland-Altman analysis showing the differences in quantification between Roche COBAS taqman and Digital PCR for the HBV DNA viral load of the samples with 2-6 Log_{10}IU/ml

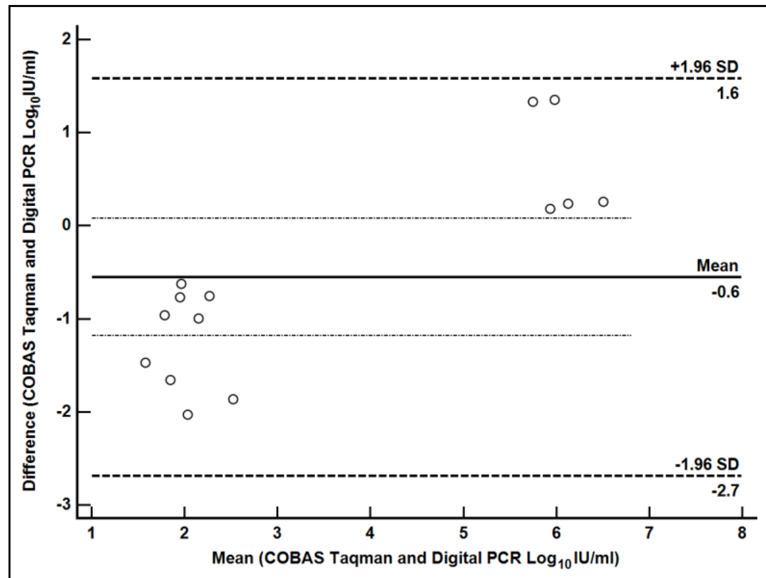


Figure 2c: Bland-Altman analysis showing the differences in quantification between Roche COBAS taqman and Digital PCR for the HBV DNA viral load of the samples with less than 2 Log10 IU/ml and more than 6 Log10IU/ml.

Box and Whisker plot for the HBV viral load samples represents the distribution of the viral load for the COBAS taqman as well as Digital PCR assay (Figure-3). The boxes represent the distribution of the middle 50% of the data and the line in the box shows the median value, while the rest 50% are denoted by the upper and lower whiskers and the ends of the whiskers indicate the minimum and maximum values. The median value for COBAS Taqman and the Digital PCR were 2.75 $\pm$ 1.73 Log_{10}IU/ml and 3.06 $\pm$ 1.19 Log_{10}IU/ml respectively showing the difference of 0.31 Log_{10}IU/ml which is less than 0.5 Log.The average viral loads (log_{10}IU/mL) in the samples measured by COBAS Taqmanand Digital PCR were 3.34 (SD - 1.73) and 3.52 (SD - 1.19) respectively (Table 2). The mean log_{10} differencebetween the two assays was 0.17 (SD -0.79) which is again less than 0.25 Log.

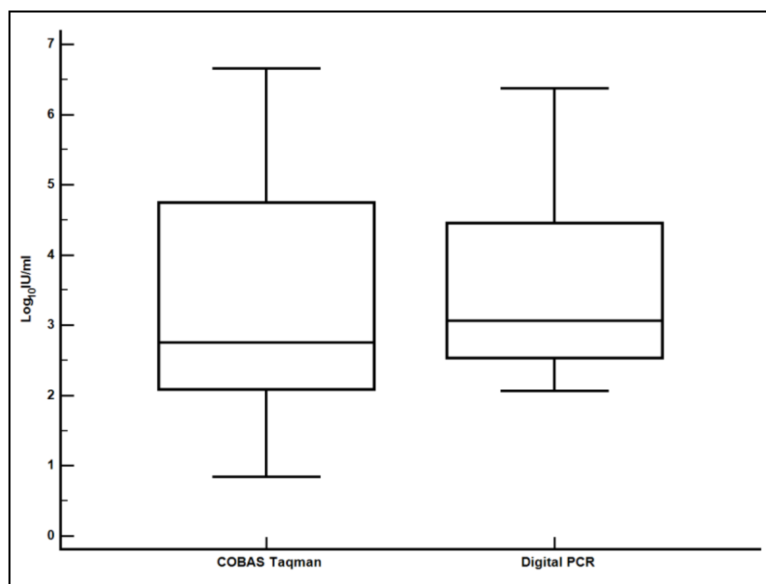


Figure 3: Box and Whisker plot for the HBV viral load samples detected by both the methods.

Table 2: Mean, Standard deviation and Coefficient of variation of the HBV viral load as detected by both the methods (Roche COBAS Taqman and Quantstudio 3D Digital PCR)

	Mean of samples (Log_{10}IU/ml)	SD	CV (%)
COBAS Taqman Method	3.34	1.73	0.51
Digital PCR Method	3.52	1.19	0.33

DISCUSSION

The real-time PCR system COBAS TaqMan™ 48 for HBV DNA has been evaluated with manual extraction platforms (Weiss et al., 2004; Gordillo et al., 2005). There are also reports of droplet digital PCR for HBV viral load detection in FFPE tissue (Huang et al., 2015)and plasma samples(Laure et al., 2014; Tang et al., 2016).No report on the Quantstudio 3D Digital PCR for HBV viral load detection and furthermore no such studies are available comparing Quantstudio 3Ddigital PCR methodology with other established or FDA approved real time technique for this study. The main aim of this study is to have a pilot project to assess the possibility of monitoring levels when a patient has cleared his viral load and to utilize this assay for detection of possible recurrence using a technology that is not only cheaper but equally sensitive as compared to COBAS Taqman real-time PCR.

Linear regression analysis in our study showed a good correlation between the two methods for HBV viral load detection ($r=0.92$, $p<0.0001$). Even with the samples of 2-6 Log_{10}IU/ml as well as less than 2 Log_{10}IU/ml and more than 6 Log_{10}IU/ml HBV viral load, higher correlation ($r=0.91$, $p<0.0001$ and $r=0.96$, $p<0.0001$ respectively) was observed between the COBAS Taqman and Digital PCR methodology. It was however noticed based on the linear regression curve, that at the lower end of the spectrum, digital PCR generally had a trend to a higher estimation of viral load compared to the Roche Taqman 48 assay. It would be important in a large scale studies to further delineate this trend and define it as a scope of limitation for either real time PCR or digital PCR and account for it in the clinical setting.

Bland Altman Plot revealed higher agreement between the two methods for the samples with the viral load of 2-6 Log_{10}IU/ml. The difference between the two methods was within 1.0 Log_{10}IU/ml while more than 70% of the samples were within 0.5 Log_{10}IU/ml for the samples with 2-6 Log_{10}IU/ml. Higher variation was observed between the two methods using Bland Altman plot for the samples with less than 2 Log_{10}IU/ml and more than 6 Log_{10}IU/ml. The variations in HBV DNA viral load was observed between the two methodologies, which might be due to the fact that different extraction and amplification procedures involved. Furthermore the variability in the analysis was in accordance with the other studies that have compared two different methods for viral load detection(Allice et al., 2008; Tung et al., 2015; Tang et al., 2016).It has already been recommended to use the same assay for a given patient to precisely monitor the efficacy of the antiviral drugs because of variations in different assays(EASL, 2009; Pawlotsy et al., 2008). Similarly, variations have also been shown in our study between the two techniques.We hence recommend using either of the assays i.e. Roche COBAS Taqman FDA approved assayor a Digital PCRTaqman assay on the Quantstudio 3D, as both of them are equally sensitive at low viral copy level.

Our resultshowever demonstrated that digital PCR is suitable for HBV detection with similar sensitivity and specificity in the clinical samples compared to a FDA approved Real Time PCR assay. Given the current scenario of HBV viral load testing which involves significant investment in standardization and management of controls, the digital PCR technologies offer a convenient way to continue processing these samples with a significantly reduced day to day running investment albeit at a slightly higher capital cost. Certain technologies such as Raindance with extremely high chip densities have significantly increased our ability to obtain higher dynamic ranges (Baker, 2012). However, the cost of the Raindance technology remains prohibitive comparatively.

CONCLUSION

Utilization of the digital PCR technology increases sensitivity and the cost per test decreases. There isalso a great scope in utilizing this technology especially in a resource scarce setting. Furthermore, the absolute HBV viral load quantification obtained from digital PCR can serve as inhouse standards or controls for real-time PCR detection technique to provide cheaper and more sensitive as well as accurate quantification for the patients. Future studies on more number of clinical samples as well as genotype analysis of the same, might help us to increase the validity of the data for the practical implementation of the technique.

Conflict of Interests

The authors declared no conflict of interests.

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