International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2015): 78.96 | Impact Factor (2015): 6.391

Clinical Performance Characteristics of Hepatitis B Virus Quantification by Combining Artus-DSP Assay with Rotor Gene Q-Real-Time PCR

Kandpal JC[1]☯, Kumar R[1], Kumar V[2], Singh PR[3]

[1]Department of Biotechnology, Kumaun University Nainital, Uttrakhand
[2]Core Diagnostics, Gurugram, Haryana
[3]Qiagen India (P) Ltd, New Delhi
[1☯]Corresponding Author: Jagdish.kandpal[at]yahoo.com

Abstract: ***Background: A simple and reliable DNA extraction & quantification method of hepatitis B virus (HBV) is critical in developing a viral load measurement for HBV infection. Current commercially available plasma Hepatitis B Virus (HBV), DNA extraction & quantification methods are less sensitive and require optimization, which restrict wide adoption in clinical laboratories. This study offers a report on the quantitative viral load- artus® HBV RG PCR assay by combining QIAamp® DSP Plasma DNA extraction with Rotor gene Q-Real-time PCR (Rotor gene Q-PCR). Methods: Plasma was separated by centrifugation after collection of whole peripheral blood, which was then stored at -20^0 C until process. DNA was extracted as per instruction & guidelines of manufacturer. The recovered eluted DNA was mixed into PCR master-mix. The Q-PCR assay performance, including linearity, diagnostic accuracy and reliability were determined. Viral load compared of the no viral load, low viral titer & high viral titer from the clinical samples for evaluation. Results: The average % yield for HBV DNA standards used was within in the range (SD 0.04 to 0.13, CV% 0.97 to 2.22). The coefficient of variation (CV) of HBV quantification for low and high titer samples was 2.23% and 2.37%, respectively. The real-time assay linearity was demonstrated with a slope of -3.03 to -3.50 and R^2 values of 0.98 to 0.99. Accuracy and reliability of the Rotor gene Q-PCR assay was confirmed with the reference panel, and there was a strong correlation between the two results (R2 = 0.99, < 0.01) & found no major difference in observed viral load. Conclusions: The Rotorgene Q-PCR performance with artus-DSP assay is reliable and wide linear range quantification method and can be used for sensitive detection of plasma HBV with low nucleic acids content.***

Keywords: Hepatitis B Virus, Quantitative Standards, DNA amplification, Taqman Technology

1. Introduction

Hepatitis B Virus infection has been known as a common cause of liver diseases in humans ranging from self-limiting acute hepatitis to active chronic hepatitis, liver cirrhosis, liver failure and hepatocellular carcinoma [6]. Approximately 95% of adults, exposure to HBV develops to an acute infection without clinical symptoms or with an inflammatory disease of the liver (acute hepatitis), which usually gets resolved in about 6 months, whereas the remaining 5 % fails to control the viral infection, developing to chronic disease or cancer [17]. Hepatitis B virus is prevalent worldwide, with an estimated 400 million people infected globally [28]. About 350 million people have chronic HBV infections [5], [11], with annually more than 750,000 deaths worldwide [12]. India has the major etiology of chronic liver diseases with more than 40 million carriers of HBV and also has ethnically diverse population [2]. With 50 million carriers, India has the second largest population of individuals with chronic Hepatitis B infection worldwide [27].

HBV is diagnosed with serological markers; include HBsAg, anti-HBsAg, HBeAg, anti-HBeAg, anti-HBc, serum alanine transaminase (ALT), aspartate transaminase (AST) tests and quantification of HBV DNA, whose measurement is applied in the management of HBV infections [3]. HBV DNA viral load play a critical role in determining the phase of infection and to decide the treatment [11]. Lower level of HBV DNA is associated with a lower risk of a hepatocellular carcinoma development than a higher level [5]. HBV DNA levels change during different phases of infection in chronically infected individuals [8]. As per guidelines of world health organization for the prevention and treatment with person of chronic HBV infection, HBV DNA quantification is recommended for the treatment [29]. HBV DNA quantification studies have helped researchers to consider guidelines for the assessment of diagnosis and treatment responses. HBV DNA monitoring is therefore a best predictor and has been recommended for efficient management of HBV [21], [23]. Various HBV DNA quantitative assays are now available and it is important to use an accurate HBV monitoring tool. Real-time PCR has became the standard diagnostic technology and is widely used for HBV detection with high sensitivity, wider linear range, and reproducibility [24], [19].For making same level results, HBV DNA load is universally reported in IU/ml that have been calibrated with the World Health Organization international standard for HBV DNA [20]. There are different viral detection limit assay available of wider range [7], but for treatment prospect of HBV, a more sensitive assay with a lower limit of detection of lower than 15 IU/ml is recommended for early detection of viral load [13], [10].

Plasma HBV DNA levels are unstable with time progress and depend upon the infection phase. HBV DNA detection in whole peripheral blood is a reliable marker of active HBV replication and is more useful in the diagnosis of infection comparison to immunoassay [15]. It had also been verified that fast processing from plasma DNA after plasma collection was necessary to produce realistic results [22].

Thus more specific method is required which can handle large sample input volume to increase sensitivity for HBV DNA detection assay so that it could reach recommended limit of detection [9]. There are a few commercial assays available which fulfill to identify the recommended lower limit viral load. Therefore, a simple, reliable DNA extraction & amplification method is important for sensitive HBV DNA detection. The HBV DNA extraction with DSP viral kit following amplification through Rotor gene Q-PCR is a validated & CE-IVD approved method. The extracted HBV DNA by DSP method is applied into artus HBV RG PCR pre-mixture and the performance characteristics of the artus-DSP assay was evaluated and promised to high DNA recoveries with good reproducibility. The method illustrated that artus-DSP Q-PCR assay is the reliable, sensitive detection assay of HBV DNA and the method also give new insight on the application of plasma samples with low DNA content.

2. Methods

Ethics statement; This study was performed & approved at Core Diagnostics, Guru-gram & the institutional review board, Kumaun University Nainital. All subjects were recommended by clinician to identify HBV DNA viral load. Test requisition form was received from all subjects before this study & their identity has not been disclosed.

2.1 Samples

For this prospective study, a total of 56 clinical samples were taken for the evaluation of HBV DNA Viral Load in the laboratory. The whole peripheral blood samples from patients suspected with HBV were collected between December 2016 to July 2017 and plasma separated immediately. All samples were stored at -20^{0}C until use. The relevant history and other details of the patients were noted from reports and test requisition form.

2.2 Extraction of HBV DNA

An assay (artus-DSP) using QIAamp® DSP virus extraction procedure was performed as per guidelines. The QIAamp® DSP virus extraction procedure allows the selective binding of viral nucleic acid in silica-based membrane. The viral DNA is purified from 500 µl of plasma and finally eluted in 50 µl of elution buffer. Reactions for amplification were performed similar to artus® HBV RG PCR. The Lower detection limit of the artus® HBV RG PCR kit using the DSP virus kit was claimed to be 10.22 IU/ml. Performance characteristics, including linear range, accuracy and analytical reliability, were studied using HBV DNA standards and clinical samples.

2.3 Real-time PCR

The Real-time HBV PCR assay with artus® HBV RG PCR kit was performed according to the manufacturer's instructions. The Q-PCR reactions were performed on a Rotor gene Q Real-Time PCR System. The cycling parameters consisted of incubation at 95^{0}C for 15 sec, 55^{0}C for 30 sec & 72^{0}C for 15 sec with 45 times repeated cycles.

2.4 Establishment of Standard Curves

For standardization of the quantitative HBV DNA detection assays, the HBV QS standards with known copy numbers ($1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$ & $1x10^5$ IU/µl) were used in the experiment. The quantitative values of standards & samples were plotted against the concentration of log10 IU/ml, which resulted in typical amplification curves.

2.5 Percent yield of HBV DNA

The HBV DNA was extracted from plasma samples with low (4.0 log10 IU/ml) and high (6.0 log10 IU/ml)–viral load using respected protocol, recommended reagents & DNA-extraction column. The amplified HBV DNA was quantified using the artus Q-PCR assay. The % yield of HBV DNA was calculated as: actual yield/ theoretical yield *100%.

2.6 Diagnostic correlation

The accuracy & reliability of the artus-DSP Q-PCR assay was determined by analyzing the known low-concentration WHO standard (3.93 log10 IU/ml) & diagnostics correlation of no titer, low titer & high titer with reference samples.

3. Statistical Analysis

The quantitative results were log transformed for further analysis. The continuous variables were expressed as mean and standard deviation (SD). The correlation analysis between the artus-DSP Q-PCR and reference method (CAP-CTM Real-time PCR-Taqman Technology) were analyzed by Pearson's product moment correlation coefficient and linear regression equation. The Bland-Altman plot was also used in order to represent the degree of agreement (đ $\pm$ 1.96*SD) between quantitative results obtained from both methods. All statistical analyses were performed using the Microsoft-excel.

4. Results

Clinical performance characteristics of among 56 clinical samples tested, 47 samples were detected by the artus-DSP assay with HBV DNA viral loads ranging 11.8 IU/ml to $4x10^8$ IU/ml (median, 2530 IU/ml). Likewise, 26 samples were analyzed by reference method (CAP-CTM Real-time PCR-Taqman technology) ranging 70 IU/ml to 20123310 IU/ml of HBV DNA viral load (median, 943 IU/ml). Out of 26 (46.4%) samples that were quantified by both method, the differences in quantification mean were 2.82 log10 IU/ml (artus-DSP) and 2.81 log10 IU/ml (reference method), respectively.

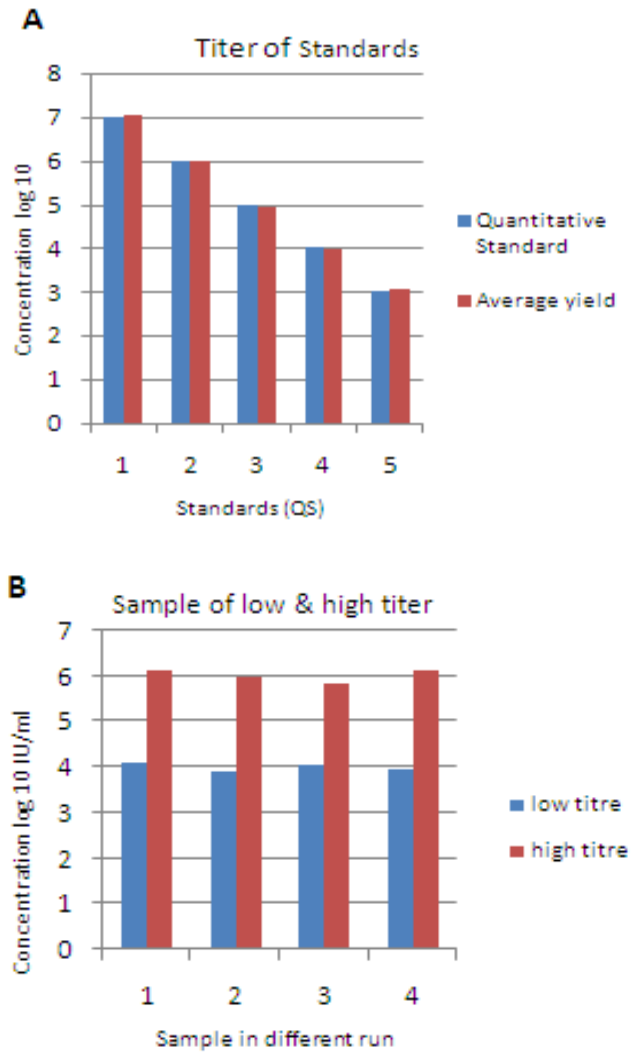

Figure 1: Yield of HBV DNA titer with standards and clinical samples; (A) The average % yield of HBV DNA concentration obtained using quantitative standards QS1 (100.57), QS2 (99.74), QS3 (99.17), QS4 (99.14), QS5 (101.67); (B) The average viral load for high-titer (6.018 log10 IU/ml) and low-titer (4.001 log10 IU/ml) for HBV DNA concentration obtained using the DNA-extraction column method.

The value of average % yield for high-titer QS1 & low-titer QS5 samples were slightly higher than rest of the QS titers, QS2, QS3 & QS4. However, the difference was not found to be statistically significant (Figure 1 A). At the same time, the average value of viral load observed for the low-titer sample and for the high-titer, obtained with the DSP DNA-extraction column were 4.001 (SD 0.089, CV% 2.239) and 6.018 (SD 0.143, CV% 2.378) respectively (Figure 1 B).

The slopes of the standard curves were generally at -3.03 to -3.50. The consistency of the standards was evaluated by a correlation coefficient (R^2) of 0.98 to 0.99, which indicates the linearity of the values plotted in the standard curves (Figure 2). All the relevant data were detected according to the standard curves in order to judge the clinical performance & characteristics of the present method including, linearity and diagnostic accuracy. Linearity was promised between 31.6 and 2 × 10^7 IU/ml in each assay, using quantitative standards. The assay linearity was assessed as the difference between the detected values and the nominal values assigned to the standards.

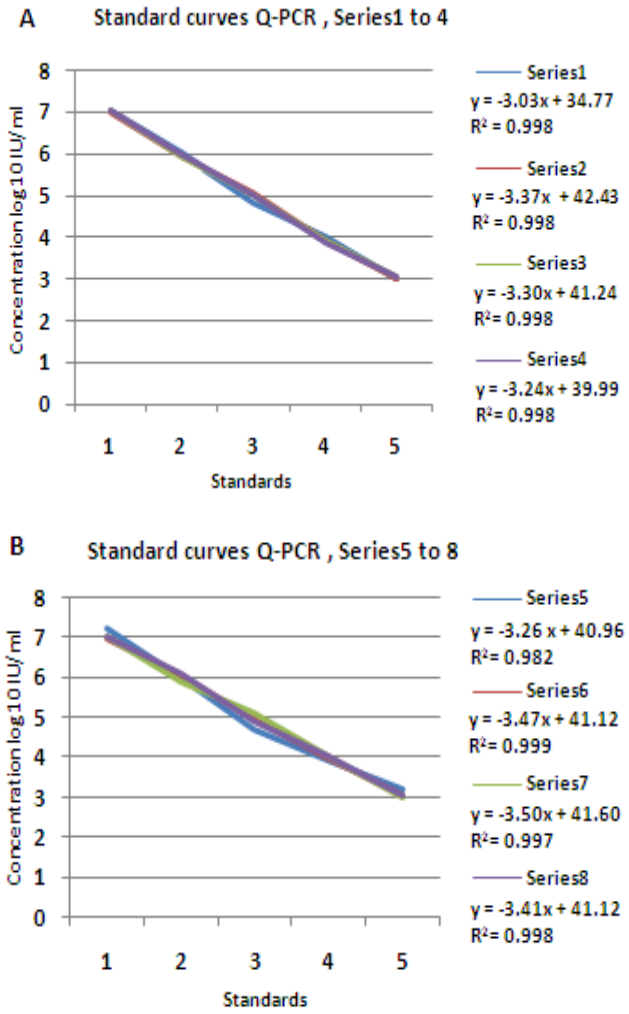

Figure 2: Performance of standards curves in Q-PCR assay; Standard curves (concentration log10 IU/ml) applied in the Q-PCR assay for the determination of results by using QS1, QS2, QS3, QS4, and QS5. (A) Series1 to 4 applied in correlation study of two methods used. (B) Series5 to 8 applied to determine viral load for low titer & high titer samples.

Diagnostic accuracy of artus-DSP Q-PCR assay was determined by analyzing the dilution of the 8500 IU/ml WHO standard. The positive value was analyzed with respect of referring concentration levels.

Correlation with 26 reference clinical samples was accessed for viral load comparison. Samples of no viral load, low viral load and high viral load were analyzed using the artus-DSP Q-PCR and the reference method using CAP-CTM Real-time PCR-Taqman Technology. As shown in Figure 3 A, correlation analysis for the paired quantitative results demonstrated a significantly positive correlation between the two assays (R^2 = 0.993, P<0.01). A Bland-Altman plot was also used to determine the agreement between the two assays (Figure 3 B). Using this method, the differences between the

HBV DNA viral load log10 IU/ml concentrations of two assays were plotted against the averages of the two methods.

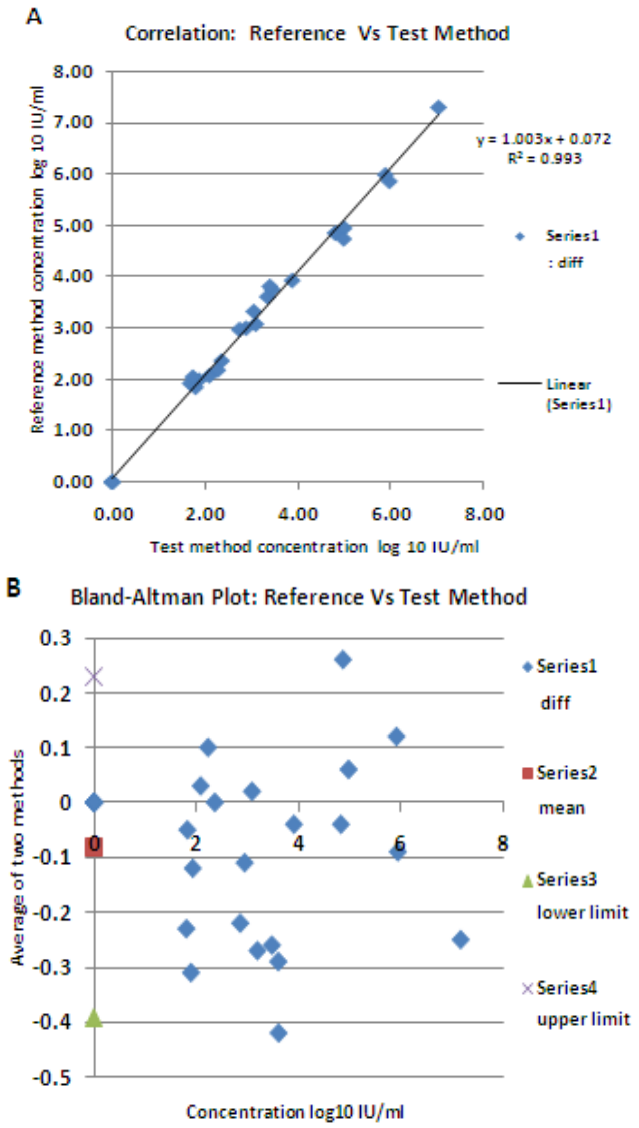

Figure 3: (A) Correlation; Comparison of paired quantitative result (R= 0.996; P = < 0.01) between the two assays observed by linear regression equation & Pearson product moment correlation coefficient. (B) Level of agreement; In Bland-Altman plot, the mean difference between artus-DSP-QPCR and the reference method calculated -0.081 log10 IU/ml (SD 0.158 log10 IU/ml) with limits of agreement of -0.391 to 0.229 log10 IU/ml.

As shown in Figure 3 B nearly all the differences of the paired viral loads were within the range of the mean difference, only two out of 26 values lies just outside the limit. The mean value of difference between these two groups was -0.081 (log10 IU/ml), with a SD of 0.158 log10 IU/ml. The Bland-Altman analysis expects the values to cluster around the mean of the differences, and certainly within 2 standard deviations of the mean. Assuming the result differences was normally distributed in a 95% prediction interval, to accept the test method depends on the level of precision needed to the particular technology. Since the range of differences between the reference and test method is very low (i.e. < 2 standard deviations of difference), hence this method can be utilized for the sensitive & wide linear detection of HBV quantification.

5. Discussion

Hepatitis B virus infection counts with a wide range of clinical symptoms and as this infection have a heavy economic and social burden for both patients and society. Therefore, there is a need for wide range reliable markers to improve the management of this infection. Lower HBV DNA level is associated with a lower risk of a hepatocellular carcinoma (HCC) development than a higher level [5]. Since the viral load of HBV patients varies greatly, Q-PCR assays with a wide detection range are highly desirable. Available real-time PCR assay for HBV DNA quantization have diverse and not the low level of limit of detection as recommended. [4]

In this study, we verified the performance specifications of Real-time HBV detection of European Conformity (CE)-marked IVD-licensed artus-DSP assay for quantification of HBV DNA. The Clinical Laboratory Improvement Amendments (CLIA) specified that clinical laboratories should verify the manufacturer's performance specifications in the respective laboratory [26]. Testing of the performance characteristics, including analytical linear range, accuracy and reliability was performed. The research has demonstrated that the detection of low level of HBV DNA was essential in order to determine the necessity of antiviral treatment [19]. The improved detection sensitivity of HBV DNA detection assays is essentially dependent upon an increased sample input volume [25]. Therefore, a large volume of sample is advantageous for the preparation of increased amounts of DNA template. The amplification efficiency of Q-PCR with DNA prepared by DSP was satisfactory, demonstrating the DSP-based DNA extraction is compatible with Q-PCR. The retention of DNA by membrane is majorly affected by DNA size and pore size of filtration membrane [1]. As reported, the plasmid DNA (3.0 to 17 kbp) transmission significantly decreased with increasing pore size of the membrane, which indicated that the pore size played a key role in affecting DNA yield [16]. In this work, HBV DNA yield obtained from artus-DSP method for low viral titer & high viral titer were satisfactory, indicating HBV DNA can be effectively retained by column devices. The gain & loss of DNA amplification signal can be described by the adsorption of DNA to the consumable items & while handling process of repeated freeze-thaw cycles [14], however it was not found any significant difference between % yields obtained. From the results of performance evaluations, it appeared that the artus-DSP Q-PCR assay was valuable method. The detection range was demonstrated of 11.8 IU/ml to $4x10^8$ IU/ml, with a slope of -3.03 to -3.50 and R^2 values of 0.98 to 0.99, indicating the reliability of the artus-DSP method. [18].The Bland-Altman analysis indicated no major difference between the two methods. The difference of the values obtained outside the limit could be due to repeated freeze-thaw of the samples [14]. Hence the results provided evidence for the performance of this assay that was compatible in analyzing plasma samples of different viral loads.

In conclusion, the artus-DSP Q-PCR assay is reliable and wide linear range quantification method. The method can be used for the sensitive detection of plasma HBV. This study finds it to be a powerful tool to achieve optimal monitoring of infection level and timely treatment advice. In addition, with its advantage in handling large volumes of sample, artus-DSP method holds the potential for dealing with low nucleic acids content.

6. Acknowledgments

The author thanks the staff of molecular laboratory of Core Diagnostics for their assistance to carry out the HBV DNA identification with the Rotor gene-Q system.

References

[1] Arkhangelsky E, Steubing B, Ben-Dov E, Kushmaro A, Gitis V. Influence of pH and ionic strength on transmission of plasmid DNA through ultra filtration membranes. Desalination. 2008; 227(1-3):111-19.
[2] Banerjee A, Datta S, Chandra PK, Chowdhury SR, Panda CK, Chakravarty R. Distribution of hepatitis B virus genotypes: Phylogenetic analysis and virological characteristics of Genotype C circulating among HBV carriers in Kolkata, Eastern India. World J Gastroenterology. 2006; 12(37): 5964-71
[3] Behzadi MA, Ziyaeyan M, Asaei S. Hepatitis B virus DNA level Among the Seropositive Afghan Immigrants, Southern Iran. Jundishapur J Microbiol. 2014; 7(5):e10127
[4] Belopolskayaa M, Avrutinb V, Firsova S, Yakovlevc A. HBsAg level and hepatitis B viral load correlation with focus on Pregnancy. Annals of Gastroenterology. 2015; 28: 379-84
[5] Chan HL. Identifying hepatitis B carriers at low risk for hepatocellular carcinoma. Gastroenterology. 2012; 142: 1057-60.
[6] Chang MH, You SL, Chen CJ, Liu CJ, Lee CM, Lin SM. Decreased incidence of hepatocellular carcinoma in hepatitis B vaccines: a 20-year follow-up study. J Natl Cancer Inst. 2009; 101(19):1348-55.
[7] Chen JL, Lin XJ, Zhou Q, Shi M, Li SP, Lao XM. Association of HBV DNA replication with antiviral treatment outcomes in the patients with early stage HBV related hepatocellular carcinoma undergoing curative resection. Chin J Cancer. 2016; 35:28
[8] Chevaliez S, Rodriguez C, Pawlotsky JM. New virologic tools for management of chronic hepatitis B and C. Gastroenterology. 2012; 142:1303-13.
[9] Ciotti M, Marcuccilli F, Guenci T, Prignano MG, Perno CF. Evaluation of the Abbott Real-time HBV DNA assay and comparison to the Cobas AmpliPrep/ Cobas TaqMan 48 assay in monitoring patients with chronic cases of hepatitis B. J Clin Microbiol. 2008; 46(4):1517-19.
[10] EASL. Management of chronic hepatitis B. EASL clinical practice guidelines (European Association for the Study of the Liver). J. Hepatol. 2009; 50:227-42
[11] EASL. Management of chronic hepatitis B virus infection. EASL clinical practice guidelines (European Association for the study of the Liver). J Hepatol. 2012; 57:167-85.
[12] Fattovich G, Bortolotti F, Donato F. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. J Hepatol. 2008; 48:335-52.
[13] Garbuglia AR, Angeletti C, Lauria FN, Zaccaro P, Cocca AM, Pisciotta M. Comparison of Versant HBV DNA 3.0 and COBAS AmpliPrep-COBAS TaqMan assays for hepatitis B DNA quantitation: Possible clinical implications. J Virol Methods. 2007; 146(1-2):274-80.
[14] Krajden M, Minor JM, Rifkin O, Comanor L. Effect of Multiple Freeze-Thaw Cycles on Hepatitis B Virus DNA and Hepatitis C Virus RNA Quantification as Measured with Branched-DNA Technology. Journal of Clinical Microbiology. 1999; 37 (6): 1683-86
[15] Lai MW, Lin TY, Tsao KC, Huang CG, Hsiao MJ, Liang KH, et al. Increased seroprevalence of HBV DNA with mutations in the s gene among individuals greater than 18 years old after complete vaccination. Gastroenterology. 2012; 143(2):400-07.
[16] Latulippe DR, Zydney AL. Salt-induced changes in plasmid DNA transmission through ultra filtration membranes. Biotechnology Bioeng. 2008; 99(2):390-8.
[17] Liaw YF, Chu CM. Hepatitis B virus infection. Lancet. 2009; 373(9663):582-92.
[18] Lindh M, Hannoun C. Dynamic range and reproducibility of hepatitis B virus (HBV) DNA detection and quantification by Cobas Taqman HBV, a Real-time semi-automated assay. J Clin Microbiol. 2005; 43 (8):4251-14.
[19] Lok AS, McMahon BJ. Chronic hepatitis B. Hepatology. 2007; 45(2):507-39.
[20] Lok A S, et al. Antiviral drug-resistant HBV: standardization of nomenclature and assays and recommendations for management. Hepatology. 2007; 46:254-265.
[21] Mackay IM, Arden KE, Nitsche A. Real-time PCR in virology. Nucleic Acids Res. 2002; 30:1292-305.
[22] Neto AS, Wroclavski ML, Freire Pinto JL, Marsicano SR, Delgado PO. Methodological Standardization for the Extraction of Free DNA in Plasma of Peripheral Blood. J Cancer Sci Ther S5. 2012; 005.
[23] Pawlotsky J M, et al. Virologic monitoring of hepatitis B virus therapy in clinical trials and practice: recommendations for a standardized approach. Gastroenterology. 2008; 134:405-15.
[24] Pawlotsky J. M. Molecular diagnosis of viral hepatitis. Gastroenterology. 2002; 122:1554-68.
[25] Ronsin C, Pillet A, Bali C, Denoyel GA. Evaluation of the COBAS AmpliPrep-total nucleic acid isolation-COBAS TaqMan hepatitis B virus (HBV) quantitative test and comparison to the VERSANT HBV DNA 3.0 assay. J Clin Microbiol. 2006; 44(4):1390-99.
[26] USDHHS. Medicare, Medicaid and CLIA programs: laboratory requirements relating to quality systems and certain personal qualifications. U.S. Department of Health and Human Services, Centers for Medicare and Medicaid Services. Final rule. 2003; Fed. Regist. 16:3640-714.

[27] WHO. Prevention of Hepatitis B in India – An Overview. World Health Organization. 2002; South-East Asia Regional Office, New Delhi.
[28] WHO. Hepatitis B. Fact sheet no. 204. World Health Organization. 2011; Accessed 04 Nov 2014; http:// www.who.int/mediacentre/factsheets/fs204/en/.
[29] WHO. Guidelines for the prevention, care and treatment of persons with chronic hepatitis B infection. World Health Organization, Geneva. 2015; Guidelines. Approved by the Guidelines Review Committee Pocket Telephone, Inc.