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Research Article

Performance Characteristic of a Simple, Rapid and Cost-Effective Micro-PCR Assay for HBV DNA Quantification

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ABSTRACT

HBV DNA detection is crucial for identifying early acute and occult HBV infections. Detection and quantification of HBV-DNA are essential to diagnose and formulate to help in the treatment of HBV infection. Micro PCR is a portable battery-operated device based on real-time PCR developed by Bigtech Bangalore, India, which is inexpensive, fast, and easily installed and can be operationalized in a remote place where diagnostic services are not easily accessible in the country. The clinical and analytical specificity of the assay was comparable i.e., 100%. Intra-assay and inter-assay coefficient of variation ranged from 0.25% to 2.85% and from 0.75% to 3.25% respectively. A strong correlation ($r=0.9563$; $p<0.001$) with the in-house TaqMan HBV-DNA test (Life River Q PCR kit) v.2. Agilent System Inc. was obtained. The count of detection using the 3rd WHO International standard for HBV DNA was 5.8 Log 10 IU/ml. The test could equally detect the most prevalent HBV genotypes (A&G). Micro PCR (Bigtech Pvt Ltd) is a sensitive, specific, precise, and accurate for quantifying serum and plasma HBV-DNA assay. Thus, this simple and fast micro-PCR device can easily be used in the field as well as in remote places where the detection of HBV-DNA is not accessible thereby as an aid in diagnosing HBV infection and monitoring drug efficacy.

Keywords: Micro-PCR; HBV-DNA; Truenat™; Trueprep™

INTRODUCTION

Viruses are tiny infectious pathogens that multiply only within the living cells of other organisms and can infect all organisms, from plants and animals to microorganisms, including bacteria and palaeontology [1]. Due to the rapid increase in virus taxonomy research, many viruses are classified into 4404 species, 735 genera, and 122 families (including 35 subfamilies and 8 orders) [2]. Hepatitis B Virus (HBV) is a member of the Hepadnaviridae family and classified into eight genotypes (A-H). Infection with Hepatitis B causes a spectrum of clinical manifestations ranging from subclinical infection and self-limiting acute viral hepatitis (AVH) to fulminant hepatic failure, chronic hepatitis, cirrhosis, and hepatocellular carcinoma [3]. HBV has a double-stranded DNA genome of approximately 3200 base pairs organized into four partially overlapping open reading frames encoding envelopes, nuclei (pronuclei/nuclei), polymerases, and X proteins. Two hundred fifty-seven million people worldwide are infected with chronic hepatitis B (CHB), with 887,000 deaths annually. Over 90% of deaths and disorders from viral hepatitis can result from CHB and chronic hepatitis C infections [4]. The World Health Organization (WHO) has approved a strategy to eliminate viral hepatitis as a public health threat by 2030, which requires a 90% reduction in new CHB and a 65% reduction in CHB-related mortality. At least one million persons die annually from chronic liver disease, including cirrhosis and liver cancer [5].

India is classified in the Intermediate Hepatitis B Virus (HBV) group, with a prevalence of 2% to 4% in the general population. Infection is thought to occur horizontally, primarily through close physical contact in early childhood, but up to 30% of cases are due to vertical transmission. India owns 10% to 15% of the world's HBV pool and has 40 million HBV carriers, of which 15% to 25% develop cirrhosis and complications, leading to medical costs and premature deaths [6]. Twenty-five million infants are born yearly, and over one million have a lifetime risk of developing chronic HBV infections [7]. Estimates indicate that annually over 100,000 Indians die due to illness related to HBV

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infection.

The currently used method for diagnosing HBV is a serum or blood test for the presence of either viral antigens (HbsAg, HBeAg) or host antibodies (anti-HBc IgM, anti-HBe, anti-HBs, or anti-HBcIgG). HBsAg is overproduced in virus-infected hepatocytes, secreted into the blood, and acts as a marker of active infection and infectivity [8]. However, the commonly used ELISA (Enzyme-Linked Immunosorbent Assay) based tests for detecting antibodies or antigens cannot determine the exact viral load. Obtaining a measure of the HBV viral load is critical considering studies highlighting the link between viremia and the occurrence of HBV-related liver damage. PCR is a frequently used method for HBV DNA, which is reasonably helpful due to its high specificity and sensitivity. Though various factors affect sometimes in visualization of qualitative image of HBV-DNA amplification.

Similarly, Real-time PCR can estimate the viral load but is not used routinely due to the expense involved. Moreover, the process is currently not adaptable to resource-limited settings where sophisticated laboratory equipment and highly skilled technical expertise are unavailable, and services are not reaching remote areas. Bigtech Labs' portable, battery-operated micro-PCR platform [9], can be used in point-of-care chip-based technology [10], and resource-limited settings to provide a fast, accurate, and cost-effective diagnosis of Hepatitis B viral infection. So, the amplification of micro-PCR for the onsite performance of the reaction of HBV DNA with serum sample could be a feasible solution for detecting of HBV-DNA.

The major challenge in reducing the disease burden is inequity in access to healthcare facilities, particularly in remote areas and rural places. Current testing methodologies are often time-consuming, cumbersome and inadaptable to resource-limited settings. Existing point-of-care tests do not have the same sensitivity and specificity as Gold standard laboratory-based tests. Samples usually need to be transported to reference laboratories for a more conclusive test like culture, PCR, and ELISA. In the meantime, the patient is still sick and capable of transmitting the infection to others. Therefore, this study was conducted to enable rapid and convenient quantitative analysis in remote areas of the country.

MATERIALS AND METHODS

Patients

107 patients were recruited with chronic HBV infection who were referred from the OPD and liver clinic of Gastroenterology, AIIMS, New Delhi. 5 ml of blood were collected as per the criteria defined for viral markers, and consent was obtained. Institutional Ethical clearance was obtained from the Ethics Committee (IEC/NP-31/2012, Dated: 3 Jan 2012).

The sample size calculation was done by using the formula given below:

$$n = \frac{\left[Z_1 \frac{\alpha}{-2} \sqrt{2\pi_o (1-\pi_o)} + Z_1 - \beta \sqrt{\pi(1-\pi) + \pi_2 (1-\pi_2)} \right]}{(\pi_2 - \pi_1)^2}$$

$$\pi_o = \frac{(\pi_2 - \pi_1)}{2a}$$

Where, α= Significance level, π_1= Sensitivity of new test, π_2= Sensitivity of reference test, 1-β= Power. The sample size was calculated to 107 in total.

Hepatitis viral markers

Serum was separated after centrifugation at 1500 rpm for 10 minutes at 4°C and stored in aliquots at -70°C until testing. Viral markers were done for all the groups on sera stored at -70°C. The commercially available ELISA kits tested for HBsAg, IgM anti-HBc, and HBeAg. Anti-HCV was tested using a third-generation kit by the ELISA method and cutoff was considered as per defined from the manufacturers. A total of 107 blood samples were collected from the Liver clinic based on the positivity or negativity of HBeAg as:

Group (a)-HBeAg positive: 31 patients were selected after qualifying the diagnostic criteria (I. HBsAg Positive, II. HBeAg positive, III. Liver function test (ALT is more than upper limit of normal)).

Group (b)-HBeAg negative: 76 patients were selected after qualifying the diagnostic criteria (I. HBsAg negative, II. The antibody to the "e" HBeAg negative, anti HBV positive, III. Possible, elevated alanine amino transferase (ALT), an enzyme that is released into blood when liver cells are damaged).

Nucleic acid extraction

Nucleic acid extraction was performed using two different methods. The HBV DNA isolated from both methods was quantified using different techniques. The first method of Viral DNA was using the TruePrep™ kit, and isolated DNA was quantified using the Truenat™ kit [11]. While in the second method Qiagen kit was used to isolate Viral DNA [12] and the purity and quality of the DNA was checked form Nano-drop (Thermos scientific).

The TruePrep™ AUTO universal cartridge-based DNA sample analysis kit protocol was followed. It uses a unique matrix encapsulated in cartridges to purify clinical-grade nucleic acids. Briefly, 500 µl of plasma, pre-treated with lysis buffer incubated for 2 minutes at room temperature, was transferred to the sample chamber of the cartridge, and run for 20 min. The reaction process contains a preloaded internal positive control (IPC) cartridge. The sample DNA/RNA binds to the matrix, and the inhibitors present in the sample were washed away. At the end of the process, the bound DNA/RNA was eluted and collected in the elution tube. All waste generated by the process is contained in the cartridge disposal area. The eluate is transferred to the Elute Collection Tube (ECT). Then, transfer 6 microliters of eluate to a Truenat ™ chip for further analysis. The brief steps of the methods have shown in Figure 1.

Primers

Primers were designed by Beacon designer software, and synthesis of primers was done from IDT, Canada. The primers' specificity was checked through 1% agarose gel, and bands were visualized under a gel documentation system. The forward and backward primers sequences are shown in the Table 1.

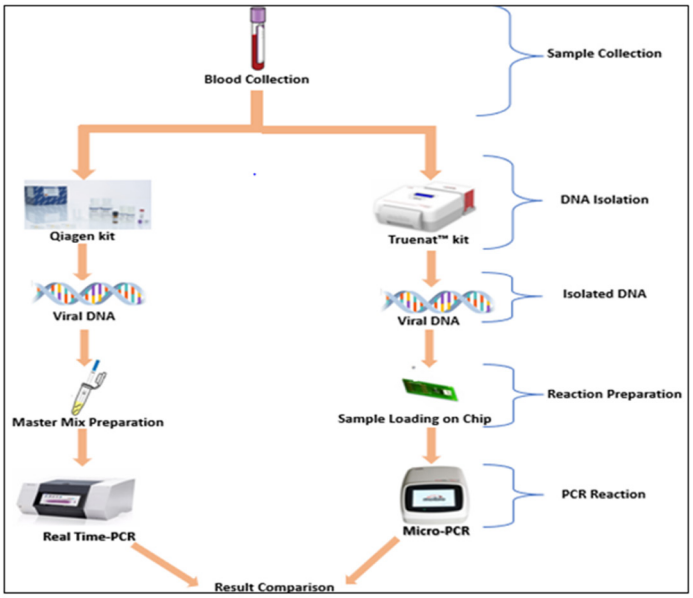

Figure 1: Flow chart of methodology.

Table 1: Sequence of primers used for Real-Time- PCR Primer Sequence.

Primer	Sequence
Forward (F)	5'-CCTGGGTGYGAAGTAATTTGG-3'
Reverse (R)	5'-TTTTARGCCCATATTAACATTGACAT-3'
TaqMan Probe	FAM 5'-AGACCCAGCATCCAGGGAATTAGTAGTCAGC- TAMRA-3'

PCR and Micro-PCR

HBV-DNA was extracted and performed PCR for genotyping analysis for Basal core region and Pre core region have shown in (Figures 2A and 2B).

Genotyping analysis was carried out by Restriction Fragment Length Polymorphism (RFLP) employing full genome/full S gene/or partial gene sequence using various restriction enzymes (AvaII, DpnII, Sau 3 AI and Bfu) for recognition of nucleotide sites in agarose gel (2%) and it was analyzed on gel documentation as shown in Figure 3.

The Amplification of HBV-DNA was done by quantitative Real Time-PCR with the thermal condition: 95°C for 3 min for one cycle, which includes 95°C for 30 sec, 57°C for 20 sec, and 72°C for 20 sec for 40 cycles. The copy number for HBV DNA was calculated using serial dilution as per defined concentrations by WHO and Acromatrix standards (IU/mL). The Truenat™ micro-PCR chip-based dried form of the reaction mixture developed from Bigtech Pvt. Ltd. Bangalore. The PCR reaction master mixture (chip) was loaded with 6 µl of extracted DNA. The thermal conditions as 95°C for 3 min for one cycle, which includes 95°C for 25 sec, 56°C for 20 sec, and 72°C for 15 sec for 40 cycles for quantification of HBV DNA.

Statistical analysis

The Bland-Altman plot conveniently displays the relationship between two pair variables using the same scale. This means we cannot get an error when making variable decisions like in a test. The Bland-Altman comparison method assumes that the two methods provide approximately the same results for each measurement date [13]. However, one method may measure more proportionally than the other. The scatter plot shows the two quantitative relationships variables measured for the same sample [14]. The Correlation Coefficient (r) measures the strength of the linear relationship between two quantitative variables.

RESULTS

Viral RNA isolated using Qiagen Kit then undergo Real Time-PCR for the amplification. WHO and Acromatrix standards were used as control while processing the reaction for Real Time-PCR. To initiate all reactions simultaneously, the enzyme master mixture was added to the sample by pipetting into a PCR tube and then spun down. HBV DNA copy counts were calculated for a 10-fold diluted series of HBV DNA included in each run as defined concentration by WHO and Acromatrix standards (IU/mL). Inputs for these standards ranged from 101 to 107 copies per reaction mixture, and nuclease-free H_2O was used as a Un Templated Control (NTC). Simultaneously, the same samples were processed for the Truenat™ micro-PCR chip to quantify Viral DNA. Among 107 samples, 31 were shown positive results while 76 were negative. The results of both methods were compared with each other. The amplification plot and Quantification of HBV viral DNA using Real Time-PCR was shown in Figure 4.

The results have shown for HBV-DNA quantification of both methods in Table 2.

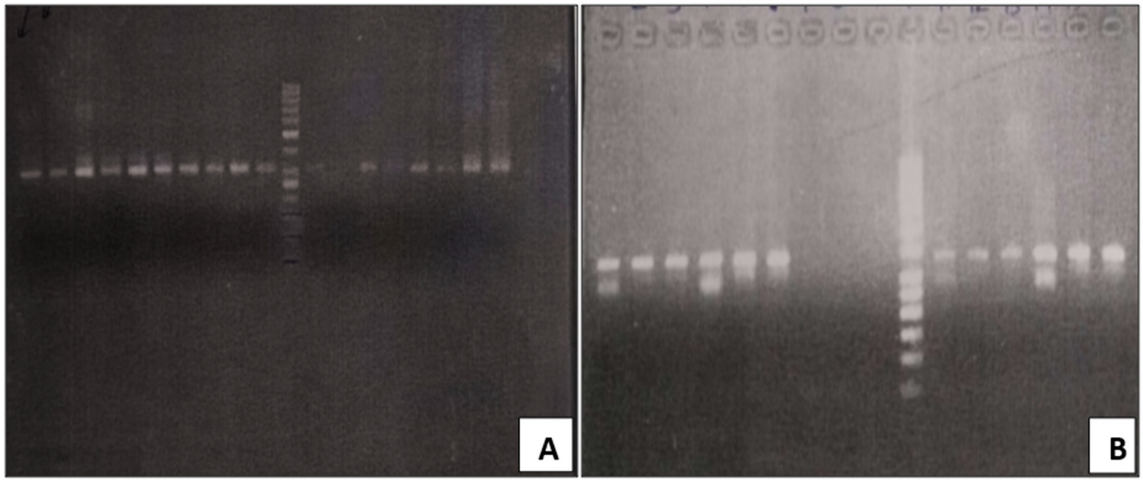

Figure 2: A) Amplification of pre-core region; B) Amplification of basal core region

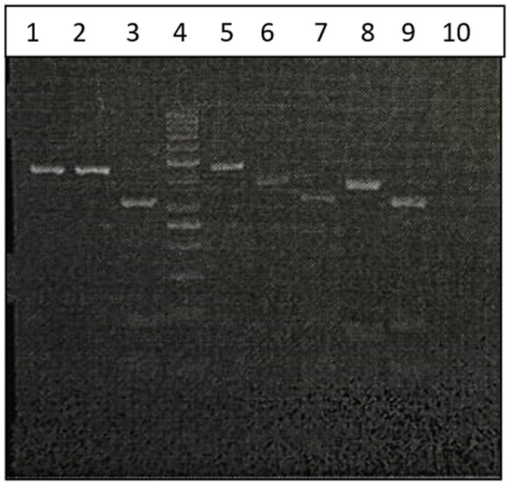

Figure 3: Genotyping of HBV-DNA; well 1-3 shows D genotype and 5-7 and 8-9 shows genotype A.

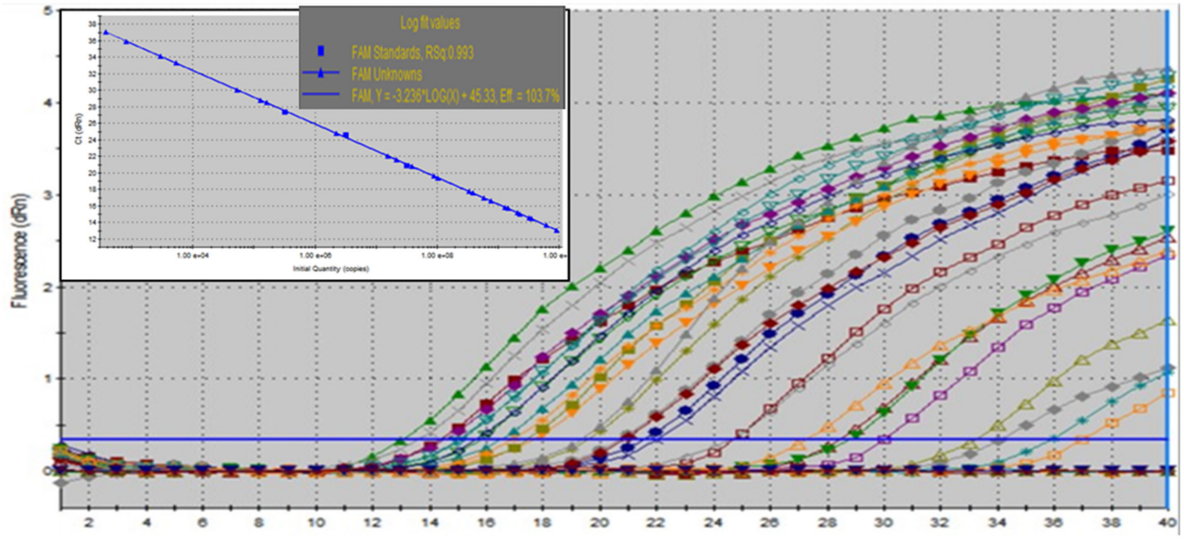

Figure 4: Real Time-PCR Amplification and Standard curve plot of HBV viral DNA. **Note:** (■) FAM Standards, RSq:0.993, (—▲—) FAM Unknowns, (___) FAM, Y=3.236*LOG(X)+45.33,Eff,=103.7%

Table 2: Quantification of HBV DNA using Truenat™ Device and AIIMS HBV Q-PCR.

S. No.	Sample ID	Truenat™ Result [IU/mL]	Truenat™ Result [Log IU/mL]	Q-PCR Result [IU/mL]	Q-PCR Result [LOG IU/mL]
1	715	6.80E+05	5.83	99000	5
2	214	8.60E+02	2.93	410	2.61
3	219	44	1.64	1300	3.11
4	288	47000000	7.67	41000000	7.61
5	292	41000000	7.61	11000000	7.04
6	215	3900	3.59	3700	3.57
7	216	13000	4.11	4300	3.63
8	273	750	2.88	420	2.62
9	274	1100000	6.04	2100000	6.32
10	285	3500	3.54	1800	3.26
11	259	30000	4.48	21000	4.32
12	267	59000	4.77	46000	4.66
13	270	150000	5.18	62000	4.79
14	291	54000000	7.73	44000000	7.64
15	260	4000000	6.6	2600000	6.41
16	261	11000	4.04	45000	4.65
17	269	77000	4.89	36000	4.56
18	262	50000000	7.7	46000000	7.66
19	223	400	2.6	470	2.67
20	251	51000	4.71	330	2.52
21	279	1100000	6.04	380000	5.58
22	281	100000000	8	27000000	7.43
23	304	140000000	8.15	12000000	7.08
24	295	990	3	360	2.56
25	18B	27000	4.43	44000	4.64
26	37B	1500000	6.18	180000	5.26
27	718	45000	4.65	25000	4.4
28	41	34000000	7.53	17000000	7.23
29	423	1300	3.11	2600	3.41
30	429	26000	4.41	12000	4.08
31	458	7600	3.88	27000	4.43

Accuracy of Truenat™ HBV assay

Accuracy was determined by DNA extraction and Truenat™ HBV-PCR for different sample titers for 5 consecutive days. The accuracy of the Truenat™ HBV assay, which quantitatively estimates the amount of HBV virus using three different lots of reagents, was also determined compared to AIIM S© in-house real-time HBV assay. The Bland-Altman plot of the method comparison was used to determine the correlation and the results reported in the Figure 5.

The mean difference between the tests is 0.23 Log IU/mL, less than the clinically significant difference of 0.5 Log IU/mL. The Correlation coefficient (r) for the micro-PCR Truenat™ method is 95% which shows a strong relationship between the methods as shown in Figure 6.

The mean value and standard deviation of Micro-PCR Truenat™ quantification were plotted against the mean value and Standard Deviation Real Time-PCR, as shown in Figure 7.

The micro-PCR Truenat™ reaction was performed on three devices of a different lot to find the inter lot deviation as shown in (Figure 8). The average deviation in HBV viral DNA estimated was 0.37 IU/mL, which is acceptable as the value is less than the clinically significant variation of 0.5 IU/mL.

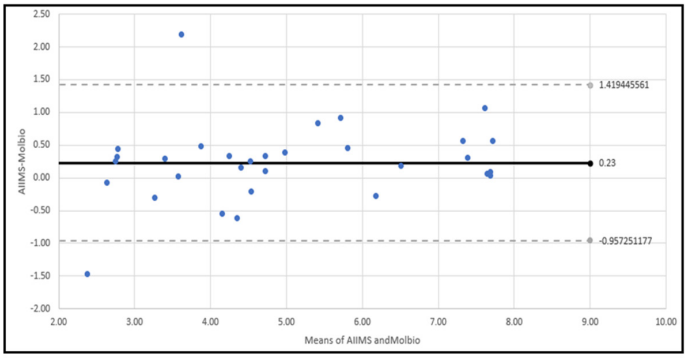

Figure 5: Bland-Altman plot for the comparison of results of Truenat™ and Q-PCR (AIIMS) result.

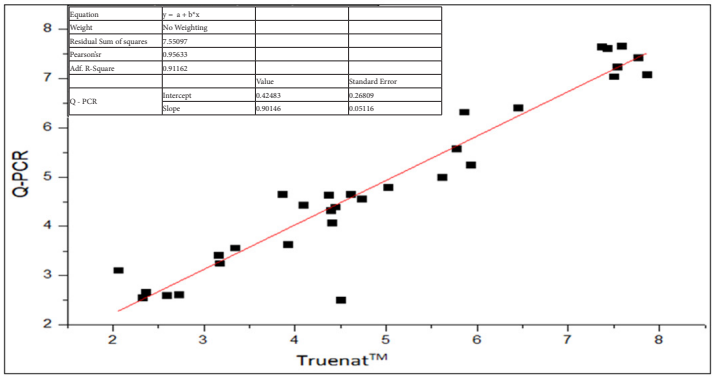

Figure 6: Scattered plot of both AIIMS and Truenat™ method.

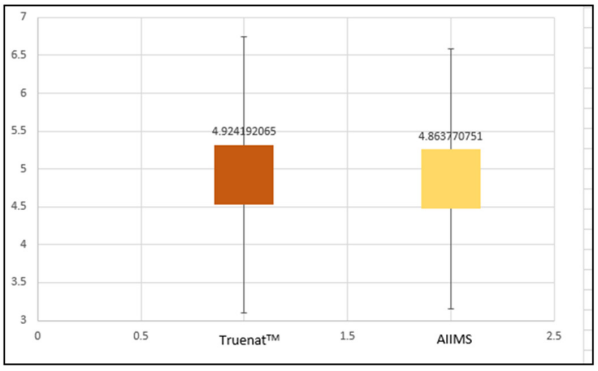

Figure 7: Mean of Truenat™ and AIIMS Real Time-PCR was plotted with error bars. **Note:** ( ) Truenat™= 4.924192065; ( ) AIIMS=4.863770751.

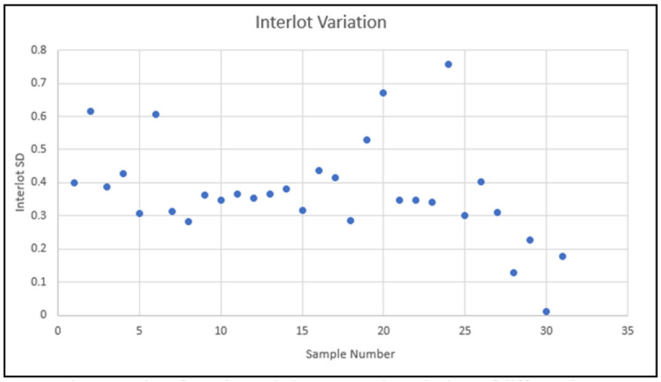

Figure 8: Plot of interlot variation among three devices of different lots.

Linearity

The linearity analysis was performed according to the CLSI guidelines. Serial dilutions of HBV DNA were performed from 5.09×10^9 copies/ml to 5.09×10^2 copies/ml. Nucleic acids were extracted 3 times with TruePrep™ Auto Sample Prep for each dilution, followed by PCR on Truenat™ (UnoDx). For HBV DNA, the assay is linear over 8 digits (5.09×10^9 copies/mL to 5.09×10^2 copies/mL)

Limit of detection

The LOD was determined by testing HBV DNA by 4th International Standard dilutions from NIBSC, U.K. Probit data analysis was used to determine the concentration of the respective DNA with a 95% probability of detection. The LOD was determined to be 55.92 IU/mL for HBV DNA

Precision

Accuracy was tested using the Truenat™ HBV Assay for high, medium and low titers of HBV DNA were administered for 5 consecutive days. Each day, PCR was performed twice for each DNA titer. The standard deviations were within the 0.5 log IU/mL range allowed by the Truenat™ HBV assay.

Assay inclusivity

Analytical responsiveness/comprehensiveness of the Truenat™ HBV assay was performed in a clinical genotype panel consisting of 6 HBV genotypes. The genotype panel was obtained from Discovery Life Sciences (DLS). After duplicate extraction of the corresponding genotype DNA using TruePrep™ Auto, PCR was performed with Truenat™ Uno Dx analyzer.

Robustness

Potential sample carryover within the Truenat™ HBV assay was assessed by alternating positive and negative samples. The number of samples run was 10 positive and 10 negatives. The results showed that there was no carryover contamination.

Clinical specificity

The 76 negative runs are correlated between the methods and show 100% specificity for the Truenat™ HBV assay.

Analytical specificity (cross reactivity)

The cross-reactivity of the test was evaluated using samples negative to HBsAg, anti-HCV, coinfection of HCV and HIV both and HBV-DNA markers but positive to the virus that may have cross-reactivity or coinfected with HBV. 10 serum samples positive to HCV RNA, 5 serums for coinfection of HCV and HIV RNA were analyzed in the cross-reactivity study.

Clinical sensitivity

Positive specimens with viral load ranging from ≈140 IU/mL to 8,50,00,000 IU/mL were tested. 31 of the 31 positive runs correlated between the methods, resulting in 100% sensitivity to the Truenat™ HBV assay.

Concordance

A satisfactory agreement (>95% within log variation) was observed between the viral load determined by the Truenat™ HBV assay and the AIIMS HBV assay.

DISCUSSION

Measuring HBV viral load is essential for monitoring infected patients with HBV. There is an increasing tendency to use combinations of nucleotide analogs, resulting in rapid viral load dynamics [15]. HBV viral load monitoring can predict the progression to cirrhosis and hepatocellular carcinoma and the rapid and sustained response to treatment as predictors of favorable outcomes [16]. It may also enable early detection of treatment failures associated with poor treatment compliance or selection of resistant viruses. The HBV quantification of viral load is routinely performed not only in diagnosing HBV but also in monitoring patients for effective anti-viral treatment through management, sometimes in the recommended intervals of the testing. Blood transfusions carry a significant risk of being infected with the hepatitis B virus (HBV). This is due to the pre-seroconversion window, immune mutant strains, and potential HBV infection [17,18]. The Real-Time-PCR based commercial assay for HBV-DNA quantification performed in clinical practice has been available worldwide for several years, but these methods are time-consuming and require well-developed infrastructure and well-trained technicians for reporting of HBV-DNA test results.

In this study, we evaluated the Micro-PCR Truenat™ test for HBV, estimated its accuracy index, and evaluated its usefulness as a diagnostic tool for early diagnosis and monitoring of HBV. Many factors affect the accuracy of quantifying HBV-DNA, such as the sample volume used for HBV-DNA isolation. Sensitivity and specificity were 100% compared to real-time PCR. Compared to PCR and real-time PCR, this assay consumes fewer resources and skills. This test is due to the device's portability, the minimal need for technician intervention for sample and reagent preparation, the use of sealed cartridges for DNA extraction, and the automatic interpretation of the results by the device's embedded firmware. The platform is suitable for adoption in medical institutions—a system of primary and secondary medical environments for early diagnosis of HBV-DNA. This device has high sensitivity and specificity of the test, even in situations where the disease rarely occurs.

Biomedical researchers have attempted to simplify PCR and real-time PCR platforms and develop simpler nucleic acid amplification methods [19]. Micro-PCR Truenat™ for *M. tuberculosis* was evaluated using the *M. tuberculosis* Growth Indicator Tube (MGIT) culture as the Gold standard [20]. As a result of the analysis, the sensitivity of Truenat™ was 94.7% (95% CI: 89.8, 97.6) reported against *M. tuberculosis*. Even Truenat™ (Truenat Uno®) for malaria was also found to have a sensitivity up to 99.3% (95% CI: 95.5, 99.9) compared to expert microscopy as a Gold Standard [21]. Micro-PCR Truenat™ for high-risk detection of human papillomavirus in the cervix had a sensitivity of 97.5% (95% CI: 86.8, 99.9) compared to the Gold Standard Hybrid Capture 2 [22]. Beta-CoV and SARS-CoV-2 Truenat™ have been evaluated for RT-PCR and found to be 100% sensitive and specific [23]. In view of the above specificity and sensitivity, micro-PCR has high sensitivity and specificity for HBV-DNA quantification which may be an alternative technique to quantify HBV-DNA levels. Micro-PCR can be used for HBV-DNA quantification in Day-to-Day patient care for diagnosis, therapeutics, monitoring and to identify high risk patients.

CONCLUSION

The healthcare system needs to be prepared to address the challenges of HBV infection. Secondary prevention, which focuses on early detection of infection and limiting harmful consequences, plays an essential role in preventing and controlling HBV.

Testing like Truenat™ micro-PCR can be a technical solution to this situation. Truenat™ micro-PCR is a 3 battery operated less complex, cheap and rapid device which can be used in remote areas where circumstances are not favorable. Using of this technology, can also be tried to quantify HCV and HEV RNA which are other major causes of liver disease in the country.

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DECLARATIONS

Author contribution

SP conceived and designed the experiments. SP, SB, and Priyatma contributed with material and analysis tools. SP, SB, Priyatma, and RA analyzed the data. SP wrote the paper in collaboration with all Co-Authors. All authors have read and approved the final manuscript.

Competing interest

The authors declare no competing interests.

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