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DRP Fonseka
Lincoln University
DTH Denipitiya
thanujah@lhd.lk

Lanka Hospitals

Short Report

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A cost-effective SYBR Green based Real-Time PCR platform for reliable detection of Hepatitis B Virus: Establishment and clinical validation.

DRP Fonseka[1], DTH Denipitiya[2*]

[1]Department of Applied Sciences, Lincoln University, Petaling Jaya, Malaysia.

[2*]Department of Molecular Diagnostics, Lanka Hospital Diagnostics, Colombo 05

dhanalifonseka@gmail.com;
*thanujah@lhd.lk

Abstract

Background:
Hepatitis B virus (HBV) remains a major global health concern, and early detection is essential to improve clinical outcomes. Conventional diagnostic methods are often limited by cost and performance. This study aimed to establish and validate a low-cost SYBR green-based real-time PCR assay for HBV detection.

Methods:
A SYBR green real-time PCR assay targeting the S gene of HBV was established. DNA was extracted from plasma using a commercial nucleic acid extraction kit. Analytical specificity was assessed against clinically relevant pathogens, and Analytical sensitivity was determined using 10-fold serial dilutions of HBV DNA (10^4–10^0 IU/mL). Clinical validation was conducted on 112 patient samples and compared with a commercial PCR kit. Statistical agreement was evaluated using Chi-square analysis.

Results:
The assay showed 100% analytical specificity and a limit of detection of 10 IU/mL. Validation with 112 samples demonstrated strong concordance with the commercial kit. The in-house assay detected 45 positives and 67 negatives, compared with 47 positives and 65 negatives by the commercial assay. Only two cases were missed, resulting in a diagnostic sensitivity of 97.01% (95% CI: 89.75–99.18) and diagnostic specificity of 100% (95% CI: 89.11–100.00). The positive predictive value was 100%, and the negative predictive value was

97.01%. Agreement was highly significant (Pearson's Chi-square = 104.03, $p < 0.001$). Precision testing showed an interassay coefficient of variation below 1%, indicating excellent reproducibility.

Conclusions:
The establishment and validation SYBR green real-time PCR assay is highly sensitive, specific, and reproducible. Its strong agreement with commercial assays and reduced cost highlight its potential as a reliable diagnostic tool as a routine laboratory method in resource-limited settings.

Keywords: SYBR green, real-time PCR, Hepatitis B virus, diagnostic specificity, sensitivity, in-house assay

1 Introduction

Hepatitis B virus (HBV) is a DNA virus belonging to the *Hepadnaviridae* family. More than 300 million individuals are affected by this viral infection (Asandem et al. 2024). According to epidemiological statistics the western Pacific region of the world is most affected by HBV (Olioso, D. et al., 2006). The second highest rates are reported from Africa. Among Asian countries, Taiwan consists of around 15-20% of individuals affected which is the highest (Liaw & Chu, 2009). HBV is an enveloped virus comprised of eight different genotypes ranging from A to H. Genotype A is widespread in Africa, sub-Saharan and Northern Europe. Genotypes B and C are common in Asia. On the other hand, genotype C is prevalent in southern Asian regions. Genotype D is prevalent in Africa, Europe and Mediterranean countries. G and H is prevalent in Germany, America and France (Manamperi et al., 2011) (MacLachlan & Cowie 2015) (Noordeen et al. 2015).

HBV virus transmits in two ways vertical transmission and horizontal transmission (Kaneko et al., 1989). Horizontal transmission takes place perinatally whereas vertical transmission takes place through unprotected sex, contaminated needles and blood transfusions (Inoue & Tanaka 2016). HBV progresses from the acute phase to the chronic phase which damages the liver hepatocytes causing cirrhosis (Lok and McMahon, 2009). The acute phase of HBV is asymptomatic; therefore, it is crucial to diagnose HBV during that stage (Trépo et al. 2014). There are different diagnostic tools and methods such as microbiological parameters, biochemical tests and molecular assays (Bello et al. 2023). Among the methods that are currently used for detecting HBV first generation assays consist of limitations such as lengthy incubation periods, high cost, large sample size or volume and high rate of false positive and cross contamination (EASL, 2017). However, due to the advancement of these techniques, cost and limitations have led in researching alternatives. During recent advancement combination of PCR fluorescence dyes such as SYBR green to detect the desired product during the step of amplification in real time PCR assays allows convenient detection. Therefore, this study explains the optimization, establishment and validation of SYBR green-based real time PCR as a qualitative assay to determine HBV in clinical samples offering a reliable and cost-effective screening method that can be used as a routine laboratory method in countries with

economic challenges.

Materials and methods

Ethical statements

Ethical approval for this study was obtained from the Ethics Committee of the Lanka Hospitals Corporations PLC. Colombo 05, SriLanka. Permission was also obtained from head of the Department of Molecular diagnostics and head of laboratories of Lanka hospitals Diagnostics (LHD) to collect clinical samples.

Hepatitis B virus and others organisms

Reference DNA of the Hepatitis B virus, extracted genomic DNA and prepared cDNA of other microorganisms were obtained from the Department of Molecular Diagnostics, LHD, Colombo 05, Sri Lanka (Table 1).

Clinical specimens

Blood samples collected to EDTA tubes of (n=112) patients were obtained from the Department of Molecular Diagnostics, LHD, Colombo 05, Sri Lanka.

In Silico Analysis of Primers

The selected primer pair (Table 1) amplifies a 98 bp fragment of the HBV S gene (nt 379–476, GenBank reference). Both primers have balanced GC content (~48–50%) and calculated Tm ≈60–62°C (Table 1). In silico hairpin analysis predicted only very weak structures: for the forward primer, the most stable hairpin had ΔG ≈ −0.6 kcal/mol (melting well below the annealing temperature), and for the reverse primer ΔG ≈ −2.9 kcal/mol – both not likely to impede priming. Self-dimer potentials were also low (ΔG ≈ −1 to −2 kcal/mol), indicating minimal risk of primer-dimer formation. Thus, the primers meet standard design criteria for secondary structure stability. BLAST searches returned no significant off-target alignments: no human or unrelated microbial sequences aligned with both primers, confirming expected specificity to HBV. Multiple sequence alignment of representative HBV genotypes A–H (including sub genotypes) confirmed that all primer 5′-end bases are conserved (100% identity) and only silent base variations occur elsewhere within the amplicon (Figure 1). This indicates that the primers should anneal effectively across diverse HBV strains.

Figure 1. The primer parameters of forward and reverse primers.

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GACAAACGGGCAACATACCTT	21	59.12	47.62	2.00	2.00
Reverse primer	GTGTCTGCGGCGTTTTATCA	20	59.21	50.00	3.00	2.00

Preparation of DNA from plasma samples
Viral DNA from human plasma (200 µl) was extracted using a commercially available kit (Geneaid viral nucleic acid extraction kit-Taiwan ,Geneaid cat # 51304) according to the manufacturer's instructions.

Real-Time PCR
Real-time PCR (rt-PCR) using the SYBR Green- I technology was performed on reference samples. Primers forward (5'GAC AAA CGG GCA ACA TAC CTT 3') and reverse (5'GTG TCT GCG GCG TTT TAT CA3') were purchased from Integrated DNA Technologies, Inc., U.S.A (411626688)(Mendy et al., 2006). rt-PCR was performed on Bio-Rad CFX 96 PCR thermal cycler (USA) and reaction conditions were optimized using reference DNA of HBV as follows: rt-PCR was carried out in a total volume of 25 µl containing 1X SYBR Green-I qPCR master mix (Applied Biological Materials Inc., Canada) containing Hot Start Taq DNA polymerase and dNTPs in an optimized PCR buffer containing a final concentration of 2.5mM $MgCl_2$. Forward and reverse primers were added to a final concentration of 0.4 µM each followed by addition of 10 µl of extracted DNA (sample). Each PCR run included negative controls in which 10 µl of nuclease free distilled water was added.

The thermal cycler profile was entered on the Bio-Rad CFX 96 PCR thermal cycler (USA) with some modifications to (Mendy et al., 2006). After an initial denaturation at 95°C for 3 minutes, amplification was performed through 40 cycles of denaturation at 95°C for 15 seconds, annealing/extension at 60°C for 45 seconds, and final extension at 65°C for 1 minute, followed by an incubation at 95°C for 3 minutes. Melting curve (Tm) analysis 65 ˚C to 95˚C with readings every 0.5 ˚C) was performed after a cooling step of 65˚C for 1 min according to the manufacturer's instructions of the SYBR Green master mix (Abm cat # 204074). The cut-off was set at Threshold Cycle (Ct) 35 which was the last cycle completely devoid of background noise (Bertoletti & Ferrari 2011). The first negative derivative of the initial melting curve (-dF/dT) was plotted against temperature for improved determination of the melting temperature (Tm). The entire program was completed in approximately 1 hour. Each PCR run in-house gene GAPDH was used as an internal control.

Evaluation of analytical specificity and sensitivity of rt-PCR assay

To determine the Analytical specificity, rt-PCR was performed on samples (n=15) including DNA and cDNA of clinically important pathogenic microorganisms in the country such as bacterial DNA and viral DNA/RNA were tested using the optimized PCR assay. BK Virus, Human immunodeficiency virus, Cytomegalovirus, ParvoB19virus, Herpes simplex virus 1/2, *Neisseria gonorrhea*, *Chlamydia trachomatis, Leptospira spp*, *Mycobacterium tuberculosis*, Toxoplasma gondii, Human Papillomavirus, SARS CoV-2, Hepatitis C virus, human genomic DNA and positive nucleic acids a were selected as a template (Table 1).

Table 1. The panel of DNA and cDNA to determine the analytical specificity.

Organism	Observed results
BK virus	Negative

Human immunodeficiency virus	Negative
Cytomegalovirus	Negative
Parvo B19 virus	Negative
Herpes simplex virus 1	Negative
Herpes simplex virus 2	Negative
Neisseria gonorrhea	Negative
Chlamydia trachomatis	Negative
Leptospira spp	Negative
Mycobacterium tuberculosis	Negative
Toxoplasma gondii	Negative
Human Papillomavirus	Negative
SARS CoV-2	Negative
Hepatitis C virus	Negative
Human genome	Negative

Known positive HBV DNA was obtained to quantify the DNA yield. Concentrations were measured using three commercially available standards (10^0, 10^1,10^3 and 10^4 IU/mL) concentrations and the copy number of the positive HBV DNA was calculated to prepare the dilution series. After performing the necessary calculations, the dilution series were subjected to optimized SYBR green real-time PCR assay to determine the minimum limit of detection (LOD) followed by melting curve analysis.

Precision studies were performed using 3 HBV clinical samples with different viral loads, 2.11×10^5 IU/mL, 9.00×10^3 IU/mL and 7.20×10^3 IU/mL. These three samples with different viral loads were tested with the in-house assay for consecutive 3 days. The Threshold cycle (Ct) values obtained were used to determine the standard deviation and coefficient of variation.

Evaluation of performance of molecular-based assay using known clinical specimen

The plasma samples (n= 112) used for the study were obtained from the Molecular Diagnostic laboratory of the Lanka Hospital (Pvt) Ltd, Narahenpita, Colombo 05, Sri Lanka. All the patient samples were laboratory-tested using a commercially available HBV real-time PCR kit previously. Blood was collected into sterile EDTA tubes from the patients. DNA extraction was carried out using patients' plasma samples using a commercially available, Geneaid viral nucleic acid extraction kit according to manufacturer's instructions. Extracted DNA from human plasma samples was subjected to SYBR green-based real-time PCR assays using optimized assay conditions. Samples that were previously laboratory confirmed by the commercially available assay were analyzed using the optimized assay. Therefore, patient plasma samples with varying viral loads were tested.

Statistical analysis

Clinical performance of the in-house rt-qPCR assay was evaluated in comparison with the commercial reference assay using a 2×2 contingency table. Diagnostic parameters including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and overall accuracy were calculated with the commercial assay taken as the gold standard.

Two-sided 95% confidence intervals (CIs) for proportions were estimated using the Wilson score method, which provides improved coverage over the traditional Wald interval, particularly in small samples.

Agreement between the two assays was assessed using Pearson's Chi-square test. All statistical analyses were performed in silico using SPSS and openepi version 3 with validated statistical functions, ensuring reproducibility.

Results

Analytical specificity and sensitivity of the rt-PCR

Analytical Specificity

The SYBR Green-based real-time PCR assay was evaluated for analytical specificity using a panel of DNA and cDNA from multiple pathogens, including BK Virus, Human immunodeficiency virus, Cytomegalovirus, ParvoB19virus, Herpes simplex virus 1/2, Neisseria gonorrhea, *Chlamydia trachomatis, Leptospira spp*, *Mycobacterium tuberculosis*, Toxoplasma gondii, Human Papillomavirus, SARS CoV-2 Hepatitis C virus and human genomic DNA. No amplification of non-target sequences was observed, indicating 100% analytical specificity for HBV detection as shown in figure 2 and 3

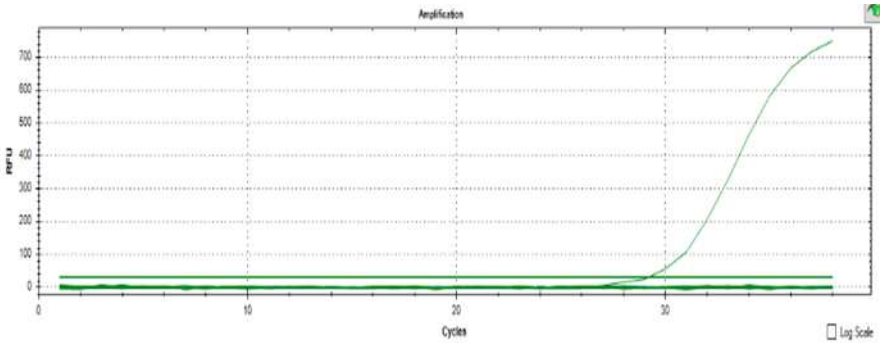

Figure 2. The florescence graph of Analytical specificity

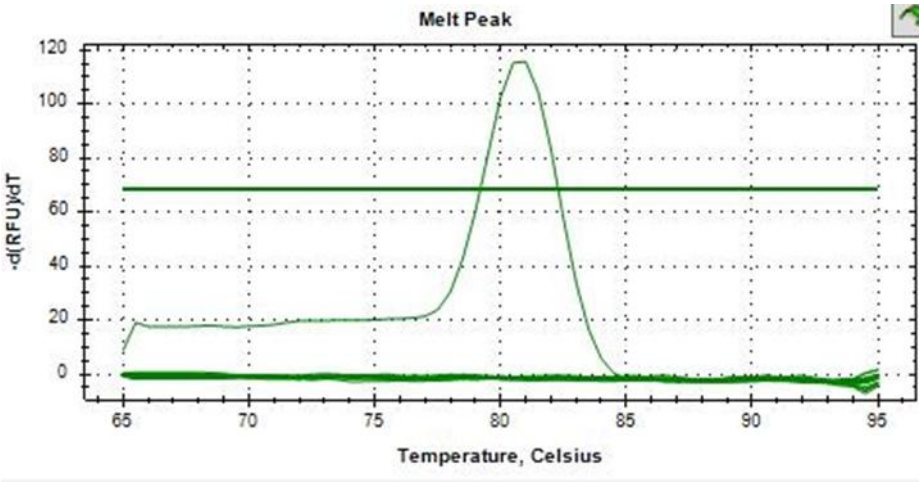

Figure 3. The melt graph of Analytical specificity

Analytical sensitivity

The limit of detection (LOD) was determined using a patient sample with a viral load of 10^4 IU/mL, subjected to 10-fold serial dilutions (10^3, 10^2, 10^1, and 10^0 IU/mL). The assay consistently detected HBV DNA at 10 IU/mL, establishing this as the LOD. As shown is Figure 4, The melt curve graph demonstrates the Analytical sensitivity of the optimized assay.

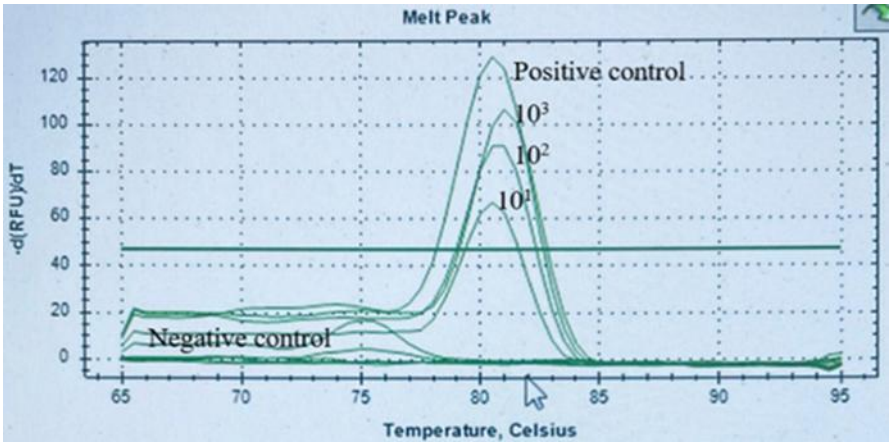

Figure 4. The melt graph obtained by Analytical sensitivity

Reproducibility

Reproducibility was assessed by testing three samples with viral loads of 2.11×10^5 IU/mL, 9×10^3 IU/mL, and 7.2×10^3 IU/mL over three consecutive days. Standard deviations were 0.28, 0.18, and 0.16, respectively, with a coefficient of variation less than 2, indicating high precision as shown in Table 2.

Table 2. The results obtained by the precision study done using 3 samples.

Sample	Concentration	Mean	Standard deviation	CV
1	2.11×10^5 IU/mL	34.58	0.28	0.83
2	9×10^3 IU/mL	28.5	0.19	0.65
3	7.2×10^3 IU/mL	26.44	0.17	0.63

Diagnostic sensitivity

Diagnostic sensitivity is a critical parameter in the evaluation of molecular diagnostic assays, reflecting the assay's capacity to accurately identify individuals harboring the target pathogen. In the context of the SYBR Green-based real-time PCR assay developed for Hepatitis B virus (HBV) detection, high diagnostic sensitivity ensures the reliable amplification of low-copy viral DNA, reducing the likelihood of false-negative results. This is particularly important for early-stage or occult HBV infections, where viral loads may be minimal. Therefore, establishing and validating a highly sensitive assay is essential for effective clinical diagnosis, patient monitoring, and public health interventions. Therefore, by the analysis of results it was determined that the diagnostic sensitivity of the in-house assay is 100% (Table 3).

Statistical analysis of in-house assay and commercial assay.

The comparison between the in-house assay and commercial assay is summarized in Table 3.

Table 3. Comparison of diagnostic accuracy of in house established real-time PCR assay with commercial real-time PCR assay.

		In-house developed assay		
		Positive	**Negative**	Total
Commercial Assay	**Positive**	45	2	47
	Negative	0	65	65

Total	45	67	112

Results of in silico analysis of the tests.

Table 4. Chi-square analysis in between in-house assay and commercial assay

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	104.033[a]	1	.000		
Continuity Correction[b]	100.088	1	.000		
Likelihood Ratio	134.374	1	.000		
Fisher's Exact Test				.000	.000
N of Valid Cases	112				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 18.88.

b. Computed only for a 2x2 table

Chi-square analysis confirmed a highly significant association between commercial and in-house classifications (Pearson's $\chi^2 = 104.03$, df = 1, $p < 0.001$). All expected cell counts exceeded the minimum threshold, supporting the robustness of the test.

Diagnostic performance

Diagnostic sensitivity and specificity were calculated using Openepi version 3 software (Table 5). The assay achieved a sensitivity of 100%, specificity of 97.01%, positive predictive value (PPV) of 95.74%, and negative predictive value (NPV) of 100%, resulting in a diagnostic accuracy of 98.21%.

Table 5. The parameters of the in-house assay were analyzed by openepi version 3.

Parameters	Estimates	Lower and upper 95% Cis	Method
Sensitivity	100%	(92.13, 100[1])	Wilson Score
Specificity	97.01%	(89.75, 99.18[1])	Wilson Score
Positive Predictive Value	95.74%	(85.75, 98.83[1])	Wilson Score
Negative Predictive Value	100%	(94.42, 100[1])	Wilson Score
Diagnostic Accuracy	98.21%	(93.72, 99.51[1])	Wilson Score

Discussion

This study describes the establishment and validation of a SYBR Green-based real-time PCR assay for the qualitative detection of hepatitis B virus (HBV) DNA in serum and plasma, offering a cost-effective diagnostic solution for resource-limited settings. Primers used in this study capable of detecting all eight HBV genotypes (A–H) (Zaghloul et al. 2016). The assay utilizes SYBR Green, a non-toxic dye that binds double-stranded DNA, absorbing blue light at 497 nm and emitting green light at 520 nm (Grimberg, 2011), to achieve precise amplification monitoring. Through comprehensive validation of analytical specificity, sensitivity, reproducibility, and diagnostic performance, the assay demonstrates performance comparable to commercial probe-based assays, making it a promising tool for HBV management in economically challenged regions.

The analytical specificity of the assay was validated against a panel of DNA and cDNA from diverse pathogens, including BK Virus, Human immunodeficiency virus, Cytomegalovirus, ParvoB19virus, Herpes simplex virus 1/2, *Neisseria gonorrhea*, *Chlamydia trachomatis*, *Leptospira spp, Mycobacterium tuberculosis, Toxoplasma gondii*, Human Papillomavirus, SARS CoV-2, Hepatitis C virus and human genomic DNA. The absence of cross-reactivity confirms the assay's ability to exclusively detect HBV DNA, a critical feature for minimizing false positives. Compared to (Aguiar et al. 2014), which tested four viral strains (HIV, HCV, HAV, and HEV), and (Coleman & Tsongalis 2022), (Darius et al. 2009), which evaluated only HIV and HCV, the broader panel of nine pathogens in this study enhances confidence in the

assay's specificity, reducing the risk of misdiagnosis and inappropriate treatment.

The analytical sensitivity, determined by a limit of detection (LOD) of 10 IU/mL through serial dilutions of a 10^4 IU/mL sample, outperforms several prior studies: Mendy et al. (2006) reported an LOD of 2.6×10^2 IU/mL, (Aguiar et al. 2014) 18 IU/mL, (Li et al. 2010) 25 copies/mL, (Ghosh et al. 2016) 30 copies/mL, and (Darius et al. 2009) 50 IU/mL. This high sensitivity, comparable to commercial assays (~10.21 IU/mL), enables detection of low viral loads, crucial for early diagnosis and monitoring of HBV patients, particularly those on antiviral therapy. Such sensitivity supports timely interventions, reducing disease progression and transmission risks, and facilitates precise viral load quantification for therapeutic decisions (Guvenir & Arikan 2020).

Reproducibility was demonstrated through repeated testing of samples with viral loads of 2.11×10^5 IU/mL, 9×10^3 IU/mL, and 7.2×10^3 IU/mL, yielding standard deviations of 0.28, 0.18, and 0.16, respectively. These low values indicate superior precision compared to (Aguiar et al. 2014), with standard deviations exceeding 5, and (Darius et al. 2009), with coefficients of variation from 1.2 to 22.7. This consistency ensures reliable results across diverse laboratory conditions, a key requirement for routine clinical diagnostics.
In silico analysis of chi square test our findings demonstrate a strong and statistically significant agreement between commercial and in-house methods, indicating that results from the two approaches are highly consistent.

The diagnostic performance of the assay was exceptional, with 100% sensitivity, 97.01% specificity, a positive predictive value (PPV) of 95.74%, and a negative predictive value (NPV) of 100%, yielding an accuracy of 98.21%. Compared to (Olioso et al. 2006), which reported 93% sensitivity, 99% specificity, 96% PPV, and 98% NPV, the in-house assay offers superior sensitivity and comparable specificity, ensuring reliable identification of HBV-positive and -negative cases.

The in-house SYBR Green-based rt-qPCR assay demonstrated high diagnostic performance, showing near-perfect agreement with the commercial reference assay. Importantly, the absence of false positives highlights the assay's strong specificity, while the minimal number of false negatives suggests only a marginal reduction in sensitivity. These findings confirm

the reliability of the assay for routine clinical detection of Hepatitis B Virus.

The primers, targeting a conserved 98-base-pair region of the HBV genome (positions 379–476) with a GC content of 47.62% (forward) and 50% (reverse) and annealing temperatures of ~59°C (Mendy et al., 2006), amplified all eight HBV genotypes, unlike those in (Olioso et al. 2006), which detected only genotypes A–E. Optimized PCR conditions, including reduced primer concentrations (0.6 µL), shortened denaturation time (15 seconds), increased annealing temperature (60°C), and fewer cycles (38), minimized non-specific amplification. DNA extraction using EDTA tubes and a commercial kit (Geneaid), with GAPDH as an internal control, ensured the determination of the inhibition of the extracted DNA (Yeh et al. 2004).

This SYBR Green-based assay offers a cost-effective alternative to commercial assays, with superior sensitivity, specificity, and reproducibility. Its ability to detect all HBV genotypes makes it a valuable tool for early diagnosis, viral load monitoring, and therapeutic management in resource-limited settings, addressing a critical need in global HBV management.

Limitations of the Study

The present study, while demonstrating strong analytical and clinical outcomes, has several limitations that should be acknowledged:

- The study was conducted using a limited number of clinical samples (n = 112) collected from a single diagnostic center, which may not fully represent the genetic diversity of Hepatitis B Virus (HBV) strains across different regions.
- The validation was limited to qualitative detection; quantitative viral load estimation was not performed. This was primarily due to the self-funded nature of the research, which restricted access to additional reagents, standards, and instrumentation required for quantitative assay development.
- Although in silico analysis confirmed primer compatibility across all HBV genotypes (A–H), experimental verification using confirmed genotype panels was not conducted, warranting further evaluation with diverse genotype samples.

- The SYBR Green chemistry, while cost-effective and efficient, can be susceptible to non-specific signal amplification if reaction parameters are not precisely optimized, underscoring the importance of strict laboratory quality control.
- Future studies supported by institutional or external funding should aim to include larger, multi-center sample sets, perform genotype-specific validations, and extend the assay to quantitative analysis for broader clinical applicability.

Conclusion

In conclusion, when the qualitative parameters are considered such as analytical sensitivity, analytical specificity, clinical sensitivity, clinical specificity and reproducibility the in-house SYBR Green-based real-time PCR assay established for HBV detection demonstrated high analytical specificity, sensitivity, and reproducibility. Most of the qualitative parameters showed similar results with the gold standard which is the commercially available kit whereas reproducibility of the in-house assay showed better results than the commercial probe-based method. Additionally, the cost is approximately 50% less and the time for the in-house assay is less compared to the commercial assay which confirms that the in-house assay is an effective and efficient alternative to economically challengeable countries like Sri Lanka. These attributes suggest that the assay is a viable alternative to commercial assays for detecting HBV in clinical samples. With further validation, this assay has the potential to be implemented as a diagnostic tool, offering reliable HBV detection across diverse clinical settings.

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the Ethics Committee of the Lanka Hospitals Pvt ltd, Colombo 05, Sri Lanka.

Informed consent was not required for this study, as only anonymized EDTA blood samples were obtained from the Molecular Diagnostics Laboratory of Lanka Hospital Diagnostics (PVT) Ltd, according to the accession numbers provided. No identifiable patient information was collected or accessed. Compliance with the Declaration of Helsinki was not required, as no identifiable human material or personal data were utilized.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

D.R.P. Fonseka conducted the research, performed laboratory work, and prepared the manuscript. D.T.H. Denipitiya supervised the project, validated results, and revised the manuscript. All authors read and approved the final manuscript.

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