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Development and Performance Evaluation of an Open-System Real-Time Quantitative PCR Assay for Decentralized Hepatitis B Viral Load Testing in Indonesia

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Research Article

Keywords: Hepatitis B virus, HBV viral load, quantitative PCR, chronic hepatitis B, clinical validation, limit of detection, Indonesia, open-system platform

Posted Date: November 17th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11212268/v1

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Additional Declarations: No competing interests reported.

Development and Performance Evaluation of an Open-System Real-Time Quantitative PCR Assay for Decentralized Hepatitis B Viral Load Testing in Indonesia

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Abstract

Background: Indonesia remains one of the countries with the highest burdens of chronic hepatitis B (CHB) in Southeast Asia. Despite this substantial disease burden, a major gap in access to quantitative hepatitis B virus (HBV) DNA testing potentially delays the diagnosis and treatment of CHB. This study aimed to develop and validate an open-system quantitative real-time PCR (qRT-PCR) assay, the INDIGEN HBV DNA qRT-PCR for detecting and quantifying HBV DNA viral load.

Methods: The assay was designed to target the S region of HBV and calibrated against the HBV World Health Organization (WHO) International Standard. Analytical performance was evaluated for linearity and limit of detection (LoD). A total of 122 HBsAg-positive samples from CHB patients were used to assess the clinical performance of our assay compared to the Xpert® HBV Viral Load (Xpert).

Results: The assay demonstrated excellent linearity across 1.0–9.0 $\log_{10}$ IU/mL ($R^2 = 0.9964$) with a LoD of 14.8 IU/mL. Compared to Xpert, the developed assay showed 100% specificity and 99.05% sensitivity with strong correlation based on Deming regression analysis.

Conclusions: The INDIGEN HBV DNA qRT-PCR assay demonstrated robust evaluation performance comparable to the Xpert, supporting its implementation as a reliable open-system assay for decentralized HBV viral load testing in Indonesia.

Keywords: Hepatitis B virus; HBV viral load; quantitative PCR; chronic hepatitis B; clinical validation; limit of detection; Indonesia; open-system platform

Registry: Dr. Cipto Mangunkusumo National General Hospital**,** TRN: ND-356/UN2.F1/ETIK/PPM.00.02/2025, Registration date: 30 May 2025

Introduction

Indonesia is classed as a moderate to high hepatitis B virus (HBV) endemic country. In the latest global hepatitis report, it was estimated that approximately 17 million people were diagnosed with chronic hepatitis B (CHB) in Indonesia. This number contributes to an estimated HBV mortality rate of 21.7 deaths per 100.000 population in Indonesia [1]. Despite this high burden, there is only a 3% chronic HBV testing rate, and 4% of the positive cases receive antiviral treatment. Considering the WHO interim target of 30% HBV detection of all people living with chronic HBV infections, this low detection rate reveals major deficiencies in national HBV care management in Indonesia [1]. Consequently, these conditions may delay the antiviral intervention for patients requiring treatment, posing a risk of accelerating disease progression. Delaying antiviral treatment and virological suppression significantly increased risks of cirrhosis, hepatocellular carcinoma case (HCC), and liver-related death, as it was reported in several prospective clinical studies [2, 3, 4]. Altogether, these reports highlight the urgent need to expand access to quantitative HBV viral load testing. Increasing HBV diagnostic capacity allows earlier treatment and ultimately reduces HBV-related morbidity and mortality.

Currently, the major commercially available HBV DNA quantification assays are run on dedicated closed-system platforms with streamlined processes of nucleic acid extraction, amplification, and result interpretation. Although the closed-system PCR platforms offer the advantage of reducing hands-on time, they are associated with several disadvantages, including substantial capital cost, reliance on specific consumables, and strict limitations on using alternative assays or reagents, thereby limiting their accessibility and long-term sustainability in low- and middle-income countries (LMICs) [5]. In contrast to closed-system platforms, greater flexibility to run different assays from multiple manufacturers on a single instrument can be achieved by the use of open-system real-time PCR (qRT-PCR) platforms. This specification allows for rapid implementation of newly developed assays and enhances resilience against supply-chain disruptions. In combating COVID-19 transmission, a total of 615 real-time PCR instruments were distributed across all 34 provinces during the pandemic in Indonesia. This instrument distribution has created a massive open-system molecular infrastructure, alongside skilled laboratorians and standardized molecular workflows. To fully leverage this established infrastructure, existing real-time PCR instruments can be reutilized for HBV DNA quantification testing that is avoiding the need for additional investment to expand access to quantitative HBV viral load testing [6].

The development of an accurate and robust open-system PCR of HBV DNA viral load assay is essential to expand access to HBV viral load testing within Indonesia's existing laboratory network. Accordingly, this study reports the complete analytical validation and clinical performance evaluation of the INDIGEN HBV DNA qRT-PCR assay against the Xpert® HBV Viral Load (Cepheid, USA) (Xpert) as the reference HBV viral load quantitation kit in Indonesia [7].

Methods

Ethical approval

This study was approved by the Ethics Committee at Dr. Cipto Mangunkusumo National General Hospital (RSCM), under ethical approval number ND-356/UN2.F1/ETIK/PPM.00.02/2025. Written informed consent was obtained from all participants prior to enrolment. All procedures were performed according to the relevant guidelines and regulations established by the WHO and the International Organization for Standardization (ISO).

Clinical samples and study design

In this prospective study, a total of 131 patients with hepatitis B were selected at the Hepatobiliary Division of RSCM between August 2025 and December 2025. Inclusion criteria included: 1) age ≥ 18 years, 2) HBsAg positivity for ≥ 6 months, 3) willingness to provide plasma specimens, 4) provision of written informed consent. Individuals who were HBsAg negative or unwilling to participate were excluded. A total of 9 patients were excluded from this study after being detected as HBsAg negative by the confirmatory testing, leaving 122 participants for the final analysis. Plasma specimens were collected in EDTA tubes, aliquoted, stored at −80°C, and processed on the same day for INDIGEN HBV DNA qRT PCR and Xpert assays. Demographic data, including age, sex, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and hepatitis B vaccination status, were recorded on standardized Case Report Forms (CRFs). This study was approved by the Ethics Committee at Dr. Cipto Mangunkusumo National General Hospital (RSCM), under ethical approval number ND-356/UN2.F1/ETIK/PPM.00.02/2025

DNA extraction

The QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany) was used to isolate the viral DNA from 200 µL of plasma and recover 30 µL of elution buffer according to the manufacturer's protocol. Quantitative PCR was performed on the same day as DNA extraction to minimize potential degradation and ensure sample integrity. To minimize the risk of false-positive results, all nucleic acid amplification procedures were conducted in accordance with strict contamination control practices [8].

Design of primers and probes

The primer and probe sequences of the INDIGEN HBV DNA qRT-PCR assay are listed in Table 1. The primers and probe target a highly conserved region within the HBV Surface (S) gene, selected for its low sequence variability across all recognized HBV genotypes (A to H) [9]. The target region was confirmed by in silico multiple sequence alignment with 5 complete sequences of each HBV genotype A-H using ClustalW algorithm implemented in Bioedit. In addition, a total of 41 sequences of major HBV genotype B and C from Indonesia were retrieved from geneBank to further evaluate the coverage of primers and probes (A to H) as shown in Table 2.

To test the capability of our assay in detecting all recognized HBV genotypes, the AccuTrak HBV DNA genotype qualification panel (Seracare, Milford, US) was used for genotype evaluation. The panel consists of seven samples of genotypes A, B, C, D, E, F, and H of HBV viral load concentrations ranging from 9.74E+04 to 3.28E+03 IU/mL. HBV amplicons are detected via HEX-labelled probe fluorescence measured in the HEX channel. The secondary primer–probe set detects the human beta-actin (ACTB) gene as an internal extraction and amplification control (IC), detected via a Cy5-labelled probe in the Cy5 channel.

Table 1. Sequences of primers and probes used for INDIGEN HBV qRT-PCR

Primer and Probe	Gene Target	Oligonucleotide Sequence (5' →3')
ACTB– Forward Primer	Human Beta-actin	ACGTCGCCAAGAACCTGTAC
ACTB - Reverse Primer	Human Beta-actin	TGATCTCTATTGTCGTGGGT
S- Forward Primer	HBV Surface region	GTGTCGTCGGCGTTTATTCA
S- Reverse Primer	HBV Surface region	GAACAACGGCGAACATCACTT
ACTB - Probe	Human Beta-actin	GACGAGATGCAAGAGGAGA

Primer and Probe	Gene Target	Oligonucleotide Sequence (5' →3')
S - Probe	HBV Surface region	CTCCTGCATCCTGCTGCATTGCCTCATC

Table 2. Accession number of HBV genotypes

HBV Genotype	Accession Number	Additional sequence from Indonesia
A	AB014370, AB064314, AB076678, AB076679, AB116076	
B	AB010289, AB010290, AB010291, AB014366, AB031267	AB033554, D00331, AP011085, GQ358136, AB033555, JQ429082, EF473971, M54923, AB713529, AB493835, EF473977, AP011088, AP011092, AP011091, GQ358138, EF473976, AP011090, AP011087, AB540582, GQ358149, GQ358152, AP011095, AB493834, AP011093, EF473975, EF473973
C	AB014360, AB014362, AB014363, AB014365, AB014367	AP011097, AB540583, AB644284, AB644283, AP011107, AP011104, AP011106, GQ358157, AB554018, AB644287, AB493840, AB644281, AB644286, AB554019, AB554020
D	AB033558, AB048701, AB048702, AB048703, AB090268	
E	AB091255, AB091256, AB106564, AB194947, AB194948	
F	AB036905, AB036909, AB036910, AB036911, AB036912	
G	AB056513, AB056515, AB064310, AB064311, AB064312	
H	AB059659, AB059660, AB059661, AB179747, AB205010	

Quantitative Calibrator Construction

Quantitative calibrator of the INDIGEN HBV DNA qRT-PCR assay was established with full metrological traceability to the WHO 4th International Standard for HBV DNA Quantification (NIBSC 10/264, assigned value: 5.98 $\log_{10}$ IU/mL), in accordance with ISO 17511:2003 (In vitro diagnostic medical devices: measurement of quantities in biological samples, metrological traceability of values assigned to calibrators and control materials) [10]. To convert the measurement unit of our assay from copy number to International Unit, the calibrator material with concentration 2.14×10^8 copies/mL was quantified using the standard curve generated from WHO HBV DNA International Standard. Calibrator standardization was

performed by analyzing serial dilutions of the WHO reference material and the HBV INDIGEN calibrator under identical PCR conditions. Linear regression analysis was performed to evaluate the amplification characteristics of the designed calibrator. Three quantitative calibrators were included in every PCR run to generate a standard curve, and the amplification efficiency was confirmed at 90–110% with $R^2 \geq 0.999$ per run.

Optimization of the quantitative PCR reaction

The qPCR reaction was carried out in a 25 µL reaction mixture containing 5 µL extracted DNA template, 0.05 unit HotStarTaq DNA polymerase, 1x PCR reaction buffer, 1.5 mM $MgCl_2$ (New England BioLabs, Beverly, USA), 8 µL nuclease-free water (Qiagen) 0.3 µM of each HBV S gene primers, 0.2 µM of HBV S gene HEX labelled probe, 0.1 µM of each *ACTB* primers and *ACTB* Cy-5 labelled probe. Primers and probes were purchased from Integrated DNA Technologies. All qRT-PCR reactions were performed on the Rotor-Gene Q real-time (Qiagen) with initial denaturation at 95°C for 15 minutes, followed by 50 cycles of 95°C for 15 seconds and 55°C for 60 seconds.

Linearity assessment

To establish the linearity of the HBV DNA quantification assay by our assay, serial dilutions of the linearity panel were prepared to obtain concentrations of 1.0 to 9.0 $\log_{10}$ IU/mL (10 to 10^9 IU/mL). All experiments were conducted in duplicate for each tested concentration in at least three independent runs. Observed concentrations were plotted against expected concentrations, and linear regression was applied to derive the coefficient of determination (R^2) and maximum deviation per concentration level. The acceptance criteria were $R^2 \geq 0.99$, consistent with CLSI EP06 guidance [11].

Limit of Detection and Lower Limit of Quantification

The LoD of our assay was determined using serial dilutions of a quantified HBV-positive plasma specimen at five different concentration levels of 90, 45, 22.5, 11.25, and 5.625 IU/mL in 20 replicate measurements at each level. The LoD was determined by Probit regression analysis of the resulting detection rate data to identify the concentration level yielding a 95% probability, as outlined in ISO 18113-2:2009 and CLSI EP17-A2 [12, 13]. The LLoQ was defined as the lowest concentration at which the total analytical error (TAE = |Absolute Bias| + 2×SD) did not exceed clinical minimum threshold for treatment HBV infected patients of 1.0 $\log_{10}$ IU/mL [13, 14].

Statistical analysis

Diagnostic performance metrics, including sensitivity, specificity, PPV, and NPV with 95% confidence intervals (CI), were derived from 2×2 contingency table analysis using the HBV viral load comparator kit as the reference HBV viral load quantitation kit in Indonesia [7]. The LoD was estimated by Probit analysis using IBM SPSS Statistics for Windows, Version 27.0 (IBM Corp., New York, USA). Correlation was assessed by Pearson correlation (R^2) and Deming regression analysis, which accounts for measurement error in both assay methods, using GraphPad Prism version 8.4.3 (GraphPad Software, San Diego, CA, United States).

Results

Assay development and qPCR optimization

The INDIGEN HBV DNA qRT-PCR primer–probe set was intended to target the *HBV Surface* (*S*) gene in all HBV genotypes (A to H). As shown in Table 3, our assay successfully detected all claimed HBV genotypes included in the panel. During the analysis, a minimal deviation (0.01 to 0.32 $\log_{10}$ IU/mL) of the measured viral load from the assigned concentration was obtained. Additionally, the optimal annealing temperature of our assay was set at 55°C. In this assay, we included a primer-probe set to target the *ACTB* gene as the internal control.

Table 3. The results of the INDIGEN HBV DNA qRT-PCR assay in reference samples

HBV Genotypes	INDIGEN HBV DNA qRT-PCR Value ($\log_{10}$ IU/mL)	SeraCare Value ($\log_{10}$ IU/mL)	SD ($\log_{10}$ IU/mL)	Deviation
A	4.67	4.66	0.01	+0.01
B	5.43	4.97	0.32	+0.46
C	5.29	5.31	0.01	-0.02
D	5.07	4.83	0.17	+0.24
E	4.82	4.72	0.07	+0.10
F	5.45	4.99	0.32	+0.46
H	3.79	3.52	0.19	-0.27

Calibrator Standardization

The calibration of the INDIGEN HBV calibrator was standardized against the WHO International Standard for HBV DNA. As shown in Figure 1, the linear regression analysis produced a good correlation between the WHO reference material ($Y = 1.002X - 0.0065$, $R^2 = 1.00$) and the HBV INDIGEN calibrator ($Y = 1.0076X - 0.0166$, $R^2 = 0.99$). Using the standard curve generated from the WHO HBV DNA International Standard ($Y = 3.377X - 41.144$, $R^2 = 0.995$), we obtained a calibration concentration of 1.36×10^7 IU/mL from an input concentration of 2.14×10^8 copies/mL. Thus, the conversion factor of our assay was determined to be 1 IU/mL = 15.7 copies/mL.

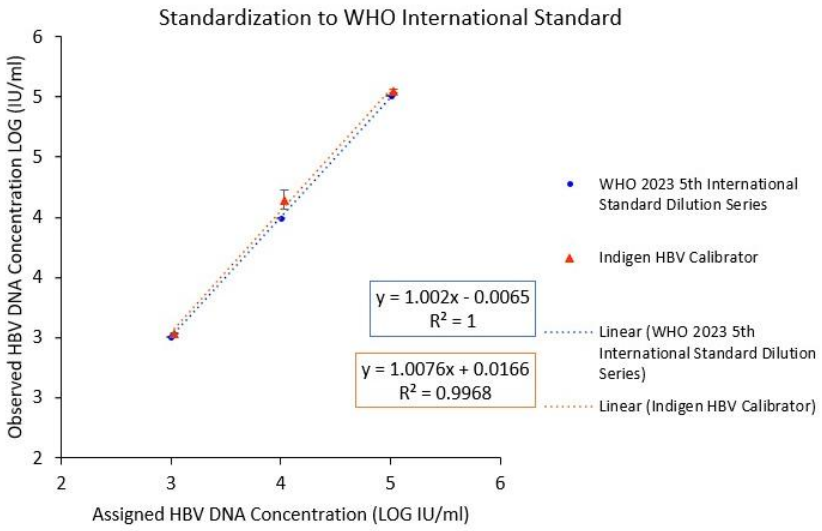


Figure 1. Linear regression of WHO International Standard and INDIGEN HBV Calibrator.

Linearity and dynamic range

Table 4 showed the quantitative linearity results of our assay against the prepared Linearity panel in nine serial dilution levels (1.0–9.0 $\log_{10}$ IU/mL). The linear regression analysis produced a coefficient of determination of $R^2 = 0.9958$ with equation $Y = 1.0285X - 0.0695$ as shown in Figure 2. Maximum deviation from expected values did not exceed 0.28 $\log_{10}$ IU/mL at any concentration level.

Table 4. Quantitative linearity of the INDIGEN HBV DNA qRT-PCR assay across ten concentration levels

Sample ID	Expected Conc. ($\log_{10}$ IU/mL)	Observed Conc. ($\log_{10}$ IU/mL)	SD ($\log_{10}$ IU/mL)	Deviation
LR-1	9.03	9.40	0.28	+0.37
LR-2	8.03	8.40	0.28	+0.37
LR-3	7.03	7.30	0.20	+0.27
LR-4	6.03	6.30	0.18	+0.27
LR-5	5.03	5.10	0.08	+0.07
LR-6	4.03	4.10	0.03	+0.07
LR-7	3.03	3.00	0.04	−0.03
LR-8	2.03	2.00	0.01	−0.03
LR-9	1.03	0.70	0.21	−0.33

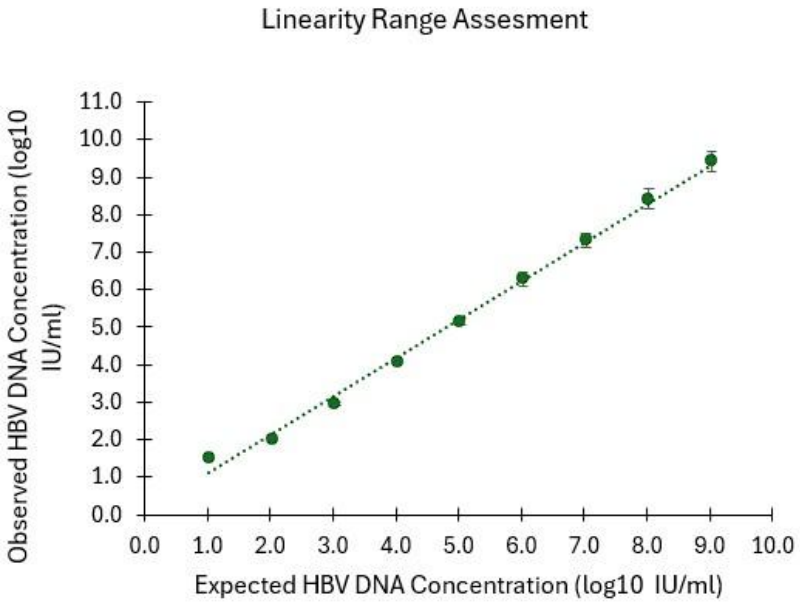


Figure 2. Linear regression of observed versus expected HBV DNA concentrations across the nine-member linearity panel.

Limit of Detection and Lower Limit of Quantification

Serial dilution of a quantified HBV-positive plasma specimen was used to determine the LoD of our assay, as presented in Table 5. Probit regression analysis produced an estimated 95% LoD for the assay of 14.8 IU/mL (1.17 $\log_{10}$ IU/mL). Additionally, the LLoQ was determined to be 22.5 IU/mL based on TAE analysis, as shown in Table 6. At the LLoQ concentration of 22.5 IU/mL, the TAE value of 1.00 $\log_{10}$ IU/mL was calculated.

Table 5. HBV DNA detection rates at serial concentration levels for probit-based LoD determination

Target Concentration (IU/mL)	Replicates Tested (n)	Positive Detections (n)	Detection Rate (%)
90	20	20	100
45	20	20	100
22.5	20	20	100
11.25	20	16	80
5.625	20	7	35

Table 6. Total Analytical Error (TAE) analysis for determination of the Lower Limit of Quantification (LLoQ)

n	Expected ($\log_{10}$ IU/mL)	Observed ($\log_{10}$ IU/mL)	SD	CV	Bias ($\log_{10}$)	TAE*
20	1.35	1.15	0.43	0.37	−0.21	**1.00**

*TAE = |Absolute Bias| + 2 × SD; acceptance criterion: TAE ≤ 1.0 $\log_{10}$ IU/mL.

Study population and subject characteristics

This study enrolled 131 participants at the RSCM Hepatobiliary Division. After excluding nine participants due to negative HBsAg results, 122 participants were included for the final analysis. The demographic data of patients were summarized in Table 7. In this study, the majority of participants were males (53.3%), with a mean age of 45.7 ± 13.3 years. Elevated ALT and AST were observed in 30 (24.6%) and 49 (40.2%) subjects, respectively.

Table 7. Demographic, biochemical characteristics, and vaccination status of the study cohort (n = 122)

Parameter	n / Value	% / Unit
Total analysed subjects	122	—
Age (years), mean ± SD	45.7 ± 13.3	—
Sex: Male	65	53.3%
Sex: Female	57	46.7%
Elevated ALT (> ULN)	30	24.6%
Elevated AST (> ULN)	49	40.2%
Vaccinated against hepatitis B	5	4.1%
Not vaccinated against hepatitis B	100	82.0%
Unknown vaccination status	17	13.9%

Clinical diagnostic performance

The comparison study of our assay against the HBV Viral Load comparator kit was shown in Table 8. The diagnostic sensitivity and specificity of our assay were 99.05% (95% CI: 94.9–100%) and 100% (95% CI: 80.5–100%), respectively. Additionally, the overall diagnostic accuracy of the assay was 99.18%. The single false-negative result was found in a specimen with an HBV DNA concentration near the lower limit of quantification of both assays, as measured by the HBV viral load comparator kit.

Table 8. Diagnostic performance of the INDIGEN HBV DNA qRT-PCR assay compared to HBV Viral Load comparator kit (n = 122)

	HBV viral load comparator kit: Detected	HBV viral load comparator kit: Not Detected	Total
INDIGEN: Detected	104	0	**104**
INDIGEN: Not Detected	1	17	**18**
Total	**105**	**17**	**122**

Correlation between the two assays

Pearson correlation analysis of the 105 specimens positive by both assays demonstrated a strong linear relationship between our assay and the comparator kit ($R^2 = 0.9667$), as shown in Figure 3. To determine

the measurement error between the two assays, Deming regression analysis produced a slope of 0.9503 (95% CI: 0.89–1.01) and an intercept of −0.058 (95% CI: −0.30 to 0.18) (Figure 4).

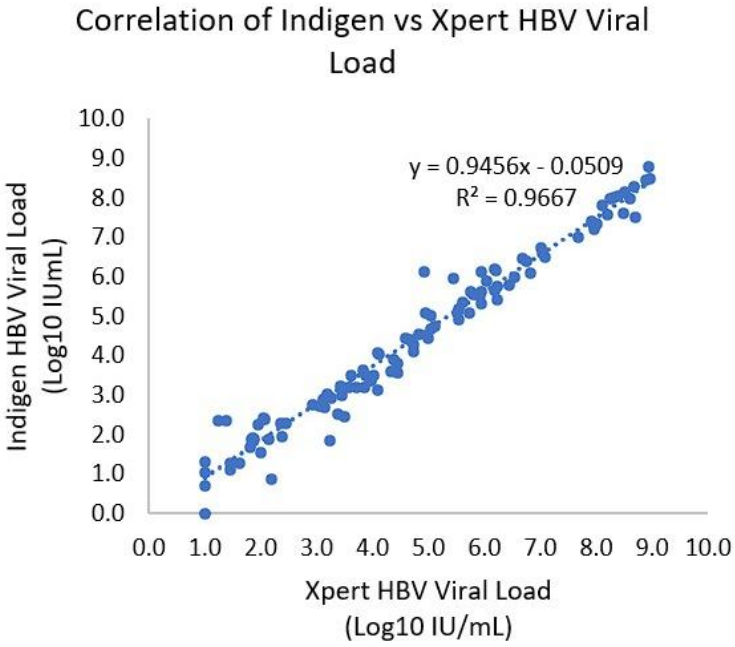


Figure 3. Pearson correlation analysis between INDIGEN HBV DNA qRT-PCR assay and HBV viral load comparator kit (slope = 0.9456, intercept = −0.0509).

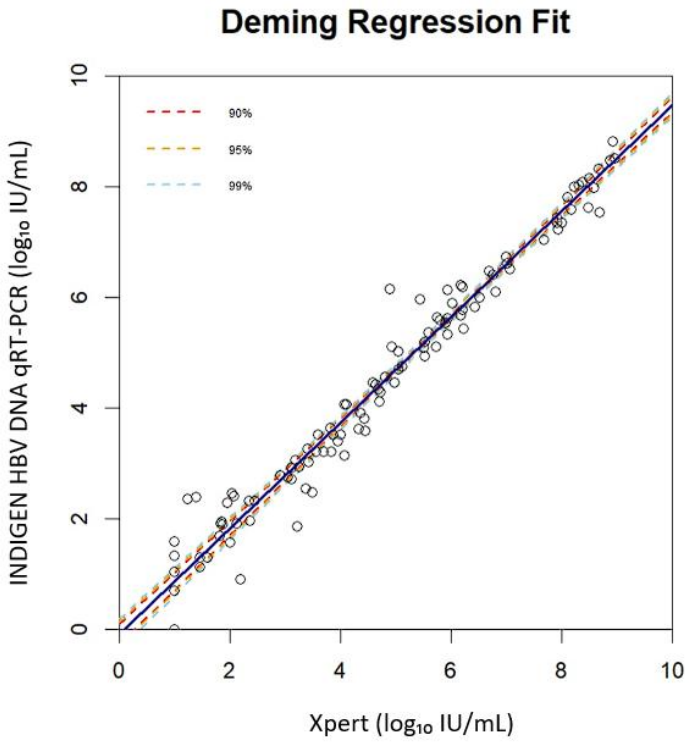


Figure 4. Deming regression analysis between INDIGEN HBV DNA qRT-PCR assay and HBV viral load comparator kit (slope = 0.9503, intercept = −0.058).

Discussion

HBV remains a public health problem in Indonesia. Despite having a high number of people living with chronic HBV infection in Indonesia, a considerably low number of HBV diagnoses poses a risk for accelerating disease progression. To increase the number of HBV diagnoses, the Ministry of Health of the Republic of Indonesia has ratified the national CHB management guidelines [7]. The guidelines recommend early HBV detection by screening high-risk individuals using serological HBsAg tests, which is followed by confirmatory HBV testing for positive results. Consequently, HBV diagnostic capacity needs to be expanded to facilitate earlier treatment initiation and reduce HBV related morbidity and mortality.

Several commercial HBV DNA quantitative assays are available and being used in Indonesia. The majority of these systems are run on dedicated closed-system platforms. This limits accessibility and sustainability of the assays in the long run due to the necessity of high investment costs and the reliance on specific consumables. In this study, we demonstrate the comprehensive analytical validation and clinical performance evaluation of a newly developed in-house HBV DNA quantitative assay, the INDIGEN HBV DNA qRT-PCR assay. This assay was developed as an open-system platform for HBV DNA detection and quantification that can be performed in any molecular laboratory supplied with an RT-PCR instrument.

We first designed the primers and probe of the assay to target a highly conserved region within the *HBV Surface (S)* gene as previously reported in several studies [15]. During the optimization of the assay, our data suggested that this assay could amplify the target S gene across HBV genotypes (A-H), including the genotypes B and C that predominate in Indonesia [16]. The low differences observed between the measured and assigned value also suggest that our primers and probe design effectively target highly conserved regions of the HBV genome. This finding showed the capability of our assay to provide broad coverage across HBV genotypes (A-H), which is essential considering the high genetic diversity of all recognized HBV genotypes.

To report the viral load results in International Units per milliliter (IU/mL) with metrological traceability, we calibrated our assay against the WHO International Standard for HBV DNA. The calibration results demonstrated excellent correlation of our assay with the WHO reference material as shown by overlapping regression lines, indicating successful calibration with minimal systematic bias and excellent agreement with the reference standard [17]. Provided the necessity to convert assay-derived copy number measurements to IU/mL, a conversion factor of 1 IU/mL = 15.7 copy/mL was applied to the INDIGEN HBV DNA qRT-PCR assay. This conversion factor allows quantitative results to be reported in the internationally standardized unit for all subsequent analytical and clinical performance evaluations. This calibration strategy is aligned with the recommendations for quantitative molecular diagnostics and the principles of ISO 17511 for establishing traceability of in vitro diagnostic assays [10].

We evaluated the capability of our assay to quantify low levels of HBV DNA by performing a linearity assessment of the assay using the standard HBV DNA panel in the range of concentrations from 1.0 to 9.0 $\log_{10}$ IU/mL. The concentration range used in this assessment represented complete clinical relevance in HBV DNA infections. The low concentrations covered the low-level viremia condition observed in inactive carrier states and therapy-suppressed patients, while the high concentrations covered

the high viral load characteristic of immune-active and immune-tolerant phases of CHB. Our linearity assessment with a produced coefficient of determination of $R^2 = 0.9965$ is comparable to several published assays. In the performance evaluation study of the Roche Cobas AmpliPrep/Cobas TaqMan HBV test version 1.0 and 2.0, the linearity of the assays was obtained to be 1.7 to 8.0 log10 IU/mL and 1.3 to 8.2 log10 IU/mL, respectively [18]. In addition, the Abbott Alinity m assay produced a similar linearity range of 1.14 to 8.14 log IU/mL [19]. The results suggest that our assay can accurately calculate viral loads within the tested concentration range that is essential for routine clinical testing. While the low measurement range provides reliable quantification close to the threshold of clinically important decisions, the high measurement range minimizes the need for retesting after specimen dilution. Together, these characteristics are essential for dynamic analysis of antiviral therapy and disease progression that requires accurate quantification over several orders of magnitude [20]. In this assessment, we observed a minor upward shift at higher viral load concentrations (LR-1 and LR-2; deviation 0.28 $\log_{10}$ IU/mL). This shift suggests slight differences in the efficiency of calibrator binding at concentrations close to the upper limit of the dynamic range. The same results have been reported in other quantitative HBV assays that are calibrated against WHO standards. Therefore, these deviations remain within clinically acceptable limits and are unlikely to influence treatment decisions [21].

The European Association for the Study of the Liver (EASL) recommends HBV DNA quantification assays with LoD values of less than or equal to 20 IU/mL [22]. According to our results, the analytical LoD of our assay was determined to be 14.8 IU/mL (1.17 $\log_{10}$ IU/mL), which meets the criteria suggested by the EASL. This performance is comparable to that reported for several established HBV DNA quantification assays, with reported LoD ranging from approximately 10 to 32 IU/mL [18, 19, 23]. The developed assay achieved a limit of detection of 14.8 IU/mL, which is consistent with the analytical sensitivity required for monitoring antiviral therapy. According to the published guidelines, an assay should have the LoD of less than 20 IU/mL to be considered as a sensitive assay for HBV DNA measurement kit [22]. Therefore, the analytical sensitivity of the developed assay is appropriate for clinically relevant monitoring of virological suppression during nucleos(t)ide analogue (NUCs) therapy [24] Additionally, the LLoQ of our assay was set to be 22.5 IU/mL (1.35 $\log_{10}$ IU/mL). This value suggests that at this concentration the total analytical error still meets the clinically acceptable threshold for evaluating the success of antiviral treatment of 1.0 $\log_{10}$ IU/mL [14].

In this study, we enrolled 122 participants to confirm the diagnostic sensitivity and specificity of our assay compared to the Xpert certified quantitative HBV DNA assay. A total of 105 participants were tested positive by the comparator kit. During our analysis, 104 participants were also positively detected by our assay, producing a diagnostic sensitivity of 99.05%. Additionally, we observed a single false-negative result that occurred in a specimen with HBV DNA near the detection boundary of both assays. This finding is consistent with the statistical expectation of near-threshold misclassification inherent to all molecular assays operating near their probit-defined LoD, and does not reflect a systematic assay deficiency [12, 13]. For the diagnostic specificity analysis, a total of 17 samples were tested negative in both assays, confirming 100% specificity of our assay in this study. Moreover, our assay produced a Pearson correlation coefficient of $R^2 = 0.9663$ and a Deming regression slope of 0.9503 with a near-zero intercept of −0.058 in a correlation study against the comparator kit using the positive samples. The observed analytical correlation suggested equivalent quantitative performance between both assays by having a strong correlation, a regression slope approaching 1.0, and an intercept near zero based on the recommended method-comparison criteria for quantitative molecular assays [25].

The INDIGEN HBV DNA qRT-PCR assay demonstrated excellent clinical sensitivity and specificity showed strong correlation with the comparator kit. Moreover, the open-system PCR system applied to this assay provides an important operational advantage over currently commercially available closed-system HBV quantification testing in Indonesia. While the majority of currently available HBV DNA testing can only be performed on specific instruments and strict workflows, our assay can be run on widely available real-time PCR platforms that will reduce limitations associated with platform-specific procurement and maintenance. Additionally, our open-system PCR also offers better scalability for HBV DNA quantification tests that can substantially increase the number of samples processed within a single analytical run compared to cartridge-based systems with a limited number of available instrument modules. This specification can improve laboratory efficiency and shorten turnaround times without reducing the analytical performance. In short, our assay can be implemented as a strategy that has the potential to improve hepatitis B disease management by expanding access to molecular diagnostics, increasing testing capacity, and accelerating treatment decisions.

A limitation in this present study is that we intentionally enrolled HBsAg-positive individuals, resulting in a high proportion of HBV DNA-positive specimens in our study cohort. Therefore, the proportion of HBV DNA-positive may not reflect the distribution encountered in general screening populations. In addition, the clinical performance evaluation of the assay did not include samples representing HBV genotypes. Further, study involves well-characterized clinical samples representing diverse HBV genotype may provide insight into the assay's performance across different HBV genotype variation.

Conclusions

In conclusion, the INDIGEN HBV DNA qRT-PCR assay is appropriate for HBV DNA quantification. This assay has demonstrated comparable analytical and clinical performance with strong agreement with the comparator HBV viral load assay, providing an open-system assay for decentralized HBV viral load testing in Indonesia. Further clinical studies are required to evaluate the performance of this assay in quantifying HBV DNA in more HBV genotypes.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Dr. Cipto Mangunkusumo National General Hospital (approval number ND-356/UN2.F1/ETIK/PPM.00.02/2025). Written informed consent was obtained from all study participants prior to enrolment. The study was conducted in compliance with ISO 20916:2019, and ICH-GCP E6 (R2).

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request, subject to applicable regulatory and ethical restrictions.

Funding

This study was funded by Stem Cell and Cancer Institute (SCI), Kalbe Farma Tbk., Jakarta, Indonesia. The funder supported the development of the investigational kit and study coordination. The clinical site and principal investigators at RSCM participated in the study independent of commercial considerations.

Authors' contributions

Rino Alvani Gani: conception and design of the study, supervision of analytical validation, principal investigator, manuscript review. Kemal Fariz Kalista: principal investigator, study design, clinical interpretation, manuscript revision. Siti Fatimah Selasih and Wisnu Aji Kuncoro: assay development, analytical validation experiments, data collection, statistical analysis, manuscript preparation and revision. Dini Budhiarko and Muhammad Yunus: primer and probe design, in silico analysis, manuscript review. Akterono Dwi Budiyati: study design, clinical study coordination, and manuscript review. Anugrah Dwi Handayu, Dela Intan Permatasari: specimen handling, data collection. All authors read and approved the final manuscript.

Acknowledgements

The authors acknowledge the research staff at the Hepatobiliary Division of Dr. Cipto Mangunkusumo National General Hospital and at Stem Cell and Cancer Institute for their contributions to data collection, specimen processing, and quality management activities. The authors thank all participants who consented to donate samples for this study.

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