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Development of a point-of-care test to detect hepatitis B virus DNA threshold relevant for treatment indication

Saran Pankaew[1], Sittiporn Pataradilokrat[1], Jantana Kampeera[2], Wansika Kiatpathomchai[2], Kiat Ruxrungtham[3], Siwaporn Boonyasuppayakorn[3,4,*]

Abstract

Background: Hepatitis B virus (HBV) has been the most prevalent blood-borne pathogen wherein utero transmission has still not been properly managed. Recent practice guidelines suggested that an antiviral drug should be administered to third-trimester pregnancies with significant viremia (>2 × 10^5 IU/mL).

Objectives: To develop a novel turbidity-based loop-mediated isothermal amplification (LAMP) coupled with heat treatment DNA extraction method that is a rapid, cost-effective, and feasible viral load assessment and could be applied to antenatal screening.

Methods: Primers and reagents were designed, turbidity-based platform and heat treatment method were added, and evaluated for optimal efficiency. Assay sensitivity was tested from serially diluted standard HBV DNA. Assay specificity was tested with six standard viral DNAs. Clinical samples were analyzed and the results were compared with those of quantitative polymerase chain reaction (qPCR) diagnostic records.

Results: The optimized condition was 60°C with no betaine, 1.4 mM deoxyribonucleotide triphosphates (dNTPs) and 6 mM of $MgSO_4$ for 60 min. The assay accurately detected samples with standard HBV DNA at >2 × 10^5 IU/mL in both distilled water and spiked serum. Results can be interpreted within 31.48 ± 1.41 min in real-time turbidimeter. The amplification is exclusively specific to HBV, but not with the other six human-specific viruses. Moreover, the assay showed comparable performance within 95% confidence interval to the previously developed HBV LAMP toward clinical specimens.

Conclusions: This newly developed method was accurate, affordable, and flexible to further implementation to large-scale third-trimester pregnancy screening.

Keywords: diagnosis; hepatitis b; pregnancy

***Correspondence to: Siwaporn Boonyasuppayakorn,** Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand, e-mail: siwaporn.b@chula.ac.th

[1]Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

[2]Bioengineering and Sensing Technology Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathum Thani 12120, Thailand

[3]Chula Vaccine Research Center, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

[4]Applied Medical Virology Research Unit, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Hepatitis B virus (HBV) is the most prevalent blood-borne pathogen infecting approximately 250 million people [1], and could potentially establish a chronic infection leading to life-threatening cirrhosis and hepatocellular carcinoma. The virus belongs to a family *Hepadnaviridae* and its 3.2 kb partially double-stranded genome is packed into an enveloped, spherical virion. According to the World Health Organization (WHO), a combination of HBV vaccine and immunoglobulin elicits 80%–95% protective effect to prevent mother to child transmissions when administrating within 24 h after childbirth. Still, 1%–9% of newborns were infected *in utero* from mother whose status were chronic HBV infection [2]. Moreover, the vaccination still limited its coverage in certain countries [3] resulting in increasing vulnerable population. Accumulated evidences suggested that treating expectant mothers with significant viremia ($\geq 2 \times 10^5$ IU/mL, or $\geq 10^2$ copies/reaction) in third trimester would prevent mother to child transmission more efficiently (reviewed by Jaffe and Brown [4]). The first-line chemotherapy was tenofovir administered from 30–32 weeks gestation to a month after delivery [5]. Following this guideline, an HBV genome detection should be included in the national program for antenatal screening in order to identify this group of high-risk pregnancy. The test was also expected to be accurate to meet the standard quantitative polymerase chain reaction (qPCR) system [6], as well as simple and cost-effective applicable in rural areas. The alternative viral load assessment method is required because of limited availability to the qPCR machine and its operating system.

The major requirements of the newly developed antenatal HBV screening would be the ability to sort out significantly viremic samples with accuracy, rapidity, feasibility, and cost-effectiveness. Turbidity-based loop-mediated isothermal amplification (LAMP) could potentially serve these specific needs [7]. LAMP utilizes a *Bst* polymerase for target amplification under a constant single temperature with a 30- to 60-min incubation [8]. The amplified product can be detected by various methods [9], but turbidity detection is the most promising means to be optimized toward both arms of accuracy and cost-effectiveness [10]. Although several HBV LAMP protocols have been described, limitations were still noted whenever applying to point of care. For example, the detection system in previous developments required a qPCR machine [11, 12] that was unavailable in the resource-limited area. Also, it is our aim to deliberately avoid chromogenic or fluorogenic substrates addition in order to minimize the cost per reaction. A recently developed in-house turbidimeter [7] can directly detect the rate of reaction by measuring magnesium pyrophosphate by-product in real time with economical price. Turbidity-based detection was generally applied to the diagnosis of various pathogens such as *Plasmodium spp.* [13], *Haemophilus influenzae* type b [14], and *Listeria ivanovii* [15]. In addition to LAMP, DNA extraction method can also be simplified to heat treatment with equal sensitivity to conventional silica-based extraction method [16]. In this study, we aimed to develop a reliable, simplified, and budget-friendly turbidity-based HBV LAMP for a routine antenatal screening.

Materials and methods

Primer design and evaluation

The whole genome of HBV, strain ayw (NC_003977) [17] was used as a template for primer design (Primer Explorer 4.0, Eiken Chemical CO., LTD., Japan). Three sets of LAMP primers (S1, S2, and X) (**Table 1**) were selected based on the following conditions: (1) the primer length of 18–25 base pairs, (2) the melting temperature of 60–65°C, and (3) the estimated Gibbs free energy of below −4 kJ/mol. Sequences were checked for self-priming and specificity before synthesis (Integrated DNA Technologies, USA). Each primer set was evaluated for amplification efficiency using a standard 2% agarose electrophoresis.

Standard HBV DNA preparation

The plasmid pUC19 containing a whole HBV genome AB246345 [18] was propagated in MAX Efficiency® DH5α™ competent cells (Thermo Fisher Scientific, USA). The plasmid was extracted and purified using a High-Speed Plasmid Mini Kit (Geneaid Biotech Ltd., Taiwan) and the concentration was measured using a NanoDrop 1000 (Thermo Scientific, USA). The plasmid was adjusted to the concentration of 1 mg/mL and stored as aliquots at −20°C until use.

LAMP reaction and turbidity detection

A HBV LAMP reaction consisted of 2.5 mL of 10× isothermal amplification buffer (New England Biolabs, USA), 150 mM $MgSO_4$, 0.14 mM dNTPs, 5 mM of F3 and B3 primers, 40 mM of forward inner primer (FIP) and backward inner primer (BIP) primers, 10 mM of loop forward primer (LF) and loop backward primer (LB) primers, eight units of *Bst* 2.0 Polymerase (New England Biolabs, USA), 2 mL of DNA sample, and deionized distilled water to a final volume of 25 mL. The reaction was incubated at 60°C for 60 min before inactivating the enzyme at 95°C for 2 min. Turbidity was detected using 8-well turbidimeter (Mobilis Automata co., Ltd., Thailand)

Table 1. Oligonucleotide sequences of three newly designed and one previously reported primer sets

Primer set	Primer name	Location	Primer sequence (5′ à 3′)	Genetic region
S1	S1_F3	470–491	CCGTTTGTCCTCTAATTCCAGG	*S* and *P* gene
	S1_B3	665–688	GCACTAGTAAACTGAGCCAGGAGA	
	S1_FIP	541–564	GGAGGGATACATAGAGGTTCCTTG-TTTT-CCTCAACAACCAGCACGGGA	
		494–513		
	S1_BIP	571–592	TGTACCAAACCTTCGGACGGAA-TTTT-CCCACTCCCATAGGAATTTTCC	
		631–652		
	S1_LF	520–540	AGCAGTAGTCATGCAGGTCCG	
	S1_LB	595–616	TGCACCTGTATTCCCATCCCAT	
S2	S2_F3	659–678	CTGCATGACTACTGCTCAAGGA	*S* and *P* gene
	S2_B3	712–731	AGCCAAACAGTGGGGGAAAG	
	S2_FIP	595–615	TGGGATGGGAATACAGGTGCA-TTTT-CCTCTATGTATCCCTCCTGTTGCT	
		548–571		
	S2_BIP	631–651	GGAAAATTCCTATGGGAGTGGG-TTTT-CCCTACGAACCACTGAACAAATGG	
		688–711		
	S2_LF	572–591	TCCGTCCGAAGGTTTGGTAC	
	S2_LB	659–678	CCCGTTTCTCCTGGCTCAGT	
X	X_F3	1493–1512	CCTTCTCCGTCTGCCGTTCC	X gene
	X_B3	1728–1747	CCCCAACTCCTCCCAGTCTT	
	X_FIP	1577–1596	GTGAAGCGAAGTGCACACGG-TTTT-CACCTCTCTTTACGCGGACTCC	
		1529–1540		
	X_BIP	1627–1646	CGCCCACCAAATATTGCCAA-TTTT-TATGCCTCAAGGTCGGTCGTTG	
		1686–1707		
	X_LF	1559–1576	TCCGGCAGATGAGAAGGC	
	X_LB	1662–1685	GGACTCTTGGACTCTCAGCAATGT	
Nyan et al. [19]	Ref_F3	530–249	TCCTCACAATACCGCAGAGT	*S* and *P* gene
	Ref_B3	402–421	GCAGCAGGATGAAGAGGAAT	
	Ref_FIP	305–326	GTTGGGGACTGCGAATTTTGGC-TTTT-TAGACTCGTGGTGGACTTCT	
		251–270		
	Ref_BIP	333–354	TCACTCACCAACCTCCTGTCCT-TTTT-AAAACGCCGCAGACACAT	
		379–396		
	Ref_LF	271–294	GGTGATCCCCCTAGAAAATTGAG	
	Ref_LB	357–378	AATTTGTCCTGGTTATCGCTGG	

F3, outer forward primer; B3, outer backward primer; FIP, forward inner primer; BIP, backward inner primer; LF, loop forward primer; LB, loop backward primer.

[7] and the results were analyzed using an LAMP-Turbidity plotter version 1.0 (National electronics and computer technology center, NECTEC, Thailand).

Sensitivity test

Assay sensitivity was tested from serially diluted standard HBV DNA to the final concentrations of 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10 copies and performed under optimized condition as described above. The reactions were incubated in turbidimeter at 60°C for 60 min and correlation between turbidity detection and HBV genome concentration was analyzed. In the spiked serum experiment, the standard HBV DNA at 10^8 to 10^2 copies was spiked into fetal bovine serum (1:100 v/v). Samples were diluted with equal amount of deionized distilled water and incubated at 95°C for 5 min and 100°C for 3 min before proceeding to turbidity-based HBV LAMP. Results were confirmed by four independent experiments.

Specificity test

The specificity of the HBV–LAMP assay was tested under optimized condition with six viral DNAs provided as positive controls as follows: HBV (Abbott, USA), human immunodeficiency virus (HIV) (Abbott, USA), Epstein–Barr virus (EBV, cytomegalovirus (CMV), hepatitis C virus (HCV), and

herpes simplex virus (HSV) (Qiagen, Germany). The LAMP products were detected using agarose gel electrophoresis. All standard viral DNAs were kindly gifted from King Chulalongkorn Memorial Hospital and were added at 2×10^5 copies to the reaction.

Analysis of clinical samples

All plasma samples and their automated qPCR results (Abbott, USA) were courtesy of Virology unit, King Chulalongkorn Memorial Hospital with IRB approval (certificate of approval no. 972/2016) from Ethical Review Board, Faculty of Medicine, Chulalongkorn University. The samples were leftovers from routine diagnosis and stored at −70°C. The frozen plasma was rapidly thawed at 37°C and the DNA was extracted from the samples using the heat treatment method [19]. Briefly, the sample was diluted with equal amount of deionized distilled water, followed by incubation at 95°C for 5 min and 100°C for 3 min. DNA was collected from supernatant after centrifugation at 12,000 g for 5 min. Turbidity-based HBV LAMP assay was performed and analyzed as previously indicated. The LAMP product was also analyzed by gel electrophoresis to confirm the end-point results. Results from turbidimeter and gel electrophoresis were compared with those of qPCR diagnostic records.

Results

Primers and reagent optimization

Three sets of primers (S1, S2, and X) were generated according to the parameters described in Materials and methods section. Efficacy of primer sets was verified using the standard HBV DNA (**Figure 1A**). Results showed that S2 and X, but not S1, primer sets expressed positive amplification bands in ladder-like pattern, similar to previous report [19]. Next, we optimized the assay condition by varying 4 parameters as follows: temperature (55°C, 60°C, 63°C, and 65°C), betaine (0 M, 0.2 M, 0.4 M, and 0.8 M), dNTPs (1.2 mM, 1.4 mM, 1.6 mM), and $MgSO_4$ (4 mM, 6 mM, 8 mM). Each assay condition was analyzed using 10-fold serially diluted HBV plasmid (10^2–10^5 copies). Results indicated that the optimal condition for S2 primer set was at 60°C with no betaine, 1.4 mM dNTPs, and 6 mM of $MgSO_4$ demonstrating the detection at 100 copies/reaction in the reaction using 2% agarose gel electrophoresis (**Figure 1B and Table 2**). Moreover, we verified the HBV DNA template as a standard positive control

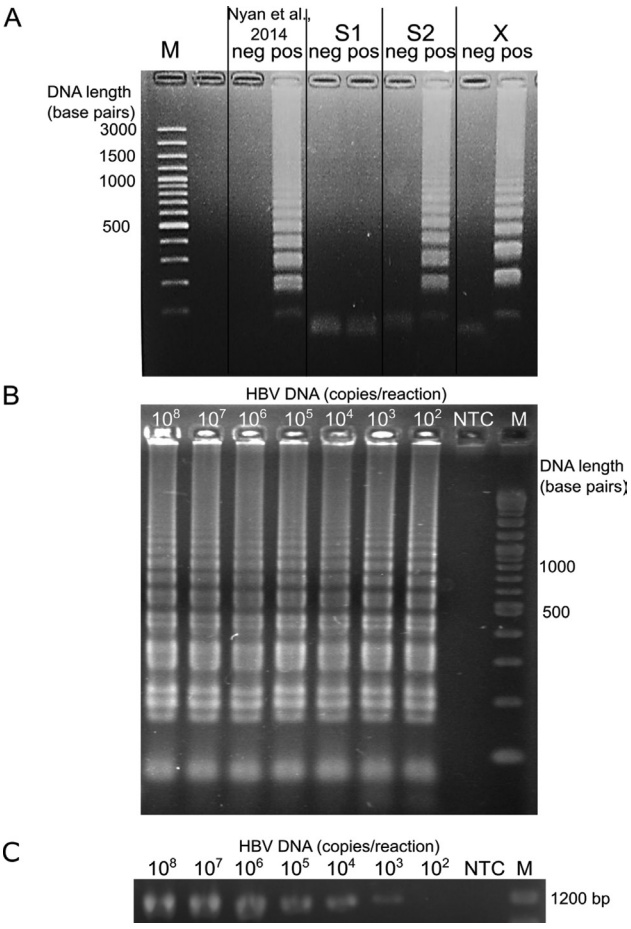

Figure 1. HBV LAMP primer tests (**A**) Three newly designed HBV LAMP primer sets (S1, S2, and X) and a previous report [19] were tested with standard HBV DNA (10^8 copies/reaction) and deionized distilled water as positive and negative controls. (**B**) S2 primer set was tested with serially diluted standard HBV DNA (10^2–10^8 copies/reaction). M and NTC were abbreviated from marker and no template control, respectively. (**C**) Sensitivity results from polymerase chain reaction using universal primers.

for further experiments by amplification with established universal primers [20]. PCR results showed that the HBV template was successfully detected by universal primers (**Figure 1C**); therefore, we reinstated that the plasmid containing HBV genome used in this assay development was a standard HBV DNA template.

Sensitivity of turbidity-based HBV–LAMP assay

The turbidity-based LAMP was set up using the optimal condition for S2 primer set as described and the turbidity was detected using hot plate-coupled, real-time turbidimeter [7]. This turbidimeter detected magnesium pyrophosphate by-product from the LAMP assay in real time. We compared sensitivity

Table 2. Sensitivity of turbidity- and gel electrophoresis-based HBV LAMP detection system

HBV DNA (copies/reaction)	Percent detection (No. of times detected/total replicated)	
	Turbidity based	Gel electrophoresis based
2000	100 (6/6)	100 (3/3)
1000	100 (6/6)	100 (3/3)
200	83 (5/6)	100 (3/3)
150	83 (5/6)	100 (3/3)
100	**100 (9/9)**	**100 (3/3)**
50	50 (3/6)	67 (2/3)
20	67 (4/6)	67 (2/3)
10	33 (1/3)	–
5	33 (1/3)	–
2.5	67 (2/3)	–
1	33 (1/3)	–
0.5	0 (0/3)	–
0.1	33 (1/3)	–

HBV, hepatitis B virus; LAMP, loop-mediated isothermal amplification.

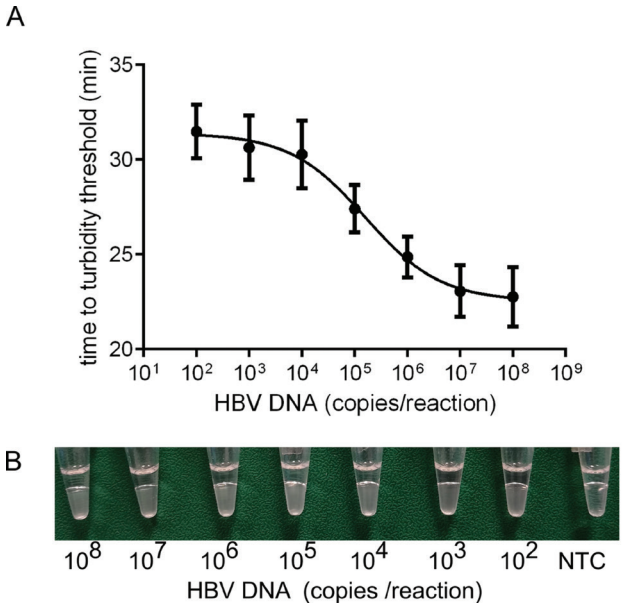

Figure 2. Turbidity-based HBV LAMP sensitivity test S2 primer set was tested with serially diluted standard HBV DNA (10^2–10^8 copies/reaction) in (**A**) turbidimeter and (**B**) direct visualization at the end point of the reaction.

of both detection systems using serially diluted standard HBV DNA (**Table 1**) and results showed equal limit of detection (LoD) at 10^2 copies/reaction. Moreover, the time to turbidity threshold at the lowest and highest concentrations (10^2 and 10^8 copies) was 1888.75 ± 84.94 s, and 1365.75 ± 94.68 s, respectively, suggesting that the positive results should be detected by this assay during 22.76 ± 1.58 and 31.48 ± 1.41 min. Also, a nonlinear correlation of HBV DNA and the threshold of time to positive turbidity were observed (**Figure 2A**) suggesting that this real-time HBV LAMP turbidity could be applicable in semiquantitative format. In addition to turbidimeter, the positive results can be detected from direct visualization and distinguishable from the no template control (NTC) (**Figure 2B**). In addition, results can also be reported in using direct visualization for turbidity at the reaction end point. This option is applicable to resource-limited area where only heat block and centrifuge were available.

Next, we tested the assay performance under the condition mimicking the actual serum samples in order to verify whether sera-derived proteins would interfere with the turbidity readout. Briefly, standard HBV DNA was serially diluted and spiked into fetal bovine serum (1:100 v/v), diluted 1:1 with deionized distilled water and heat treated before proceeding to turbidity-based HBV LAMP. The objective was to study possible turbidity interference from other contents in the serum when processing samples with heat treatment method. Our results were in accordance with the previous report comparing the heat treatment and standard DNA extraction methods [16]. LoD of this experiment was read at 10^2 copies/reaction with no significant difference from that of the sensitivity test previously described (P = 0.6571, Mann–Whitney test). In addition, the conventional HBV PCR amplified by universal primers (**Figure 1C**) was successfully detected the template at 10^3 copies per reaction. We concluded that the heat treatment method coupled with turbidity-based LAMP analysis was adequate for analysis of clinical samples.

Specificity test

The HBV LAMP was tested with the standard DNA of HBV, HIV, HCV, CMV, EBV, and Herpes viruses standardized to 2×10^5 copies/reaction as described in Materials and methods section. The assay was performed in turbidimeter for 60 min and the products were analyzed by gel electrophoresis. Positive results, or a ladder-like pattern, were found only in the HBV sample (**Figure 3**) suggesting that the HBV LAMP assay was exclusively specific to HBV.

Evaluation with clinical samples

A total of 270 clinical samples consisted of 162 significant (<2×10^5 IU/mL) and 108 nonsignificant (<2×10^5 IU/mL) viremia according to the automated qPCR results. Samples were thawed and DNA was extracted using heat treatment

method. HBV LAMP was performed in real-time turbidimeter and results were obtained using turbidity measurement (A_{650}) and gel electrophoresis. The results were analyzed using 2 × 2 table for an accuracy of a diagnostic test [21] and compared with the diagnostic results from automated qPCR system. The turbidity-based LAMP method was sensitive, specific, and accurate at 90.12%, 83.33%, and 87.40%, respectively (**Table 3**), whereas results from the gel electrophoresis showed sensitivity, specificity, and accuracy of the test at 95.06%, 75.00%, and 87.03%, respectively. Interestingly, gel electrophoresis detection was more sensitive but less specific comparing to turbidity detection; in other words, the gel electrophoresis increased both detection rate and false-positive results. We concluded that the turbidity-based LAMP assay is applicable to discriminate the samples containing high viral titers (≥2 × 10^5 IU/mL) from those of low viral titers (<2 × 10^5 IU/mL) and negative results (undetectable) previously determined by qPCR (Abbott, USA). Moreover, the sensitivities, specificities, and accuracies of the tests were calculated using the other cutoff values (**Table 4**). Results showed that the test elicited the maximal accuracy at 10^5 IU/mL.

In addition, significant viremic samples were further analyzed for correlation with time to turbidity thresholds derived from turbidity-based LAMP assay [12]. Unfortunately, linear regression pattern was not found; therefore, we concluded that the time to turbidity threshold could not be used to quantify HBV viral load in turbidity-based LAMP assay.

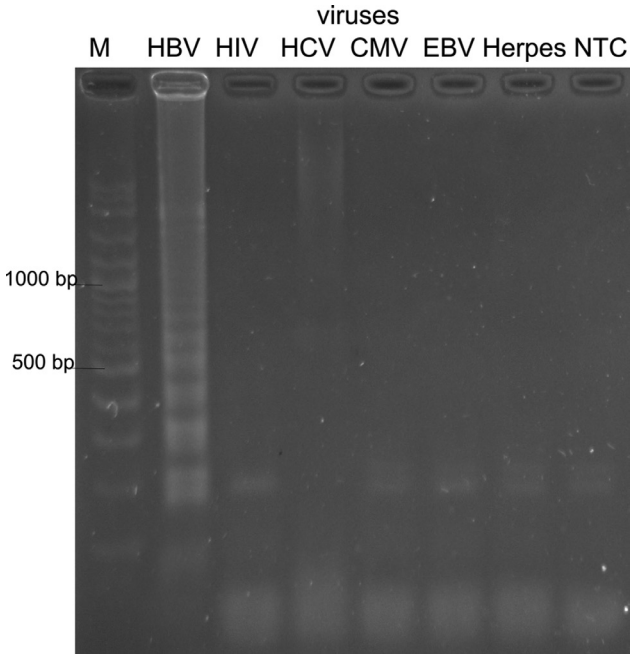

Figure 3. Specificity test S2 primer set was tested with 2 × 10^5 copies of standard DNAs of hepatitis B virus (HBV), human immunodeficiency virus (HIV), Epstein–Barr virus (EBV), cytomegalovirus (CMV), hepatitis C virus (HCV), and herpes simplex virus (HSV) and analyzed by 2% agarose gel electrophoresis.

Cost and method comparison

The major cost difference between qPCR and LAMP was the equipment used in each method. A quantitative thermal cycler machine price ranges between 30,000 and 50,000 USD, whereas a turbidimeter price used in this optimization was at 2,000 USD. In our design, we expected the local facilities to use a heat block (200–1200 USD), or a waterbath (50–100 USD). Since LAMP relied on an equipment capable of maintaining a single temperature for 30–60 min, the more economical choices of equipment selection can be considered. Moreover, the heat treatment method was not only feasible and time-saving, but also economical for omitting the DNA extraction process. Currently, DNA extraction kit price ranges between 2 and 5 USD/sample and requires about 2 h processing time. Cost and method comparison were summarized in **Table 5**.

Discussion

This assay development was mainly determined to answer the obstetrician's request for an antenatal screening in rural area. Turbidity-based HBV LAMP coupled with heat treatment method was chosen because of acceptable accuracy, simplified methods, rapidity, and cost-effectiveness. The assay sensitivity to detect HBV DNA was 100 copies/reaction (2 × 10^5 IU/mL) and no cross detection to other viruses was

Table 3. Performance of turbidity-, and gel electrophoresis-based HBV LAMP assay at the 2 × 10^5 IU/mL cutoff titer of HBV qPCR results

Methods	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
Turbidity-based	90.12 (146/162)	83.33 (90/108)	89.02 (146/164)	84.90 (90/106)	87.40 (236/270)
Gel electrophoresis	95.06 (154/162)	75.00 (81/108)	85.08 (154/181)	91.01 (81/89)	87.03 (235/270)

HBV, hepatitis B virus; LAMP, loop-mediated isothermal amplification; qPCR, quantitative PCR.

Table 4. Performance of turbidity-, and gel electrophoresis-based HBV LAMP assay at other cutoff titers of HBV qPCR results

Cutoff	Sensitivity		Specificity		PPV		NPV		Accuracy
Turbidity									
10^6	131/139	94.2%	74/105	70.5%	131/162	80.9%	74/82	90.2%	84.0%
5×10^5	140/152	92.1%	70/92	76.1%	140/162	86.4%	70/82	85.4%	86.1%
10^5	146/162	90.1%	66/82	80.5%	146/162	90.1%	66/82	80.5%	86.9%
5×10^4	150/170	88.2%	62/72	83.8%	150/162	92.6%	62/82	75.6%	86.9%
10^4	156/185	84.3%	53/59	89.8%	156/162	96.3%	53/82	64.6%	85.7%
10^3	157/202	77.7%	37/42	88.1%	157/162	96.9%	37/82	45.1%	79.5%
Gel electrophoresis									
10^6	135/139	97.1%	63/105	60.0%	135/177	76.3%	63/67	94.0%	81.1%
5×10^5	147/152	96.7%	62/92	67.4%	147/177	83.1%	62/67	92.5%	85.7%
10^5	154/162	95.1%	59/82	72.0%	154/177	87.0%	59/67	88.1%	87.3%
5×10^4	157/170	92.4%	54/67	73.0%	157/177	88.7%	54/67	80.6%	86.5%
10^4	163/185	88.1%	45/59	76.3%	163/177	92.1%	45/67	67.2%	85.2%
10^3	168/202	83.2%	33/42	78.6%	168/177	94.9%	33/67	49.3%	82.4%

HBV, hepatitis B virus; LAMP, loop-mediated isothermal amplification; qPCR, quantitative PCR; PPV, positive predictive values; NPV, negative predictive values.

Table 5. Cost comparison between quantitative PCR and LAMP

Methods	Price (per equipment)	Price (per reaction)			Price (per test, include positive and negative controls)
	Equipment	DNA extraction	Amplification reagents	Detection	Total
Quantitative PCR	qPCR 30,000–50,000 USD	5 USD	30 USD	–	105 USD
LAMP	Turbidimeter 2000 USD, or heat block (200–1200 USD), or waterbath (50–100 USD)	–	20 USD	–	60 USD

LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction.

observed. The positive results can be determined by turbidimeter between 22.76 ± 1.58 and 31.48 ± 1.41 min. In clinical samples, the assay performed 90.12% sensitivity, 83.33% specificity, and 87.40% accuracy comparable to previously developed HBV LAMP assays. Meta-analysis of 12 HBV LAMPs literatures performing 494 cases revealed the average assay sensitivity, and specificity at 0.922 (95% confidence interval [CI]: 0.905–0.937), and 0.860 (95% CI: 0.818–0.896), respectively [22]. Therefore, we concluded that our turbidity-based HBV LAMP coupled with heat treatment method was similarly accurate to the previously developed HBV LAMP methods and can be implemented to the national-level HBV antenatal diagnostic test.

Our turbidity-based HBV LAMP interpreted positive and negative results at the 2×10^5 IU/mL HBV DNA cutoff value at 89.02% and 84.90%, respectively (**Table 2**). The 2×10^5 IU/mL cutoff value was clinically defined as significant viremia of which initiating an antiviral therapy was highly recommended [4]. Previous HBV LAMP development utilized the fluorescent dye to detect the amplified product [11, 12] and the results were proven equally sensitive and specific to standard qPCR method. However, those fluorescent-based LAMP assays were also developed under qPCR equipment that was unavailable in point-of-care setting. The pricing of qPCR machine and real-time turbidimeter were approximately 15–25 times difference at 30,000–50,000 USD and 2,000 USD, respectively. Moreover, in remote area where turbidimeter could not be procured, the turbidity-based assay coupled with heat treatment method can still be performed using only a heat block and a micro-centrifuge. Positive results can be alternatively detected from direct visualization or spectrophotometer (A_{650}). In addition, we also utilized the heat treatment method to substitute the standard DNA extraction of clinical samples with the objectives to reduce (1) analytical period, (2) dependency of laboratory equipment, (3) the risk of cross-contamination, and (4) the expense of DNA extraction kit. The heat treatment did

not interfere with turbidity readouts according to our spiked sera results and the previous report [19].

Several detection platforms were integrated to LAMP such as lateral flow dipstick, enzyme-linked immunosorbent assay (ELISA), and microfluidic chip using antibody-labeled streptavidin–biotin, fluorescent-labeled probes, giant magnetoresistive (GMR) sensors, probe-functionalized nanoparticles, magnetic nanoclusters (MNCs), and line probe assay (LiPA) [9, 23]. Incorporating one of the platforms into LAMP detection system could potentially increase the sensitivity, as well as the cost per reaction. Further assay development toward field implementation might emphasize on reducing interpersonal variation by transferring to lateral flow dipstick or microfluidic chip platform while maintaining with the low price for affordability.

Author contributions. All the authors contributed substantially to the conception and design of this study. SP1, JK, WK, and SB contributed substantially to the acquisition of data. SP1, JK, WK, KR, and SB analyzed and interpreted the data. All the authors drafted the manuscript. SP1, SP2, KR, and SB contributed substantially to its critical revision. All the authors approved the final version submitted for publication and take responsibility for the statements made in the published article.

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Conflict of interest statement. The authors have completed and submitted the International Committee of Medical Journal Editors Uniform Disclosure Form for Potential Conflicts of Interest. None of the authors disclose any conflict of interest.

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