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Original Article

Development of Molecular-Based Screening Test for Hepatitis B Virus in Human Plasma Samples

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ABSTRACT: Despite regular administration of hepatitis B virus (HBV) vaccine in several countries, the mortality rate associated with HBV remains significant. The antiviral medications available for the treatment of HBV infection do not suffice for the serious complications related to chronic HBV infection. Additionally, the serological tests fail to detect early viral replication preventing early treatment response. Recently, many studies have demonstrated the significant advantages of loop-mediated isothermal amplification (LAMP) over serological testing and polymerase chain reaction (PCR), for the rapid detection of microbial pathogens. Here we developed a rapid, sensitive, and portable system-integrative LAMP assay for the detection of HBV DNA in plasma samples. The final optimized assay was achieved with an amplification time of less than 45 min at 62°C. The assay showed 100% specificity, 92.20% sensitivity, and a detection limit of 10 copies/μL in 77 HBV-positive plasma samples with known Cq values. Our results showed that the colorimetric LAMP assay is sensitive, efficient, and supremely reliable for rapid detection of HBV, and may be potentially used as a screening test in areas with poor laboratory facilities and limited resource availability.

INTRODUCTION

Hepatitis B virus (HBV) is known to infect over 2 billion people worldwide, with approximately 240 million chronic carriers, causing an estimated 500,000–700,000 deaths annually. According to the data from World Health Organization (WHO), it is estimated that 296 million people were living with chronic hepatitis B infection, and there were 1.5 million new infections each year (1). In 2019, an estimated 820,000 deaths occurred, mostly due to complications such as cirrhosis, and hepatocellular carcinoma (HCC), that are associated with HBV infection. Chronic HBV infection not only leads to chronic liver failure and cirrhosis but also increases the risk of developing HCC by 100 times. Therefore, early diagnosis and treatment are crucial (2,3). Traditional serological tests and polymerase chain reaction (PCR) have been widely used for diagnosis; however, they have limitations, particularly in detecting early viral replication which hampers early treatment response. Consequently, there is an urgent need for innovative diagnostic techniques that offer improved sensitivity, specificity, and rapid detection of HBV infection (4,5).

In recent years, loop-mediated isothermal amplification (LAMP) has emerged as a promising alternative for the rapid and efficient detection of microbial pathogens. Numerous studies have demonstrated the advantages of the LAMP method over traditional serological testing and PCR. LAMP offers several benefits including simplicity, rapidity, and cost-effectiveness. It operates under isothermal conditions, eliminating the need for expensive thermal cycling equipment and enables testing in resource-limited settings with poor laboratory facilities (6,7). In this study, we aimed to develop a rapid, sensitive, and portable LAMP-based assay to detect Hepatitis B DNA in plasma samples.

HBV has 9 subtypes determined by HBsAg antigenic determinants, with more than 8% variation in their entire genome sequences. These subtypes named A–J, for a total of 10 genotypes. Molecular epidemiological studies have revealed significant differences in the geographical distribution of HBV genotypes. Studies covering extensive geographical regions indicate that genotypes A, C, and D are dominant. Türkiye is a region with intermediate endemicity of HBV infection (2–7%), and research has indicates that genotype D is dominant in this area (8,9,10). With a 99% similarity rate, our LAMP primers identified genotype D as the most common genotype in Türkiye.

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Table 1. Information of designed LAMP primers used in this study

Primer setting	Primer sequence 5′ to 3′	Length (mer)	GC contents (%)
HbS-F3	TCCAGGATCATCAACCACCA	20	50.0
HbS-B3	CGAACCACTGAACAAATGGA	20	45.0
HbS-FIP	GGTGCAGTTGCATCCGAAGGTCAGAACCTGCGCACTCC	38	60.5
HbS-BIP	ATTCCCATCCCATCGTCCTGGGCAAGAGAAACGGGCTGAGG	41	58.5
HbS-LF	GGATACATAGAGGTTCCTTGAGCA	24	45.8
HbS-LB	CTTTCGGAAAATCCTATGGGAGT	23	43.5
HbC-F3	TCCAGCCTATAGACCACC	18	55.6
HbC-B3	ACAGTAGAAGAATAAAAGTCCAGTA	25	32.0
HbC-FIP	CCTCGGTCTCGTCGTCTAACAAATGCCCCTATCTTATCAAC	41	48.8
HbC-BIP	CAGACGAAGATCTCAATCGCCGACCTTATGAGTCCAAGGA	40	50.0
HbC-LB	CGTCGCAGAAGATCTCAATCTC	22	50.0
HbX-F3	ACCTCTCTTTACGCGGTCTC	20	55.0
HbX-B3	CCCAACTCCTCCCAGTCTT	19	57.9
HbX-FIP	CTCCATGCAACGTGCAGAGGTGCCGTCTGTGCCTTCTCATC	41	58.5
HbX-BIP	CATCAGGTCCTGCCCAAGGTCGTAGGCCTCAAGGTCGGT	39	61.5
HbX-LF	AGCGAAGTGCACACGGACC	19	63.2
HbX-LB	TTACATAAGAGGACTCTTGGACTCT	25	40.0

LAMP, loop-mediated isothermal amplification; HbS, hepatitis B virus surface protein; HbC, hepatitis B virus core protein; HbX, hepatitis B virus X protein.

We achieved an amplification time of less than 45 min at 62°C by optimizing the assay parameters. The findings of our study, which included the analysis of 77 blood samples that tested positive for HBV, revealed a high level of sensitivity (92.20%) and a detection limit of 10 copies/μL. Additionally, we observed 100% specificity in the analysis of 22 negative samples. These results highlight the potential efficacy of colorimetric LAMP as a reliable screening method for the rapid detection of HBV infection, particularly in regions with restricted access to laboratory facilities (6).

MATERIALS AND METHODS

Sample collection: Under the approval of the Ethics Committee from Istanbul Medipol University's Genetic Diseases Assessment Center (Medigen, Türkiye), a total of 99 DNA isolates were obtained within the scope of ethical approval (no: E-10840098-), comprising 77 HBV-positive and 22 HBV-negative cases. The HBV DNA was extracted using an isolation robot. The copy numbers and Cq values of the positive samples were determined using the Bosphore HBV Quantification Real-Time PCR kit (Anatolia Geneworks, Istanbul, Türkiye). The isolates were transported under cold chain conditions and stored at –20°C until further processing.

Design of LAMP primers: Design of LAMP primers is a crucial step in assay development. PrimerExplorer V.5 software (Fujitsu Japan Limited, Tokyo, Japan) was used to design LAMP-specific primers based on three different highly conserved regions: the surface protein (101–781 nt), X protein (1474–1690 nt), and core protein (1901–2347 nt) of HBV (GenBank accession number: MZ093431.1). Several key factors were considered to meet the criteria for successful primer design. Primers should have stability at the 3′ end, with a free energy value (-ΔG) of −4 kcal/mol or less. The GC content of primer should be 40–60%. Melting temperature (Tm) is the temperature at which half of the DNA duplex dissociates and becomes single-stranded, indicating duplex stability. Primers with Tm above 65◦C have a tendency for secondary annealing. The GC content of the sequence provides a fair indication of the Tm. Primers with Tm 52–58°C generally produced the best results. In addition, the optimal Tm of the primers should be similar to ensure compatible annealing conditions. It is also important to consider the amplicon length, which should be approximately 200 base pair (bp) (11). To evaluate potential secondary structures, such as homodimers, hairpins, and heterodimers, the designed primers were analyzed using the web-based free software program OligoAnalyzer (Version 3.1; Integrated DNA Technologies, Coralville, IA, USA). The presence of complementary regions among primer pairs can lead to primer dimers and hairpins, which can negatively affect the availability of primers for annealing to the template, resulting in poor or no product yield (12,13). Three primers were designed to target the HbS: Surface protein (S), HbC: Core protein (C), and HbX: X protein regions (X). To verify the specificity of the designed primers, the nucleotide Basic Local Alignment Search Tool (BLAST) (National Library of Medicine, Bethesda, MD, USA) was used for sequence comparison with known sequences in the database. Based on BLAST alignment analysis, our LAMP primer regions exhibited similarities of 93% for genotype A (A1-A2-A3), 92% for genotype B, 95% for genotype C, 99% for genotype D, 94% for genotype E, 91% for genotype F, 92% for genotype G, and 93% for genotype H. Our primer region was 99% similar to genotype D, the most common genotype in Türkiye (8).

After completing the primer design procedure, the

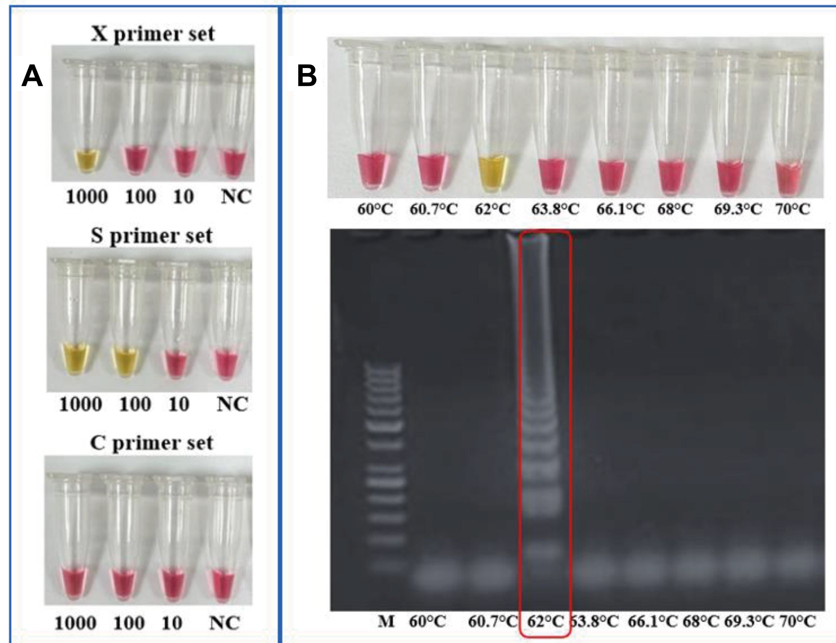

Fig. 1. (Color online) Selection of the most suitable LAMP primer sets based on results. (A) HBV DNA dilution series (1,000, 100, and 10 pg/µL) results of colorimetric LAMP reaction using X primer, S primer, and C primer sets. The negative control tube (NC) remained pink in color while yellow appeared in the positive reaction tubes. (B) Upper tubes represent colorimetric HBV-LAMP results using the S primer set at 8 reaction temperatures (60°C, 60.7°C, 62°C, 63.8°C, 66.1°C, 68°C, 69.3°C, and 70°C). Lower panel shows detection results in agarose gel electrophoresis. Lane M indicates 50 bp DNA ladder.

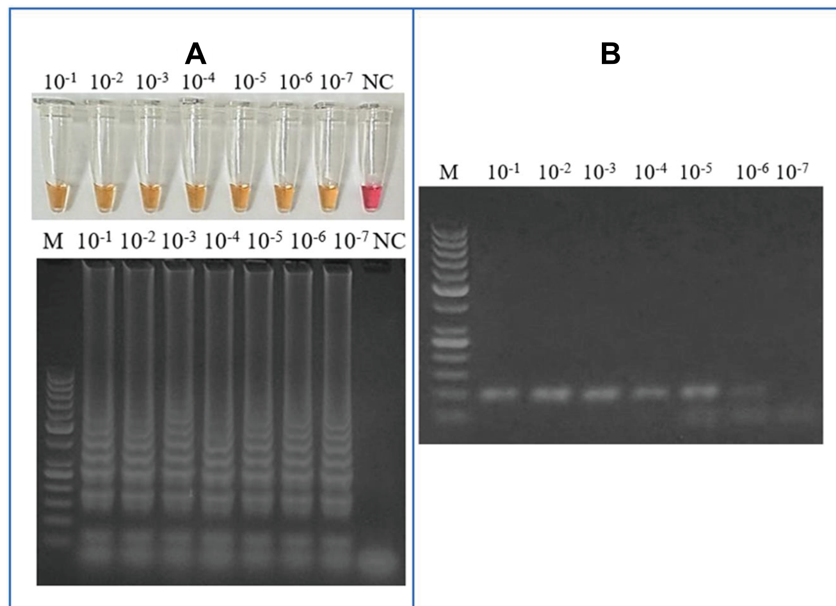

Fig. 2. (Color online) Sensitivity studies of LAMP assay and conventional PCR using the S primer with a 10-fold serial dilution of the template. (A) Sensitivity study of the LAMP assay colorimetric analysis; yellow indicates a positive reaction, pink represents a negative reaction. Lower panel represents the assay results of agarose gel electrophoresis. NC, negative control. (B) The assay using agarose gel electrophoresis for demonstrating the sensitivity of a conventional PCR assay. Lane M indicates 50 bp DNA ladder.

oligomers were synthesized by Oligomer Biyoteknoloji (Ankara, Türkiye) and are listed in Table 1. The 100 µM dried primer pellets were dissolved in sterile nuclease-free deionized water, diluted to a concentration of 10 µM as working primer stocks and stored at –20°C. The detection limit study was conducted by serial dilution of the same sample, on a single day, for 3 distinct primer sets mentioned above to achieve optimum results and to select the most suitable LAMP primer set, as shown in Fig.1.

LAMP assay optimization: Another significant factor influencing the specificity and sensitivity of the LAMP test was the buffer conditions. The concentrations of primers, dNTPs, and magnesium ions were adjusted to ensure efficiency and effectiveness of the LAMP test. The LAMP assay was conducted in a 25 µL reaction

Table 2. Results of the limit of detection (LOD) in the HBV LAMP assay

Serial dilutions of HBV DNA	DNA concentration (pg/μL)	RT-PCR Cq value	Detection rate[1] of LAMP assay (%)
10^{-1}	100	13.76	100
10^{-2}	10	16.78	100
10^{-3}	1	20.27	100
10^{-4}	0.1	24.09	100
10^{-5}	0.01	27.69	100
10^{-6}	0.001	31.03	98
10^{-7}	0.0001	34.46	95

[1]: The detection rate was determined by repeated tests in triplicate for each sample.
HBV, hepatitis B virus; LAMP, loop-mediated isothermal amplification; RT-PCR, real-time polymerase chain reaction; Cq, quantification cycle.

mixture consisting of 12.5 μL colorimetric LAMP 2× Master Mix (New England Biolabs, Ipswich, MA, USA), 0.4 μM each of the F3 and B3 outer primers, 1.6 μM each of the FIP and BIP inner primers, 0.8 μM each of the LF and LB loop primers, 1 μL DNA template, and the remaining volume was filled with ddH_2O to reach 25 μL. Negative controls were prepared using sterile water.

The LAMP assay was performed using a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). To optimize the reaction conditions, the following parameters were varied: the ratio of inner to outer primers was adjusted from 1:4 to 2:1, amplification temperatures ranging from 60°C to 70°C were tested, and different time gradients (30, 35, 45, 50, and 60 min) were examined. Each reaction was performed in triplicate, and the amplified products were visually detected with the naked eye.

Positive results appeared as yellow, negative results appeared as pink-fuchsia, and the products were separated using 2.5% agarose gel electrophoresis, as shown in Fig. 2A.

Specificty and sensitivity of LAMP assay: To validate the cross-reactivity of the LAMP assay, nucleic acid samples from HIV1 (ATCC VR-3245SD), HCV (ATCC VR-3233SD), *E. coli* (ATCC 25922), *Salmonella enterica* (ATCC 14028), *Legionella pneumophila* (ATCC 33152), and *Mycobacterium tuberculosis* (ATCC 27294) were used (data not shown). Additionally, HBV-positive plasma DNA samples and non-infected plasma DNA samples were included for specificity analysis. The sensitivity of the LAMP assay's detection limit was assessed by performing 10-fold serial dilutions of purified clinical HBV DNA, ranging from 1,000 pg/μL to 0.0001 pg/μL, which corresponds to 10^7 to 10 copies/μL. The reactions were conducted at 62°C for various time durations (30, 35, 45, 50, and 60 min). The results were verified by comparing the outcomes of thecolorimetric LAMP and conventional PCR tests (Fig. 2B). Positive reactions in both assays were detected either by agarose gel electrophoresis or by a visual color change.

RESULTS

The Primer selection and optimization: The performance of the three primer sets targeting different regions (S, C, and X) was tested using the HBV DNA dilution series (1,000, 100, and 10 pg/μL). Colorimetric LAMP reactions were performed at 65°C for 60 min following the manufacturer's recommendations (New England Biolabs). The S primer set had a limit of detection (LOD) of 100 pg/μL, while the X primer set had a LOD of 1,000 pg/μL. No amplification was observed with the C primer set (Fig. 1A). The S primer set was selected for further analysis based on evaluation of the LAMP reaction results.

A temperature gradient study comprising the range of 60–70°C was carried out to identify the optimum operating temperature range of the S primer. By visually inspecting the color change in the reaction tubes throughout a temperature range of 60–70°C, the best reaction temperature for the HBV LAMP test was determined. A ladder-like pattern of LAMP products was obtained by agarose gel electrophoresis. Brighter colorimetric results were obtained at 62°C for 60 min (Fig. 1B).

The LAMP assay was performed at the predetermined optimal reaction temperature of 62°C for various durations, including 30, 35, 45, and 60 min. The results demonstrated that a reaction time of 45 min yielded the same detection as the recommended 60-min reaction time at 62°C. The detection limits did not differ significantly between the two reaction times. The optimal reaction conditions for the LAMP assay were determined to be 62°C for 45 min (data not shown).

The analysis of primer specificity and sensitivity: Almost all HBV-positive samples were positive in the LAMP specificity analysis, whereas all HBV-negative samples were deemed negative. These results indicate the ability of the assay to accurately differentiate between positive and negative cells. Additionally, no cross-reactivity was observed for HIV1, HCV, *E. coli*, *S. enteritidis*, *L. pneumophila*, or *M. tuberculosis* (data not shown).

Performance of colorimetric LAMP assay: The LOD of the LAMP assay was determined by analyzing three replicates of serially diluted 100 pg/μL HBV DNA samples (Table 2). Distinct color changes were observed for templates ranging from 10^6 to 10 copies/μL. However, no color change was observed for samples with less than 10 copies/μL and the negative control (Fig. 2A). As a result, the colorimetric detection sensitivity was determined to be 10 copies/μL (0.0001

Table 3. Comparison between colorimetric LAMP results and Cq values of RT-PCR or copy numbers

Sample ID	Colorimetric LAMP result	RT-PCR Cq value[1)]	Copy number[1)] (IU/mL)	Sample ID	Colorimetric LAMP result	RT-PCR Cq value[1)]	Copy number[1)] (IU/mL)
HB1	P	27.33	11,750	HB51	N	N	-
HB2	P	29.00	4,702	HB52	P	26.29	39,480
HB3	P	26.27	40,130	HB53	P	19.41	7,829,000
HB4	P	11.60	50,000,000	HB54	N	31.00	1,506
HB5	P	16.41	24,525,886	HB55	P	24.43	8,355
HB6	P	16.09	43,214	HB56	P	28.31	519
HB7	P	13.34	685,957	HB57	P	34.00	7,443
HB8	P	18.20	11,325	HB58	P	30.00	735
HB9	P	24.29	184,900	HB59	P	31.48	20,850
HB10	P	20.02	4,712,000	HB60	P	27.13	236
HB11	P	17.34	1,564,000	HB61	N	N	-
HB12	N	N	-	HB62	N	N	-
HB13	P	29.00	4,702	HB63	N	N	-
HB14	P	21.48	7,462,000	HB64	N	N	-
HB15	N	N	-	HB65	P	33.00	5,000,000
HB16	N	N	-	HB66	P	16.00	6,215
HB17	N	N	-	HB67	P	28.00	153
HB18	N	N	-	HB68	P	33.01	28,380
HB19	P	26.00	9,875	HB69	P	28.80	8,355
HB20	P	9.00	500,000,000	HB70	P	22.80	9,305,000
HB21	P	20.64	20,548	HB71	N	30.00	2,203
HB22	P	17.52	1,088,000	HB72	P	33.40	1,016
HB23	N	N	-	HB73	N	30.44	1,624
HB24	P	11.00	500,000,000	HB74	N	30.00	1,443
HB25	P	22.15	235,004	HB75	P	23.94	240,400
HB26	P	23.23	3,265,810	HB76	P	19.74	6,106,000
HB27	P	29.84	1,587	HB77	N	30.41	2,305
HB28	P	25.66	37,590	HB78	N	34.00	80
HB29	P	22.62	666,600	HB79	P	27.79	652,085
HB30	N	N	-	HB80	P	24.00	79,040
HB31	N	N	-	HB81	P	25.55	69,800
HB32	N	N	-	HB82	P	27.30	17,210
HB33	N	N	-	HB83	P	27.75	12,880
HB34	N	N	-	HB84	P	12.00	18,596,420
HB35	N	N	-	HB85	P	11.00	50,000,000
HB36	P	28.12	9,731	HB86	P	11.00	50,000,000
HB37	P	21.88	1,174,000	HB87	P	16.99	95,870
HB38	P	21.34	1,776,000	HB88	P	27.18	5,485
HB39	P	12.00	500,000,000	HB89	P	15.00	851,287
HB40	N	N	-	HB90	P	13.01	536,874
HB41	N	N	-	HB91	P	11.41	23,584,725
HB42	P	14.00	500,000,000	HB92	P	28.09	11,025
HB43	P	26.22	41,870	HB93	P	26.27	69,584
HB44	N	N	-	HB94	P	24.99	68,574
HB45	P	25.68	310	HB95	P	24.56	884,527
HB46	P	16.00	63,380	HB96	P	25.76	32,568
HB47	N	N	-	HB97	P	14.00	20,554,750
HB48	N	N	-	HB98	P	28.50	55,628
HB49	P	15.30	50,000,000	HB99	P	29.33	2,587
HB50	P	16.53	13,890,000				

[1)]: The Cq values and copy numbers were obtained using a Bosphore HBV Real-Time PCR kit which detects HBV genotypes A to H. LAMP, loop-mediated isothermal amplification; Cq, quantification cycle; RT-PCR, real-time polymerase chain reaction; P, positive; N, negative.

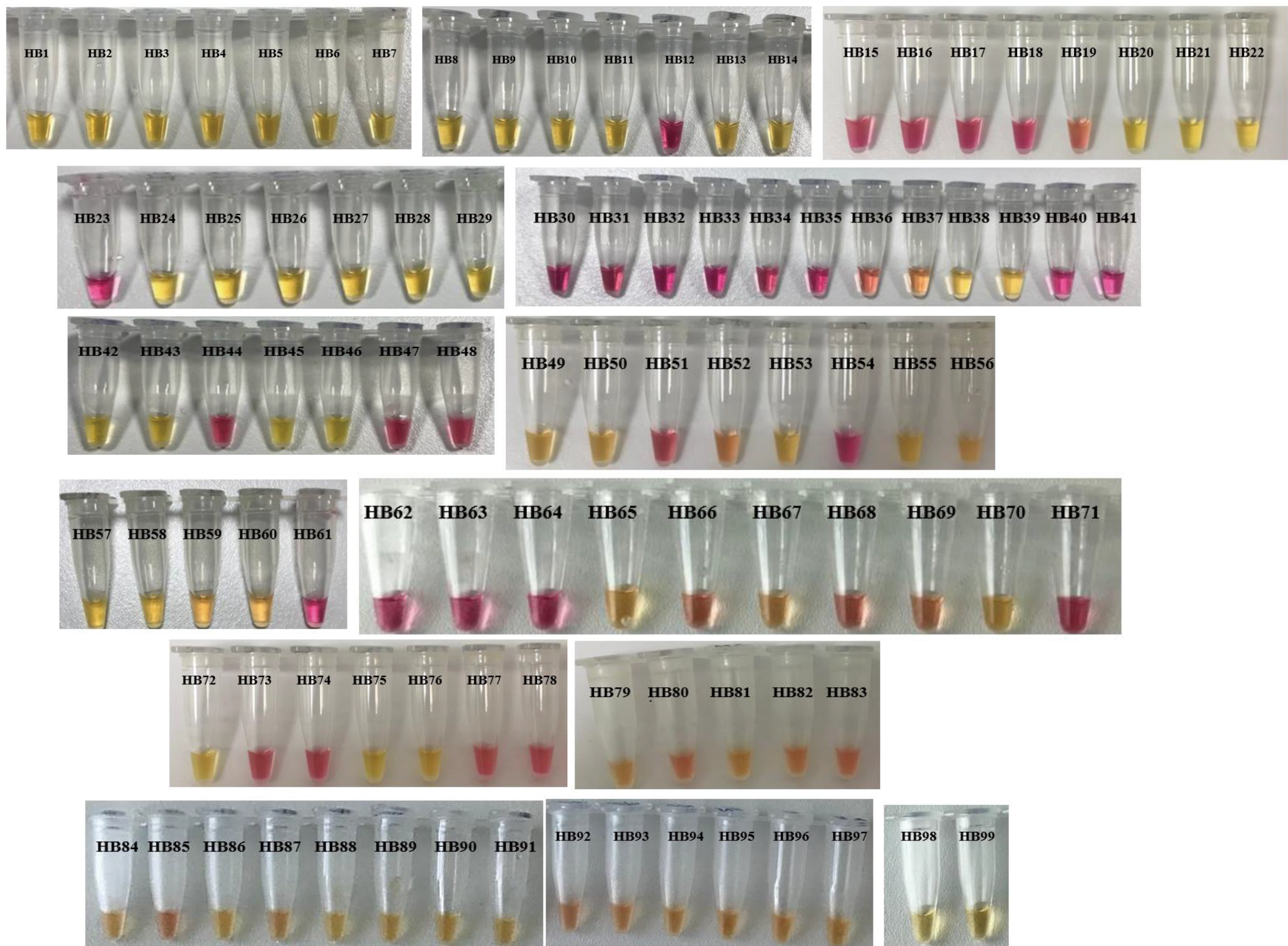

Fig.3. (Color online) Colorimetric evaluation of 99 samples with naked eyes by LAMP analysis.

pg/µL HBV genomic DNA).

The clinical evaluation of the samples included 77 positive plasma specimens. The color change of the LAMP products was compared with the Cq values and copy numbers obtained from the Bosphore HBV detection kit (Anatolia Geneworks), as shown in Table 3. Positive samples exhibited Cq values ranging from 11 to 35. Comparative analysis of the Cq values between real-time PCR and LAMP assays revealed that clinical samples with Cq values below 30 exhibited a concordance rate of 92%, whereas samples with Cq values over 30 demonstrated 81% concordance rate. Both assays were performed on the same day to ensure consistent sample conditions. Table 4 provides an overall result obtained from both assays. After LAMP analysis of the 99 samples, the positive and negative tubes were evaluated colorimetrically with the naked eye (Fig. 3).

DISCUSSION

In recent years, LAMP detection has gained attention as it provides results in a shorter time as compared to real-time PCR and can be performed with a simple heater or water bath. Assay sensitivity was increased using six different specific primers, preventing false-positive and nonspecific amplification results. LAMP has been used to detect pathogens such as bacteria and viruses in samples with low LODs (14). In future, LAMP will be one of the most important molecular techniques for the detection of infectious agents in the field, and to provide potential point-of-care testing (POCT) (15). In this study, we established a naked-eye visual LAMP assay to detect HBV in blood samples. Primers were designed to target three HBV-conserved regions, and our results showed that the S-LAMP primer set was the best to evaluate the LAMP assay. The best primer region optimized in the LAMP assay was confirmed by a previous study which showed that the S gene is the most conserved region, with 96% similarity among all HBV genotypes 1 (12). In other LAMP assays, researchers have designed LAMP primers based on the S gene, which shows that this region is optimal for primer design (16,17).

In our study, the LAMP assay detected 71 of 77 HBV-positive plasma samples with 92.20% sensitivity and healthy plasma samples (n = 22) showed no amplification. The sensitivity of our optimized LAMP primers was higher than the previous reports of 92%, 91%, and 63% respectively (18,19,20). Several studies have shown that HBV LAMP reaction mixtures can be incubated at constant temperature for 1 h (16,21). A recent study used real-time turbidimetric LAMP to identify HBV DNA from eight HBV genotypes. The

Table 4. Overal sensitivity of hepatitis B virus (HBV) DNA detection by RT-PCR and LAMP assays

RT-PCR		LAMP positive (*n*)	Sensitivity (%)[2)]
Copy no. (IU/mL)[1)]	Positive (*n*)		
Greater than 2,000	30	30	100
Between 100 and 2,000	38	36	94
Less than 50	8	5	62.5
Total	76	71	93.4

1): Copy no. (IU/mL) was determined by a Bosphore HBV Quantification Kit.
2): LAMP positive/RT-PCR positive.
RT-PCR, real-time polymerase chain reaction; LAMP, loop-mediated isothermal amplification.

Table 5. Details on selected HBV LAMP studies that were published between 2008 and 2021

Reference	Year	Comparison method	LAMP method	Primer Target Region	LAMP reaction mix	Time (min)	Sample	LOD	Sensitivity (%)
This study		RT- PCR	Colorimetric LAMP	Surface region	NEB[1)]	45	Plasma	10 copies/μL	92
Vanhomwegen et al. [22]	2021	PCR	Real-time Turbidimetric LAMP	Pan-genotypic primers	Loopamp[2)]	60	Plasma	40-400IU/mL (among 8 genotypes)	91.1
Chen et al. [23]	2019	PCR	RTF- LAMP	Surface region	In-house	60	Plasma	10 copies/reaction	84
Quoc et al. [20]	2018	RT-PCR	LAMP analyzed by melting curve	Not mentioned	Optigene[3)]	30	Blood	2.218 pg/μL	63
Nyan et al. [18]	2014	Procleix Ultrio Plus Assay	Colorimetric LAMP	Surface region	In-house	60	Plasma	10 IU/reaction	92
Izadi et al. [15]	2012	PCR	Colorimetric LAMP	Surface region	In-house	60	Plasma	Not mentioned	86
Cai et al. [17]	2011	RT-PCR	Colorimetric LAMP	Surface region	In-house	60	Serum	323 copies/mL (for genotype B), 515 copies/mL (for genotype C)	50.5 (genotype B), 36 (genotype C)
Moslemi et al. [16]	2009	RT-PCR (COBAS)	Colorimetric LAMP	Surface region	In-house	60	Serum	Not mentioned	97
Cai et al. [21]	2008	PCR	RTF-LAMP	Core Region	In-house	60	Blood	210 copies/mL	84

1): WarmStart® Colorimetric LAMP 2× Master Mix.
2): Loopamp DNA Amplification reagent LMP207.
3): OptiGene Isothermal Mastermix.
HBV, hepatitis B virus; LAMP, loop-mediated isothermal amplification; LOD, limit of detection; RT-PCR, real-time polymerase chain reaction; RTF-LAMP, real-time fluorescence LAMP.

reaction time required to achieve a detection limit of 40–400 IU/mL was approximately 60 min (22). By comparison, the reaction time was reduced to 45 min. However, a sensitive fluorescence-based HBV LAMP assay was reported with a significant amplification time (30 min) and melting curve analysis step but thedetection limit of serially diluted HBV DNA was 2.218 pg/μL (20).

In contrast, the HBV DNA detection limit in our assay was 0.0001 pg/μL which corresponds to 10 copies per reaction. It was reported an HBV LAMP assay with 10 copies/μL LOD and overall sensitivity of 84% (107 of 127 samples). Although our detection limit was the same as that used in this study, only heat-extracted DNA without a purification step was used (23). Details of the HBV LAMP studies are presented in Table 5.

In addition, our results showed that time-consuming gel electrophoresis was not required at the end of the LAMP reaction. The results were visualized with the naked eye. The colorimetric LAMP assay has the potential to be used as a screening test in areas with insufficient laboratory environment and limited

resources, and in situations requiring urgent intervention to ensure safety against blood-borne infections. Our assay is suitable for on-site detection.

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Conflict of interest None to declare.

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