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Closed tube loop-mediated isothermal amplification assay for rapid detection of hepatitis B virus in human blood

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Abstract

Recently, many studies have demonstrated the significant advantages of loop-mediated isothermal amplification (LAMP) based methods over serological tests and PCR for rapid detection of microbial pathogens. Here, a rapid LAMP assay was developed to detect the hepatitis B virus (HBV) from DNA, and particularly, blood samples from infected patients using a commercially available master mix and portable real-time fluorometer. The final optimized fluorescence-based LAMP assay provided significant amplification time of less than 15 minutes compared with over 1 hour for PCR and an opened tube LAMP system described previously. Results indicated that fluorescence-based LAMP assay was more sensitive than PCR as a rapid, sensitive, efficient, and highly reliable approach for rapid detection of HBV.

Keywords: Medicine, Evidence-based medicine, Infectious disease, Pathology, Biochemistry, Bioengineering, Microbiology, Virology

1. Introduction

Hepatitis B virus (HBV) is considered to be one of the major causes of acute hepatitis, chronic hepatitis, and hepatocellular carcinoma (HCC). According to the World Health Organization, 2 billion people have been infected globally and 686,000 die as a result of hepatitis B every year (WHO, 2016). Based on the similarity of genomic sequences, HBV can be divided into 8 different genotypes denoted from A to H following the order of discovery, with a difference of 8% over the entire genome (Okamoto et al., 1988; Norder et al., 1994; Kramvis et al., 2005). Genotype distribution also depends on geography (Okamoto et al., 1988; Norder et al., 1993; Sunbul, 2014). Limited development of HBV therapy worldwide has contrasted with considerable advances in the epidemiology of the virus. Thus, finding and accurately detecting HBV genotypes is vital to reduce global HBV infection rates and boost both prevention and treatment.

Various techniques including enzyme-linked immunosorbent assay (ELISA) and real-time PCR have been considered for detecting HBV surface antigens, antibodies and DNA-HBV (Abe et al., 1999; Usuda et al., 1999; Yeh et al., 2004; Wu et al., 2008). However, such methods are normally both labor and time intensive and can only be undertaken in well-equipped labs (Kao, 2008; Caliendo et al., 2011; Wang et al., 2012). Therefore, an alternative approach known as loop-mediated isothermal amplification (LAMP) has been intensively studied and developed worldwide as a rapid, simple and low-cost diagnostic tool. The LAMP assay can detect clinical microbial pathogens less than half an hour including sample treatment and is appropriate for poor and developing countries. Development of LAMP based detection of all the major HBV genotypes in peripheral blood has been previously reported, with amplification results of plasma and HBV DNA samples evaluated based on multiple inverted repeats of target amplicons (ladder-like banding pattern) and colorimetric assays (Nyan et al., 2014; Moslemi et al., 2009). This LAMP technique is considered as an opened tube system with the possibility of causing false positive results. It is also time-consuming due to cross DNA contamination (multiple cap opening) and running agarose gel for electrophoresis, indicating some disadvantages for portable detection of HBV at the point of care. Using commercially available master mix and a real-time fluorometer such as Genie II (OptiGene, UK), various studies using portable devices to avoid the obstacles of an opened LAMP system reported rapid detection of human viral pathogens, measuring the amplification and melting curves of target amplicons in real-time (Mahony et al., 2013; Randhawa et al., 2013). Here, a modified sensitive LAMP assay was developed for the rapid detection of hepatitis B virus (HBV) using the Genie III system (OptiGene, UK) and compared with LAMP testing for HBV DNA and heat-treated donor blood samples to provide diagnosis results in less than 30 minutes.

2. Material and methods

2.1. Specimens and DNA preparation

A total of 30 healthy and HBV-infected blood samples were obtained from the medical clinic/hospital in Ho Chi Minh City and clinically validated by real-time PCR. Written informed consent was attained from all patients and the Human Research Ethics Committee, Oncology Hospital (#08/BVUB-HDDD) approved the research protocol. DNA extraction was performed using a DNA extraction kit (ZR Viral DNA Kit™, USA) according to the manufacturer's protocol. DNA was extracted from 200 μL of plasma standards and eluted in 800 μL of ZR Viral DNA buffer. DNA was measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA), aliquoted and stored at $-20\text{ }^{\circ}\text{C}$ until needed for testing. The DNA concentration of HBV positive sample for PCR assays and serial dilutions was 22.18 ng/ μL .

2.2. Heat treatment of donor blood and plasma samples as substrate

Substrate for HBV-LAMP was prepared by heat treatment of donor blood and plasma samples without DNA extraction (Nyan et al., 2014). For plasma specimens, 25 μL of samples were diluted 2-fold with nuclease-free water. The mixture was then centrifuged at $12,000\times g$ for 3 minutes. The supernatant was reserved and 3–10 μL were used in the isothermal amplification reactions. Heat treatment for the plasma samples was applied in a similar manner to the blood samples.

2.3. PCR reaction and electrophoresis

PCR reaction was carried out in a total of 25 μL reaction mixture containing 12.5 μL Go Taq Master Mix (Promega, USA), 1 μmol of each forward and reverse primers, and 2 μL of template DNA. PCR cycling parameter incubation cycles consisted of denaturation at $95\text{ }^{\circ}\text{C}$ for 30 s, annealing at $53\text{ }^{\circ}\text{C}$ for 60 s, and extension at $74\text{ }^{\circ}\text{C}$ for 30 s, repeated for 35 cycles. The PCR amplified product was electrophoresed on 1% agarose gel ($0.5\times\text{TBE}$).

2.4. LAMP reaction and amplification condition

The sequence of primers in Table 1 was used as described previously (Nyan et al., 2014). LAMP reaction was carried out using a total of 25 μL of the reaction mixture containing 0.5 μL each of HBU-F3 and HBU-B3 primers (25 pmol/ μL) equivalent to 5 μM final concentration, 2.0 μL each of HBU-FIP and HBU-BIP primers (25 pmol/ μL) equivalent to 20 μM final concentration, and 1.0 μL each of HBU-LF and HBU-LB primers (25 pmol/ μL) equivalent to 10 μM final concentration. Fifteen microliters of Isothermal Master Mix (OptiGene, UK) and 3 μL DNA was added as a

Table 1. Sequences of LAMP and PCR primers (Nyan et al., 2014; Gunter et al., 1998).

Primer	Primer sequence	Reference
HBU-F3	5' TCCTCACAATACCGCAGAGT 3'	Nyan et al., 2014
HBU-B3	5' GCAGCAGGATGAAGAGGAAT 3'	
HBU-FIP	5' GTTGGGGACTGCGAATTTTGGCT TTTTAGACTCGTGGTGGACTTCT 3'	
HBU-BIP	5' TCACTCACCAACCTCCTGTCCTT TTTAAAACGCCGACACACAT 3'	
HBU-LF	5' GGTGATCCCCCTAGAAAATTGAG 5'	
HBU-LB	5' AATTGTCCTGGTTATCGCTGG 3'	
ATL-F	5' CCCTGATGAAGAACTGTATCTC 3'	Gunter et al., 1998
ATL-R	5' GAAATTACACACATAGGTGGCACT 3'	

template. The LAMP assay was run at 63 °C for 30 minutes with a melting curve analysis step (annealing curve 98°–80 °C ramping at 0.1 per min) in a portable real-time fluorometer like the Genie III (OptiGene, UK). A no template (water) control and DNA extracted from HBV genotypes were used as negative and positive controls, respectively.

2.5. Detection of HBV from DNA, plasma and blood samples using Genie III

Detection of hepatitis B virus (HBV) was performed by LAMP reactions as described above with HBV-DNA templates extracted from plasma, heat-treated plasma and blood samples. Positive and negative results of samples were evaluated based on amplification plots, time to positivity (less than 15 minutes) and melting curves of target amplicons analyzed automatically by the Genie III system (OptiGene, UK). To check the reliability of the Genie III system, LAMP reaction products were run on 1% agarose gel electrophoresis in 1 × TBE buffer at 100 V for 50–55 minutes, followed by staining with GelRed DNA dye (TBR, Vietnam) and visualized with a UV transilluminator at 302 nm.

2.6. Analytical sensitivity of HBV assays with DNA, heat-treated plasma and blood samples

Analytical sensitivity was evaluated by testing 10-fold serial dilutions of HBV DNA in LAMP reactions. Assay detection limit was determined by analyzing 4–7 replicates of serially diluted HBV DNA. All LAMP reactions were run using the Genie III system (OptiGene, UK). PCR reactions were also conducted with HBU-F3 and HBU-B3 (Table 1) to compare with the LAMP technique, followed by sequencing for evaluation. A pair of ATL1 primers was also used as internal amplification

control in PCR assays to guard against poor quality DNA and PCR inhibition, nucleic acid extraction failure or template degradation (Table 1).

3. Results

3.1. Detection of HBV using different templates for PCR and LAMP assays

Templates used for PCR reaction in this assay included HBV DNA extracted from plasma, heat-treated plasma, and blood samples of HBV-infected patients. Electrophoresis analysis of amplified amplicons showed successful detection of HBV DNA extracted from plasma (263 bp) with a pair of universal HBV primers (F3 and B3) (Table 1). No amplified PCR products were observed for heat-treated plasma and blood templates (Fig. 1A). Results of BLAST running indicated that the sequence of PCR-amplified product from DNA template was HBV-DNA (data not shown).

To compare detectable capacity with PCR assay, LAMP products were amplified with a universal set of LAMP primers at 63 °C for 30 mins using the Genie III system, run on 1% agarose gel with 1 × TBE and UV visualized with GelRed dye. The appearance of a unique laddering pattern of amplicons observed for all three different templates revealed that they were positive for HBV DNA, resulting in a detectable capacity of LAMP assays with templates that could not be amplified by conventional approaches due to the inhibition of chemicals in blood or plasma specimens (Fig. 1B).

3.2. Limits of HBV-DNA detection between PCR and LAMP assays

To compare the limits of HBV detection by PCR and LAMP assays, at least 7 replicates of serially diluted HBV DNA (10^{-1} – 10^{-7}) extracted from HBV-infected

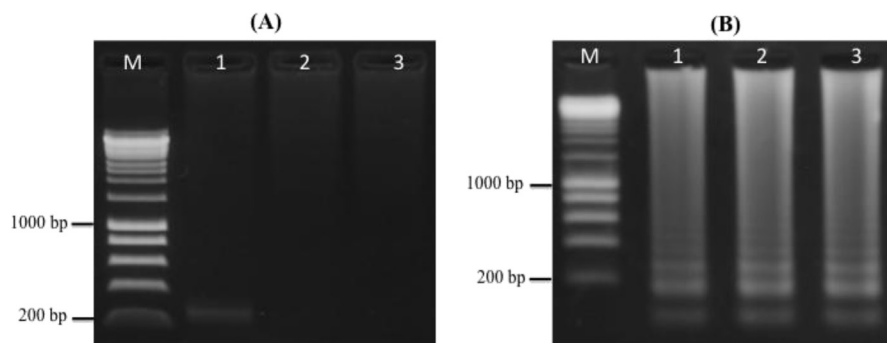


Fig. 1. Gel electrophoresis of PCR and LAMP assays with different templates. (A) PCR reactions with HBV-DNA (lane 1), heat-treated plasma specimen (lane 2), heat-treated blood sample (lane 3). (B) LAMP assays with HBV-DNA (lane 1), heat-treated plasma specimen (lane 2), heat-treated blood sample (lane 3).

plasma sample were assayed and analyzed. Concentrations of HBV-DNA corresponding to above serial dilutions were from 2218 pg/ μ L to 0.002218 pg/ μ L, respectively. Results of LAMP assays indicated a 100% detection rate from 10^{-1} to 10^{-7} of HBV DNA molecules per reaction (Table 2). For PCR assays, 100% rates of HBV detection were observed from 10^{-1} to 10^{-4} HBV DNA, whereas 10^{-5} and 10^{-6} – 10^{-7} diluted HBV DNA templates were detected at 57% and 0%, respectively (Table 2).

3.3. Evaluation of HBV LAMP assays with blood samples

The use of both extracted DNA and heat-treated plasma samples for the detection of HBV DNA was described previously (Nyan et al., 2014). Here, experiments were conducted to determine a detectable capacity of HBV with donor blood samples by LAMP assay in the shortest possible time. Aliquots of identical blood samples were also heat-treated (without DNA and plasma extraction) within 3 minutes and directly tested in the LAMP reactions. HBV DNA extraction of these blood samples was conducted to confirm the results of LAMP based assay using polymerase chain reaction (PCR) with universal HBV primers (F3 and B3). Amplicons, amplified with ATL1 primers known as internal amplification control, indicated good quality DNA for PCR and LAMP assays (Fig. S1) (Gunter et al., 1998). Six of the seven blood samples showed amplification plots within 9–13 minutes, revealing positive results for amplified HBV DNA (Fig. 2A). LAMP products were also confirmed by electrophoresis analysis. Results were consistent with amplification plots displayed in the Genie III system (Fig. 2B.) and PCR amplification (Fig. 2C).

3.4. Time of LAMP based detection with heat-treated HBV-infected blood samples

Ten-fold serial dilutions of heat-treated HBV-infected blood samples were tested using LAMP assays with three replicates. Results indicated that assay detection of

Table 2. Environment of HBV DNA detection by PCR and LAMP assays.

Serial diluted HBV-DNA	DNA concentration (pg/ μ L)	PCR			LAMP		
		No. of replicates tested	No. of detections	Detection rate (%)	No. of replicates tested	No. of detections	Detection rate (%)
10^{-1}	2218	7	7	100	7	7	100
10^{-2}	221.8	7	7	100	7	7	100
10^{-3}	22.18	7	7	100	7	7	100
10^{-4}	2.218	7	7	100	7	7	100
10^{-5}	0.2218	7	4	57	7	7	100
10^{-6}	0.02218	7	0	0	7	7	100
10^{-7}	0.002218	7	0	0	7	7	100

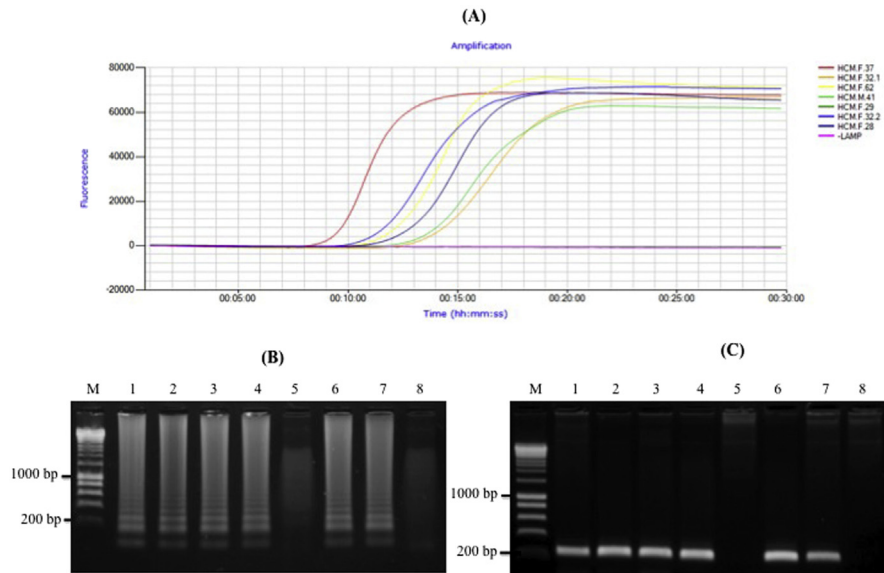


Fig. 2. Detection of Hepatitis B Virus (HBV) from heat-treated blood samples using the Genie III system (OptiGene, UK). (A) Electrophoresis result of LAMP to blood heat-treatment. (B) Amplification plots of the LAMP assays from blood heat-treatment. (C) Electrophoresis results of HBV-DNA from blood samples using PCR method with F3 and B3 primers. Lane M = 1 kp marker; Lanes 1–7 = *HCM.F.37*, *HCM.F.32.1*, *HCM.F.62*, *HCM.M.41*, *HCM.F.29*, *HCM.F.32.2*, *HCM.F.28* DNA-HBV samples; Lane 8 = *Negative LAMP assay*.

HBV DNA appeared after approximately 10 minutes using blood samples directly, compared with less than 14 minutes at 10^{-5} dilution for heat-treated HBV-infected blood samples (Table 3). To evaluate the productivity and diagnostic sensitivity of LAMP assay, donor blood samples ($n = 30$) were tested. Results revealed that LAMP assays detected 19 out of 30 donor blood samples (63%) as HBV positive (Fig. S2) and demonstrated the closed tube HBV-LAMP assay as a rapid and sensitive diagnostic tool for screening blood donors and on-site testing of patient specimens (Table S1).

Table 3. Times for LAMP based detection with heat-treated HBV-infected blood samples.

Serial dilution of heat-treated HBV-infected blood samples	Peak values			Mean of peak values	Standard deviation
	Run 1	Run 2	Run 3		
Positive sample	09:45	10:15	09:45	09:55	0.01
10^{-1}	10:15	11:00	09:15	10:10	0.04
10^{-2}	11:15	11:45	08:45	10:35	0.07
10^{-3}	13:00	13:00	09:00	11:40	0.1
10^{-4}	12:15	13:00	10:15	11:50	0.06
10^{-5}	12:00	18:15	10:45	13:40	0.17

4. Discussion

Advantages of LAMP based HBV detection were described previously as a simple and rapid diagnostic and screening tool that is useful for undeveloped and developing countries with limited resources, and also in other global regions with high HBV prevalence (Nyan et al., 2014; Jain et al., 2012; Stramer et al., 2011). However, although LAMP universal primers for the detection of HBV genotypes (A-F) were successfully designed and showed high detection specificity and accuracy in clinical diagnostics, many reports highlighted major drawbacks of the opened tube LAMP technique, as cross contamination when the lids of the reaction tubes were opened at the end of the reaction for gel electrophoresis, or when adding dye for result visualization (Nyan et al., 2014; Parida et al., 2008; Njiru et al., 2008; Lau et al., 2010; Angamuthu et al., 2011). In addition, running gel electrophoresis at the end of the LAMP reaction requires this step to be conducted in the lab which is time-consuming and, therefore, not suitable for on-site rapid detection.

To surmount such obstacles of an opened tube LAMP system, closed tube LAMP assays using a fluorometer (Genie II/III systems) and commercially available master mix (OptiGene, UK) were developed and validated for rapid detection of HBV in DNA and blood samples. This approach has been demonstrated by few previous studies; the visualization of LAMP reaction via closed tube system decreased cross contamination (Tao et al., 2011; Karthik et al., 2014; Prusty et al., 2016). Furthermore, the LAMP master mix can be used for Genie II/III systems (OptiGene, UK) and also for other mobile/molecular devices such as Smart-DART (Diagenetix, USA), PCR, real-time PCR, and even colorimetric assays (Reddy et al., 2011; Davoodian and Sadeghifard, 2011). The sensitivity and clinical applicability of closed tube LAMP assays for HBV detection were validated and compared with different templates including HBV-DNA, plasma specimens, and blood samples. The HBV LAMP assay detected all these templates, whereas the PCR method detected HBV-DNA but not HBV-infected plasma specimens and blood samples that could be interfered by PCR reaction inhibitors. Sensitivity of the HBV LAMP assay over the PCR method gave the following advantages: (i) the assay did not require well-equipped laboratories, (ii) it reduced time to within 15 minutes for the detection of HBV, without the necessity of HBV DNA or plasma extraction from blood samples, (iii) it was performed on a closed tube LAMP system operated simply, accurately and quickly to reduce cross contamination, and (iv) the amplification plots and melting curves could be easily and directly observed via the Genie III system, with reduced time for generating results and the potential for differentiating genotypes of HBV. These advantages indicated that closed tube HBV LAMP assay is applicable for on-site detection and useful for developing or developed economies with limited resources to conduct affordable, quick and accurate diagnoses for HBV infection. Costs of LAMP and PCR assays were calculated by

Table 4. Relative costs of LAMP and PCR assays.^a

Assay type	No. of tests/run	HBV-DNA extraction kit cost (\$)	Reagent cost/run (\$)	Cost of consumables and primers (\$)	Hands-on-time (h)	Labor cost/run ^b (\$)	Depreciation of equipment (\$)	Total cost/test (\$)
LAMP	8	17.6	16	16	0.5	0.63	8	7.3
Modified LAMP	8	0	16	16	0.5	0.63	8	5.1
PCR	8	17.6	8	16	3	3.75	10	7.0

^a All costs are in US dollars.

^b Based on the basic salary of technicians in Vietnam.

determining reagent component cost, consumables and primer cost, depreciation of equipment and a technologist's salary of \$1.25/hour in Vietnam. Cost of HBV-DNA extraction was calculated using the purchase price of the HBV-DNA extraction kit included in the PCR cost determination. Costs were based on a run size of 8 samples in each assay format. There were no significant differences between costs of PCR and LAMP assay with plasma based HBV-DNA extraction; however, the advantage of LAMP was observed in hands-on-time at 30 minutes compared with 3 hours using PCR assay (Table 4). Specificity and accuracy of primers in LAMP assays were reported by Nyan et al. (2014), and closed tube LAMP assays were developed to detect heat-treated blood samples directly. This approach does not require plasma extraction which reduces the time for sample preparation, running cost at \$5.1/test, and requires a smaller blood sample volume (approximately 20–25 μ l) (Table 4). Healthy donor blood samples ($n = 11$) showed no amplification of HBV DNA, whereas HBV-infected blood samples gave amplification of HBV DNA within 10–15 minutes. LAMP based assays were consistent with clinical validation of these samples using real-time PCR (Table S1).

This approach can, therefore, be used to investigate HBV prevalence rapidly, accurately and economically. Furthermore, the master mix (OptiGene, UK) is adaptable for LAMP assays using various mobile devices including Genie II, Genie III, Smart-DART, PCR, and real-time PCR. Master mix is stable and robust under thermally stressed conditions and applicable for in-field environments and on-site detection of HBV-DNA.

5. Conclusion

The closed tube HBV-LAMP assay reported here could be used directly with blood samples; it is specific with high reliability, low cost, and easy implementation for rapid detection of the hepatitis B virus (HBV). This method is particularly applicable for on-site detection of HBV infection for accurate treatment and prognosis of HBV-infected patients.

Declarations

Author contribution statement

Nguyen Bao Quoc: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Nguyen Doan Nguyen Phuong: Performed the experiments.

Nguyen Ngoc Bao Chau: Analyzed and interpreted the data.

Do Thi Phuong Linh: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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