Simple and visible detection of duck hepatitis B virus in ducks and geese using loop-mediated isothermal amplification

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ABSTRACT In this study, loop-mediated isothermal amplification (**LAMP**) was used to establish a rapid, specific, and visual detection method for duck hepatitis B virus (**DHBV**). The design and synthesis of 4 specific LAMP primers were based on the conserved gene region of the DHBV genome, and the optimum temperature and time of the LAMP reaction were 63°C and 50 min, respectively. The LAMP assay was confirmed to be specific for DHBV detection and had the same sensitivity as the quantitative PCR assay. A visual detection method for rapid determination of results was developed using a color indicator containing phenol red and cresol red. A color change was produced based on a pH change in the reaction system, indicating a positive reaction. For the detection of samples from ducks and geese, the LAMP method has the advantages of simplicity, high sensitivity and specificity, good visibility, and low cost. Moreover, it is more practical and convenient than PCR-related assays for the clinical detection of DHBV.

Key words: DHBV, visible detection, loop-mediated isothermal amplification, simplicity

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INTRODUCTION

Duck hepatitis B virus (**DHBV**) is an avian liver DNA virus belonging to the *Hepadnaviridae* family, which includes human hepatitis B virus (**HBV**) and woodchuck hepatitis virus (Marion et al., 1984; Yugo et al., 2016). The virus particles are spheroidal, with a diameter of 45 to 65 nm and a capsule that is embedded in the lipid bilayer membrane of the virus surface antigen (DHBsAg) and the host cell (Zhang, 2015). The genome of the virus is a double-stranded circular DNA with a single unequal length area, and the length of its long chain or negative chain is fixed at approximately 3021 to 3027 nt (Molnar-Kimber et al., 1984; Li et al., 2016). As another related liver DNA virus, HBV is a worldwide human health problem, and carriers are at risk of developing cirrhosis and hepatocarcinoma. Interestingly, the morphology, genome size, replication mode and relative hepatotropism of DHBV and HBV are similar (Marion et al., 1984). Therefore, ducks infected with DHBV have been used as an animal model to study HBV infection and pathogenesis (Sauerbrei et al., 2012). After infection with DHBV, ducks may sustain pathological changes in the liver, such as infiltration of inflammatory cells, necrosis of liver cells, radiating extension of bile duct hyperplasia, hyperplasia of fibrous tissue, hyper-proliferation, carcinogenesis, and fatty degeneration of the liver (Summers et al., 1978). Furthermore, congenitally infected ducks are at risk of developing hepatoma or secondary amyloidosis due to chronic stimulation of the immune system (Duflot et al., 1995). Unlike DHBV, duck hepatitis A virus (**DHAV**), as the most important pathogen related to hepatitis in ducks, has generated increased concern because DHAV usually causes more malignant disease in ducklings. In contrast, most ducks naturally infected with DHBV display no obvious clinical symptoms but carry persistent viral infections (Narayan et al., 2006). However, DHBV has been reported to not only infect ducks but also to cause some diseases in geese (Funk et al., 2007). The infection rate of DHBV is very high in eastern China based on avian virus surveillance (Qiu et al., 2019). The characteristics of DHBV call for increased attention to related diseases.

Detection methods for DHBV include molecular assays (PCR and other related assays based on PCR) and serological assays (such as enzyme-linked immunosorbent assays) (Liu et al., 2014). At present, PCR is the most commonly used method for the detection of DHBV and is simple and sensitive (Wang et al.,

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Table 1. Primer sets for the loop-mediated isothermal amplification assay.

Primer name	Sequence 5′-3′
DHBV-F3	TGCAATGCG*Y*[1]TTTCCAAGA
DHBV-B3	GAGAAAAGGGCTGAGACCG
DHBV-FIP	CA*R*AGAAATCCTGGGCATCCCC**GAATTC**[2]CTGGAGCCCAAA*Y*CTC*W*CC
DHBV-BIP	TGCTAGTAGCAGCAGGCT*Y*GC-AC*K*CCCATTGGAGCTTTCCT

([1] Italic text indicates degenerate oligonucleotides, Y = C + T, R = G + A, W = A + T, K = G + T; [2]Bold text represents the *Eco*R I restriction site added between the F1c and F2).

2002). However, routine PCR operation has a high equipment demand, and the sophisticated procedures required for product analysis may lead to cross contamination that causes false positive results. Therefore, fluorescence quantitative PCR (**FQ-PCR**) was further used to detect DHBV (Wang et al., 2013). Fluorescence quantitative PCR only partially solves the problem of false positive results and complex operation of conventional PCR technology because it cannot be quantified and has easily contaminable products. Additionally, FQ-PCR requires more expensive equipment and sophisticated operator skills. To solve these problems of molecular detection, Notomi (Notomi et al., 2000) developed a new nucleic acid amplification method called loop-mediated isothermal amplification (**LAMP**) that can amplify nucleic acids in a short time (usually 1 h or less) under isothermal conditions (60 to 65°C). At present, the technology has been widely used for the identification of bacteria, viruses, and parasites and even for molecular pathology analysis (Li et al., 2018; Salim et al., 2018; Yang et al., 2018). In this paper, we first developed a LAMP assay for DHBV diagnosis, and the DHBV-LAMP assay can be visualized using phenol red and cresol red as color indicators (Tanner et al., 2015).

MATERIALS AND METHODS

Viruses and Clinical Samples

The livers of dead ducks and geese suspected to be infected with DHBV were washed with 1 × PBS and immersed in liquid nitrogen for grinding. A 20% suspension was prepared by adding PBS and freezing and thawing repeatedly three times. Serum samples from duck and goose farms were isolated from the jugular vein blood using aseptic techniques. All of the tissue and serum samples were centrifuged at 5,000 rpm for 10 min. Then, 0.2 mL of the supernatant was removed for nucleic acid extraction, and the remaining supernatant was stored at −80°C.

Extraction of DNA and RNA

Total DNA and RNA was extracted with an extraction kit (EasyPure Viral DNA/RNA Kit; TransGen Biotechnology, Inc., Beijing, China) according to the manufacturer's instructions. The purity and concentration of the DNA/RNA samples were determined by biological spectrophotometry, and the samples were stored at −20°C until use.

Primer Design

Primers used for the LAMP assay were designed using the online primer software Explorer V5 (http://primerexplorer.jp/e/v5_manual/index.html) and based on the conserved regions of the S gene of the DHBV virus genus, which are distinct from genes of other duck pathogens. Four primers were designed, including an external primer pair (F3 and B3) and an internal primer pair (FIP and BIP), and a primer pair used for PCR amplification of the DHBV gene was also designed. The oligonucleotide sequences of the primers are shown in Table 1.

Optimization of LAMP Reaction Conditions

Each LAMP reaction included each outer primer (F3 and B3, 0.2 μM), each inner primer (FIP and BIP, 1.6 μM), 1.2 mM dNTP mixture, 0.6 M betaine, 6 mM $MgSO_4$, 8 U Bst DNA polymerase (New England Biolabs, Hitchin, UK), 10 mM $(NH4)_2SO_4$, 50 mM KCl, 0.1% v/v Tween-20, 1 μL of template DNA, and 1 μL of dye (consisting of 0.025 mM phenol red and 0.08 mM cresol red). In addition, ddH_2O was added to bring each reaction to a total volume of 25 μL. Then, 25 μL of mineral oil was added to prevent volatilization of the LAMP products. To obtain the optimal system and conditions for LAMP amplification, gradient optimization was performed in a water bath at reaction temperatures of 61, 62, 63, 64, and 65°C. The DHBV-LAMP assay was optimized using a time gradient of 30 min, 40 min, 50 min and 60 min. The reaction product was observed by electrophoresis on a 2% agarose gel, and the optimal reaction time and temperature were determined according to the clarity and brightness of the obtained bands.

SYBR Green Dye-Based Quantitative PCR for Comparative Analysis

SYBR green quantitative PCR (**qPCR**) was performed for DHBV detection using a CFX96 Touch detection system (BIO-RAD, Hercules, CA, USA) with a qPCR mixture containing 1 μL SYBR PCR buffer, 0.2 mM (each) dATP, dCTP, and dGTP, 0.5 mM dUTP,

and the specific primer; the reaction procedure was performed as described previously (Wang et al., 2002).

Visual Observation and Sensitivity of the LAMP Assay

A DHBV plasmid (**pDHBV**) used as a standard DNA template was constructed by ligation of a genome sequence from a Chinese DHBV strain in pMD 18-T (TaKaRa Biotech Corporation, Dalian, China). The limits of detection of the LAMP and qPCR assays were evaluated by serial 10-fold dilutions of the pDHBV from 1.09×10 to 1.09×10^7 copies/μL, which were performed 3 times to properly assess the detection limits of the 2 methods. Furthermore, a color change was observed in the LAMP product due to the addition of a colored dye; yellow indicated a positive result, and purple-red indicated a negative result.

Specificity of LAMP Products

DNA extracted from goose parvovirus (**GPV**), duck enteritis virus (**DEV**), duck adenovirus type 3 (**DAdV-3**) and DHBV and cDNA converted from RNA of Newcastle disease virus (**NDV**), duck hepatitis A virus subtype 1 (**DHAV-1**), duck hepatitis A virus subtype 3 (**DHAV-3**), duck reovirus (**DRV**) and duck Tembusu virus (**DTMUV**) was detected by the LAMP assay to evaluate the specificity of the DHBV primers.

Additionally, amplified LAMP products were visualized by agarose gel electrophoresis, which cannot be used to determine whether the amplified fragment is the target fragment. Therefore, appropriate enzymes should be selected to identify the amplified products. The amplified fragments in this experiment contained *Eco*R I restriction endonuclease sites; therefore, *Eco*R I restriction endonucleases were used to produce specific nucleic acid fragments of LAMP-amplified products by enzyme digestion.

Detection of DHBV in Clinical Samples

To evaluate the diagnostic accuracy of the DHBV-LAMP assay, 11 previously isolated DHBV strains, 108 liver samples of dead ducks or geese and 164 serum samples from different farms located in Henan, Anhui and Hubei provinces in central China were detected by LAMP and qPCR assays within the optimized conditions, and the positive detection rates using the 2 methods were calculated and compared.

Ethics Statement

The serum collection and autopsy protocols for dead birds were conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Approval for the use of animals during this study was obtained from the South China Agricultural University Committee for Animal Experiments (approval ID: SYXK-2014-0136).

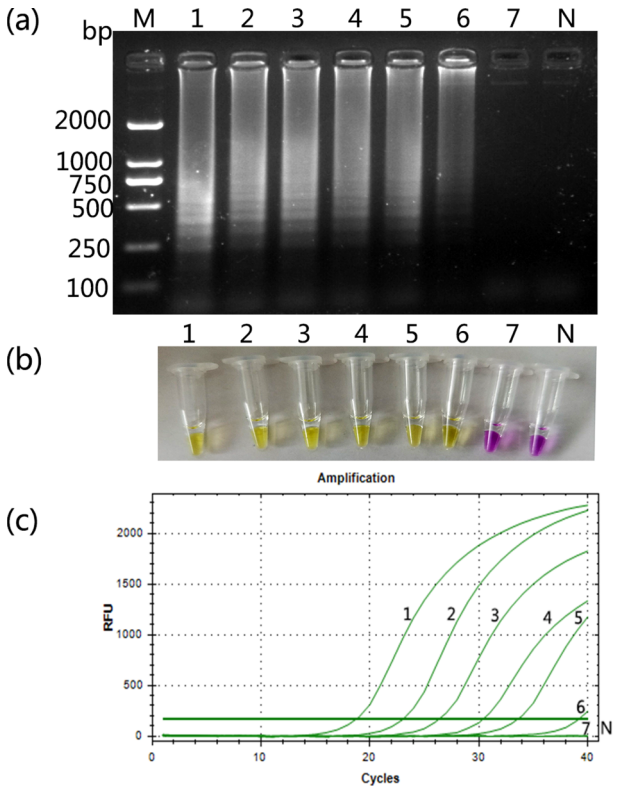

Figure 1. Sensitivity of the loop-mediated isothermal amplification (LAMP) and quantitative PCR assays. Lanes 1 to 7: 10-fold serial dilution of the template (1.09×10^7 to 1.09×10 copies); M: molecular size marker DL2000; N: negative control. (a) Agarose gel electrophoresis demonstrating the sensitivity of the LAMP assay using 10-fold serial dilutions of the standard plasmid. (b) Colorimetric analysis using the color indicator demonstrating the sensitivity of the LAMP assay; yellow represents a positive reaction, and purple represents a negative reaction. (c) Amplification plot demonstrating the sensitivity of the PCR assay using 10-fold serial dilutions of viral DNA.

RESULTS

Optimized Reaction Temperature and Time for the LAMP Assay

The temperature and reaction duration of the LAMP reaction are the 2 parameters that are critical for a successful LAMP reaction for specific primers and target genes. According to the electrophoresis diagrams, the 63°C reaction yielded the brightest ladder-like bands, and the brightness of the bands peaked at 50 min. Therefore, the optimal conditions for the LAMP assay were determined to be 63°C for 50 min.

Sensitivity Test Results for the LAMP Method

At the end of the reactions, 5 μL of the LAMP and PCR reaction products were subjected to agarose gel electrophoresis, and the remaining volume was examined by the naked eye (Figure 1). The DNA template

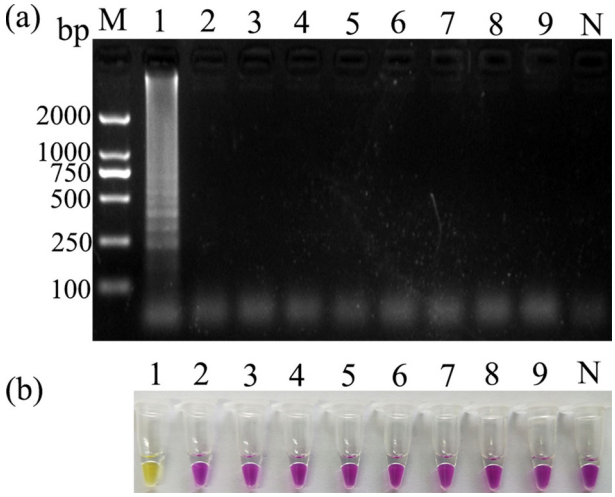

Figure 2. (a) Loop-mediated isothermal amplification (LAMP)-specific products after 2% gel electrophoresis. Lane 1: duck hepatitis B virus (DHBV); Lanes 2 to 10: goose parvovirus (GPV), goose parvovirus (DEV), duck adenovirus type 3 (DAdV-3), Newcastle disease virus (NDV), duck reovirus (DRV), duck Tembusu virus (DTMUV), duck hepatitis A virus subtype 1 (DHAV-1), and duck hepatitis A virus subtype 3 (DHAV-3); M: molecular size marker DL2000; N: negative control. (b) Visual detection of negative and positive LAMP amplification products. Lane 1: DHBV; Lanes 2 to 10: GPV, DEV, DAdV-3, NDV, DRV, DTMUV, DHAV-1, and DHAV-3; M: molecular size marker DL2000; N: negative control; yellow represents a positive reaction, and purple represents a negative reaction.

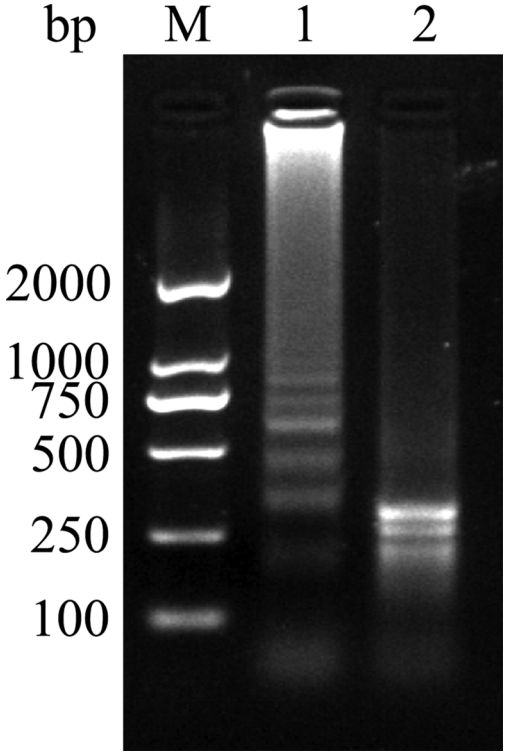

Figure 3. Identification of loop-mediated isothermal amplification (LAMP) reaction products by enzyme digestion. M: molecular size marker DL2000; Lane 1: LAMP products; Lane 2: enzyme digestion of the LAMP product.

was serially diluted 10-fold. The image shows that the band brightness decreases with decreasing DNA concentrations. The LAMP detection method could detect DNA concentrations as low as 1.09×10^2 copies, and the qPCR detection method detected the same template concentration, indicating that the LAMP assay is as sensitive as the qPCR assay. The results of the DHBV-LAMP can be observed directly with the naked eye as amplified products because positive tubes display a yellow color. These results were in agreement with the results of agarose gel electrophoresis and suggested that phenol red and cresol red were sensitive color indicators for visual inspection of the DHBV-LAMP results.

Specificity of the LAMP Assay

The amplified products of the positive DHBV strain and other viruses were detected by agarose gel electrophoresis (Figure 2). Only DHBV exhibited clear bands, which indicated that the LAMP method has good specificity.

According to theoretical calculations, DHBV-amplified products should be digested by the endonuclease *Eco*R I, which should produce 3 main bands of 207 bp, 234 bp and 312 bp. The results exhibited good agreement with the theoretical values, indicating that the detection method established in this study has good specificity, as shown in Figure 3.

Evaluation of the DHBV-LAMP Assay Using Clinical Samples

A total of 11 positive isolations, 69 clinical tissue samples and 88 serum samples were confirmed to be positive by qPCR, and the LAMP assay showed 100% agreement with the qPCR assay. The results are summarized in Table 2. The results also suggested that DHBV infection is very common in geese in China.

DISCUSSION

In this study, DHBV-LAMP was shown to be as sensitive as qPCR, displayed remarkable accuracy with good specificity, and could distinguish DHBV from other reference pathogens related to hepatitis. The DHBV-LAMP detection method established in this experiment requires less than 60 min for the reaction and has been validated to work well at a relatively wide range of temperatures (from 62 to 65°C), which makes it more applicable in the field, as repeated heating and cooling are not needed, and use of a simple thermostat device is sufficient. The molecular assays used for the detection of DHBV described previously, including routine PCR, qPCR, and FQ-PCR, require highly precise thermal cycling during DNA amplification and elaborate procedures for detection of the amplified products (Wang et al., 2013; Yugo et al., 2016). Furthermore, because PCR and LAMP amplification products are detected by agarose gel electrophoresis, tube opening

Table 2. Comparison of quantitative PCR (qPCR) and loop-mediated isothermal amplification (LAMP) for the detection of duck hepatitis B virus in clinical samples.

	Positive detection rates for assays				
Method	Positive Isolations	Tissues from ducks	Serum from ducks	Tissues from geese	Serum from geese
qPCR	11/11*	39/57	51/93	30/51	37/71
LAMP	11/11	39/57	51/93	30/51	37/71

(11/11* indicates positive results/the number of samples).

is more likely to cause experimental contamination, especially for the LAMP assay, because the amplification efficiency of the LAMP reaction is higher than that of PCR. Mori proposed determining whether the amplification reaction occurred by observing the turbidity produced by the byproduct magnesium pyrophosphate in the LAMP reaction tube; however, determination of turbidity by the naked eye is subjective, and using a turbidimeter to analyze the products adds to the procedure and equipment cost (Mori et al., 2001). Therefore, the dye method is superior to the turbidity method with regard to convenience, accuracy, and sensitivity. Hydroxyl naphthol blue can be used to indicate the results according to whether the reaction system remains a violet color, but the color change did not display a satisfactory dissimilarity (Choopara et al., 2017). LAMP reaction products detected using SYBR Green I dye could display a satisfactory dissimilarity between positive and negative reactions, but the dye must be added at the end of reaction and cannot be added before the reaction starts because of an inhibitory effect (Zheng et al., 2018). In this study, a color indicator containing phenol red and cresol red was chosen for product analysis based on a previous report (Tanner et al., 2015). The result is determined according to a color change caused by a pH change, which is caused by the formation of byproducts including a pyrophosphate moiety and hydrogen ions. With the indicator, the color of a positive reaction changes from purple-red to yellow, but the negative reaction tube remains the original color. In addition, rapid detection for the purpose of further saving time is achieved by adding the color indicator.

In this study, all field isolates with positive results determined by DHBV-qPCR could be accurately detected using the LAMP assay, which again demonstrated the sensitivity and specificity of the newly developed assay. Meanwhile, the qPCR assay requires nearly 90 min for the reaction and uses a sophisticated real-time PCR quantitative system, which limits its practical application. Therefore, the LAMP assay developed in this study could be a satisfactory method for clinical detection.

In conclusion, the new successfully developed DHBV-LAMP assay is a simple, rapid, specific, highly sensitive, and visible method that would be a good alternative method for the clinical diagnosis of DHBV.

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