Development of a Loop-mediated Isothermal Amplification Method for Rapid Detection of Beak and Feather Disease Virus in Parrots

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Abstract

Background: There are no effective antiviral treatments for Beak and Feather Disease virus (BFDV); thus, rapid diagnosis is critical for effective control of the disease. Recent development of a novel Loop-Mediated Isothermal Amplification (LAMP) technique that amplifies nucleic acids rapidly with high specificity and sensitivity under isothermal conditions has overcome some of the deficiencies of nucleic acid-based diagnostic tests and has made on-site diagnosis possible.

Methods and finding: We established a LAMP method for rapid detection of BFDV using 2 pairs of primers that were designed from BFDV and compared its sensitivity and specificity with PCR. Amplification by LAMP was optimal at 63°C for 60 min. The detection limit was nearly 3.5 fg of BFDV DNA— as sensitive as PCR. There was no cross-reaction with porcine circovirus type 2 (PCV2), pigeon circovirus (PiCV) or avian polyomavirus under the same conditions. The assay also successfully detected BFDV DNA in the tissues of infected parrots.

Conclusion: This is the first report indicating that LAMP is a valuable, rapid method of detecting BFDV with high sensitivity and specificity.

List of abbreviations

BIP: Backward Inner Primer; FIP: Forward Inner Primer; LAMP: Loop-Mediated Isothermal Amplification; PCR: Polymerase Chain reaction; BFDV: Beak and Feather Disease Virus; PCV2: Porcine Circovirus Type 2 Virus

Introduction

Psittacine Beak and Feather Disease (PBFD) is a viral disease that was first discovered in 1975 in cockatoos in Australia [1]. Since then, several outbreaks have been reported in parrots in many countries, including New Zealand [2], South Africa [1], Europe [3], Asia [4-6] and USA [7,8]. The causative agent, BFDV, has a circular ssDNA ambisense genome [9] with two major open reading frames (ORF). ORF V1 encodes for the replication-associated protein (Rep) and ORF C1 encodes for the capsid protein [10]. A third ORF has been identified, but its function remains unknown [8].

The symptoms of chronic PBFD consist of feather loss and abnormalities in feather or beak shape resulting from damage to the epidermis of the feathers and beak [11]. Acquired immunodeficiency as a result of BFDV infection often leads to fatal secondary infections [12].

Several methods have been established to detect BFDV infection, including Haemagglutination (HA) and Haemagglutination Inhibition (HI) assays [13], histology [11], blocking ELISA [14], polymerase chain reaction (PCR) [1, 8, 15-18], real-time PCR [19] and quantitative real-time PCR [20]. However, these methods are time-consuming and require expensive instruments.

Loop-Mediated Isothermal Amplification (LAMP) is a molecular method that is used to amplify DNA or RNA under isothermal conditions and was developed by Notomi et al. [21]. Several studies suggested that LAMP could be a rapid and highly specific assay that amplifies DNA with high specificity, efficiency and

rapidity under isothermal conditions [21-24]. The LAMP reaction is an auto-cycling strand amplification carried out by a Bst DNA polymerase and two pair of specific primers that recognize six sequences on the target DNA. The final LAMP products have stem-loop DNA with various lengths that can be detected by a ladder pattern of bands on a DNA agarose gel or can be visualized as precipitates in a turbid solution with no specific reagent or equipment requirement for the detection of amplified DNA [21, 25]. On the other hand, the entire duration of the LAMP assay, including DNA extraction, the reaction, and the final visualization of amplification products, is less than 2.5 h whereas at least 5 h is needed for a conventional PCR-based method [21,26]. In this study, we established a LAMP assay to detect BFDV, for which we determined its sensitivity and specificity.

Materials and Methods
Plasmid and clinical samples

Porcine Circovirus Type 2 (PCV2), pigeon circovirus and Avian Polyoma Virus (APV) specimens were obtained from field and identified by PCR and sequencing. A total of 11 suspected BFDV liver and spleen specimens from different areas of Taiwan were used for diagnostic purposes.

Confirmed clinical samples of PCV2, PiCV and APV were used as controls for specificity experiments. APV is a common virus that causes psittacine species diseases [27-30]. The nucleotide sequence homology in the ORF C1 region between BFDV and PCV2 is approximately 49.1~59% [31]. The recombinant plasmid yT&A-BFDV was used for temperature, time and sensitivity test of LAMP reaction. Eleven suspected PBFD samples were used for clinical sample test of LAMP. A 10% suspension of mixture of liver and spleen from same samples was prepared in PBS (pH7.2) for DNA isolation. All samples were stored at -80°C.

Plasmid extraction

Following the manufacturer's instruction, plasmid extraction was done using a DNA mini plasmid kit (Qiagen, Hilden, Germany). The purified plasmid was eluted in 60 μL of sterile water and stored at -20°C until later use.

DNA extraction

First, 500 μL of tissue samples that have been frozen and thawed three times was digested by 50 μL of 10% SDS and 10 μL of proteinase K (20 mg/mL) at 55°C for 2 h. Then, DNA was extracted with TRIS saturated phenol, chloroform and absolute ethanol according to the manufacturer's instruction (Thermo Fisher Scientific, CA, USA). Lastly, DNA was washed with 75% ice-cold ethanol. The precipitated DNA was dissolved in 20 μL of sterile water and stored at -20°C for later use.

LAMP primer design

Forty-one complete genomes, including the 14 subgroups (A to N) from Varsani's study [35], were used for primer design. A set of four primers was designed according to the conserved region (200-900) of 41 published full length BFDV genome (GQ396652.1, GQ396653.1, GQ396654.1, AF311298.1, AF311302.1, AF311297.1, AY450434.1, AY450435.1, AY450436.1, AF311300.1, AF311301.1, FJ685980.1, FJ685978.1, GU015012.1, GU015013.1, GU015023.1, AF311299.1, FJ685989.1, FJ685970.1, HM748919.1, AY521236.1, DQ397818.1, GU047347.1, GQ120621.1, AY521238.1, FJ685985.1, AF311296.1, AY521235.1, HM748929.1, HM748928.1, HM748927.1, HM748939.1, HM748938.1, HM748918.1, HM748925.1, AY450439.1,AY450438.1, AY450442.1, GQ165757.1, AB277746.1 and AB277747.1). He primers were designed using Primer Explorer ver. (http://primerexplorer.jp/elamp4.0.0/index.html). A forward primer (LAMP-F3), a backward primer (LAMP-B3), a forward inner primer (LAMP-FIP) and a backward inner primer (LAMP-BIP) were used for the LAMP method. The sequences and binding sites of the primers are shown in **Table 1**.

LAMP reaction

The LAMP assay was performed using the Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Tokyo, Japan). In brief, the assay was performed with the following optimized reaction mixture: 25 μL of a mixture containing 12.5 μL of reaction mix buffer, 1 μL of the template DNA, 40 pmol (each) of primers FIP and BIP, 5 pmol of primers B3 and F3, and 1 μL of Bst DNA polymerase. To determine the optimal conditions for sensitivity and selectivity, the LAMP reactions were performed at a range of temperatures (60°C- 65°C) for different time periods (30 - 60 min). After each incubation time and temperature, reactions were stopped by heating at 80°C for 5 min. LAMP products were detected using 2% agarose gel electrophoresis with ethidium bromide staining.

PCR

Three primer pairs (PCV2 ORF2F and PCV2 ORF2R for PCV2; PiCV-Pet32aF and PiCV-Pet32aR for PiCV; APV-VP1F and APV-VP1R for APV) were used to study the specificity of LAMP when compared with PCR. PCR was carried out in a 25 μL reaction volume containing final concentrations of 0.4 μM of each primer, dNTP mixture (0.2mM each dATP, dCTP, dGTP, dTTP) (Fermentas, CA, USA), 1.5 mM MgCl2 (Fermentas, CA, USA), 2.5 μL of PCR buffer (Fermentas, CA, USA), 1 μL of extracted DNA and 2.5 U of Taq DNA polymerase (Fermentas, CA, USA). The PCR conditions were as follows: an initial activation at 95°C for 10 min, and then 35 cycles of amplification (3 min at 95°C, 45 s at 53°C, 30 s at 72°C). The PCR products were analyzed by 1.5% agarose gel electrophoresis.

Sensitivity and specificity of LAMP

The BFDV template was diluted serially 10-fold in double-distilled water (diluted from 3.5 pg to 3.5 fg) to determine the detection limit. To compare the sensitivity of LAMP with that of PCR, both LAMP (LAMP-F3, LAMP-B3, LAMP-FIP and LAMP-BIP primer pairs) and PCR (PBFDV-ORFF and PBFDV-ORFR primer pair) were carried out under optimized condition with. The final optimized condition of LAMP was 63°C for 60 min. DNA of PCV2 (PCV2 ORF2F and PCV2 ORF2R primer pair), PiCV (PiCV-Pet32aF and PiCV-Pet32aR primer pair) and APV (APV-VP1F and APV-VP1R primer pair) were examined to assess the specificity of LAMP and conventional PCR. The sequences of the primers are shown in **Table 1**. Furthermore,

Table 1 The specific primers for following diseases.

Primer	Type	Length	Sequence	Product	Pathogen	Genome position	References
PBFDV-ORFF	Forward	18	AACCCTACAGACGGCGAG	717	*Psittacine Beak and feather disease virus*	182-898	15
PBFDV-ORFR	Reverse	20	GTCACAGTCCTCCTTGTACC				
PCV2 ORF2F	Forward	21	ACGTATCCAAGGAGGCGTTAC	703	*Porcine Circovirus-type-2*	1031-1733	26
PCV2 ORF2R	Reverse	20	AGGGTTAAGTGGGGGGTCTT				
PiCV-Pet32aF	Forward	27	GAATTCCAGATGAGAAGGCGGAGATTC	820	*Pigeon Circovirus*	1166-1985	26
PiCV-Pet32aR	Reverse	31	CTCGAGCATCTGCAAAACACTGGTTACAATC				
APV-VP1F	Forward	24	CTTATGTGGGAGGCTGTCAGTGTT	550	*Avain polyomavirus*	2183-2732	30
APV-VP1R	Reverse	24	TACTGAAATAGCGTGGTAGGCCTC				
LAMP-F3	Forward Outer	18	TTGTGGCGAGAGACGGTC	-	*Psittacine Beak and feather disease virus*	462-479	This research
LAMP-B3	Backward Outer	20	CCGTAGATGACGTCAACCTC			637-656	
LAMP-FIP	Forward Inner	39	AACTCTCGCGCGACTTCCTTCGCTTTCGACGGAGCTGTT			459-477+505-524	
LAMP-BIP	Forward Inner	40	CATGGGCGGGGCTTGCATAAAGTCTTGAAATCACGTGGGC			544-563+589-608	

11 clinical samples were tested simultaneously by LAMP and PCR. Sterile water was used as negative control.

Clinical specimens

During the period 2012-2013, 11 clinical specimens of suspected PBFD samples were collected from different parrot farms in southern Taiwan. A 10% suspension of mixture of liver and spleen from the same sample was prepared in PBS (pH 7.2) for DNA isolation. All samples were stored at −80°C. The use of animals in this research complies with the Taiwan animal welfare law.

Results

Optimization of BFDV LAMP assay

A one-step LAMP assay for the rapid detection of BFDV was developed using a set of 4 primers designed based on highly conserved regions of BFDV. Plasmid yT&A-BFDV was used to optimize the assay reaction temperature and time. In the first step, the LAMP reaction was carried out for 60 min at 60, 61, 62, 63, 64, and 65°C. As shown in **Figure 1a**, ladder pattern of products were generated at 60-65°C. Further experiments showed that there was significant amplification with reaction times for 30, 40, 50 and 60 min at 63°C, yielding ladder pattern of bands on a DNA agarose gel (**Figure 1b**); thus, 60 min at 63°C was used as the optimal reaction conditions in subsequent assays.

Sensitivity of LAMP and PCR for BFDV

Using serially diluted DNA from recombinant plasmid as template from 3.5 pg to 3.5 fg, LAMP assay and conventional PCR for PiCV were performed to compare their detection limits. All amplicons were analyzed by agarose gel electrophoresis. The detection limit of LAMP (**Figure 2b)** was 3.5 fg/μL of DNA versus 3.5 fg/μL for conventional PCR (**Figure 2a**). Thus, the LAMP assay was as sensitive as conventional PCR.

Specificity of LAMP for BFDV

To determine the specificity of the LAMP assay, PiCV, PCV2 and avian polyoma virus samples were tested for cross-reactivity. Viral genomic DNA was used as template for LAMP and conventional PCR detection. By agarose gel electrophoresis, only BFDV gave a positive reaction—a ladder-like pattern of bands **(Figure 3b).** Moreover, PCR was positive with specific primers for PiCV, PCV2 and avian polyoma virus (**Figure 3a**). Thus, the LAMP assay was specific for BFDV. Four primers were needed to recognize 6 distinct regions in the LAMP reaction to amplify the target DNA with high specificity.

LAMP of clinical samples

To confirm its applicability for detecting BFDV in field-obtained samples, the LAMP assay was used to detect BFDV in 11 liver and spleen mixture samples from parrots. All 4 (4/11; 36.3%) samples were positive by LAMP assay, and 7(7/11; 63.6%) samples were negative (Fig 4b). To validate these results, PCR was performed on the same samples. Only 2 (2/11; 18.1%) samples were positive by conventional PCR assay, and 9 (9/11; 81.8%) samples were negative **(Figure 4a).** These results suggest that the BFDV LAMP assay is more sensitive than PCR in detecting clinical PBFD samples.
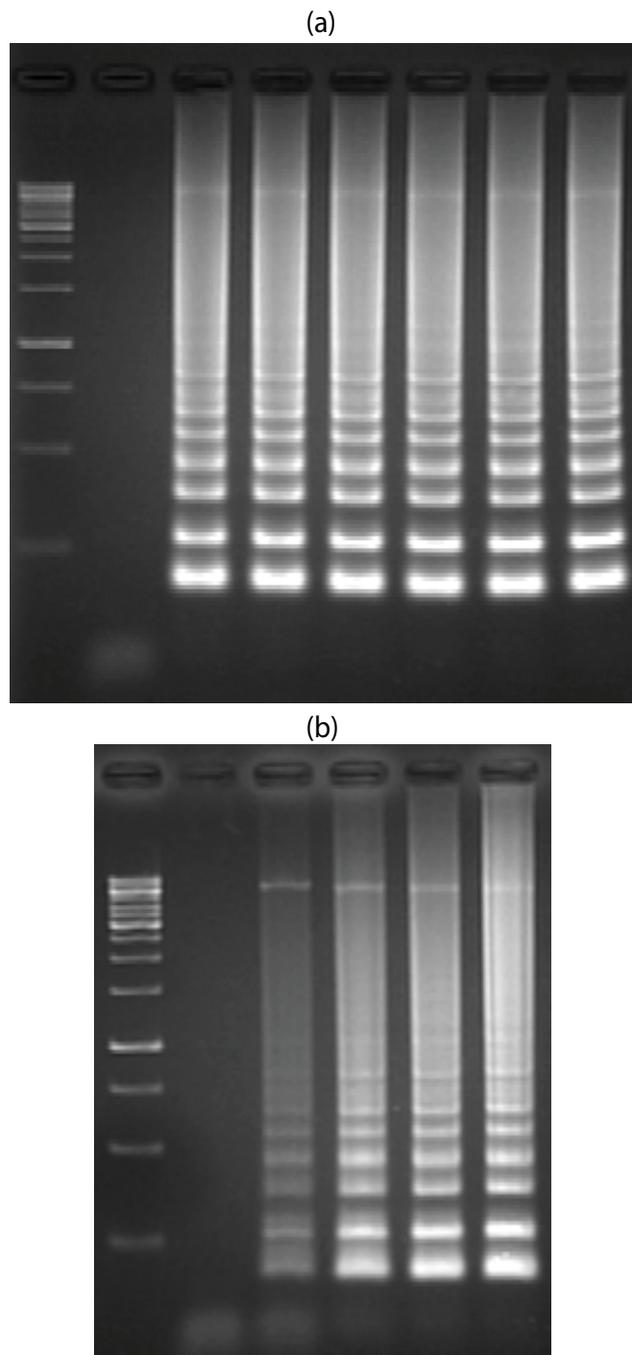
(a)

(b)

Figure 1 Optimization of LAMP reaction temperature and time of BFDV.
Different reaction temperature (a) and time (b) were test to determine the optimal LAMP reaction temperature. Lane M, DNA marker, lane N negative control, lanes 1-6, reaction temperature of 60 , 61, 62, 63, 64 and 65℃. Lanes 1–4 are samples of reaction times of 30, 40, 50 and 60 min.

Discussion

BFDV is a significant viral pathogen in parrot species, causing feather loss and abnormalities in feather or beak shape resulting

from damage to the epidermis of the feathers and beak [11]. Acquired immunodeficiency as a result of BFDV infection often leads to fatal secondary infections [12]. Amplification methods that are based on PCR, such as conventional PCR and real-time PCR, have been used to detect and quantify this virus [32-34], but application of the recently developed LAMP assay for rapid detection of BFDV has not been reported. As described for many
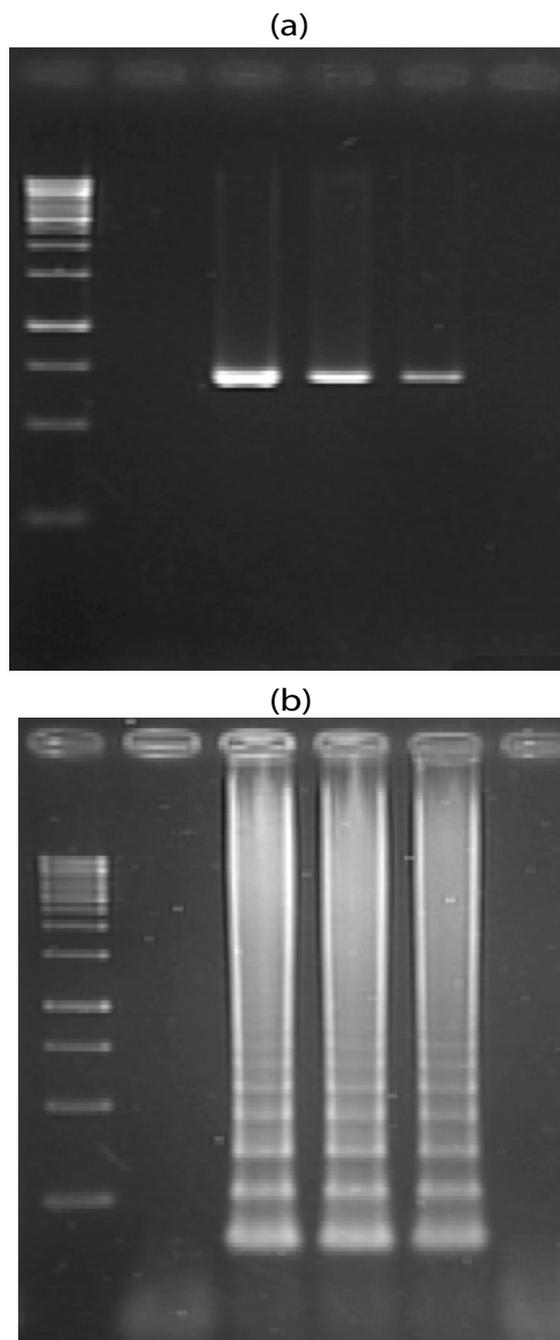
(a)

(b)

Figure 2 Sensitivity of BFDV detection by PCR (a) and LAMP (b) assay.
A 10-fold serial dilution of BFDV DNA was used to determine the sensitivity of the
LAMP assay. Lane M, DNA marker; lane N, negative control, lanes 1–5, amplification products obtained with DNA templates of 3.5 pg ~ 3.5 fg
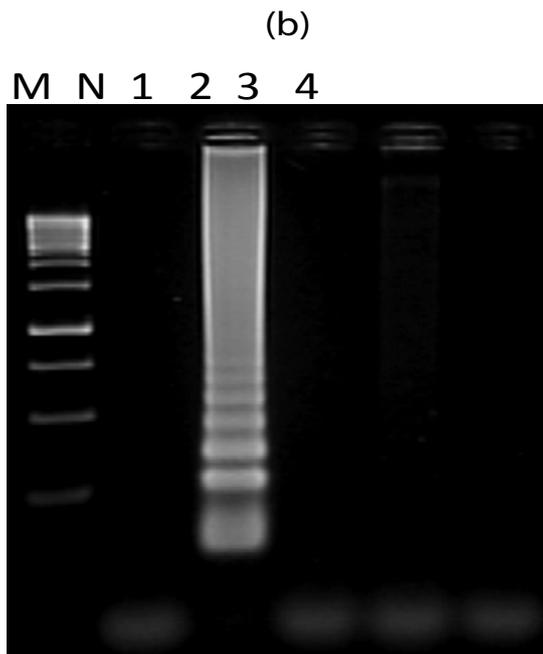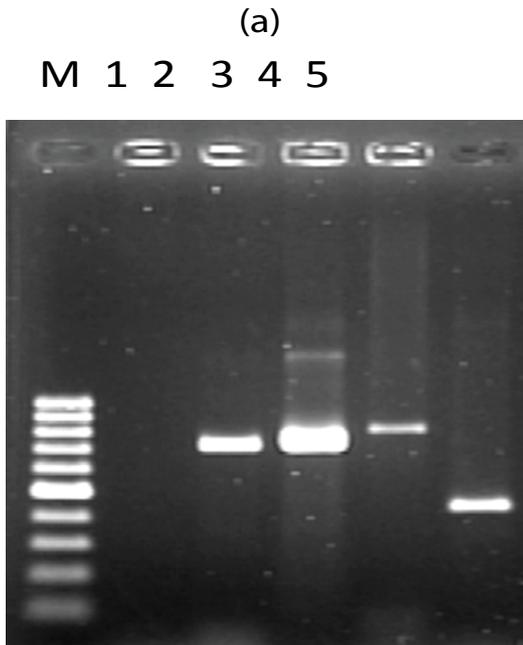
(a)

M 1 2 3 4 5

(b)

M N 1 2 3 4

Figure 3 Specificity of of PCR (a) and LAMP (b).Lane M: marker, lane N: negative control, lane 1-4 (BFDV, PCV2, PiCV and APV respectively.

viral pathogens [26], the LAMP assay shows good sensitivity in detecting viruses. The entire duration of the LAMP assay, including DNA extraction, the reaction, and the final visualization of amplification products, is less than 2.5 h whereas at least 5 h is needed for a conventional PCR-based method [26]. Thus, LAMP is a more rapid method of detecting BFDV than PCR. In addition, LAMP can be performed with common and inexpensive equipment. The LAMP reaction is executed in a single tube and only requires a water bath to provide a constant temperature; moreover, the amplification products can be detected by the

naked eye. Further still, there are several studies reporting on a direct LAMP assay: by a simple boiling and chilling treatment, no nucleic acid extraction was necessary for the detection of EV71 in nasopharyngeal swab specimens, alpha herpesvirus in skin infection, canine parvovirus in suspected faecal samples, and *Mycobacterium tuberculosis* in L-J media cultured bacteria [35-38]. It means that on-site diagnosis using LAMP is possible. Based on these advantages, the LAMP assay is considerably more applicable than PCR, particularly in the field.

The optimal conditions for the LAMP reaction in detecting BFDV DNA were 63°C and 60 min. In this study, the limit of detection of LAMP for BFDV was 3.5 fg of viral DNA, suggesting that the LAMP assay is as sensitive as a PCR-based assay. In detecting BFDV from experimental and clinical samples, the LAMP method was as least as sensitive as PCR. Also, the specificity of the LAMP reaction was extremely high because it employs 4 primers that recognized 6 distinct regions in the target DNA. In this study, other pathogens, including PCV2, PiCV, and avian polyoma virus, were not detected by LAMP, indicating the high specificity of this assay for BFDV.
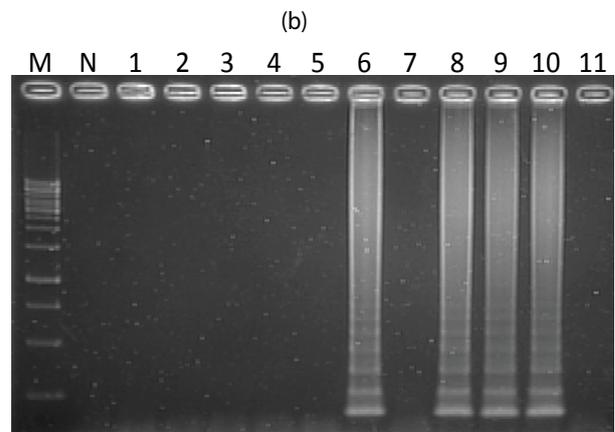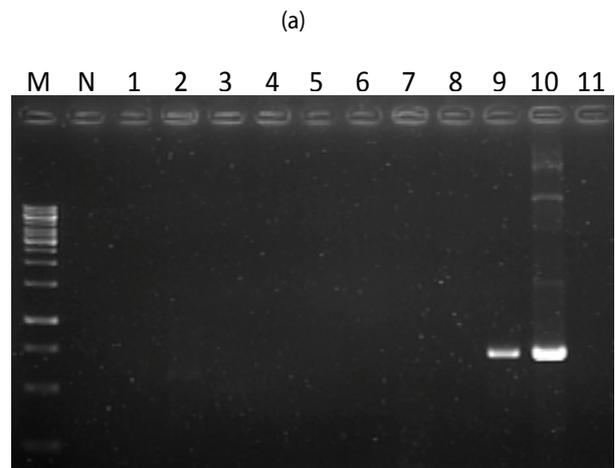

(a)

M N 1 2 3 4 5 6 7 8 9 10 11

(b)

M N 1 2 3 4 5 6 7 8 9 10 11

Figure 4 Clinical samples tested by LAMP and PCR. Eleven clinical bursa samples were assayed for BFDV by LAMP and PCR. (a) LAMP products: lane M, DNA marker; lanes 1–11, LAMP products from the 11 clinical samples. (b) PCR products. For the 11 clinical samples, PCR was carried out to validate the presence of virus in the bursa. Lane M, DNA marker; lanes 1–11, PCR products from the 11 clinical samples.

Our LAMP assay detected BFDV not only from template DNA but also from tissues of infected parrots, indicating the feasibility of rapid diagnosis in early infection. The final LAMP products have stem-loop DNA with various lengths that can be detected by a ladder pattern of bands on a DNA agarose gel or can be visualized as precipitates in a turbid solution with no specific reagent or equipment requirement for the detection of amplified DNA [21,25]. On the other hand, the entire duration of the LAMP assay, including DNA extraction, the reaction, and the final visualization of amplification products, is less than 2.5 h whereas at least 5 h is needed for a conventional PCR-based method [21,26]. The assay is simple, has high sensitivity and specificity, and is cost-effective as it does not require special reagents or sophisticated equipment. The LAMP assay may be useful for rapid diagnosis in the early stages of BFDV infection in parrots, accelerating implementation of disease control protocols.

Conclusion

This is the first report indicating that LAMP is a valuable, rapid method of detecting BFDV with high sensitivity and specificity.

Acknowledgements

Conflict of interest

All authors have no financial or personal relationships with other people or organizations that could inappropriately influence or bias their work.

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