1 **A novel one-step reverse transcription loop-mediated isothermal**

2 **amplification method for detection of infectious bursal disease virus**

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11

1 **Abstract**

2   Infectious bursal disease virus (IBDV) is an economically important viral

3 pathogen that affects young chickens worldwide and causes high mortality and

4 immunosuppression. An very fast, sensitive and specific reverse transcription

5 loop-mediated isothermal amplification (RT-LAMP) assay was developed involving a

6 single tube and a one step reaction for detecting IBDV infection. A set of four specific

7 designed RT-LAMP primers were used for the amplification of specific cDNA from

8 the IBDV genome, specifically the VP2 gene. Amplicons were successfully amplified

9 using *Bst* DNA polymerase under isothermal condition, and were verified by digestion

10 with the restriction enzyme *BamH* I. The amplified LAMP products were detected by

11 DNA electrophoresis and by direct observation using the naked eye in the presence of

12 SYBR Green I. The sensitivity of the RT-LAMP was determined to be 0.01 fg of

13 IBDV viral RNA. This RT-LAMP assay for IBDV is more sensitive than the

14 conventional RT-PCR assay, which has a detection limit of 1 ng. The specificity of the

15 LAMP assay was also assessed and it was found that it could precisely discriminate

16 between positive and negative test samples. This newly established LAMP assay,

17 combined with reverse transcription (RT) into RT-LAMP, is a practical diagnostic tool

18 because IBDV infected and uninfected clinical samples collected from an

19 experimental farm could be discriminated. Full verification of a sample's IBDV status

1   was obtained within 40 min of extracting the viral RNA, which could be then directly

2   added to the RT-LAMP reaction mixture.

3

4   **Keywords:** infectious bursal disease virus (IBDV), reverse transcription

5   loop-mediated isothermal amplification (RT-LAMP), rapid detection, VP2, diagnosis

6

Introduction

8   Loop-mediated isothermal amplification (LAMP) is a novel method that able to

9   rapidly amplify a specific nucleic acid with high specificity under isothermal

10   conditions using four to six specifically designed primers [1]. This amplification

11   occurs by auto cycling strand displacement DNA synthesis, which is catalyzed by *Bst*

12   DNA polymerase. The LAMP reaction process has no denaturation step, which is

13   different from conventional PCR, and DNA amplification occurs by means of the

14   strand displacement activity of the *Bst* DNA polymerase [1,2]. So far, the LAMP

15   method has been applied to the detection of various microbes and pathogens in the

16   environmental, food and clinical samples including protozoa, bacteria, and viruses

17   [3-10]. Moreover, several uses of LAMP for pathogen detection have been

18   commercialized into LAMP kits [11]. When detecting the RNA genome of a pathogen

19   such as a RNA virus, LAMP has been merged with reverse transcription (RT) into

4

1   RT-LAMP to allow nucleic acid amplification [12]. For example, for medical

2   purposes, RT-LAMP has been employed in minimal residual disease (MRD)

3   monitoring of the WT1 mRNA expression level [13].

4   Infectious bursal disease virus (IBDV) is an important veterinary pathogen

5   that infects young chickens. The virus belongs to the genus birnavirus of the family

6   Birnaviridae and contains two segments of double-stranded RNA genome, which are

7   designated A and B [14]. Epidemiological studies have shown that almost all 2 to

8   8-weeks-old chicks are susceptible to IBDV infection. IBDV infects the lymphoid

9   cells in the bursa of Fabricius (BF). Young chickens show various clinical signs

10   including whitish and watery diarrhea, anorexia, depression, trembling, ruffled

11   feathers and severe prostration on infection with IBDV [15]. General speaking, the

12   mortality rate may ranged from 1% to 50% across various outbreaks. In broilers,

13   infection may result in up to 50% morbidity, but mortality is seldom greater than 3%

14   in flocks aged 3-6 weeks. In addition to mortality, IBDV also often causes

15   immunosuppression in the chickens due to destruction of lymphoid tissue. This makes

16   these birds more susceptible to other pathogens, which can result in various secondary

17   infections [15]. At present, there are several conventional methods that can be used to

18   diagnose the IBDV pathogen including agar gel precipitation (AGP), virus

19   neutralization (VN), enzyme-linked immunosorbent assay (ELISA), dot blot

1 hybridization assay, indirect immunofluorescence (IIF) and electron microscopy

2 [16-21]. In addition, RT-PCR (reverse transcriptase polymerase chain reaction),

3 real-time RT-PCR, RT-PCR-RE (analysis of PCR product by restriction endonuclease)

4 and RT-PCR-RFLP (analysis of PCR product by restriction fragment length

5 polymorphism) have also been commonly used as molecular diagnostic methods to

6 detect IBDV [22-26] Among the assays mentioned above, RT-PCR is the most

7 convenient to apply because it allows the testing of a large number of samples.

8 However, the traditional RT-PCR method requires a relatively well-equipped

9 laboratory, needs well-trained staff and involves multiple reaction steps in order to

10 amplify the nucleic acid. Furthermore, the sensitivity of the method can be affected by

11 the fact that template RNA is limited. Therefore, there is a need to develop an

12 alternative assay that is more efficient, has greater sensitivity and has better specificity

13 that will be more economical when used for the diagnosis of IBDV infection. Recently,

14 the use of RT-LAMP for detection of the IBDV RNA genome has been reported; this

15 used M-MLV RTase (Moloney Murine Leukemia Virus Reverse Transciptase) and *Bst*

16 DNA polymerase in a two-stage reaction that needed at least 70 min [27]. This

17 two-step reaction is not very efficient and involved tedious processing. Nonetheless, it

18 had a sensitivity limit of 4.78 fg, which is about 100 times better than RT-PCR

19 detection of IBDV.

1   In this study, we described an improved RT-LAMP assay method to detect the

2   IBDV genome. This approach is characterized by a high sensitivity, a high specificity

3   and a very rapid procedure and is catalyzed by AMV RTase (Avian Myelobastosis

4   virus reverse transcriptase) and *Bst* DNA polymerase; the reaction involves one-step

5   process in a single tube. This newly established RT-LAMP should be a very valuable

6   and applicable tool for the detection of IBDV infection in the future.

7

8   **Materials and methods**

9   **Virus strain, IBDV-infected bursa tissue and the plasmid used**

10   Infectious bursal disease virus P3009 (Genebank Accession No. AF109154) was

11   provided by Professor Min-Ying Wang of National ChungHsin University (Taichung,

12   Taiwan). To propagate the virus, it was inoculated into specific pathogen free (SPF)

13   chickens that were one week old. The IBDV infected bursa of Fabricius from these

14   chickens were collected. This was done at 3-days post-infection; this involved

15   removing the individual bursa of the sacrificed chickens and homogenizing them

16   individually in phosphate-buffered saline. The tissue homogenate was then collected

17   and stored at -80ºC until required. The recombinant plasmid, pFastBac-VP2, which

18   harbors the VP2 gene of P3009, has been used previously [28]. In this study, this

19   plasmid was used to produce VP2 cDNA as a template for testing the LAMP assay.

1   The VP2 cDNA was amplified by PCR using the previously described primers, P4F

2   and 1323R [28].

3

4   **RNA extraction from the chicken bursa specimens**

5   Total viral RNA genomes were extracted from 100 µl of homogenized bursa

6   tissue infected with infectious bursal disease virus using the Axyprep[TM] Body Fluid

7   Viral DNA/ RNA Miniprep Kit (Axygen, USA) according to the manufacturer's

8   protocol. The RNA was eluted from the column in a final volume of 50 µl of elution

9   buffer and stored at $-20^{o}C$ until required. In addition, 21 clinical samples were

10   obtained from an experimental broiler farm, 6 of these were infected with IBDV and

11   15 were uninfected; these were also extracted as described above.

12

13   **Design of the primers for the RT-LAMP assay**

14   Four specific RT-LAMP primers (F3, B3, FIP, and BIP) for infectious bursal

15   virus detection were designed based on the sequence of the VP2 gene of infectious

16   bursal virus P3009 (Genebank Accession No. AF109154) using Primer Explorer V3

17   software (http://primerexplorer.jp; Eiken Chemical Co. Ltd, Japan). F3 and B3 were

18   the outer RT-LAMP primers, while FIP and BIP were inner RT-LAMP primers. FIP

19   consisted of 19 nucleotides of F2 and a 22nucleotides of F1c, which were linked by a

1 TTTT spacer. BIP consisted of 20 nucleotides of B2 and 20 nucleotides of B1c, which

2 were also linked by a TTTT spacer. The schematic positions on the VP2 gene are

3 showed in Figure 1. These primers were used in LAMP assay using VP2

4 complementary DNA as template as well as the viral assay itself. The sequences of the

5 primers are shown in Table 1.

6

7 **LAMP/ RT-LAMP reaction conditions**

8 The LAMP reaction was carried out in a total volume of 25µl. A reaction mixture

9 containing 12.5µl of 2×LAMP reaction buffer [20 mM Tri-HCl (pH8.8), 10 mM KCl,

10 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$ and 0.2% Tween 20], 8U of Bst DNA polymerase

11 (NEW England Biolabs, Germany), 10µM of each F3 and B3 primers, 10µM of each

12 FIP and BIP primers, and 10µM of 2M betaine was used. After that, 2µl of the target

13 DNA was added to each LAMP reaction. The mixtures were incubated at $60^{\circ}C$ for 40

14 min in a heating block (PCR Mechanics). For the RT-LAMP reaction, reverse

15 transcription and LAMP were carried out in single tube using a one step reaction that

16 was catalyzed by 10U (AMV) enhanced avian reverse transcriptase (Invitrogen, USA)

17 and 8U of Bst DNA polymerase at $60^{\circ}C$ for 40 min.

18

19 **Detection of the LAMP/RT-LAMP product**

1 An aliquot of 1 μl of LAMP product was subjected to DNA electrophoresis on a

2 1.6 % agarose gel and visualized under ultraviolet light after ethidium bromide

3 staining. Alternatively, the LAMP products were also detected using naked eye

4 observation of the color change during the LAMP reaction. SYBR Green I reagent

5 (Invitrogen, USA) was added to the LAMP reaction mixture before the reaction was

6 started. Finally, based on the target sequence, which contains a *BamH* I restriction

7 enzyme site, restriction digestion with *BamH* I was carried out for 1 hr at 37°C in a

8 water bath to test the amplification specificity of the target sequence.

9

10 **Sensitivity and specificity of the LAMP assay**

11 The sensitivity of the LAMP assay was evaluated using different amounts of

12 complementary DNA from the IBDV VP2 gene as template in a LAMP reaction. The

13 DNA consisted of the VP2 gene of IBDV, which had been amplified from the plasmid

14 pFastBac-VP2 [28]. The specificity of the LAMP assay for IBDV detection was tested

15 using the VP2 gene DNA of chicken anemia virus (CAV) and the H6 gene of avian

16 influenza virus H6N1 and the results were compared to those of the VP2 gene of

17 IBDV. These two additional virus templates had been created in our laboratory

18 previously.

19

1   **RT-PCR for IBDV detection**

2   Reverse transcription PCR (RT-PCR) was performed in one-tube contained

3   reverse transcriptase and *Taq* DNA polymerase. The RT-PCR reaction mixture was

4   carried out in a 25 μl total volume that contained 1x ThermoPol Reaction Buffer (20

5   mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM MgSO4, 0.1% Triton X-100,

6   pH8.8 at 25℃), 0.4 mM of dNTPs, 5 pmol each of F3 and B3, 4U *Taq* DNA

7   polymerase (New England BioLabs), 25 U *Reverse*-iT™ RTase Blend (ABgene) and

8   5 μl RNA template; the mixture was made up to 25 μl by DEPC-treated water. The RT

9   conditions was 45^oC for 30 min, and the reaction program of following PCR was

10   94^oC for 2 min at pre-denaturation step, then followed by 35 cycles of 94^oC for 20 sec,

11   55^oC for 20 sec and 72^oC for 20 sec, and a final extension cycle at 72^oC for 10 min.

12

13   **Western blotting analysis**

14   Bursa samples containing IBDV VP2 protein were resolved on polyacrylamide

15   slab gels, and then were incubated in Tris-glycine running buffer (48 mM Tris-HCl,

16   39 mM glycine, 0.0375% SDS). The resulting gels containing the specific proteins

17   were transferred to a polyvinylidene difluoride (PVDF) membrane in a wet

18   electroblotter cassette using 100V for 60 min. Blots were then treated with blocking

19   buffer (5% skim milk in PBS) for 30 min and with 2000-fold PBS diluted primary

1   rabbit antibodies against IBDV VP2 protein for 2 hrs. The membrane was washed

2   three times in PBS-T buffer (1X phosphate-buffered saline + 0.5% Tween 20) and

3   incubated with an alkaline phosphatase-conjugated goat anti-rabbit antibody (ABcam)

4   for 2 hrs. Membranes were again washed three times and then finally incubated with

5   NBT and BCIP for color developing.

6

7   **Results**

8   **Establishing the LAMP assay for detection of the IBDV complementary genome**

9   To establish the reverse transcription LAMP (RT-LAMP) method for IBDV

10   detection, primer explorer software was used to design the primers F3, B3, FIP and

11   BIP. These were then used to examine the amplification efficiency-using LAMP.

12   Using IBDV complementary VP2 cDNA as template, the LAMP reaction was carried

13   out using *Bst* DNA polymerase at 65°C for 1 hr. As illustrated in lane 3 of figure 2A,

14   visually distinct DNA ladder-like fragments could be seen on the ethidium

15   bromide-stained agarose gel after DNA electrophoresis. In contrast, LAMP reaction

16   mixtures without *Bst* DNA polymerase or target DNA template did not have LAMP

17   products present after the assay had been carried out. These results indicate that these

18   specifically designed LAMP primers are suitable for amplifying the target nucleic acid,

19   and are able to discriminate between the positive and negative test samples. To further

1   identify the presence of amplified LAMP products, the LAMP products were digested

2   with *BamH* I. As shown in lane 4 of figure 2A, *BamH* I digestion of the LAMP

3   products resulted in 108 bp and 80 bp DNA fragments. Theoretically, the LAMP

4   amplicon consists of a 188 bp length of DNA that is generated by the outer primers F3

5   and B3. The target sequence of *BamH* I is present within this DNA region. Therefore,

6   these results clearly demonstrated that the amplified LAMP products did indeed

7   originated from the IBDV VP2 gene. In addition to DNA electrophoresis, the LAMP

8   products were also detected by having SYBR Green I present. Tube 3 of figure 2B

9   shows that the LAMP products were stained and could be detected as strong

10   fluorescence under UV excitation. In contrast, the negative control showed only slight

11   background fluorescence. In addition, the reaction time and temperature for LAMP

12   were also determined in this study. The optimized reaction time and temperature were

13   determined over a range from 20-40 min and from 60-63°C as illustrated in figure 2C.

14   The LAMP product's DNA ladder-like fragments were easily produced and detected

15   under all of these reaction conditions and   optimized reaction conditions were

16   identified as .

17

18   **Sensitivity and specificity of the LAMP assay**

19   To determine the sensitivity of the LAMP assay for detecting IBDV genome,

1   complementary DNA encoding the IBDV VP2 gene was serially diluted 10-fold and

2   used as template DNA. VP2 gene amounts ranging from 1 ng to 1 fg were used in the

3   LAMP reaction. After performing the LAMP reaction, LAMP products were detected

4   over the range of DNA from 1 ng to 10 fg; this is shown in figure 3A. There has no

5   LAMP products detected by DNA electrophoresis when less than 10 fg of template

6   DNA was added to the LAMP assay. Therefore, the sensitivity of LAMP when

7   detecting complementary DNA from the IBDV genome was determined to be 10 fg.

8   This is more sensitive than conventional PCR amplification of the IBDV VP2 gene.

9   Figure 3B shows that at least 1 pg of VP2 gene is required to amplify DNA by PCR in

10   order to detect IBDV infection. Next, the specificity of the LAMP assay was

11   evaluated using the H6 gene of avian influenza virus (AIV) and the VP2 gene of CAV;

12   the results are shown in figure 4. No LAMP products were generated in these

13   reactions and only reaction mixtures containing IBDV VP2 gene cDNA gave a

14   positive signal by LAMP assay. These results suggest that this newly established

15   LAMP assay is highly specific and sensitive when detecting the IBDV complementary

16   genome.

17

18   **Detection of the IBDV genome by RT-LAMP**

19   Having established that the LAMP assay can be used to detect the IBDV

1   complementary genome, it was necessary to establish whether the approach could be

2   expanded to detect the double-stranded RNA viral genome of IBDV. To do this,

3   reverse transcription (RT) is required to form complementary DNA from the viral

4   RNA genome before performing the LAMP assay. Thus, the LAMP assay was

5   combined into a RT-LAMP reaction by adding a reverse transcription step for cDNA

6   synthesis. As illustrated in lane 3 of figure 5A, the RT-LAMP reaction was performed

7   in a single tube and using one step. The results showed that a typical LAMP pattern

8   could be observed by DNA agarose gel electrophoresis using extracted viral RNA

9   from IBDV-infected bursa tissue that was added to the RT-LAMP reaction. In this

10   case, the negative sample (mock-infection) gave no LAMP product. Moreover,

11   IBDV-RT-LAMP did not amplify the RNA extracted from bursa tissue not infected

12   with IBDV (lane 2 of figure 5). Furthermore, it also did not amplify RNA extracted

13   from AIV or CAV infected liver tissue (data not shown). In terms of the amplification

14   limit, when the RT-PCR was performed using a 10 fold dilution series of viral RNA

15   from 1 ng to 0.001 fg, the IBDV viral RNA could be detected in all samples, which is

16   a RT-LAMP sensitivity of 0.01 fg of viral RNA. The results are shown in figure 5B.

17

18   **Application of the RT-LAMP assay as a diagnostic technique**

19   Based on the above results, we evaluated the method for use as a practical tool

1   using 21 clinical bursa specimens from chickens. As shown in Figure 6, the genomic

2   viral RNAs were extracted from the bursa of six IBDV infected and fifteen IBDV

3   non-infected broilers; these were then analyzed by one-step RT-LAMP. The results

4   showed that six out of six of the clinical samples were positive when RT-LAMP

5   analysis was used (upper panel of figure 6). The IBDV non-infected samples were all

6   negative by RT-LAMP assay (lower panel of figure 6). These results completely agree

7   with the clinical testing by conventional Western blot assay.

8

9   **Discussion**

10   Infectious bursal disease virus (IBDV) causes an acute and highly contagious

11   viral disease. Once chickens are infected with this virus, it may result in high

12   mortality and immunosuppression. Therefore, IBDV is an economically important

13   viral pathogen that needs to be controlled within the poultry industry worldwide.

14   However, a simple, sensitive, specific and rapid assay is needed to detect IBDV

15   infection. In the past, several methods have been developed to diagnosis IBDV

16   infection. These methods include TEM observation, serological methods such as AGP

17   and ELISA and molecular diagnosis tools such as reverse transcription PCR

18   (RT-PCR). Among these methods, nucleic acid-based amplification techniques, such

19   as RT-PCR, are more rapid, sensitive and specific than the other approaches to IBDV

1   detection. However, even using real time RT-PCR, the detection limit can be as high

2   as 100 pg when SYBR Green I was used in the reaction [29]. In addition, there are a

3   number of shortcomings to these diagnosis methods, namely they are time-consuming,

4   tedious, have a low amplification efficiency, and need the use of a thermal cycler. In

5   this study, we have successfully developed a one-step RT-LAMP method for

6   detecting the IBDV RNA genome. The one-step approach involves combining the

7   LAMP method with AMV reverse transcriptase. In our study, the one-step RT-LAMP

8   method enable specific detection of IBDV infection in broilers using the optimized

9   condition of 60°C and a reaction time as short as 40 min. The assay can be performed

10   using avian reverse transcriptase and *Bst* DNA polymerase at the same time in a water

11   bath tank under isothermal conditions. The initial reverse transcription reaction uses

12   AMV RTase rather than M-MLV RTase because of the former enzyme's broad range

13   of reaction temperatures, namely 42°C to 60°C. This allows the reaction to be carried

14   out with both enzymes, AMV RTase and *Bst* DNA polymerase, at the same time. The

15   simplicity of this approach together with its speed will allow this assay to be applied

16   to the detection of IBDV during infection screening or routine evaluation of

17   immunization efficacy. For the one-step reaction, the temperature chosen was 60°C

18   and therefore there is no necessity to pre-heat as is needed in conventional RT-PCR.

19   Overall, RT-LAMP is easier to carry out and save time compared to RT-PCR.

1   For RT-LAMP assay, the VP2 gene in segment A of the IBDV genome was

2   chosen and the designed primer set is able to recognize specifically a highly

3   conserved region of VP2. The RT-LAMP product gave the characteristic ladder-like

4   pattern by gel electrophoresis; this is specific because the reaction produces a mixture

5   of stem-loop structured DNA products with various lengths. The identity of the

6   amplified products was clearly confirmed by *BamH I* restriction enzyme digestion. To

7   save time when detecting the RT-LAMP products, SYBR Green I is the most

8   common approach for field operations.. When SYBR Green I is exposed to UV light,

9   it is possible to determine directly whether the RT-LAMP assay is positive or negative

10   by the naked eye.

11   The sensitivity of one-step RT-LAMP was established as 0.01 fg of viral RNA

12   using specifically designed RT-LAMP primers. This contrasts with a much poorer

13   sensitivity using RT-PCR of at least 1 ng of viral RNA. Thus, our results show that

14   the sensitivity of the RT-LAMP assay for IBDV detection is about 10^8 fold higher

15   than the RT-PCR assay. Based on the molecular weight of the IBDV genome at 4.7

16   MDa [30], this means that the detection limits for the RT-LAMP and RT-PCR assays

17   are approximately 1 and 10^8 copies of the IBDV genome, respectively. In addition,

18   there was complete agreement of the RT-LAMP results with the Western blot assay

19   results, which indicates that the one-step RT-LAMP method is both highly sensitive

1   and accurate. In order to test specificity, the VP2 gene of chicken anemia virus (CAV)

2   and hemagglutinin gene of H6 avian influenza virus (H6-AIV) were used. The results

3   showed that the RT-LAMP assay was specific to IBDV compared to these other two

4   viruses. IBDV is the sole member of the genus birnavirus, which is part of the

5   birnaviridae family. This genus also contains pancreatic necrosis virus (IPNV).

6   However, there is low sequence similarity between IBDV and IPNV and therefore

7   there is little likelihood of cross-reactivity between these two viruses. Based on the

8   specificity results, we suggest that the RT-LAMP assay will make a very good clinical

9   test for IBDV.

10   One of major problems with RT-PCR is that the assay needs an accurately

11   controlled thermocycling system to repeatedly perform the three temperatures shifts

12   needed to amplify using reverse transcriptase and *Taq* DNA polymerase over 2-3 hrs.

13   The one-step RT-LAMP needs no such expensive system, only a temperature

14   controlled waterbath or heating block. Conventional RT-PCR is currently used as the

15   "golden standard method" for IBDV detection, but the need for both the

16   thermocycling system and an elaborate method of detecting the product after

17   amplification is a disadvantage. Product detection using the RT-LAMP method is

18   easier than with RT-PCR and can be done by either using a fluorescence staining

19   reagent such as SYBR Green I, as was done in this study, or even by just observing

19

1 the turbidity [11]. Therefore, using RT-LAMP, a well-equipped laboratory,

2 well-trained staff, and multiple reaction steps are not needed, which makes this assay

3 more suitable to large-scale field investigations.

4   Xu et al. also used a RT-LAMP method to detect IBDV [27]. This method needed

5 1.5 hours and a two-step heating process to allow detection of the virus. Compared to

6 this earlier study, the sensitivity of our assay is 100 fold greater, as well as being

7 much faster and a one-step process. The primers used in Xu et al. targeted the VP3

8 gene of IBDV, while our assay targets the VP2 gene. In addition, in order to optimize

9 the primers, we focused on low free energy criteria. We suggested that the low free

10 energy of the primers used in our assay allowed us to achieve higher sensitivity.

11 Overall, the assay developed in this study is a sensitive, time saving and easier

12 one-step process for the detection of IBDV than the approach described in Xu et al.

13 Consequently, this one step RT-LAMP method can be easily applied to clinical

14 samples when performing IBDV diagnosis during epidemiological investigations and

15 to the assessment of immunization efficacy after vaccination.

16   In conclusion, the IBDV diagnostic method using one-step RT-LAMP assay

17 described here has the potential to become a valuable diagnostic system and could be

18 made into a simple-to-use diagnostic kit. This is the first report to demonstrate the

19 application of an improved and one-step RT-LAMP assay to the field diagnosis of

1   IBDV. In the future, the RT-LAMP assay for IBDV detection could be developed into

2   a portable mini-laboratory system for the on-site epidemiological detection of

3   infectious bursal disease virus infection among farm-bred young chickens.

4

5   **Acknowledgements**

8

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5

1 **Legends**

2

3 **Figure 1. Schematic presentation of the primers used in the RT-LAMP assay for**

4 **IBDV detection.** The partial nucleotide sequence of the VP2 cDNA of IBDV, the six

5 target regions for primers annealing, and the four specifically designed RT-LAMP

6 primers used in the assay are indicated. The underlined sequence is the *BamH* I

7 restriction enzyme site.

8

9 **Figure 2. Analysis of the LAMP products using agarose gel electrophoresis and**

10 **SYBR Green I staining.** (A) The LAMP products were detected by agarose gel

11 electrophoresis and the products was also digested with *BamH* I. LAMP in some cases

12 as described in Materials and Methods. Lane M, the 100 bp DNA ladder as size

13 marker. Lane 1, LAMP reaction mixture without *Bst* DNA polymerase; Lane 2,

14 LAMP reaction mixture without target DNA template; Lane 3, complete LAMP

15 reaction mixture; Lane 4, LAMP products after digestion with *BamH* I. (B)

16 Visualization of LAMP products by staining with SYBR Green I and using UV

17 excitation. Tubes 1-3 are the LAMP products showed in lanes 1 to 3 of (A). (C)

18 LAMP was performed for different reaction times and at various temperatures. Lane C,

19 LAMP reaction without target DNA as a negative control.

20

1   **Figure 3. Sensitivity of the LAMP assay (A) and compare with PCR (B) for**

2   **detecting the cDNA of IBDV VP2 gene**. The PCR was carried out using the outer

3   LAMP primers, F3 and B3. A 188 bp length fragment of the IBDV VP2 DNA was

4   obtained after the PCR reaction. Lane 1-7, 100 pg, 10, pg, 1 pg, 100 fg, 10 fg, 1 fg,

5   0.1 fg of VP2 cDNA were added to the LAMP reactions; Lane M, 100 bp DNA ladder;

6   Lane C, negative control.

7

8   **Figure 4. Specificity of the LAMP assay for the detection of the VP2 gene of**

9   **IBDV. The** LAMP reaction was performed at 65°C for 1 hr. Specific viral

10   complementary DNA from the three viruses tested was prepared as described in the

11   Materials and Methods. IBDV, VP2 gene of infectious bursal disease virus added as

12   the template. CAV, VP2 gene of chicken anemia virus added as the template. AIV,

13   H6 gene of avian influenza virus added as the template. Lane C, negative control.

14

15   **Figure 5. The one-step RT-LAMP assay for detecting IBDV-infected bursa tissue**

16   **from chickens.** The RT-LAMP primers were used with genomic RNA for the

17   diagnosis of IBDV in a one step RT-LAMP reaction (A). Lane M, 100 bp DNA ladder

18   marker; lane 1, RT-LAMP reaction mixture containing no template; lane 2, RT-LAMP

19   reaction mixture containing no reverse transcriptase; lane 3, extracted IBDV viral

20   RNA added to the RT-LAMP reaction; lane 4, IBDV VP2 complementary DNA added

1   to the RT-LAMP reaction. (B) One-step RT-LAMP and RT-PCR were performed after

2   adding various amounts of viral RNA. Upper panel is the RT-LAMP pattern, lower

3   panel is the RT-PCR pattern. Lane 1-10 were 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg,

4   1 fg, 0.1 fg, 0.01 fg, 0.001 fg of IBDV total RNA, respectively. Lane NC, negative

5   control.

6

7   **Figure 6. Detection of IBDV-RNA from experimental chickens by RT-LAMP.** 1

8   ng of viral genomic RNA extracted from the bursa was used for each RT-LAMP and

9   RT-PCR assay. The RT-LAMP and RT-PCR patterns were analyzed using 1.6%

10   agarose electrophoresis gel. The numbers on each of the lanes represent the different

11   clinical collected bursa samples from either IBDV infected or non-infected chickens.

12   These samples were also analyzed by Western blotting using antibody against the

13   IBDV VP2 protein. Lane NC, negative control for the RT-LAMP and RT-PCR assays.

14   WB, Western blotting assays.

15

Table 1. Details of oligonucleotide primers used for one-step RT-LAMP to amplify VP2 gene of IBDV.

Primer name	Type	Length(s)	Genome position	Sequence(5'→3')
F3	Forward outer	20-mer	999-1018	AGCAGTGACAATCCATGGTG
B3	Reverse outer	18-mer	1169-1186	TCATGGCTCCTGGGTCAA
FIP	Forward inner (F1c-TTTT-F2)	45-mer(F1C, 22-mer; F2, 19-mer)	F1C, 1075-1096 F2, 1035-1053	CGACCGTAACGACGGATCCTG TTTTTCCTCCGTCCCGTCACAC TA
BIP	Reverse inner (B1C-TTTT-B2)	44-mer(B1C, 20-mer; B2, 20-mer)	B1C, 1097-1116; B2, 1148-1166	CCGGGGTGAGCAACTTCGAGT TTTT CGGCCGTATTCTGTAACCA
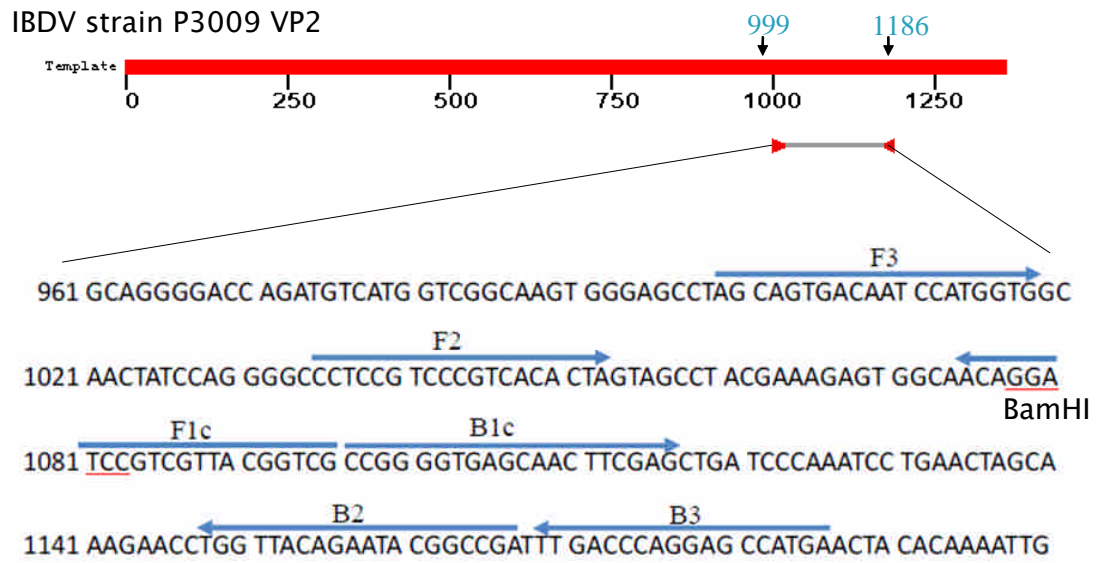
Figure 1. Schematic presentation of the primers used in the RT-LAMP assay for IBDV detection. The partial nucleotide sequence of the VP2 cDNA of IBDV, the six target regions for primers annealing, and the four specifically designed RT-LAMP primers used in the assay are indicated. The underlined sequence is the *BamH* I restriction enzyme site.
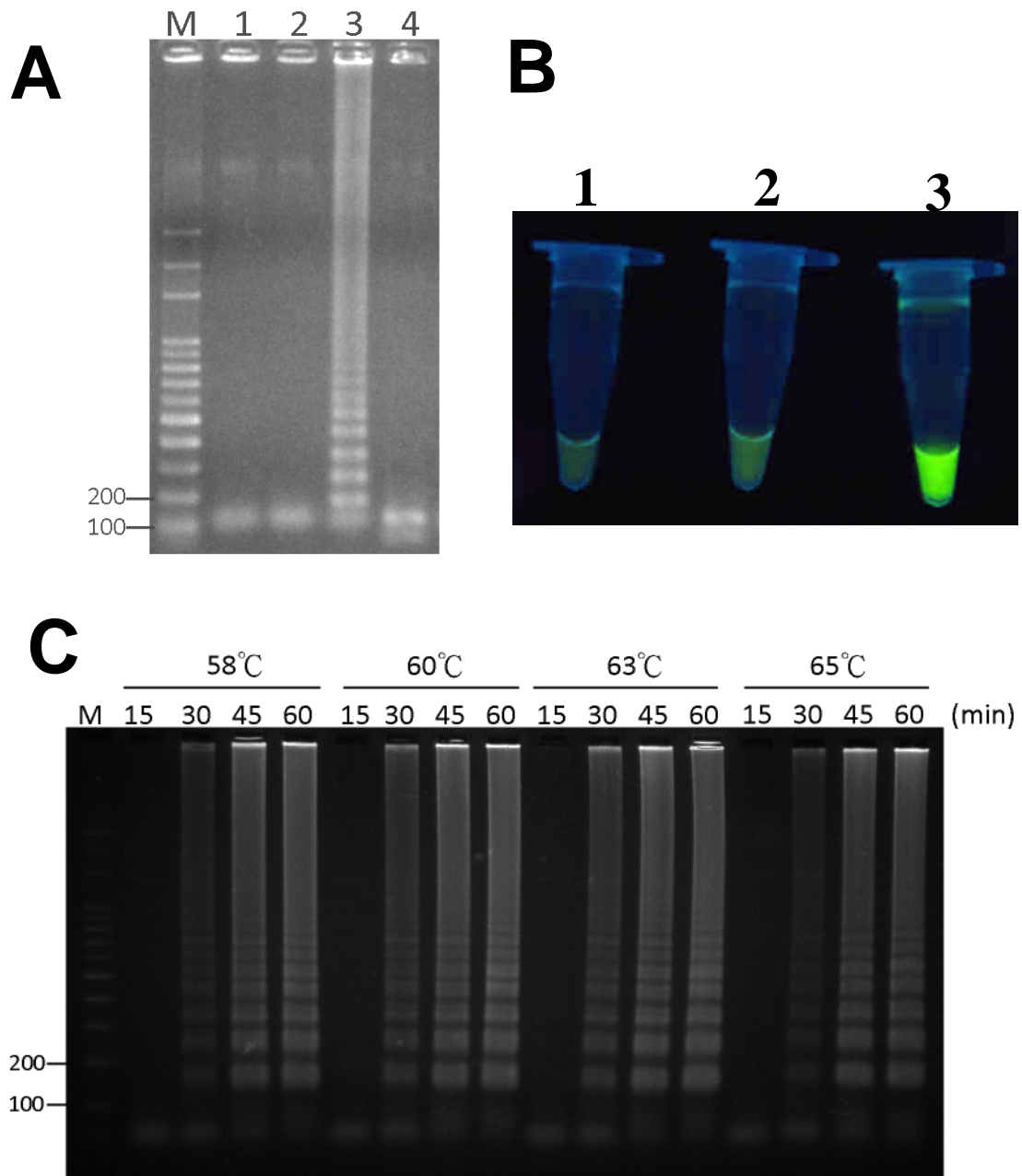
Figure 2. Analysis of the LAMP products using agarose gel electrophoresis and SYBR Green I staining. (A) The LAMP products were detected by agarose gel electrophoresis and the products was also digested with *BamH* I. LAMP in some cases as described in Materials and Methods. Lane M, the 100 bp DNA ladder as size marker. Lane 1, LAMP reaction mixture without *Bst* DNA polymerase; Lane 2,

LAMP reaction mixture without target DNA template; Lane 3, complete LAMP

reaction mixture; Lane 4, LAMP products after digestion with *BamH* I. (B)

Visualization of LAMP products by staining with SYBR Green I and using UV

excitation. Tubes 1-3 are the LAMP products showed in lanes 1 to 3 of (A). (C)

LAMP was performed for different reaction times and at various temperatures. Lane
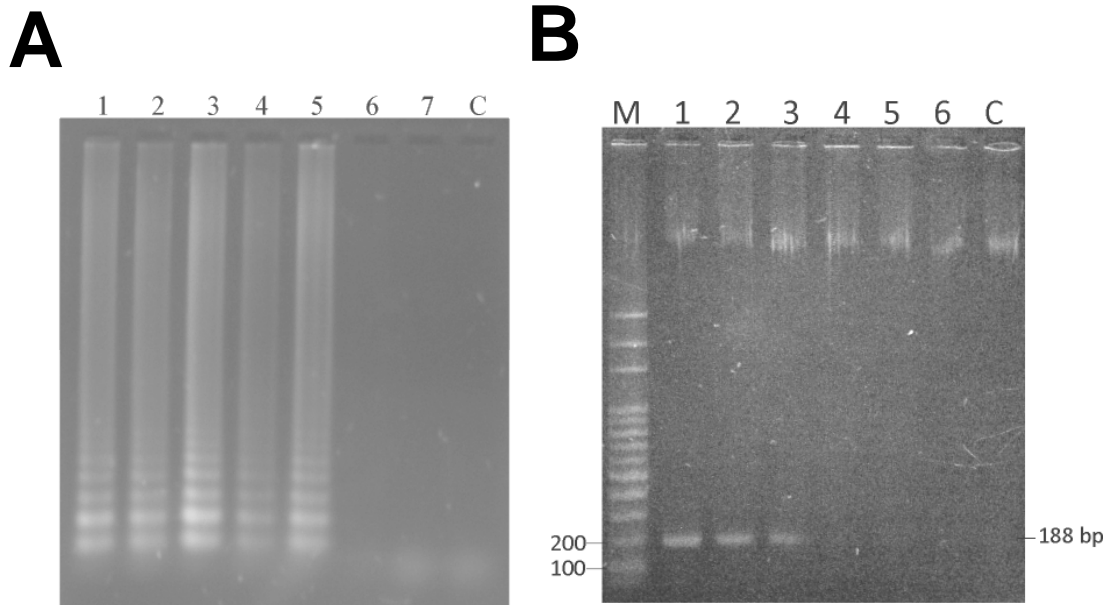
C, LAMP reaction without target DNA as a negative control.

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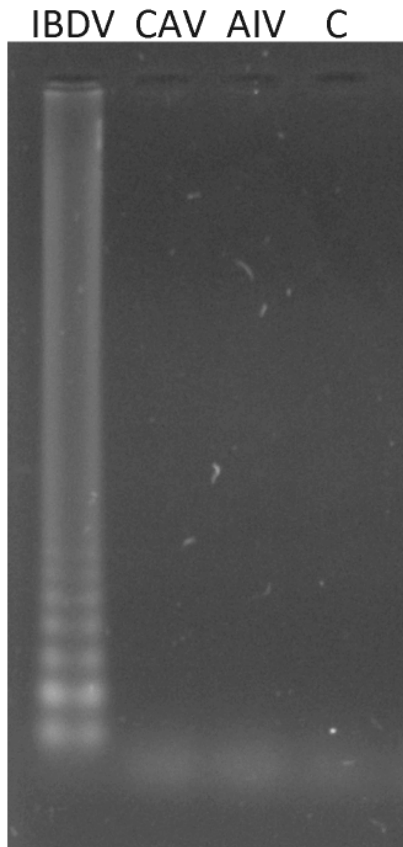
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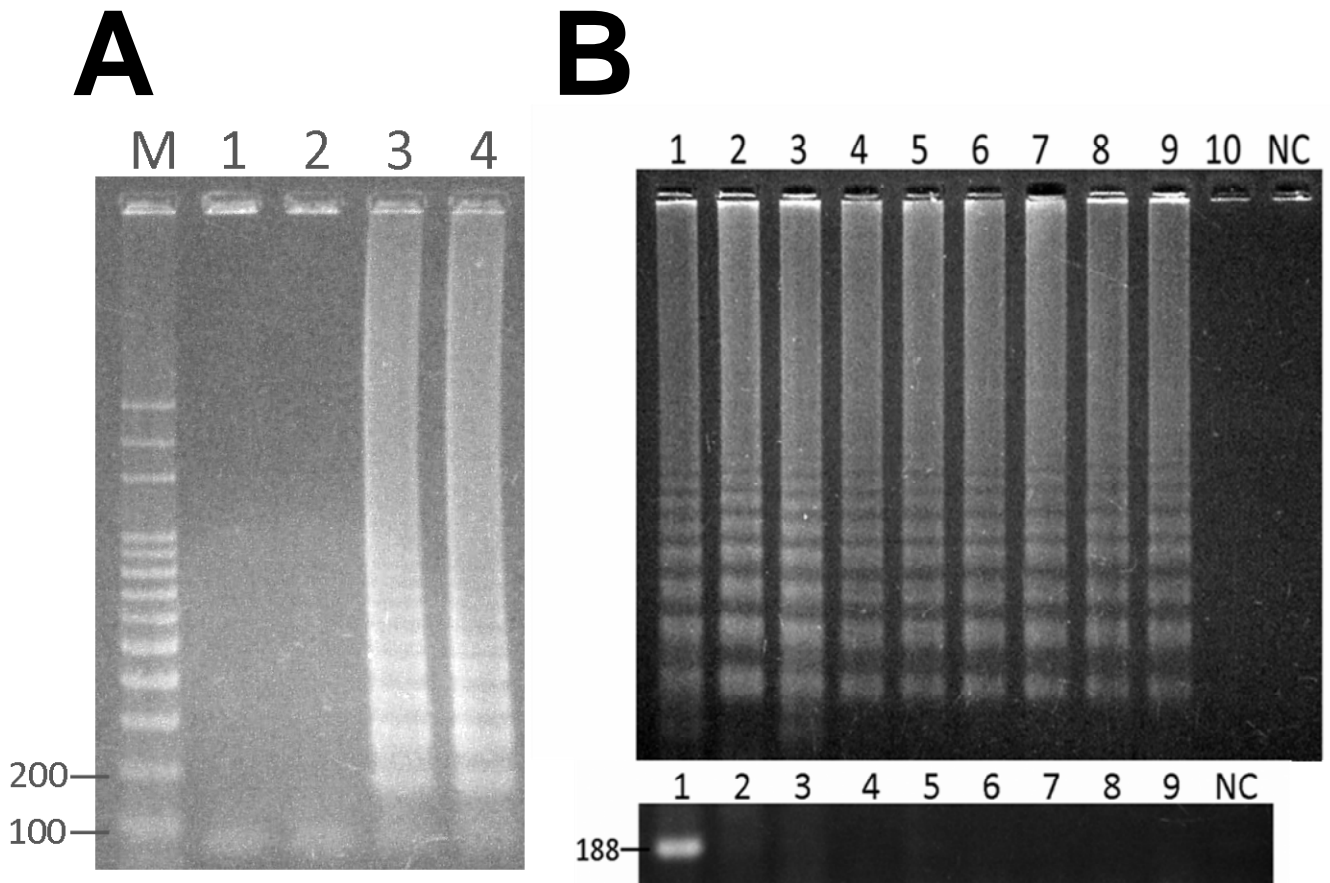
Figure 5. The one-step RT-LAMP assay for detecting IBDV-infected bursa tissue from chickens. The RT-LAMP primers were used with genomic RNA for the diagnosis of IBDV in a one step RT-LAMP reaction (A). Lane M, 100 bp DNA ladder marker; lane 1, RT-LAMP reaction mixture containing no template; lane 2, RT-LAMP reaction mixture containing no reverse transcriptase; lane 3, extracted IBDV viral RNA added to the RT-LAMP reaction; lane 4, IBDV VP2 complementary DNA added to the RT-LAMP reaction. (B) One-step RT-LAMP and RT-PCR were performed after adding various amounts of viral RNA. Upper panel is the RT-LAMP pattern, lower panel is the RT-PCR pattern. Lane 1-10 were 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg,

1 fg, 0.1 fg, 0.01 fg, 0.001 fg of IBDV total RNA, respectively. Lane NC, negative
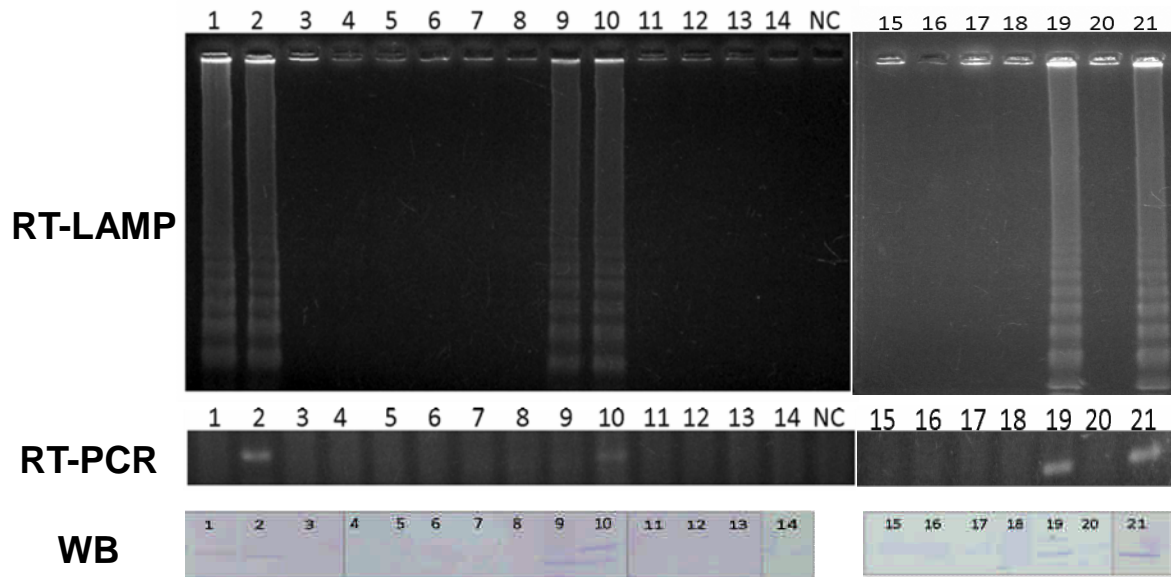
control.

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