

# Research Note: Application of reverse-transcription recombinase-aided amplification-lateral flow dipstick method in the detection of infectious bursal disease virus

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**ABSTRACT** Infectious bursal disease (IBD) is a highly contagious viral disease caused by infectious bursal disease virus (IBDV) in chickens. The consequent immunosuppression and secondary infection affect the healthy development of chicken industry. In this study, specific primers and probes were screened in the conserved region of IBDV VP2 gene sequence, and reverse transcription-recombinase-aided amplification (RT-RAA) was combined with lateral flow dipstick (LFD) for establishing RT-RAA-LFD method for detection of IBDV in chickens. The reaction conditions of RT-RAA-LFD assay were optimized, and the specificity, sensitivity, and repeatability were verified. The results showed that the RT-RAA-LFD method could amplify the IBDV

target fragment at 37°C for 15 min, and the required primer and probe concentration was 1,250 nmol/L. The detection results were directly observed by the dipstick, the lowest detectable limit (LDL) for IBDV was 10 copies/ $\mu$ L, and there was no cross reaction with several common immunosuppressive pathogens in poultry. The total coincidence rate of sample test results between RT-RAA-LFD and reverse transcription-polymerase chain reaction (RT-PCR) was 95.83%. Due to advantages of high sensitivity, strong specificity, easy operation, fast detection, the established RT-RAA-LFD method can provide some technical support and new solutions for local laboratory to detect IBDV.

**Key words:** infectious bursal disease virus, reverse-transcription recombinase-aided amplification, lateral flow dipstick

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## INTRODUCTION

Infectious bursal disease (IBD) is an acute and highly contagious disease caused by infectious bursal disease virus (IBDV) in chickens (Swayne et al., 2020). IBDV attacks the B lymphocytes in the bursa of Fabricius of young chickens, leading to immunosuppression (Xu et al., 2020). IBDV infection in chickens can affect the efficacy of various vaccines, resulting in vaccination failure. Chickens infected with IBVD are more susceptible to other bacterial, viral, and parasitic diseases. IBD is easy to cause necrotizing dermatitis, inclusion body hepatitis and anemia syndrome, ultimately affecting the performance and economic value of chickens. At present, the outbreak of IBD shows the characteristics of

asymptomatic infection, silent infection, mixed infection, virus variation, atypical symptoms, and virulent strains, which brings greater challenges to the prevention and control of IBD (Fan et al., 2019; Li et al., 2020). The establishment of a rapid, sensitive, and specific diagnostic method is the key to effectively preventing and controlling IBD.

The common methods for detection of IBDV include reverse transcription-polymerase chain reaction (RT-PCR), real-time fluorescence-based quantitative polymerase chain reaction (RFQ-PCR), multiplex polymerase chain reaction (MPCR), immunocapture-polymerase chain reaction (IC-PCR), polymerase chain reaction-enzyme-linked immunosorbent assay (PCR-ELISA), indirect ELISA, reverse transcription-loop-mediated isothermal amplification (RT-LAMP), and colloidal gold immunochromatographic assay (GICA).

Recombinase-aided amplification (RAA) is a new isothermal nucleic acid amplification technique, which can amplify the target gene rapidly under isothermal conditions, and the addition of the probe ensures the specificity and sensitivity of detection (Gurusamy et al., 2021). The results can be presented by diversified means such

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as agarose gel electrophoresis (**AGE**), RFQ-PCR or lateral flow dipstick (**LFD**). RAA can be used to detect RNA/DNA of a variety of pathogens, and has been successfully used to detect Newcastle disease virus (**NDV**) (Wang et al., 2020), African swine fever virus (**ASFV**) (Zhao et al., 2021), and *Staphylococcus aureus* (**SA**) (Hou et al., 2021).

In this study, RT-RAA was combined with LFD to establish a rapid visual detection method for IBDV. The principle of the RT-RAA-LFD method established: a nfo probe was added on the basis of RT-RAA reaction, and the probe was labeled with 6-carboxyfluorescein (**FAM**) at its 5' end, and labeled with tetrahydrofuran (**THF**) residue at a distance of more than 30 bp from the 5' end. The 5' end of the downstream primer was labeled with biotin. After the reaction, a double-labeled product with biotin and fluorescein was generated, and the results were visible to the naked eye by LFD detection. This study provides a new technique for early and rapid diagnosis of IBDV.

## MATERIALS AND METHODS

### Extraction of Virus DNA/RNA

IBDV, Marek's disease virus (**MDV**), chicken infectious anemia virus (**CIAV**), reticuloendotheliosis virus (**REV**), and Avian leukosis virus (**ALV**) were identified and preserved in our laboratory. Virus genomic DNA/RNA extraction kit (Tiangen Biotech Co., Ltd., Beijing, China) was used to extract virus DNA or RNA, stored at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$ .

### Primer Screening and Probe Design

GenBank was searched for the VP2 gene sequence of IBDV (E05443.1, MN700903.1, MT215084.1, MT215085.1, MT215086.1, MT215087.1, MN652177.1, AY780423.1, LM651365.1). DNAMAN was used to compare multiple sequences and determine the conserved region. Three pairs of primers were designed according to the RT-RAA primer and probe design principles. RAA-AGE and BLAST in NCBI were used for

specificity analysis and comparison, and the optimal primers were selected. The probe was designed and modified according to the selected primers. The primer and probe were sent to a biotechnology company (Sangon Biotech Co., Ltd., Shanghai, China) for synthesis and labeling (Table 1).

### Construction of Standard Plasmids

The IBDV cDNA was used as the template for PCR amplification, recovered by agarose electrophoresis gel, purified, and connected to T-Vector pMD 20 vector, and introduced into the DH5 $\alpha$  competent cells. After incubation at  $37^{\circ}\text{C}$  for 1 h, 100  $\mu\text{L}$  of bacterial solution was evenly coated on LB solid medium containing ampicillin resistance, and incubated overnight at  $37^{\circ}\text{C}$ . After blue and white screening, white colonies were selected and placed in LB broth medium for overnight culture at  $37^{\circ}\text{C}$ . The plasmid was extracted and the concentration was measured. The DNA copy number contained in the plasmid per unit volume was calculated according to Moore's law. The calculation formula is as follows:

Plasmid copy number (copies/ $\mu\text{L}$ ) = [plasmid concentration (g/ $\mu\text{L}$ )  $\times$   $6.02 \times 10^{23}$ ] / [total fragment length (bp)  $\times$  660 g/mol]; total fragment length = vector length (bp) + fragment length (bp).

The constructed standard plasmid was diluted to a concentration gradient of  $10^9$  to  $10^0$  copies/ $\mu\text{L}$  by 10-fold dilution method and stored at  $-20^{\circ}\text{C}$  for later use.

### Establishment of RT-RAA-LFD Method

According to the VP2 gene sequence of IBDV (MN652177.1), the requirements of RT-RAA kit (Qitian Biological Technology Co. Ltd., Nanjing, China), a 50  $\mu\text{L}$  reaction system was established: 25  $\mu\text{L}$  buffer, 16.7  $\mu\text{L}$  purified water, 2.1  $\mu\text{L}$  forward primers, 2.1  $\mu\text{L}$  reverse primers, 0.6  $\mu\text{L}$  probe, 1  $\mu\text{L}$  template, 2.5  $\mu\text{L}$  magnesium acetate, reaction at  $39^{\circ}\text{C}$  for 30 min. Ten  $\mu\text{L}$  of product and 90  $\mu\text{L}$  of buffer were put into a 1.5 mL centrifuge tube, and then the LFD was inserted into the centrifuge tube for detection. After 3 min, the test results could be observed.

**Table 1.** Primers and probes for detecting IBDV by RT-RAA-LFD and RT-PCR.

Primers and probes		Sequence(5'-3')	Size (bp)	Gene localization
RT-RAA	F1	TAACCGTTCTCAGCTTACCCACATCATAACG	30	455–484
	R1	CGCAGTCCCATCAAAGCCTATAAGGTAGATT	31	744–774
	F2	ACGATCTCGGGTATGTGAGGCTTGGTGACC	30	482–511
	R2	AGTACAAGGTTTTGGATGCTGGTTTTGAAC	30	705–734
	F3	CTGCTGTAGGGCTCGACCCAAAGATGGTAGC	31	518–548
	R3	ATGTTGGCTGAGAACAGTGTGATTGTTACC	30	636–665
RT-RAA-LFD	F2	ACGATCTCGGGTATGTGAGGCTTGGTGACC	30	482–511
	R3	Biotin-ATGTTGGCTGAGAACAGTGTGATTGTTACC	30	636–665
	Probe	FAM-TACACCATAACTGCAGCCGACAATTACCAATTCT/idSp/ ATCACAGTACAAGACAG-P	52	580–631
RT-PCR/RFQ-PCR	F	ACGATCTCGGGTATGTGAGG	20	482–501
	R	CGCAGTCCCATCAAAGCCTA	20	755–774

Abbreviations: IBDV, infectious bursal disease virus; RT-RAA, reverse transcription-recombinase-aided amplification; RT-RAA-LFD, reverse transcription-recombinase-aided amplification-lateral flow dipstick; RT-PCR, reverse transcription-polymerase chain reaction; RFQ-PCR, real-time fluorescence-based quantitative polymerase chain reaction.

Judging criteria: the test result was positive when there were 2 lines on the LFD, one in the quality control area (blue), the other in the detection area (red); the test result was negative when only a blue line appeared in the quality control area.

### **Optimization of RT-RAA-LFD Reaction Conditions**

The above reaction system was used to optimize the reaction temperature, reaction time, and the concentration of primers. The reaction temperature was set to 35, 36, 37, 38, 39, 40, 41°C, respectively; the reaction time was set to 5, 10, 15, 20, 25 min, respectively; the concentration of primers was set to 10,000, 5,000, 2,500, 1,250, 625 nmol/L, respectively. Judging criteria for optimal reaction conditions: clear quality control line, lowest reaction temperature, shortest reaction time, and lowest primer or probe concentration.

### **Specific Test of RT-RAA-LFD**

With the DNA or RNA of IBDV, MDV, CIAV, REV, and ALV as templates and purified water as negative control, the optimized reaction system and conditions were used to evaluate the specificity of RT-RAA-LFD in the detection of IBDV.

### **Comparison of Sensitivity of Three Methods in Detecting IBDV**

Different concentrations of plasmids were used as templates for IBDV detection by RT-RAA-LFD, RT-PCR, RFQ-PCR. With ddH<sub>2</sub>O as negative control, the lowest detectable limit (LDL) of the 3 detection methods was compared.

RT-RAA-LFD used 10<sup>5</sup> to 10<sup>0</sup> copies/μL plasmids as template, and the optimized reaction conditions and system were employed.

RT-PCR used 10<sup>7</sup> to 10<sup>0</sup> copies/μL plasmids as template. 25 μL reaction system: 12.5 μL 2 × Taq Mix (Takara Co., Ltd., Dalian, China), 0.5 μL forward primers, 0.5 μL reverse primers, 9.5 μL ddH<sub>2</sub>O, 2 μL DNA template. Reaction system: predenaturation at 94°C for 5 min; denaturation at 94°C for 30 s; annealing at 53°C for 30 s; extending at 72°C for 30 s, 34 cycles for amplification; extending at 72°C for 5 min, preservation at 4°C. The results were observed by electrophoresis in 2% agarose gels.

RFQ-PCR used 10<sup>6</sup> to 10<sup>0</sup> copies/μL plasmids as template. 25 μL system (primers same as those in RT-PCR): 12.5 μL TB Green Premix Dimer Eraser (2X), 0.75 μL forward primers, 0.75 μL reverse primers, 2 μL template, the final volume was made up to 25 μL with ddH<sub>2</sub>O. Reaction system: predenaturation at 95°C for 30 s, denaturation at 95°C for 5 s, annealing at 55°C for 30 s, extending at 72°C for 30 s, the results were observed after 40 cycles.

### **Repeatability Test of RT-RAA-LFD**

The established RT-RAA-LFD method for the detection of IBDV was tested by intragroup and intergroup repeatability tests respectively. In the intragroup repeatability test, the IBDV standard with the dilution concentration of 10<sup>6</sup> copies/μL-10<sup>2</sup> copies/μL was used as the template for 3 parallel replicates under the same reaction system and reaction conditions. Inter-group repeatability test, 3 different batches of IBDV standards with dilution concentration of 10<sup>6</sup> copies/μL-10<sup>2</sup> copies/μL were used as templates for 3 times under the same reaction system and reaction conditions to verify the repeatability and stability of the established RT-RAA-LFD method for the detection of IBDV.

### **Detection of Clinical Samples**

The bursa of Fabricius of 48 chickens with clinical symptoms suspected of IBD infection from 15 chicken farms in Hebei Province were detected by RT-RAA-LFD and RT-PCR, and the coincidence rate of the 2 methods was compared.

## **RESULTS AND DISCUSSION**

### **Screening of RT-RAA Primers**

According to the designed 3 pairs of forward primers and 3 pairs of reverse primers were combined without repetition (F1&R1, F1&R2, F1&R3, F2&R1, F2&R2, F2&R3, F3&R1, F3&R2, F3&R3). IBDV was detected by RT-RAA reaction system, and the product was determined by AGE. The results show that the 6 band was clear and accords with the position of the target segment, so the best primer combination selected was F2&R3.

### **Optimization of RT-RAA-LFD Reaction Conditions**

The temperature was 35 to 41°C, and a faint red line appeared at 36°C. When the temperature increased, the definition of the band was the same. A faint red line appeared at 10 min, the line was clear when the time was prolonged. Red lines were shown for all primer and probe concentrations except 625 nmol/L. Based on the abovementioned judging criteria, the optimal reaction conditions for IBDV detection by RT-RAA-LFD were determined as temperature of 37°C, reaction time of 15 min, primer and probe concentration of 1250 nmol/L.

### **Specificity of RT-RAA-LFD in Detecting IBDV**

IBDV and common viruses that can easily cause immunosuppression in chickens were detected by RT-RAA-LFD at the same time. Under the same reaction conditions, only the LFD detection area of IBDV showed a red line, and the result was positive. No redline was found in other viruses (MDV, CIAV,

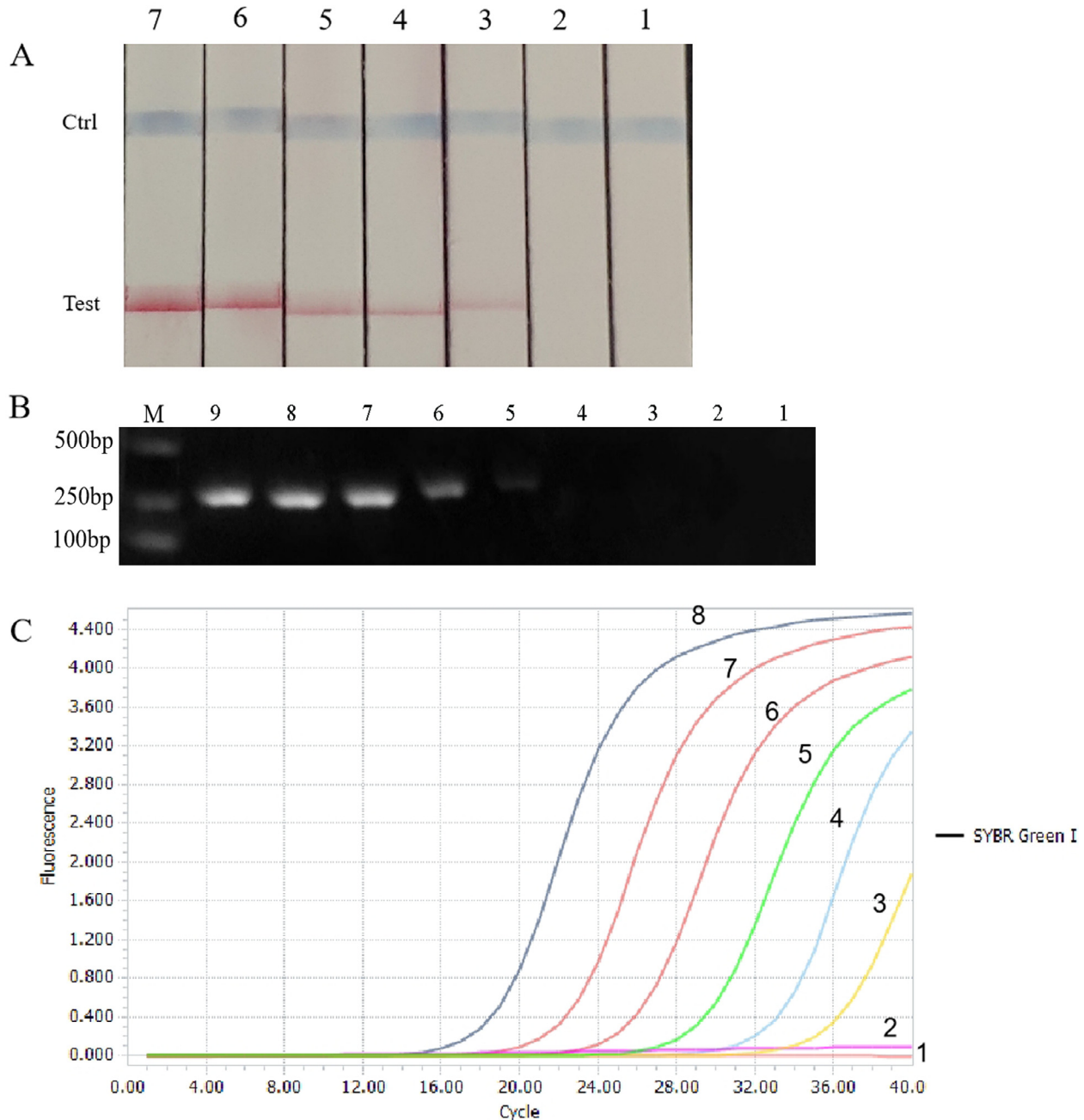
ALV, REV) and control group, and the results were all negative. The results showed that IBDV had no cross reaction with other pathogens, and RT-RAA-LFD had strong specificity.

### Comparison of Sensitivity in Detection Methods

The established RT-RAA-LFD, RFQ-PCR and RT-PCR were used to detect IBDV, respectively, and the

sensitivity of the 3 methods was compared. The LDL was  $10^3$  copies/ $\mu\text{L}$  for RT-PCR, 10 copies/ $\mu\text{L}$  for both RT-RAA-LFD and RFQ-PCR. The results are shown in Figure 1.

At present, RT-PCR and RFQ-PCR are the main detection methods of IBDV. The sensitivity of the RFQ-PCR method for IBDV detection established by Zhang Weiye (Zhang et al., 2018) can reach 10 copies/ $\mu\text{L}$ , but the analysis of results requires professional staff and equipment, which is not conducive to rapid detection in production. The RT-PCR method for IBDV detection



**Figure 1.** Comparison of sensitivity of three methods in detecting IBDV. (A) Sensitivity of IBDV detected by RT-RAA-LFD. 7-2 were  $10^5$  to  $10^0$  copies/ $\mu\text{L}$  of plasmid template, respectively, 1: negative control, LDL: 10 copies/ $\mu\text{L}$ . (B) Sensitivity of IBDV detected by RT-PCR. M: Marker, 9-2 were  $10^7$  to  $10^0$  copies/ $\mu\text{L}$  of plasmid template, respectively, 1: negative control, LDL:  $10^3$  copies/ $\mu\text{L}$ . (C) Sensitivity of IBDV detected by RFQ-PCR. 8-2 were  $10^9$  to  $10^0$  copies/ $\mu\text{L}$  of plasmid template, respectively, 1: negative control, LDL: 10 copies/ $\mu\text{L}$ . Abbreviations: IBDV, infectious bursal disease virus; LDL, lowest detectable limit; RT-RAA-LFD, reverse transcription-recombinase-aided amplification-lateral flow dipstick; RT-PCR, reverse transcription-polymerase chain reaction; RFQ-PCR, real-time fluorescence-based quantitative polymerase chain reaction.

established by Li Jijuan (Li and Zhang, 2018) has a sensitivity of 100 pg/ $\mu$ L, but it takes 6 h to complete the detection, which is time-consuming. The RT-RAA-LFD method for IBDV detection established in this experiment could be completed at 37°C for 15 min, and the LDL was 10 copies/ $\mu$ L, which was 100 times that of RT-PCR (10<sup>3</sup> copies/ $\mu$ L), equal to that of RFQ-PCR, while the reaction time required was shorter and the detection results could be more easily observed than those by RFQ-PCR.

### **Repeatability of RT-RAA-LFD in Detecting IBDV**

The repeatability test of the method of RT-RAA-LFD for the detection of IBDV was carried out. The results of three replicates within the group and 3 replicates between the groups were positive for the standard of IBDV with the dilution concentration of 10<sup>6</sup> copies/ $\mu$ L-10<sup>2</sup> copies/ $\mu$ L, indicating that the establishment of the method of RT-RAA-LFD for the detection of IBDV had good stability and repeatability.

### **Test of Clinical Samples**

Viral DNA/RNA extraction kit was used to extract nucleic acid from 48 clinical samples, which were detected by RT-RAA-LFD and RT-PCR. Forty one cases were positive and 7 cases negative by RT-PCR, 43 cases were positive and 5 cases negative by RT-RAA-LFD. The total coincidence rate was 95.83% between RT-RAA-LFD and RT-PCR. It can be seen that RT-RAA-LFD can be used for clinical IBDV detection.

The RT-RAA-LFD method established in this experiment can save the detection time of IBDV. Under the same sample preparation operation, RT-RAA-LFD can be completed in half an hour, while RT-PCR and agarose gel electrophoresis were about 3 h. The RT-RAA-LFD method has high specificity and sensitivity, and the reaction results can be observed and judged by naked eyes, which is suitable for disease diagnosis on the farm and field environment, and provides a new method for the detection of IBDV.

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### **DISCLOSURES**

The authors declare that they have no competing interests, the manuscript has not been published or submitted to other journals previously.

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