Recombinase-aided amplification–lateral flow dipstick assay—a specific and sensitive method for visual detection of avian infectious laryngotracheitis virus

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ABSTRACT The purpose of this study was to explore a specific, simple, and sensitive method for diagnosis of avian infectious laryngotracheitis virus. Recombinase-aided amplification (**RAA**) and lateral flow dipstick (**LFD**) were combined for labeling the optimized RAA probe with 6-carboxyfluorescein (FAM) and the 5′-end of the downstream primer with biotin, respectively. By optimizing the reaction time, temperature, and primer concentration of RAA, a RAA–LFD assay, which could be used for detection of infectious laryngotracheitis, was established. After the specificity and sensitivity test, the target gene fragments could be amplified by RAA–LFD assay in 20 min under isothermal conditions (37°C), and the amplification products could be visually observed and determined by LFD within 3 min. There was no cross-reaction with nucleic acids of other avian pathogens, the lowest detectable limit of RAA–LFD was 10^2 copies/μL, and the sensitivity of this method was 100 times higher than that of conventional PCR with the lowest detectable limit of 10^4 copies/μL. The results showed that RAA–LFD assay was highly sensitive, easy to use, and more suitable for clinical detection.

Key words: avian infectious laryngotracheitis virus, recombinase-aided amplification, lateral flow dipstick

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INTRODUCTION

Avian infectious laryngotracheitis (**ILT**) is a highly infectious respiratory disease caused by avian infectious laryngotracheitis virus (**ILTV**) (Ali et al., 2019). It is one of the main infectious diseases endangering the poultry industry (Zhao et al., 2013; Menendez et al., 2014). Once infected, it will cause a large area of disease and cause serious economic losses. The virus mainly exists in the tracheal tissue and exudates of diseased chickens. Acute onset and rapid transmission are characteristic of ILT, and chickens infected with ILTV will experience shortness of breath and have a runny nose, cough, and other symptoms (Song, 2019). With further development of disease, runny nose–infected birds develop nasal exudates and cough bloody exudates from the trachea. Some affected chickens die of asphyxiation owing to blockage of the respiratory tract (Shan, 2019). Infectious laryngotracheitis spreads quickly and is highly infectious and difficult to eliminate from the chicken house environment.

The clinical symptoms and pathological changes of ILT are similar to those of Newcastle disease (**ND**), infectious bronchitis (**IB**), and low pathogenic avian influenza, and it is very easy to be misdiagnosed, which brings some difficulties to clinical diagnosis (Zhang, 2019). At present, the main methods for detection of ILTV include PCR, real-time fluorescence-based quantitative-PCR (**RFQ-PCR**) (Zhang et al., 2018), ELISA, conventional virus isolation, and loop-mediated isothermal amplification. However, PCR needs special instruments, and agarose gel electrophoresis mostly uses dyes that are harmful to the body; RFQ-PCR involves expensive instruments and complex operations; conventional virus isolation may not give accurate results and takes a long time; ELISA has a problem of nonspecific reaction in the detection of antibodies (Meulemans and Halen, 1982); loop-mediated isothermal amplification reaction requires multiple pairs of primers and high requirements for target genes (Poon et al., 2005; Li et al., 2015).

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Consequently, there is an urgent need for a simple, fast, specific, and user-friendly detection method that requires no expensive instrument. Recombinase-aided amplification (**RAA**) is a rapid, specific, sensitive, and reliable technique for isothermal gene amplification (Daher et al., 2016). The recombinase combines with primers in the RAA reaction system to form a complex, moving to look for homologous DNA sequences in the genome template, followed by strand displacement, which realizes easy and rapid nucleic acid detection (Piepenburg et al., 2006).

In this experiment, using RAA combined with lateral flow dipstick (**LFD**), the downstream primer was labeled with biotin, and the designed probe was modified with a FAM fluorophore at its 5′-end as per the requirements of the kit. (The probe generally needs to be 46- to 52-bp long, with at least 30 bases from the 5′-end to the position modified with tetrahydrofuran [**THF**] residue and at least 15 bases from the position modified with THF residue to the 3′-end.) There was a single-base gap at a position 30 bp from the 5′-end, which was modified with THF residue, and the 3′-end was blocked by phosphorylation. A compatible probe was helpful to improve the specificity of RAA reaction. The double-labeled products amplified by RAA could be detected directly by LFD and observed with naked eye. The LFD assay is a combination of immunization, molecular hybridization, colloidal gold labeling, and lateral flow immunoassay, using the working principle of sandwich ELISA for rapid detection of RAA amplification products, and the 2 strands of the amplification products contain 2 antigens, biotin and fluorescein isothiocyanate, respectively, to bind with the antibodies on dipstick. Through this method, the reaction could be completed at a constant temperature (37°C–39°C), and the amplification process did not require complex instruments. In this experiment, a method for the detection of ILTV by RAA–LFD assay was established, and it was verified by the specificity test, sensitivity test, and comparison with the coincidence rate of PCR test results. This experiment provided a convenient new technique for rapid visual detection and identification of ILTV as well as the rapid field test by grassroots quarantine departments in future.

MATERIALS AND METHODS

Primer and Probe Design

Compared with the thymidine kinase gene sequence of ILTV, primers and probes were designed as per the requirements of the RT-RAA nucleic acid amplification kit (Qitian Gene Biological Technology Co., Ltd., Hangzhou, China). Different from PCR primer design, the RAA primer is about 30–35 bp in length, which is longer than the PCR primer. The downstream primer was labeled with biotin. The probe was modified with a FAM fluorophore at its 5′-end. There was a single-base gap at a position 30 bp from the 5′-end, which was modified with THF residue, and the 3′-end was blocked by phosphorylation. Primers and probes were synthesized and labeled by Sangon Biotech (Shanghai) Co., Ltd (Sangon Biotech Co., Ltd, Shanghai, China).

Establishment of RAA–LFD Assay

The reaction system was prepared as per the kit: DNA extracted from ILTV (strain Wanggang AV195 was purchased from China Institute of Veterinary Drug Control) was used as a template. Ⅵ reaction buffer 25 μL, upstream and downstream primers (10 μM) 2.1 μL each, probe 0.6 μL, template 1 μL, purified water 16.7 μL, and magnesium acetate 2.5 μL. The aforementioned reaction tube was put into a 39°C water bath to react for 20 min. Ten microliters of RAA products was transferred to the test area, the LFD (Ustar Biotechnologies Co., Ltd., Hangzhou, China) was inserted into a 2-mL centrifuge tube with 100 μL of buffer, and the results were observed after 3 min. The dipstick showed positive results when 2 red bands appeared: one in the quality control area and the other in the test area. The positive results indicated that the sample contained nucleic acid fragments to be detected. If there was a red band in the quality control area while no red band was found in the test area, the dipstick had no quality problem and the sample tested negative, suggesting that no nucleic acid fragments were tested.

Optimization of Reaction Conditions

Optimization of Reaction Temperature Temperature was controlled at 33°C, 35°C, 37°C, 39°C, and 41°C as per optimization time.

Optimization of Reaction Time Reaction time was limited to 2, 5, 8, 10, 12, 14, 16, 18, and 20 min at 37°C.

Optimization of Reaction Primer and Probe Concentrations After reaction time and temperature were optimized, primer and probe concentrations were optimized on the basis of 10,000, 5,000, 2,500, 1,250, and 625 nmol/L.

Reaction conditions were the best when the test line appeared clearly, reaction time was the shortest, and primer or probe concentration was the lowest.

Specificity Test

The RNA of IB virus, ND virus, and Avian influenza virus (**AIV**) (3 kinds of virus preserved in our laboratory after nucleic acid detection) were extracted by using the virus genomic DNA/RNA extraction kit (Tiangen Biotech Co., Ltd., Beijing, China), and the corresponding cDNA was obtained by reverse transcription; DNA was extracted from ILTV; purified water was used as the negative control. The specificity of this method was verified by the RAA reaction system with the addition of a specific probe.

Table 1. Primers and probe.

Method	Primers and probe	Sequences (5′–3′)
RAA–LFD	F1	CAGTATCTGGCATCGCCTCATTTCTTTCTA
	R1	Biotin-CTCATCACTATCCTCCTCAACCTCCTCCTC
	T	FAM-TTCCCCCGGCCGGAACTCCTCCACGACCCTC-THF-AGACGTTACTACAAG/C3-spacer/
PCR and RFQ-PCR	F2	CAGTATCTGGCATCGCCTCAT
	R2	CTCATCACTATCCTCCTCAAC

Abbreviations: LFD, lateral flow dipstick; RAA, recombinase-aided amplification; RFQ-PCR, real-time fluorescence-based quantitative-PCR.

Sensitivity Test

Preparation of Recombinant Plasmids The extracted ILTV genomic DNA was used as the template DNA, and the length of the primer was modified as per the requirements of conventional PCR (Table 1). The PCR reaction system (50 μL) consisted of the following: 2×Taq Mix, 25 μL; DNA template, 2 μL; and upstream and downstream primers (10 μM), 1 μL each, replenished with 21 μL of ddH_2O. The reaction system was predenaturalized at 94°C for 5 min; denaturalized at 94°C for 45 s; annealed at 56°C for 45 s; extended at 72°C for 60 s, a total of 35 cycles of amplification; extended at 72°C for 10 min, and then stored at 4°C. The purified target fragment was ligated to pMD20 (Takara Biomedical Technology Co., Ltd., Beijing, China) plasmid after gel extraction. The standard plasmid was selected after purification and screening, and the DNA copy number per unit volume of plasmid was calculated as per Moore's law.

Plasmid copy number (copies/L) = [plasmid concentration ($g \cdot \mu L^{-1}$) × 6.02×10^{23}]/[total fragment length (bp) × 660 g/mol], total fragment length = vector length (bp) + fragment length (bp).

The standard plasmid was diluted to different gradients (10^0–10^7 copies/μL); RAA–LFD assay, conventional PCR assay, and RFQ-PCR assay were carried out; and the replicate was set.

The RAA reaction system was based on the optimized reaction conditions.

The PCR system (25 μL) consisted of the following: 2×Gflex PCR Buffer (Mg^{2+}, dNTP plus), 12.5 μL; upstream and downstream primers (10 μM), 0.5 μL each; TksGflex DNA Polymerase (1.25 units/μL), 0.5 μL; template, 1 μL. The final volume was made up to 25 μL with ddH_2O. The reaction system was predenaturalized at 94°C for 1 min; denaturalized at 98°C for 10 s; annealed at 55°C for 15 s; extended at 68°C for 30 s, a total of 30 cycles; and finally extended at 68°C for 5 min.

The RFQ-PCR system (25 μL) (primers the same as those in conventional PCR) consisted of the following: TB Green Premix DimerEraser (2×), 12 μL; upstream and downstream primers (10 μM), 0.75 μL each; template, 2 μL. The final volume was made up to 25 μL with ddH_2O. The reaction system was predenaturalized at 95°C for 30s; denaturalized at 95°C for 5 s; annealed at 55°C for 30 s; and extended at 72°C for 30 s, a total of 40 cycles.

Detection of Clinical Samples

Forty-five suspected ILTV throat swab samples were detected by RAA–LFD assay and conventional PCR assay, and the coincidence rate of the 2 methods was compared. The throat swab was collected from 7 unvaccinated chicken farms in Hebei Province, China, and the diseased chickens were diagnosed with ILT by symptom observation and clinical dissection.

RESULTS AND DISCUSSION

Optimization of Reaction Conditions

In this experiment, the reaction time, temperature, and primer concentration were optimized. Compared with the results at 39°C, the band color was similar, but the reaction temperature was lower at 37°C. After 14 min, the band appeared in the detection area, and after 20 min, the band was clear. The detection line was clear, and the required concentration was the lowest when the concentration of primers was 1,250 nmol/L in the reaction system.

Finally, the optimum reaction conditions were determined as follows: reaction temperature, 37°C; reaction time, 20 min, and primer concentration, 1,250 nmol/L (Figure 1).

Specificity Test

Using the cDNAs of DNA, IB virus, ND virus, and AIV of ILTV as templates, the products were amplified in a reaction system using the RT-RAA nucleic acid amplification kit. After LFD detection, it was found that the test line and quality control line appeared on dipstick at the same time for the RAA products of ILTV, whereas only the quality control line appeared for the products of other viruses and the negative control (Figure 2), showing that this system had no cross-reaction with other viruses and had good specificity.

Sensitivity Test

The standard plasmid was diluted by 10-fold gradient, obtaining 10^0–10^7 copies/μL for testing. The results showed that the lowest detectable limit (**LDL**) was 10^4 copies/μL for conventional PCR, 10^1 copies/μL for RFQ-PCR, and 10^2 copies/μL for RAA–LFD assay

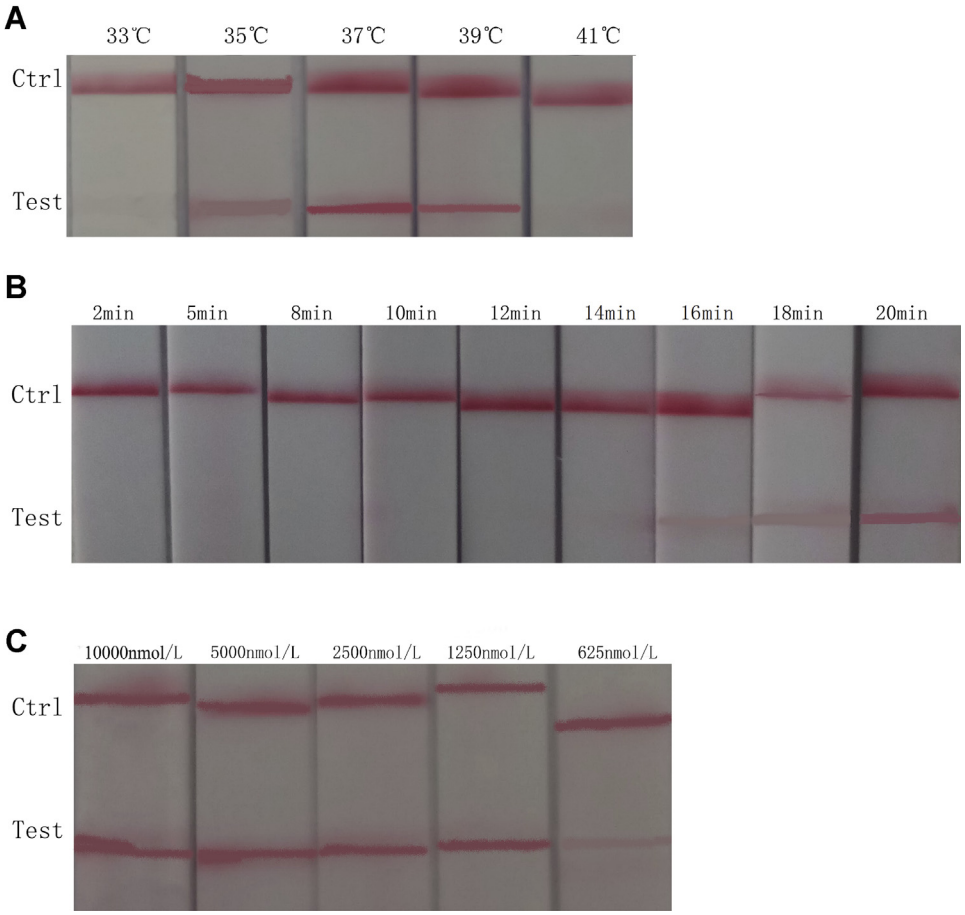

Figure 1. Screening and determination of the best reaction conditions for the detection of ILTV by RAA–LFD. (A) Optimization of RAA–LFD reaction temperature for ILTV detection. The amplified reaction works effectively within a wide range of reaction temperatures from 35°C to 39°C. A light colored band appeared in the detection area at 35°C. With the increase of temperature, the amplification product increased, and the band color darkened. The amplification product decreased, and the band color became lighter at 41°C. The optimum reaction temperature was determined to be 37°C. (B) Optimization of RAA–LFD reaction time for ILTV detection. After 20 min, the amplified reaction works effectively. After 14 min, the band appeared in the detection area, and the band of the detection line became clearer after 20 min. Considering the experimental time and the reliability of the results, the best reaction time was determined to be 20 min. (C) Optimization of primer and probe concentrations of RAA–LFD assay for ILTV detection. The amplified reaction works effectively within a wide range of primer concentration of 1,250 nmol/L–10,000 nmol/L. When the concentration of primer is 1,250 nmol/L, the detection line was clear and the required concentration was the lowest, so the best concentration of the primer and probe was determined to be 1,250 nmol/L. Abbreviations: ILTV, infectious laryngotracheitis virus; LFD, lateral flow dipstick; RAA, recombinase-aided amplification.

(Figure 3). The results of the repeated tests were the same. The sensitivity of RAA–LFD assay was lower than that of RFQ-PCR but higher than that of conventional PCR.

Detection of Clinical Samples

A total of 45 suspected clinical samples were detected; of which, 35 were positive and 10 were negative by RAA–LFD assay; 32 were positive and 13 were negative by conventional PCR assay. The coincidence rate of the RAA and PCR methods was 93.33% (Table 2), indicating that the RAA–LFD assay established in this study could be used for detection of ILTV.

Compared with conventional PCR, RAA is easy to transport and use and highly sensitive. Recombinase-aided amplification places particularly low demands on operating temperature, and the reaction does not require thermal or chemical dissolution at a temperature of 37–42°C, so expensive device is not required. PCR needs to go through the process of denaturation, annealing, and extension and depends on temperature control equipment. Recombinase-aided amplification reaction is much faster than PCR reaction and can usually be carried out within 15–20 min. Recombinase-aided

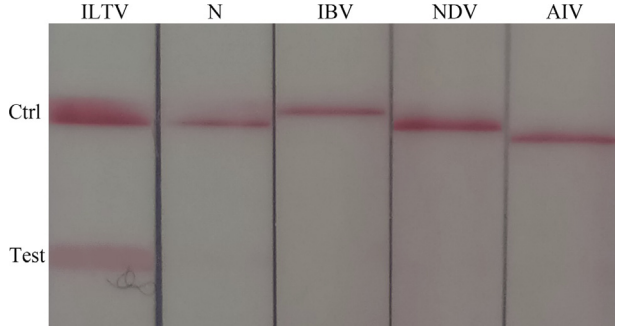

Figure 2. Specificity test by RAA–LFD assay for ILTV detection. It was found that the test line and quality control line appeared on dipstick at the same time for the RAA products of ILTV, whereas only the quality control line appeared for the products of other viruses (IB virus, ND virus, and AIV) and the negative control (N). Abbreviations: IB, ILTV, infectious laryngotracheitis virus; LFD, lateral flow dipstick; ND, Newcastle disease; RAA, recombinase-aided amplification.

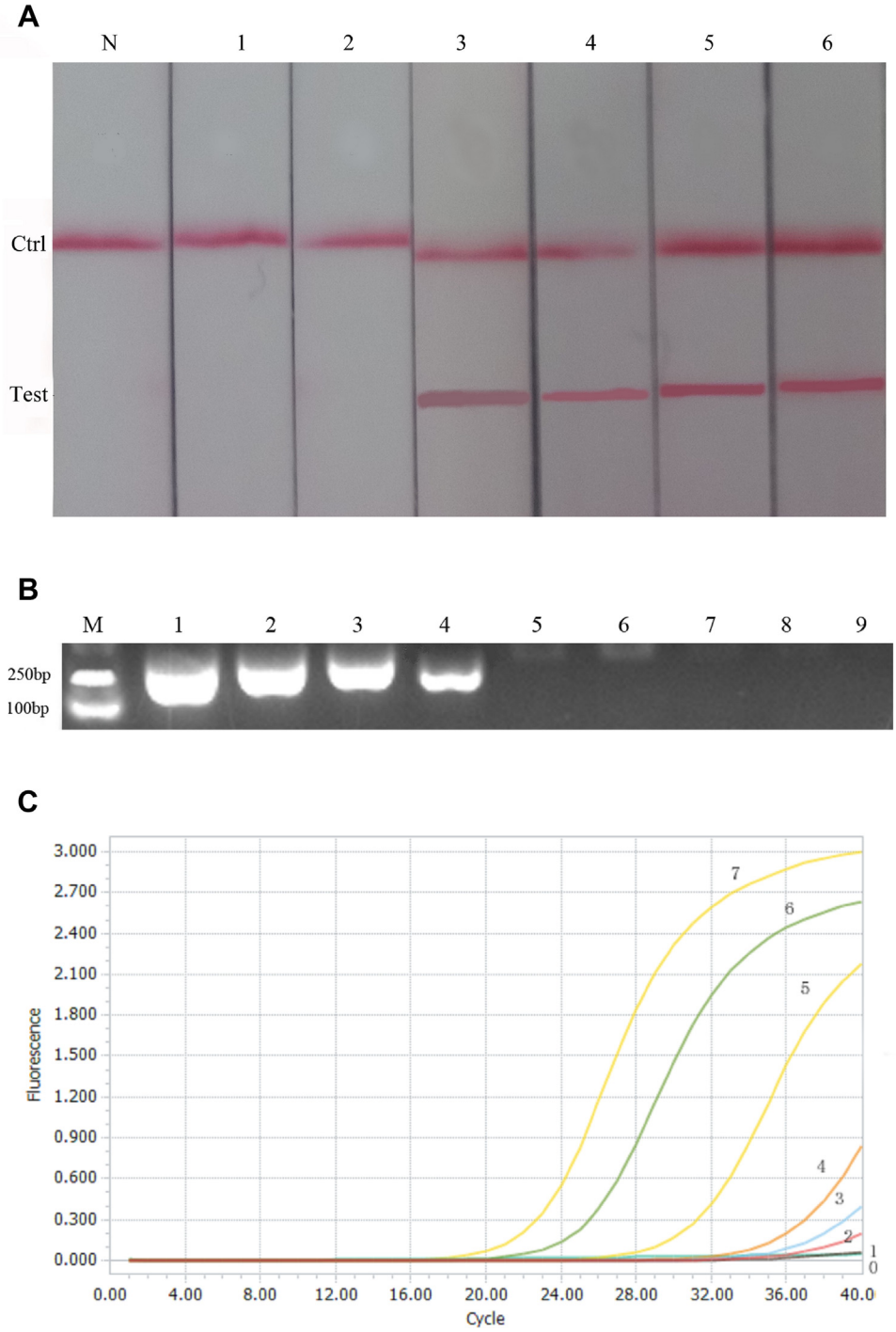

Figure 3. Compare the sensitivities of the 3 methods for ILTV detection. (A) Sensitivity of RAA–LFD assay for ILTV detection. N, negative control; 1–6, template concentration of 10^0–10^5 copies/μL. The results showed that the lowest detectable limit was 10^2 copies/μL for RAA–LFD assay. (B) Sensitivity of PCR assay for ILTV detection. M, marker; 1–8, concentration of 10^7–10^0 copies/μL; 9, negative control. The results showed that the lowest detectable limit was 10^4 copies/μL for PCR. (C) Sensitivity of RFQ-PCR assay for ILTV detection. 0, negative control; 1–7, 10^0–10^6 copies/μL. The results showed that the lowest detectable limit was 10 copies/μL for RFQ-PCR. Abbreviations: ILTV, infectious laryngotracheitis virus; LFD, lateral flow dipstick; RAA, recombinase-aided amplification; RFQ-PCR, real-time fluorescence-based quantitative-PCR.

amplification is preserved with lyophilized reagents, and it has a simple workflow and does not require professional training (Yang et al., 2018). At present, this technology has been widely used in many fields such as human diseases, veterinary medicine, food industry, and agriculture (Lu et al., 2010; Chen et al., 2018).

In this experiment, the visual detection of ILTV was realized by combining RAA with LFD. The results showed that when the concentration of the primer and probe was 1,250 nmol/L, the specificity of RAA–LFD assay (37°C, 20 min) was good and the sensitivity of RAA–LFD assay (10^2 copies/μL) was 100 times higher than that of PCR and slightly lower than that of RFQ-PCR. The coincidence rate of RAA–LFD detection results and PCR test results was 93.33%, which proved that RAA–LFD assay was an effective detection

Table 2. The coincidence rate of the 2 methods for ILT clinical detection.

Case	PCR, positive	PCR, negative	The coincidence rate (%)*
RAA–LFD, positive	32[a]	3[c]	93.33
RAA–LFD, negative	0[d]	10[b]	

Abbreviations: ILT, infectious laryngotracheitis; LFD, lateral flow dipstick; RAA, recombinase-aided amplification.

*The coincidence rate (%) = (a+b)/(a+b+c+d)%.

technique. Recombinase-aided amplification–lateral flow dipstick assay really realized the visual detection of ILTV, provided more convenient technical support for the prevention and control of ILT, and had a certain practical significance for the development of poultry industry. Recombinase-aided amplification–lateral flow dipstick assay shortens the detection time, and efficient detection helps prevent and control ILT.

CONCLUSION

The RAA–LFD assay had good specificity for detection of ILT and had no cross-reaction with the nucleic acids of other kinds of avian pathogens. Recombinase-aided amplification–lateral flow dipstick assay had an LDL of 10^2 copies/μL, and its sensitivity was 100 times higher than that of conventional PCR (LDL of 10^4 copies/μL). It was easy to operate, and the results could be visually observed and determined by LFD within 3 min after 20-min reaction under isothermal conditions (37°C), so RAA–LFD assay was very suitable for clinical veterinary research and grassroots laboratory use.

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DISCLOSURES

The authors declare no conflicts of interest.

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