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Simultaneous and visual detection of infectious bronchitis virus and Newcastle disease virus by multiple LAMP and lateral flow dipstick

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ABSTRACT Infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) are both important viruses seriously affecting poultry industry worldwide. In this study, reverse-transcription LAMP (RT-LAMP) was combined with lateral flow dipstick (LFD) forming a novel detection tool which could simultaneously detect IBV and NDV visually. Primers targeted the 5'-untranslated region (5'-UTR) of IBV genome and the conserved region of NDV large polymerase gene (LP). The specificity and sensitivity of this multiple reverse transcription-LAMP-LFD (mRT-LAMP-LFD) assay were compared with those of conventional RT-PCR, nested RT-PCR (nRT-PCR), quantification RT-PCR (qRT-PCR), and RT-LAMP monitored by electrophoresis. No non-specific amplifications were observed when the assays were tested with unrelated

viruses. According to the sensitivity study, when detecting IBV or NDV alone, the lowest detection limits of mRT-LAMP-LFD were $10^{0.8}$ IBV RNA copies/reaction and $10^{0.7}$ NDV RNA copies/reaction. Furthermore, when detecting IBV and NDV simultaneously, the lowest detection limit was the same as that of the single detection assays. In the clinical sample study, mRT-LAMP-LFD performed the best among these assays. When tested with IBV or NDV single infected samples, the mean detection rates were 98.65% and 97.25%, respectively. In the IBV and NDV co-infected sample study, the mean detection rates of IBV and NDV were both 95%. This study showed that mRT-LAMP-LFD was a promising qualitative detection tool suitable for field single or multiple IBV and NDV detection.

Key words: infectious bronchitis virus, Newcastle disease virus, multiple pathogen detection, LAMP-LFD

2019 Poultry Science 98:5401–5411
<http://dx.doi.org/10.3382/ps/pez372>

INTRODUCTION

Infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) are 2 of the most important viruses seriously affecting the poultry industry and causing huge economic losses worldwide (Bande et al., 2017; Brown and Bevins, 2017). IBV and NDV belong to the *Gammacoronavirus* of the *Coronaviridae* family and the *Avulavirus* of the *Paramyxoviridae* family, respectively (<http://www.ictv.global>). The genome of IBV is about 27.6 kb in length. It encodes 15 non-structural proteins, and 4 structural proteins: spike glycoprotein (S), small membrane protein (E), membrane glycoprotein (M), and phosphorylated nucleocapsid protein (N). At the 5' and 3' ends of the genome, there is an untranslated region (UTR) each (Armesto et al., 2009). NDV possesses a 15 kb long genome comprising 6 genes

which individually encode the nucleocapsid (N), matrix protein (M), phosphoprotein (P), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large polymerase protein (LP) (de Leeuw and Peeters, 1999). IBV and NDV both have high mutation rates, making their prevention and control difficult. Quick and accurate detection of IBV and NDV is important for preventing the viruses from spreading.

A wide variety of diagnostic assays for IBV and NDV have been developed, including virus isolation, and serological and molecular assays (Bande et al., 2016; Brown and Bevins, 2017). Costs, requirements of stringent techniques, and time required limit the use of virus isolation as a routine virus detection assay (Bande et al., 2016). Serological assays, such as hemagglutination inhibition and ELISA, are faster and simpler than virus isolation, but tend to lack specificity and sensitivity, especially in the case of IBV, and poor cross-reactions between serotypes makes serological tests less applicable (Cavanagh, 2007; Miller et al., 2010). In view of their high sensitivity, specificity, and reduced flow time, molecular assays are the most commonly used

© 2019 Poultry Science Association Inc.

Received April 18, 2019.

Accepted June 9, 2019.

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methods for IBV and NDV monitoring. According to previous studies, both IBV and NDV quantification RT-PCR (**qRT-PCR**) detection methods were highly specific, and the lowest detection limits were 10^2 – 10^4 genome copies indicating that these qRT-PCR methods were highly sensitive (Callison et al., 2006; Farkas et al., 2009; Wise et al., 2004). Another highly specific and sensitive molecular method is nested RT-PCR (**nRT-PCR**) which involves 2 rounds of PCR amplifications. As previously reported, the lowest detection limits of IBV and NDV nRT-PCR assays were $10^{1.9}$ and $10^{4.0}$ EID₅₀/mL, respectively (Nguyen et al., 2013). While PCR assays are widely applied in pathogen detection, the conduct of PCR requires sophisticated laboratory equipment and observation of PCR product requires electrophoresis, making PCR assays unsuitable for point-of-care and visible detections, especially in some low-resource regions.

Loop-mediated isothermal amplification (**LAMP**) amplifies DNA under isothermal conditions by the *Bst* DNA polymerase large fragment (Notomi et al., 2000). Numerous studies have demonstrated that the amplification efficiency of LAMP is quite high (Khan et al., 2017; Zhang et al., 2014). Moreover, the specificity of LAMP is also satisfactory as there are 4 specially designed primers recognizing 6 distinct regions on the target DNA (Asiello and Baeumner, 2011; Zhang et al., 2014). Furthermore, unlike conventional PCR assays, only simple devices are needed during LAMP, such as a water bath or a heat block. LAMP is thought to revolutionize molecular biology not only because of its excellent performance on DNA amplification but also due to its diverse, simple, and intuitional reaction monitoring methods. Several naked eye monitoring approaches have been applied, such as adding color indicators into reactions and combining with immunochromatographic techniques (Parida et al., 2008; Zhang et al., 2014). Lateral flow dipstick (**LFD**), an immunochromatographic technique, utilizes antibody capture followed by secondary antibody labeling (Chen et al., 2016; Zhang et al., 2014). LAMP combined with LFD (**LAMP-LFD**) could be used for highly sensitive, simple, visual, and multiple pathogen detections (Chen et al., 2016). LAMP products can be labeled by employing biotin/FITC modified FIP/BIP primers, and subsequently, these biotin-FITC double labeled LAMP products can be captured by biotin-antibodies and immobilized at specific locations on LFD strips (test line). Subsequently, FITC at the other end of the products can specifically combine with gold particles labeled with FITC-antibodies, thus making the results readable using the naked eye (Nimitphak et al., 2008). However, no previous studies have reported multiple detection of avian pathogens using LAMP-LFD.

Both IBV and NDV are pathogens that cause avian respiratory diseases, and single or multiple infection by them may cause similar clinical signs. Studies have shown that multiple conventional RT-PCR could be used for detecting and differentiating respiratory dis-

ease pathogens in poultry diseases (Pang et al., 2002; Rashid et al., 2009). However, the sensitivity of multiple conventional RT-PCR is not satisfactory. Multiple nested RT-PCR is much more sensitive than multiple conventional RT-PCR but time-consuming (Nguyen et al., 2013). Furthermore, these assays are not suitable for on-site pathogen detection, because products of RT-PCR need to be monitored by electrophoresis and qRT-PCR need to be conducted with highly accurate instruments. Here, we developed a visual multiple RT-LAMP-LFD (**mRT-LAMP-LFD**) assay which could simultaneously detect IBV and NDV and be easily carried out and monitored by the naked eye. To evaluate this novel detection method, PCR assays (including conventional RT-PCR, qRT-PCR and nRT-PCR) and reverse-transcription LAMP (**RT-LAMP**) monitored by electrophoresis were also conducted and the specificity and sensitivity of the assays were compared with those of the mRT-LAMP-LFD assay.

MATERIALS AND METHODS

Virus Strains and Tissue Samples

A total of 13 IBV strains, 7 NDV strains, and the PCR and LAMP target sequences of 6 NDV and 1 turkey coronavirus strains (**TCoV**) synthesized by Sangon Biotech (Shanghai, China) Co, as well as 6 other avian virus strains, were used for the determination of the specificities of RT-PCR and RT-LAMP assays. The GenBank numbers of IBV and NDV strains were labeled in Figure S1 (Supplementary Information); the TCoV strain and the 6 other avian virus strains used in the specificity study were listed in Table S1 (Supplementary Information). Tissue samples used in this study were stored at -80°C .

RNA Extraction and cDNA Synthesis

RNA in the samples was extracted with TRIzol (Invitrogen, Carlsbad, CA). Subsequently, cDNA was synthesized using PrimeScript RT reagent Kit (TaKaRa, Beijing, China) following the manufacturer's instructions. Briefly, the reverse transcription reaction mixture consisted of 2 μL 5 \times PrimeScript Buffer, 0.5 μL PrimeScript RT Enzyme Mix I, 0.5 μL Oligo dT Primer, 0.5 μL Random 6 mers, the RNA of the virus, and RNase Free dH₂O, thus creating a final volume of 10 μL .

Primer Design

Complete genome sequences of 224 IBV strains and 331 NDV strains available in GenBank were aligned using MEGA 6 software. Subsequently, to determine conserved regions in the IBV and NDV genomes, aligned results were used for similarity plotting analysis with the Simplot program 3.5.1. Primers for RT-PCR, nRT-PCR, qRT-PCR, and RT-LAMP assays were designed

Table 1. Details of PCR and LAMP primers.

Assay	Primer name	Primer sequence (5'-3')	Reaction or annealing temperature
IBV LAMP	IB-FIP	TGACCCCGTATTTTCACACAA-CGGGTGTGTGGAAGTAGC	65°C
	IB-BIP	TCCCCCACATACCTCTAAGGG-GCCGACCTTATGCGAGAA	
	IB-F3	CTGGTTCATCTAGCGGTAGG	
	IB-B3	ACCCCTACAAACGTCGTAT	
IBV qPCR	qIB-F ¹	GTTGCTGGTATCACTGCTTG	56°C
	qIB-R ¹	ACCGCTAGATGAACCAGA	
IBV nPCR	nIB-outF	CTCATAGGTGTTCCATTGC	53°C
	nIB-outR	CGTATAGCCGACCTTATG	
IBV LAMP-LFD	Bio-IB-FIP ²	Biotin-TGACCCCGTATTTTCACACAA-CGGGTGTGTGGAAGTAGC	65°C
	FITC-IB-BIP ²	FITC-TCCCCCACATACCTCTAAGGG-GCCGACCTTATGCGAGAA	
	IB-F3	CTGGTTCATCTAGCGGTAGG	
	IB-B3	ACCCCTACAAACGTCGTAT	
	ND-FIP	CAGGAACCTGTGTCTGGGGTCGG-CAATACGCTTTTCTCAAATGAGA	
	ND-BIP	CAGTCGTTTATAGGAATTTACAGGC-ATGGACGGAATTCCTGGA	
NDV LAMP	ND-F3	TGCATGTGCCACATGAGACT	67°C
	ND-B3	CTTTCCTCTGTATTCTCTCTCC	
	qND-F ¹	GAAACTCCTATACTTAGCAGAAG	
	qND-R ¹	GGTACTTCCGCCTGTAAATT	
NDV nPCR	nND-outF	CCTCTTGGTATAAGGCGTCCC	58°C
	nND-outR	TAATGGACGGAATTCCTGGA	
NDV LAMP-LFD	Dig-ND-FIP ²	Digoxigenine-CAGGAACCTGTGTCTGGGGTCGG-CAATACGCTTTTCTCAAATGAGA	67°C
	FITC-ND-BIP ²	FITC-CAGTCGTTTATAGGAATTTACAGGC-ATGGACGGAATTCCTGGA	
	ND-F3	TGCATGTGCCACATGAGACT	
	ND-B3	CTTTCCTCTGTATTCTCTCTCC	

¹Primers designed for qPCR assays were also used in conventional PCR and nPCR assays (act as inner primers).

²In the LAMP -LFD assays, IB-FIP and ND-FIP primers were individually 5' modified by biotin (Bio) and digoxigenine (Dig), IB-BIP and ND-BIP were both 5' modified by fluorescein isothiocyanate (FITC).

on base of IBV and NDV genome conservative regions: IBV primers targeted the 5'-UTR region, and NDV primers located in the conserved region of LP gene. Primers for RT-PCR, nRT-PCR, and qRT-PCR were designed by Primer Premier 6 software (Premier Inc., Palm Desert, CA); primers for RT-LAMP assays were designed by PrimerExplorer V4 software (Fujitsu, Tokyo, Japan). In mRT-LAMP-LFD reactions, modified FIP and BIP primers were used: Bio-IB-FIP was modified by biotin on the 5'-end, Dig-ND-FIP was modified by digoxigenin on the 5'-end, and FITC-IB-BIP and FITC-ND-BIP were modified by FITC on the 5'-end. The sequences of the primers are listed in Table 1.

PCR Assays

The most appropriate annealing temperatures for each pair of primers were determined using gradient PCR and listed in Table 1. Conventional RT-PCR reaction mixture consisted of 12.5 μ L 2 \times M5 Pfu PCR MasterMix (Mei5, Beijing, China), 10 pmol of each primer, 1 μ L template and double distilled water (ddH₂O) creating a final volume of 25 μ L. The PCR parameters included an initial denaturation step for 5 min at 94°C followed by 32 cycles of denaturation at 94°C for 50 s, annealing for 50 s, extension at 72°C for 10 to 30 s

depending on the sizes of the products and a final extension step at 72°C for 10 min.

nRT-PCR involved 2 rounds of amplifications. The reaction mixture and parameters of each round of amplification were the same as that of conventional RT-PCR. The products of conventional RT-PCR and nRT-PCR were monitored by electrophoresis in 1% agarose gels.

qRT-PCR assays were conducted with 10 μ L 2 \times SsoFast EvaGreen Supermix (BIO-RAD, Hercules, CA), 10 pmol of each primer, 1 μ L template and ddH₂O making the final volume to 20 μ L. The parameters were: initial denaturation for 30 s at 95°C followed by 39 cycles of denaturation at 95°C for 5 s, annealing/extension for 5 s, and a final melting curve at 65 to 95°C with increment 0.5°C/5 s. Data was analyzed by Bio-Rad CFX Maestro 1.1 software (BIO-RAD, Hercules, CA). Plate read was added during the extension and melting curve steps. To establish IBV and NDV qRT-PCR standard curves, fragments amplified using qIB-F/R and qND-F/R were individually cloned into *pEASY-T1* vector (Transgen, Beijing, China). Plasmids were extracted using TIANprep Mini Plasmid Kit (TIANGEN, Beijing, China). Subsequently, IBV plasmids were 10-fold serial diluted from 5.09×10^9 to 5.09×10^4 copies/ μ L and NDV plasmids were 10-fold serial diluted from 3.97×10^9 to 3.97×10^4 copies/ μ L. Diluted plasmids were used as standard samples during the establishment of

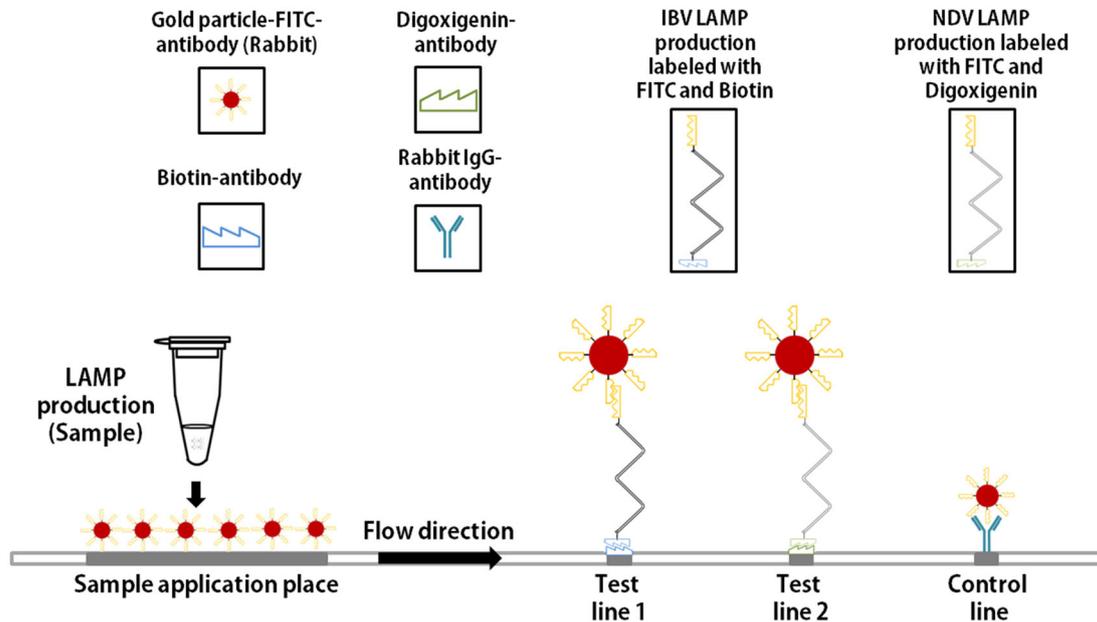


Figure 1. Principle of mRT-LAMP-LFD. IBV and NDV LAMP amplify products were labeled with FITC/Biotin and FITC/Digoxigenin, respectively. Gold particles modified with FITC-antibodies can combine with the labeled IBV and NDV products. Subsequently, IBV products will be captured by Biotin-antibodies immobilized on the test line 1, NDV products will be captured by Digoxigenin-antibodies immobilized on the test line 2, and the free gold particles will be immobilized on the control line. Thus, the products are visualized.

IBV and NDV qRT-PCR standard curves. Slopes and intercepts of standard curves, amplification efficiency (E) and corresponding correlation coefficients (R^2) were generated using Bio-Rad CFX Maestro 1.1 software (BIO-RAD, Hercules, CA).

LAMP Assays

The RT-LAMP reactions were conducted under gradient temperatures (50 to 68°C) to determine optimal reaction temperatures. The concentration of $MgSO_4$ (4 to 10 mM) and dosage of *Bst* 2.0 WarmStart DNA Polymerase (New England Biolabs, Ipswich, MA) (1 to 8 U) were also optimized. The optimal reaction mixture contains 2.5 μ L 10 \times Isothermal Amplification Buffer (contains 2 mM of $MgSO_4$), 4 mM of $MgSO_4$ (6 mM of $MgSO_4$ total), 1.4 mM of each dNTP, 1.6 μ M of FIP/BIP, 0.2 μ M of F3/B3, 8 U of *Bst* 2.0 WarmStart DNA Polymerase, 1 μ L template, and ddH₂O creating a final volume of 25 μ L. The mixture was incubated for 60 min. Products of LAMP were monitored visually using 2% agarose gel electrophoresis.

mRT-LAMP-LFD was performed to detect IBV and NDV either singly or simultaneously. The reaction mixture of mRT-LAMP-LFD contained 2.5 μ L 10 \times Isothermal Amplification Buffer (contains 2 mM of $MgSO_4$), 4 mM of $MgSO_4$ (6 mM of $MgSO_4$ total), 1.4 mM of each dNTP, 1.6 μ M of each modified primer (Bio-IB-FIP, Dig-ND-FIP, FITC-IB-BIP, and FITC-ND-BIP), 0.2 μ M of F3/B3, 8 U of *Bst* 2.0 WarmStart DNA Polymerase 1 μ L template, and ddH₂O creating a final volume of 25 μ L. After incubation for 60 min, 20 μ L of reaction product and 80 μ L of HybridDetect Assay Buffer (Milenia biotec, Gießen, Germany) were

mixed in a new tube, and subsequently an LFD strip (Milenia biotec, Gießen, Germany) was dipped into this mixture. After 3 to 5 minutes, test lines appeared in positive reactions (Figure 1).

Specificity and Sensitivity Studies

To evaluate the specificities of the assays, the phylogenetic analyses on PCR and LAMP target sequences of 224 IBV and 331 NDV strains were conducted. According to the phylogenetic trees, 224 IBV strains were grouped into 4 clades, and 331 NDV strains were grouped into 6 clades. A total of 13 IBV strains were used to determine the specificities of the assays distribute in all 4 clades, likewise, 13 NDV strains were used to determine the specificities distribute in all 6 clades (Figure S1, Supplementary Information).

In addition, cDNA of avian reovirus (ARV), infectious bursal disease virus (IBDV), and avian influenza virus (AIV), DNA of gallid alphaherpesvirus 2 (GaHV-2) and fowl adenovirus (FAV), as well as synthesized sequence of 1 TCoV strain were used as templates for further evaluation of the specificities of the assays (Table S1, Supplementary Information).

To determine the lowest detection limits of the assays in terms of RNA copy numbers, 10-fold serial diluted in vitro-transcribed RNA of target regions was used as templates. Briefly, fragments containing IBV 5'-UTR and NDV LP target regions were separately amplified using primer pairs 5'-ATCACACTAGCCTTGC GCTAGA-3'/5'-GCAAAGCATCAGCGTAATCC-3' and 5'-AATCTGTATTACATGTCTAGG-3'/5'-AGA GAGAATATATCCTTTTCGC-3'. Subsequently, the fragments were separately ligated downstream T7

promoter. In vitro-transcriptions were conducted using HiScribe T7 High Yield RNA Synthesis Kit (NEB, Beijing, China). The concentrations of the RNA transcripts were measured using NANO DROP 2000 (Thermo, Shanghai, China), and the copy numbers of IBV and NDV RNA molecules were calculated following the formula reported previously (Fronhoffs et al., 2002). The copy numbers of IBV and NDV RNA molecules were $10^{6.8}$ and $10^{5.7}$ copies/ μL , respectively. IBV RNA was 10-fold serial diluted into $10^{6.8}$ to $10^{0.8}$ copies/ μL ; NDV RNA was 10-fold serial diluted into $10^{5.7}$ to $10^{0.7}$ copies/ μL . Whereafter, 1 μL RNA from each concentration was used in reverse transcription reaction (10 μL reaction volume). After reverse transcription, 1 μL cDNA was used in the PCR and LAMP assays. Thus, the concentrations of serial diluted IBV and NDV RNA, which was finally used as templates, were separately $10^{5.8}$ to $10^{-0.2}$ copies/reaction and $10^{4.7}$ to $10^{-0.3}$ copies/reaction.

Furthermore, serial diluted IBV and NDV RNA was mixed to test the lowest detection limit of mRT-LAMP-LFD when simultaneously detecting IBV and NDV. Negative control reactions in specificity and sensitivity studies were conducted with total RNA extracted from allantoic fluid of health specific pathogen-free (SPF) chick embryo as templates.

IBV and NDV Clinical Samples

IBV-positive samples, including 144 tissue samples (78 tracheas and 66 lungs), 124 swabs (82 oral swabs and 42 cloacal swabs), NDV-positive samples, including 87 tissues (52 tracheas and 35 lungs), and 76 swabs (40 oral swabs and 36 cloacal swabs) were used to examine the performance of the assays in detecting IBV and NDV in clinical samples. In addition, to further investigate the specificities of the assays when detecting clinical samples, 33 negative tissues (including 10 tracheas, 10 lungs, and 13 kidneys), 10 FAdV positive livers, and 7 AIV H9N2 positive lungs were also tested by the assays. All these samples were collected from 32 chicken farms distributed in 7 provinces, China, during our routine monitor on avian diseases.

To investigate whether the mRT-LAMP-LFD assay could detect IBV and NDV in co-infected samples, both accurately and simultaneously, ten 4-wk-old SPF chickens were inoculated with $10^{2.7}$ EID₅₀ IBV M41 and $10^{2.4}$ EID₅₀ NDV F48E9 by the nasal route to mimic IBV-NDV co-infected chickens. Oral and cloacal swabs were collected on 3 and 6 days-post-infection (dpi) from each bird. On 6 dpi, all the chickens were sacrificed, and lungs and tracheas were collected.

The animal experiment in this study was approved by the Animal Ethics Committee of the College of Life Sciences, Sichuan University (license: SYXK-Chuan-2013-185). All experimental procedures and animal welfare

standards strictly followed the guidelines of Animal Management at Sichuan University.

Statistical Analysis

Statistical significance differences in the mean detection rates of the assays, when detecting different kinds of samples, were evaluated by One-way ANOVA using GraphPad Prism version 6 (GraphPad Software Inc., San Diego, CA). Differences were considered to be significant at $*P < 0.05$.

RESULTS

Optimization of PCR, nPCR, qPCR, LAMP, and LAMP-LFD Assays

Under optimal annealing temperatures, RT-PCR and nRT-PCR amplified specific products of the expected lengths, and there were no non-specific bands observed in the negative controls (Figure 2A).

Both in IBV and NDV qRT-PCR assays, fluorescent signals were detected with the target IBV or NDV templates, while no fluorescent signals were detected in negative control reactions (Figure 2B). According to the standard curves, C_t values (y), and log of copy numbers (x) were linearly correlated (IBV: $y = -3.323x + 41.249$, $E = 99.9\%$, $R^2 = 0.999$; NDV: $y = -3.369x + 41.444$, $E = 98.1\%$, $R^2 = 0.999$). The melting curve showed a single peak indicating no primer dimer formed (Figure 2C).

After IBV and NDV RT-LAMP amplification, symbolic ladder-like bands were observed in a 2% agarose gel (Figure 3A). As for mRT-LAMP-LFD assays, IBV-positive reactions generated test line 1 and the control line; NDV-positive reactions generated test line 2 and the control line; and test lines 1, 2, and the control line appeared when both IBV and NDV were present; only the control line was generated when neither virus was present (Figure 3B).

Specificity Study

As Figure 4A shows, there were no positive reactions observed when IBV assays were tested with other pathogen templates except for TCoV. The results are not unexpected, because TCoV and IBV are very closely related in terms of both antigenic and genomic characterizations (Guy, 2000). Target sequence (5'-UTR) nucleotide identities between TCoV and 224 IBV strains are 91.3 to 97.7% (data not shown). Moreover, according to the new 2018 taxonomy of viruses published by International Committee on Taxonomy of Viruses, IBV, and TCoV are classified as 1 specie (<http://www.ictv.global>).

NDV detection assays were specific to the NDV templates (Figure 4B). Conventional RT-PCR, nRT-PCR, and RT-LAMP assays yielded specific products only

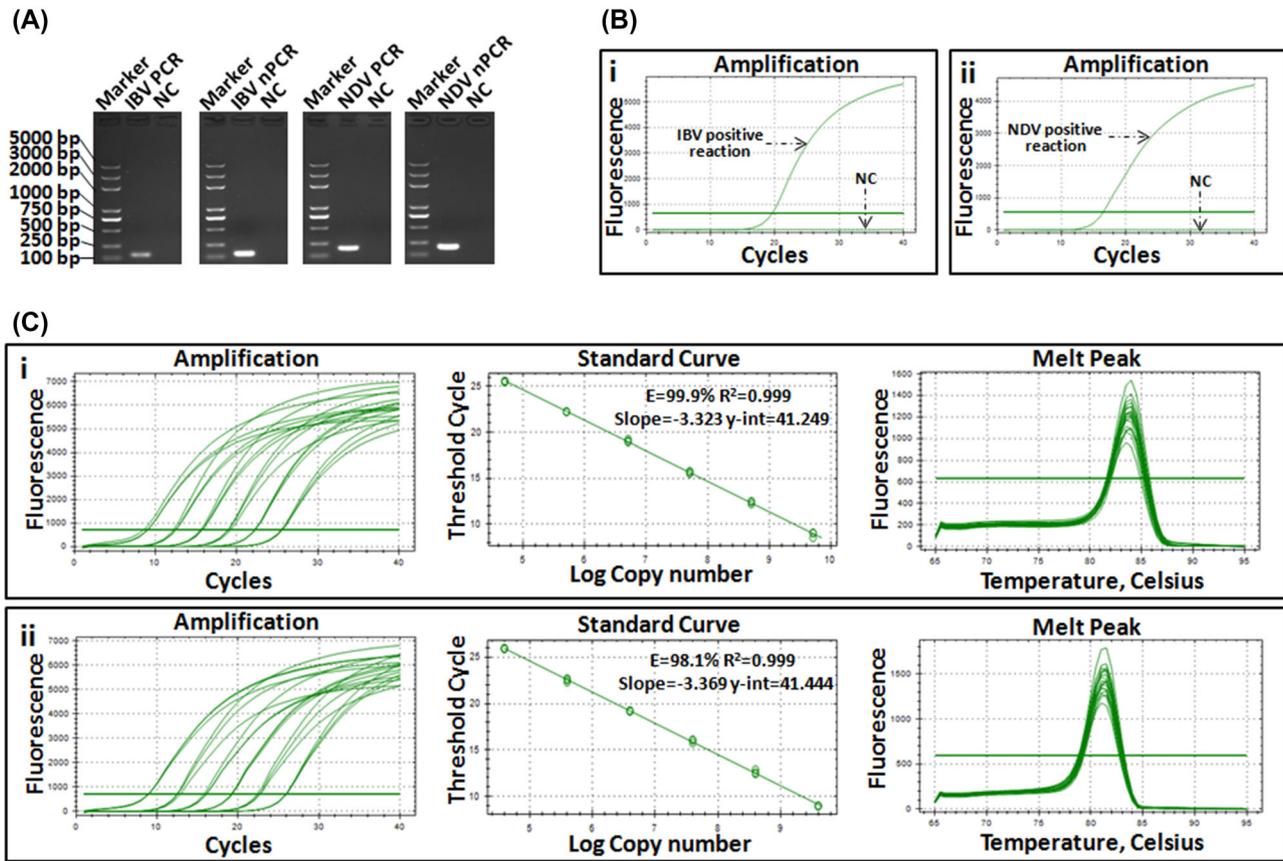


Figure 2. RT-PCR, nRT-PCR, and qRT-PCR assays. (A) Product of conventional RT-PCR and nRT-PCR analyzed by electrophoresis. The molecular weights of the marker were labeled on the left. All the RT-PCR and nRT-PCR amplified specific bands with expected lengths, 130 bp for IBV and 190 bp for NDV. (B) Amplification signals were detected in IBV and NDV positive qRT-PCR reactions. NC in (A) and (B) represents negative control. (C) The standard curves of IBV and NDV qRT-PCR. The standard curves were generated by plotting the C_T (y) values vs. log copy numbers (x). The reaction efficiencies (E) of IBV and NDV qPCR were 99.9% and 98.1%, respectively. For IBV, $y = -3.323x + 41.249$; for NDV, $y = -3.369x + 41.444$.

when tested with target NDV templates. In mRT-LAMP-LFD assays, test lines were observed when target templates were contained in the reaction mixture. As for qRT-PCR assays, fluorescent signals were detected only when tested with the NDV templates.

Sensitivity Study

The lowest detection limits of IBV conventional RT-PCR, nRT-PCR, qRT-PCR, RT-LAMP, and mRT-LAMP-LFD assays, when detecting IBV alone, were $10^{3.8}$, $10^{0.8}$, $10^{1.8}$, $10^{0.8}$, and $10^{0.8}$ copies/reaction, respectively (Figure 5A). The lowest detection limits of NDV conventional RT-PCR, nRT-PCR, qRT-PCR, RT-LAMP, and mRT-LAMP-LFD assays, detecting NDV alone, were individually $10^{3.7}$, $10^{0.7}$, $10^{1.7}$, $10^{0.7}$, and $10^{0.7}$ copies/reaction (Figure 5B).

When simultaneously detecting IBV and NDV, mRT-LAMP-LFD produced clear visible test lines at concentration of $10^{0.8}$ IBV + $10^{0.7}$ NDV copies/reaction, and the lowest detection limit was the same as that of mRT-LAMP-LFD when detecting IBV or NDV alone (Figure 5C).

Detection of IBV and NDV from Clinical Samples

As Figure 6A shows, mRT-LAMP-LFD exhibited the highest mean detection rates in the detection of different types of clinical samples when conducting IBV or NDV single detection, 98.65% for IBV and 97.25% for NDV. Statistical significance difference studies showed that the mean detection rates of mRT-LAMP-LFD were significantly higher than that of conventional RT-PCR assays when detecting IBV or NDV alone ($P < 0.05$). No positive results were observed when the assays were tested with negative tissues, FAdV positive livers, and AIV positive lungs.

To further evaluate mRT-LAMP-LFD, 10 chickens were experimentally co-infected with IBV and NDV. Results showed that mRT-LAMP-LFD could not only detect 2 pathogens simultaneously, but also showed higher mean detection rates than the other assays presented here. The mean IBV and NDV detection rates of different samples, detected by mRT-LAMP-LFD, were both 95%, and were significantly higher than those detected by conventional RT-PCR and qRT-PCR ($P < 0.05$, Figure 6B).

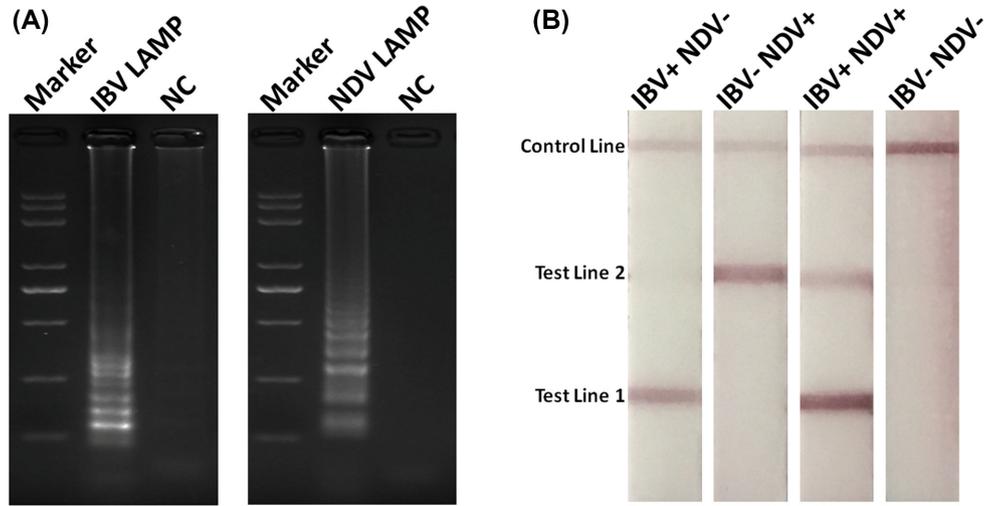


Figure 3. The RT-LAMP and mRT-LAMP-LFD. (A) The RT-LAMP products monitored by electrophoresis. The Marker is the same as that used in Figure 2A. NC represents negative control. (B) mRT-LAMP-LFD singly detect IBV or NDV, or simultaneously detect IBV and NDV. Test line 1 corresponds to IBV positive sample and test line 2 corresponds to NDV positive sample.

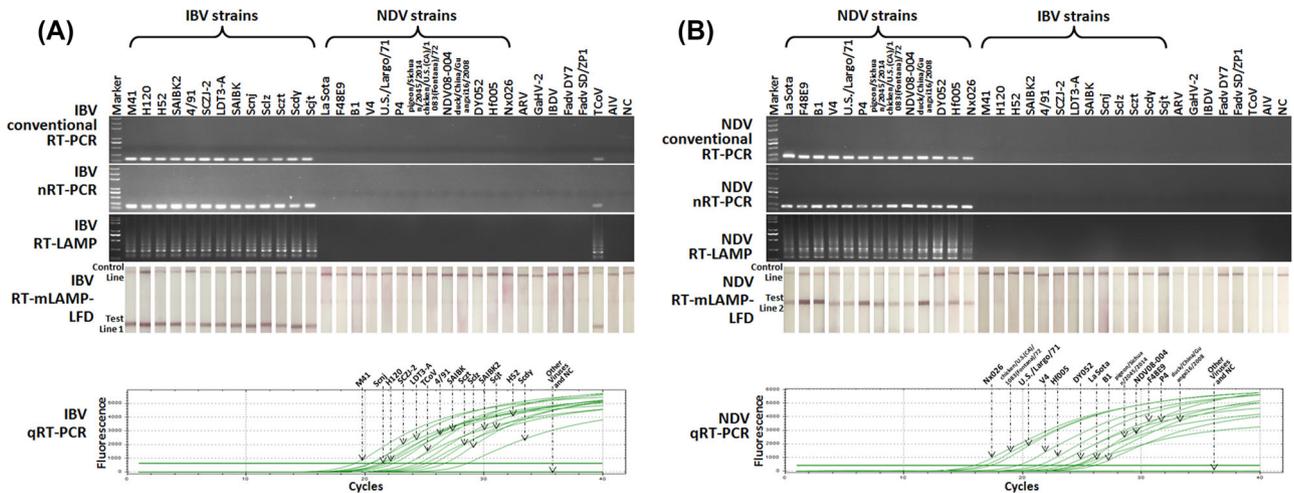


Figure 4. Specificity study of PCR and LAMP assays of (A) IBV and (B) NDV. In specificity study, cDNA of 13 IBV, 7 NDV, 1 ARV, 1 IBDV, and 1 AIV and DNA of 1 GaHV-2 and 2 FAdV, as well as the synthesized PCR and LAMP target sequences including 6 NDV and 1 TCoV were used as templates. In the IBV detection assays, positive results were detected with IBV and TCoV templates. In IBV qRT-PCR, other viruses include NDV, ARV, GaHV-2, IBDV, FAdV, and AIV. As for NDV detection assays, positive results were observed only when NDV templates existed. In NDV qRT-PCR, other viruses refer to IBV, ARV, GaHV-2, IBDV, FAdV, TCoV, and AIV. NC means negative control reactions which were conducted using total RNA extracted from allantoic fluid of healthy SPF chick embryo as template. Markers in the electrophoresis were the same as that in Figure 2A.

DISCUSSION

Timely and accurate diagnostic methods are very important for the control of infectious diseases, especially for IBV and NDV which are 2 of the most important contagious viruses seriously affecting the poultry industry. Furthermore, IBV and NDV produce clinical picture somewhat resembling each other, it is very much crucial not only to detect but also differentiate simultaneously. Existing IBV and NDV diagnostic methods, including virus isolation and PCR assays, are specific and sensitive. However, they are not suitable for timely on-site pathogen detection. Although portable PCR machines are gradually applied in the field, in most areas, especially in undeveloped and developing countries,

these sophisticated equipments are too expensive to be popularized, and the sensitivity of PCR is not satisfactory. Multiple RT-LAMP-LFD developed in this study could detect and differentiate IBV and NDV, both simultaneously and accurately. When IBV and NDV cDNA co-exist in the same reaction system, an IBV and NDV double-positive result was observed. To evaluate the sensitivity of mRT-LAMP-LFD, conventional RT-PCR, nRT-PCR, qRT-PCR, and RT-LAMP assays detecting IBV or NDV alone were also conducted to compare with mRT-LAMP-LFD. When detecting IBV or NDV alone, mRT-LAMP-LFD performed as sensitive as nRT-PCR and RT-LAMP did, in a directly visual way. It is always thought that qRT-PCR methods provided high sensitivity during pathogen diagnoses.

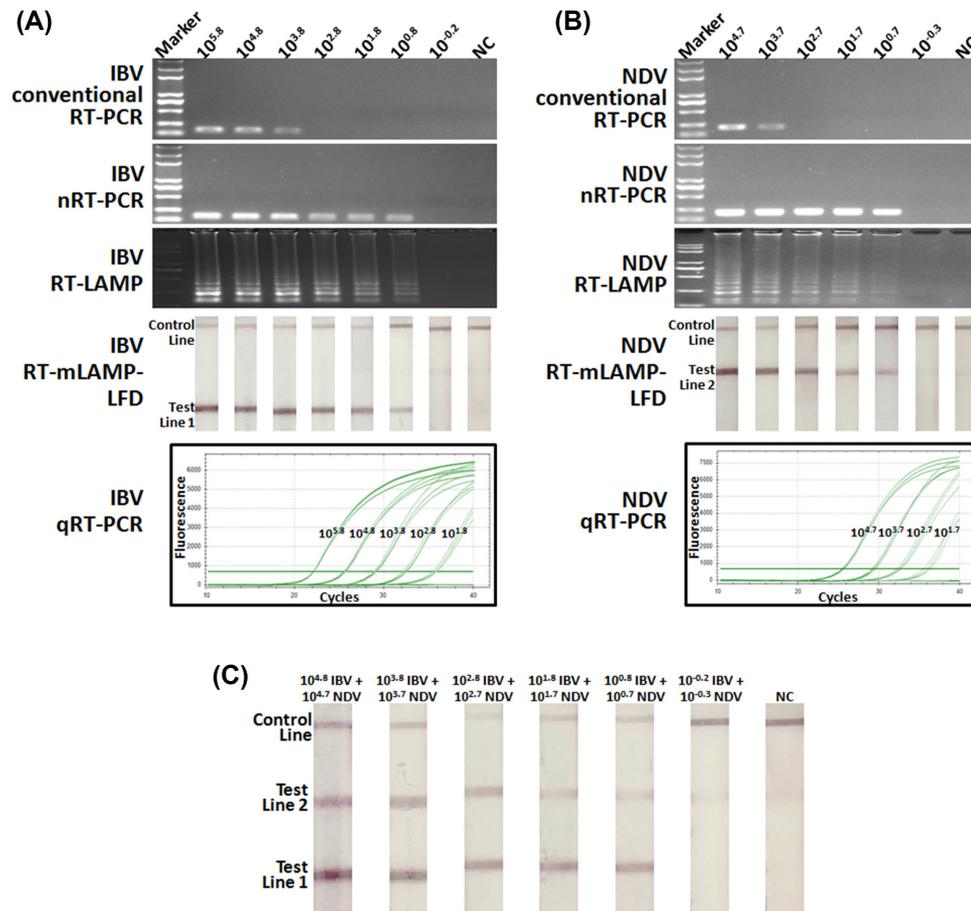


Figure 5. Sensitivity study of PCR and LAMP assays of individually detecting (A) IBV and (B) NDV, or (C) simultaneously detecting IBV and NDV. The copy numbers of IBV and NDV RNA used in each reaction were labeled on the top of (A), (B), and (C). NC refers to negative control reactions. Markers in the electrophoresis were the same as that in Figure 2A.

According to the result of detection limit study, nRT-PCR, and LAMP assays, established in this study, possessed 10 times higher sensitivity than qRT-PCR. In the first round of amplification of nRT-PCR, the original template was amplified with the outer primers. Thus, the number of the fragments containing the inner primer target sequence is greatly improved compared with the original template. As a result, the number of templates in the second round of amplification of nRT-PCR is much higher than that in qRT-PCR. This is the reason why nRT-PCR could detect lower concentration of original templates than qRT-PCR do. Similarly to our results, previous study conducted by Weng and Chen indicated that nPCR showed higher sensitivity than real-time PCR when detecting *Phytophthora infestans* (Khan et al., 2017). LAMP is one of the most widely used isothermal nucleic acid amplification techniques (INATs). Several studies on the diagnostic methods of other pathogens had showed that these INATs possessed equal or even higher sensitivities compared with qPCR assays (Gao et al., 2018; Khan et al., 2017; Yang et al., 2015). Our results indicated that LAMP assays possessed higher sensitivities than qPCR when detecting IBV and NDV (Figure 5).

Several multiple RT-PCR assays detecting avian respiratory pathogens have been developed in previous studies, while the sensitivity of these multiple assays was lower than single pathogen detection assays (Nguyen et al., 2013; Pang et al., 2002). This may be due to the competition among different sets of primers. However, when detecting with IBV and NDV co-existing samples, the lowest detection limit of mRT-LAMP-LFD was the same as that of mRT-LAMP-LFD when detecting a single pathogen (i.e., $10^{0.8}$ copies/reaction for IBV and $10^{0.7}$ for copies/reaction for NDV), indicating that the sensitivity of mRT-LAMP-LFD was not affected when the components of the reaction system became more complex.

The purpose of this study was to develop a novel detection tool which could simultaneously accurately detect IBV and NDV on site. Previous studies on PCR methods detecting IBV and NDV showed that these PCR methods are specific and sensitive, but the need for expensive thermal cycling equipments makes them not suitable for on-site IBV and NDV detection. Portable PCR machines are gradually applied in the field. While, in most areas, especially in undeveloped and developing countries, these sophisticated

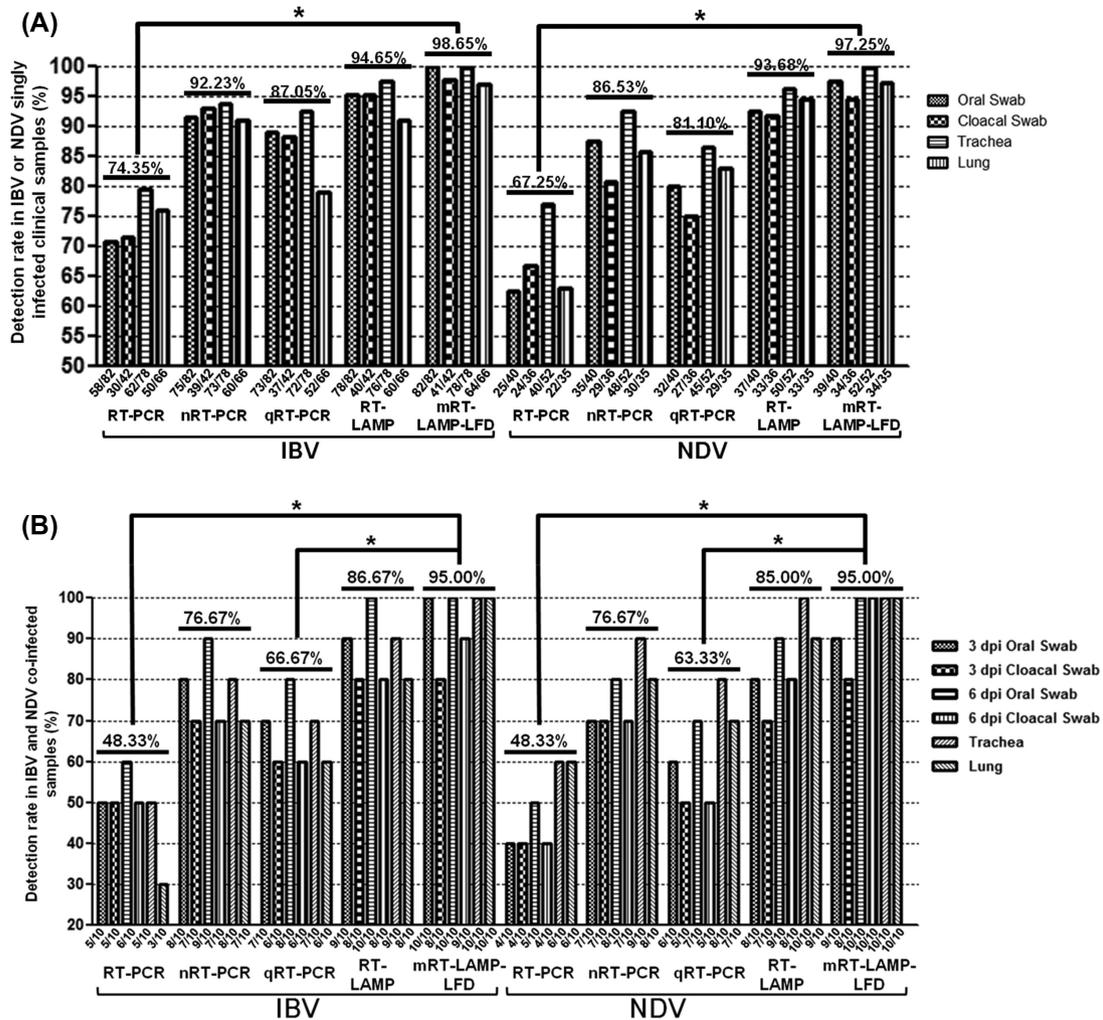


Figure 6. Detection rates of the assays when detecting with IBV- or NDV-infected samples (A) and IBV-NDV co-infected samples (B). (A) Different kind of IBV and NDV positive samples collected from the field were used to examine the performance of the assays in detecting IBV and NDV in clinical samples. (B) To further evaluate mRT-LAMP-LFD, 10 chickens were experimentally co-infected with IBV and NDV. The mean detection rates of the assays, when detecting different kinds of samples, are labeled above the histograms. The fraction numbers under the X-axis represent (positive sample numbers detected by the assays)/(total sample numbers). * indicate $P < 0.05$.

equipments are too expensive to be popularized. Thus, simpler and visible IBV and NDV detecting methods are urgently needed. High specific and sensitive single IBV and NDV RT-LAMP assays have been developed in previous studies (Chen et al., 2010; Pham et al., 2005). In these assays, products were visualized by electrophoresis or by adding color indicators. When detecting multiple pathogens, the products must be easily differentiated. When visualized by electrophoresis, LAMP products could be distinguished by observing bands with different molecular weights, however, this method is not suitable for on-site pathogen detection. By adding color indicators, the change in color could be easily observed, but this change is non-specific. Obviously, these two LAMP monitoring methods are not applicable for on-site multiple pathogens detection. Multiple LAMP-LFD has been applied in the detection of some pathogens, and it showed great advances compared with common PCR and LAMP assays, such

as the requirement for little equipment, short reaction time, and the ability to detect multiple genes or pathogens (Chen et al., 2016; Lalle et al., 2018). To our knowledge, RT-LAMP-LFD has not been applied to IBV or NDV detection and no studies on an mRT-LAMP-LFD technique that detects multiple avian respiratory viruses have been reported. In this study, the products generated in IBV and NDV RT-LAMP were differentiated by Biotin/FITC and Digoxigenin/FITC labeling, respectively. The products could bind with Biotin- or Digoxigenin-antibodies fixed on different test lines on the LFD strip, and then products were visualized by combining with gold particles modified with FITC-antibodies (Figure 1). When tested with clinical samples, the mean detection rate of mRT-LAMP-LFD was higher than that of the other assays. These results indicate that mRT-LAMP-LFD is not only able to detect IBV and NDV simultaneously, but is also suitable for field testing in both technical (more sensitive

than PCR and LAMP assays) and practical aspects (more simple to put out and no specialized equipment needed).

This study combined RT-LAMP and LFD conducting a novel IBV and NDV mRT-LAMP-LFD detection assay which is specific and sensitive in detecting IBV and NDV simultaneously. Furthermore, mRT-LAMP-LFD does not require specialized instrumentations, making it suitable for on-site detection. In conclusion, mRT-LAMP-LFD is a promising qualitative detection tool, and is even applicable in some low-resource locations.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

Table S1. The TCoV strain and 6 other avian virus strains used in the specificity study

Figure S1. Phylogenetic analyses based on PCR and LAMP target sequences of (A) IBV and (B) NDV. Strains used in specificity study are in bigger font. Wild strains detected during our avian virus monitoring, of which genome sequences have not been submitted to GenBank, were labeled by solid circles; artificially synthesized sequences were labeled by solid triangles. According to the trees, 224 IBV strains were grouped into 4 clades, and the IBV strains used in this study distribute in all 4 clades; 331 NDV strains were grouped into 6 clades, and the NDV strains used in this study distribute in all 6 clades.

ACKNOWLEDGMENTS

This research is funded by the National Key R&D Program of China (2017YFD0500703), the China Agriculture Research System (CARS-40), the National System for Layer Production Technology (CARS-40-K14), and the Project for Science and Technology Support Program of Sichuan Province (2018NZ0009).

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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