Reverse transcription recombinase-aided amplification assay combined with a lateral flow dipstick for detection of avian infectious bronchitis virus

Wenjing Wang,[*,†,1] Chunguang Wang,[*,1] Peng Zhang,[*] Shanshan Yao,[*] Jingru Liu,[*] Xianghe Zhai,[*] and Tie Zhang[*,2]

[*]*College of Veterinary Medicine, Hebei Agricultural University, Baoding 071001, China; and* [†]*Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou 730046, China*

ABSTRACT The study was conducted to develop a specific, simple, and sensitive method for diagnosis of avian infectious bronchitis virus (**IBV**). In this experiment, the selected downstream primer was labeled with biotin and the 5' end of RAA probe was labeled with FAM by reverse transcription recombinase-aided amplification (**RT-RAA**) combined with lateral flow dipstick (**LFD**). A RT-RAA-LFD assay that could be used for detection of IBV was established after optimization of RT-RAA reaction time, reaction temperature, and primer concentration. This method did not need reverse transcription of IBV template under isothermal condition (37°C), the amplification of target gene fragments could be completed within only 24 min, and the amplification products could be visually observed and determined by LFD within 3 min. The specificity test demonstrated that there was no cross reaction with the nucleic acids of other similar common pathogens. The lowest detectable limit for IBV was 10^2 copies/μL, and this method was 100 times more sensitive than conventional PCR (10^4 copies/μL), as verified by sensitivity test. The results showed that RT-RAA-LFD assay with strong specificity and high sensitivity was simple and easy to operate, and could be used for rapid detection of IBV in clinical diagnosis.

Key words: avian infectious bronchitis virus, reverse transcription recombinase-aided amplification, lateral flow dipstick, specificity, sensitivity

2020 Poultry Science 99:89–94
http://dx.doi.org/10.3382/ps/pez559

INTRODUCTION

Avian infectious bronchitis (**IB**) is a viral respiratory disease in chickens caused by avian infectious bronchitis virus (**IBV**) (Cook and Heijden, 2010). Infectious bronchitis virus is an enveloped, non-segmented, single-stranded, positive-sense RNA virus belonging to the genus *Gammacoronavirus* of the family Coronaviridae (Cheng et al., 2018; Haji-Abdolvahab et al., 2019). Infectious bronchitis virus genome is unstable and prone to point mutation, deletion, insertion, etc., which leads to continuous isolation of gene mutants. In addition, there are many serotypes of IBV, and the cross-protection between different serotypes or genotypes is very weak (Leghari et al., 2016), which makes the immune control of this disease constantly challenged (Faruku et al., 2015). Therefore, although the disease has been reported for more than 60 yr, its prevention and control is still not good (Cook et al., 2012). The incidence of IBV is high, broilers infected with this virus will grow slowly and feed conversion ratio (**FCR**) will decline, and if laying hens are infected with IBV, it can lead to the decline of egg production and egg quality, so IB is one of the main infectious diseases detrimental to China's poultry industry.

IB is characterized by respiratory signs, kidney lesions, and proventriculus lesions (Zhao et al., 2014). The respiratory signs include coughing, sneezing, gasping, and tracheal rales, and there may be gray feces for some diseased chickens (Wu et al., 1998). Necropsy showed catarrhal and hemorrhagic inflammatory lesions of the trachea. Chickens of all ages can be infected with IBV, which is mainly found in respiratory epithelial cells, digestive tract, and other tissues such as kidney, fallopian tube, and testes (Cavanagh, 2007). With clinical symptoms and pathological features similar to those of Newcastle disease (**ND**), avian influenza (**AI**), and infectious laryngotracheitis (**ILT**), IB is easy to be misdiagnosed (Giovanni et al., 2017). The current methods for detection of IBV are conventional virus isolation (**CVI**), reverse transcription-polymerase chain reaction-agarose gel electrophoresis (**RT-PCR-AGE**), enzyme-linked immunosorbent assay (**ELISA**), and loop-mediated isothermal amplification (**LAMP**). However, these methods often require expensive devices and professional operators, making it very

Received August 11, 2019.
Accepted September 11, 2019.
[1]Wenjing Wang and Chunguang Wang contributed equally to this study and share first authorship.
[2]Corresponding author: zhangtie1998@163.com

© 2019 The Authors. Published by Elsevier on behalf of Poultry Science Association Inc.
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Table 1. Selection of primers for RT-RAA reactions.

Primers	Sequences (5′-3′)	Gene location
IBV-1-F	TCCGTTGGGATTTTATTCCTCTGAATCGTG	468 to 498
IBV-1-R	TACTGGTCTGGTATAGCACATGGTTGGGTC	683 to 703
IBV-2-F	AGTATCTGGCATCGCCTCATTTCTTTCTAACC	238 to 270
IBV-2-R	CCTACCACGATTCAGAGGAATAAAATCCCAACG	502 to 535
IBV-3-F	GCCGATTTTGTAAACGTACCATTCCACCAG	684 to 711
IBV-3-R	AACACGCCCATCCTTAATACCTTCCTCATT	814 to 844
IBV-4-F	CGTCTTATAACTGGTAGATTGTCATCACTC	989 to 1019
IBV-4-R	ATTACCACAAAAGGAGTACCTAGTAGACTG	1142 to 1172

inconvenient for veterinarians at the grass roots level in many regions.

Reverse transcription recombinase-aided amplification (**RT-RAA**), following the principle of recombinase polymerase amplification (**RPA**), is employed for simulating the enzymatic process of DNA replication in vivo, and amplifying the DNA template. Using this technique, the reverse transcription of RNA and DNA amplification can be completed in a short time at a constant temperature of about 37 to 39°C. At the same time, the probes were labeled with 6-carboxyfluorescein (**FAM**) and primers were labeled with biotin in the experiment to form double-labeled amplification product that could be visually observed and determined by lateral flow dipstick (**LFD**) with FAM antibody test line and biotin antibody control line. This method with high specificity and sensitivity was easy and convenient to use, and thus had broad application prospects in clinical diagnosis (Kim and Easley, 2011).

MATERIALS AND METHODS

Materials

Infectious bronchitis virus standard strain (AV1511) and infectious laryngotracheitis virus (**ILTV**) strain Wanggang (AV195) were purchased from China Institute of Veterinary Drug Control, and avian influenza virus (**AIV**) and Newcastle disease virus (**NDV**) were preserved after clinical detection in our laboratory. Virus genomic DNA/RNA extraction kit was bought from Tiangen Biotech Co., Ltd.; RT-RAA nucleic acid amplification kit was bought from Qitian Biotechnology Co., Ltd.; LFD (with FAM antibody test line and biotin antibody control line) was bought from Ustar Biotechnologies Co., Ltd.; T-Vector Pmd20 plasmid was bought from Takara Biomedical Technology Co., Ltd.; primers and probes were synthesized and labeled by Sangon Biotech Co., Ltd.

Methods

Design and Screening of Primers According to the requirements of RT-RAA nucleic acid amplification kit, 4 pairs of primers were designed by comparison with the sequences of IBV N gene (GenBank: EF602441.1) and IBV S_2 gene (GenBank: EF602461.1) (Table 1). Primers 1 to 3 were designed according to the sequence of IBV N gene, and primer 4 was designed according to the sequence of IBV S_2 gene. RNA of IBV was extracted as a template. Reaction system: Reaction buffer 25 μL; upstream and downstream primers (10 μM) 2.1 μL each; template 1 μL; purified water 17.3 μL; magnesium acetate 2.5 μL. After 30 min reaction at 39°C, electrophoresis in 2% agarose gels was used to test RT-RAA reaction products, and a pair of primers from which single and clear band as big as the target segment was amplified, were selected. The selected downstream primer was labeled with biotin at its 5' end. The selected primers were compared with the gene sequence, and the probes were designed and labeled based on the requirements of kit. Probe sequence: 5′-GTCACAACAGCGTGAGTTAGCCACGCAAAAG /idSp/ATTAATGAGTGTGTTA-3′. The probe was modified with a FAM fluorophore at its 5' end, there was a single-base gap at a position 31 bp from the 5' end, which was modified with tetrahydrofuran residue, and the 3' end was blocked by phosphorylation. The labeled primers and probes were used in follow-on experiment.

Establishment of RT-RAA-LFD Assay Reaction system: 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; magnesium acetate 2.5 μL. For the negative control, purified water was used instead of template. After 25 min reaction at 39°C, 10 μL of RT-RAA products were transferred to the test area of dipstick, the dipstick was inserted into a 2 mL centrifuge tube with 100 μL of buffer, and the results were observed after 3 min.

Dipstick criteria: It was positive when 2 red bands appeared, one in the quality control area and the other in the test area, and positive results showed that nucleic acid fragments to be detected were contained in the sample; it was negative if there was a red band in the quality control area while no red band was found in the test area, and negative results showed that no nucleic acid fragment for detection was contained in the sample.

Optimization of Reaction Conditions Optimization of reaction temperature: 33, 35, 37, 39, and 41°C were selected as reaction temperatures, respectively.

Optimization of reaction time: At the optimal temperature of 37°C, 16, 18, 20, 22 and 24 min were selected as reaction time, respectively.

Optimization of primer and probe concentration: At the optimized temperature and reaction time, primer concentration and probe concentrations were optimized on the basis of 10,000, 5,000, 2,500, 1,250, 625 nmol/L.

Criteria for the best reaction conditions: Clear and obvious quality control line, shortest reaction time, lowest primer or probe concentration.

Specificity Test The genomes of ILTV, NDV, and AIV were extracted by the virus genomic DNA/RNA extraction kit. RNA of IBV, NDV, and AIV and DNA of ILTV were used as templates, and the specificity of RT-RAA-LFD was verified by RT-RAA reaction and LFD assay.

Sensitivity Test Preparation of recombinant plasmids: The template cDNA was obtained by reverse transcription of IBV genomic RNA, and primer was IBV-4-F/IBV-4-R. It was the optimal primer obtained by screening, the primer length was modified according to the requirements of conventional PCR, and sequence was F:5′-CGTCTTATAACTGGTAGATTG-3′, R:5′-ATTACCACAAAAGGAGTACCT-3′. 50 μL reaction system: 2 × Taq Mix 25 μL; cDNA template 2 μL; upstream and downstream primers (10 μM) 1 μL each; replenished with 21 μL of ddH2O. Reaction system: It 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 plasmid after gel extraction of PCR products. The standard plasmid was constructed after purification and screening, and the DNA copy number in the standard plasmid was calculated after the nucleic acid content was determined.

$$\text{Plasmid copy number (copies/L)} = [\text{plasmid concentration } (\text{g} \cdot \mu\text{L}^{-1}) \times 6.02 \times 10^{23}] / [\text{total fragment length (bp)} \times 660\text{ng/mol}];$$

$$\text{Total fragment length} = \text{vector length (bp)} + \text{fragment length (bp)}.$$

The standard plasmid constructed was diluted to a concentration gradient of 10^0 to 10^7 copies/μL, and the sensitivity of conventional PCR assay, RT-RAA-LFD assay, and RFQ-PCR assay to IBV was compared.

Conventional PCR system (25 μL): 2 × Gflex PCR Buffer (Mg^{2+}, dNTP plus) 12.5 μL; upstream and downstream primers (10 μM) 0.5 μL each; Tks Gflex DNA polymerase (1.25 units/μL) 0.5 μL; template 1 μL; the final volume was made up to 25 μL with ddH_2O. Reaction system: It 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; finally extended at 68°C for 5 min.

RFQ-PCR system (25 μL) (primers the same as those in conventional PCR): TB Green Premix DimerEraser (2X) 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. Reaction system: It was predenaturalized at 95°C for 30 s; denaturalized at 95°C for 5 s; annealed at 55°C for 30 s; extended at 72°C for 30 s, a total of 40 cycles.

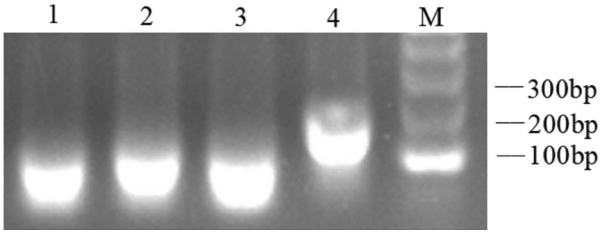

Figure 1. Reverse transcription recombinase-aided amplification primer screening. M: marker, 1: IBV-1-F/IBV-1-R, 2: IBV-2-F/IBV-2-R, 3: IBV-3-F/IBV-3-R, 4: IBV-4-F/IBV-4-R.

Reverse transcription recombinase-aided amplification reaction system was based on the optimized reaction conditions, and the template in the reaction system was standard plasmid with a concentration gradient of 10^0 to 10^7 copies/μL.

Clinical Sample Test A total of 57 suspected IBV samples in clinical diagnosis were tested by RT-RAA-LFD assay and conventional PCR assay, and the coincidence rate of the 2 assays was compared.

RESULTS

RT-RAA Primer Screening

Four pairs of primers were designed in accordance with the requirements of RT-RAA nucleic acid amplification. After the products were amplified by RT-RAA nucleic acid amplification kit, they were screened by 2% agarose electrophoresis. The results showed that only IBV-4-F/IBV-4-R amplified a single and clear band of the expected size (target segment 153 bp). Therefore, IBV-4-F/IBV4-R was selected as the primer for the follow-on experiment. The results are shown in Figure 1.

Optimal Reaction Conditions

The reaction time, temperature, and primer concentration were optimized in this experiment. There were optimal reaction conditions when quality control line was clear, reaction time was the shortest, and primer concentration was the lowest. Finally, the optimal reaction conditions were determined as follows: reaction time 24 min; reaction temperature 37°C; primer concentration 1,250 nmol/L (Figures 2 to 4).

Specificity Test

With the RNA of IBV, NDV, AIV and the DNA of ILTV as templates, the products were amplified in RT-RAA reaction system, and by LFD assay, only the RT-RAA product of IBV showed both test line and quality control line on dipstick. However, the products

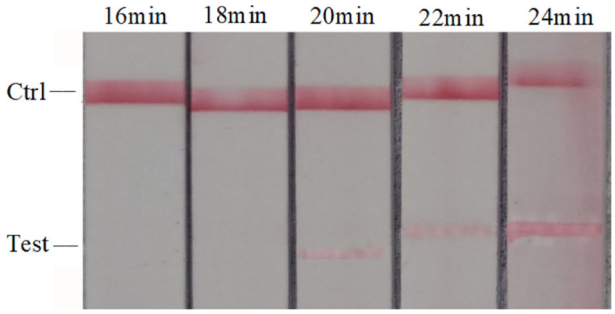

Figure 2. Optimization of reaction time.

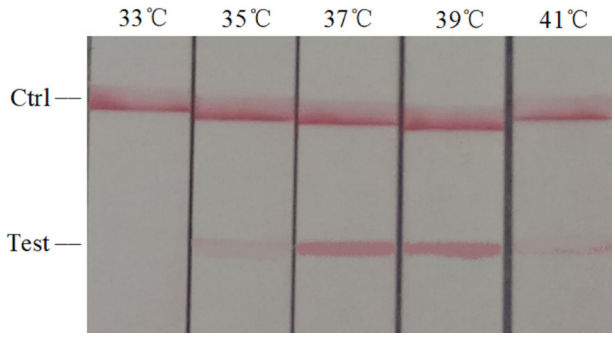

Figure 3. Optimization of reaction temperature.

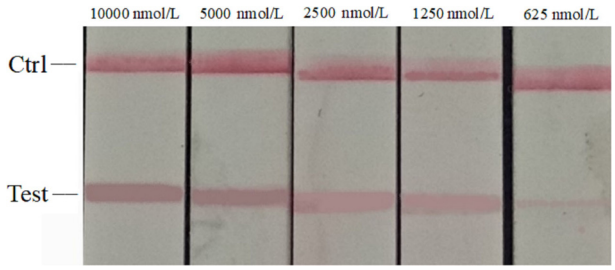

Figure 4. Optimization of primer and probe concentrations.

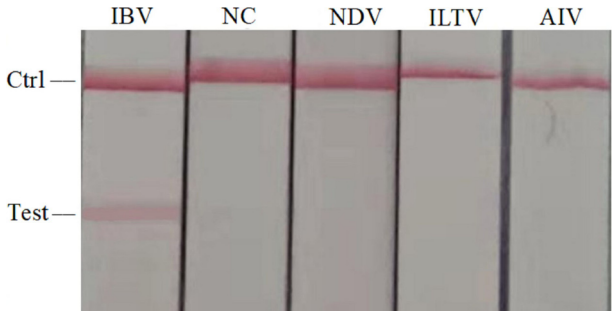

Figure 5. Specificity test by RT-RAA-LFD.

of other viruses and negative control could only show quality control line, which indicated that the specificity of RT-RAA-LFD assay was good, and this method was tenable. The results are shown in Figure 5.

Sensitivity Test

The standard plasmid was diluted by 10-fold gradient, and the sensitivity of conventional PCR, RFQ-PCR and RT-RAA-LFD was compared based on the concentration of 10^0 to 10^7 copies/μL. The results showed that

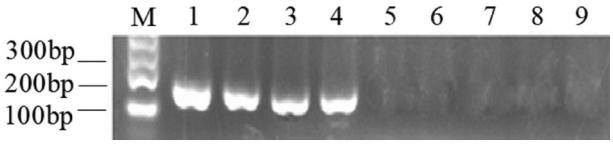

Figure 6. Sensitivity of conventional PCR assay. M: marker, 1: 10^7copies/μL, 2: 10^6 copies/μL, 3: 10^5 copies/μL, 4: 10^4 copies/μL, 5: 10^3 copies/μL, 6: 10^2 copies/μL, 7: 10^1 copies/μL, 8: 10^0 copies/μL, 9: negative control.

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 RT-RAA-LFD. The sensitivity of RT-RAA-LFD assay was 100 times higher than that of conventional PCR assay, but lower than that of RFQ-PCR assay (Figures 6 to 8).

Clinical Sample Test

A total of 57 suspected IB samples from chickens with clinical respiratory symptoms were detected, of which 48 were positive and 9 were negative by RT-RAA-LFD, 43 were positive and 15 were negative by conventional PCR. The detection rate of RT-RAA-LFD assay was higher than that of conventional PCR, which indicated that the RT-RAA-LFD assay established in this study could be used for detection of IBV.

DISCUSSION

IB is responsible for substantial economic loss within the poultry industry, so it is very important to explore a specific, convenient, and sensitive detection method to better prevent and control IB.

The clinical symptoms and pathological changes of IB are similar to those of ND and ILT, so it is easy to be misdiagnosed, which brings some difficulties to clinical diagnosis. Conventional virus isolation (CVI) is the most classical and reliable method for the diagnosis of IB, but it has the disadvantages of long period and high technical requirements. Serological diagnosis is also a common technique in the actual detection of IB, but most of the commercial IBV antibody kits are coated with whole virions, having a strong cross-reaction with local strains of different serotypes, as a result, the application of commercial IBV antibody detection kit is limited (Lin et al., 1991). Conventional PCR and RFQ-PCR are sensitive and specific in nucleic acid diagnosis, but they still have the shortcomings of long period, carcinogen EB pollution and need for expensive instruments (Zhang et al., 2018). Reverse transcription recombinase-aided amplification is a rapid, specific, sensitive, and reliable technique for isothermal gene amplification (Daher et al., 2016). The whole reaction is simple, rapid, and does not require high temperature cycling, so it is particularly suitable for use in non-laboratory testing sites with a large number of samples (Piepenburg et al., 2006).

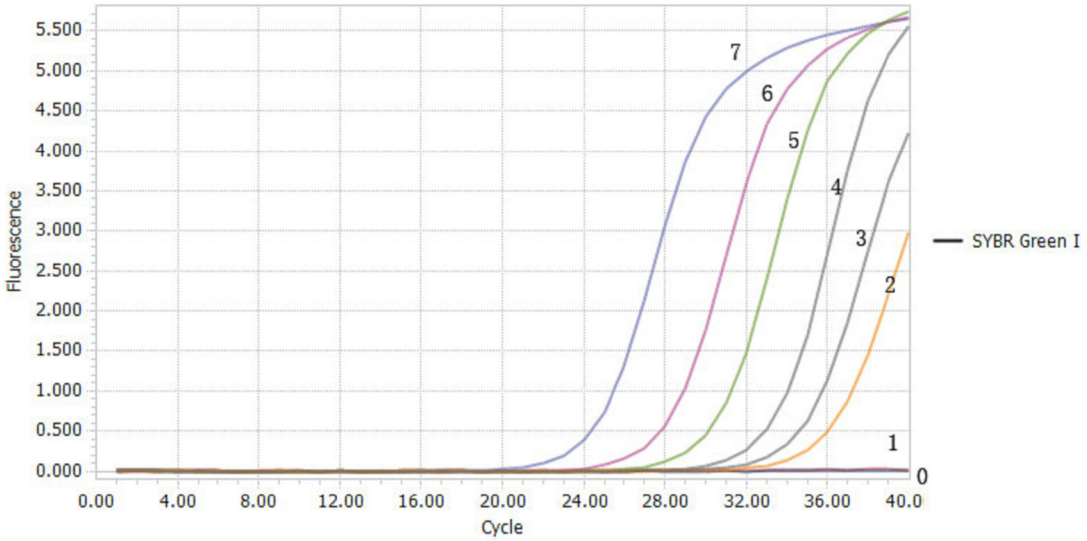

Figure 7. Sensitivity of RFQ-PCR assay. 7: 10^6 copies/μL, 6: 10^5 copies/μL, 5: 10^4 copies/μL, 4: 10^3 copies/μL, 3: 10^2 copies/μL, 2: 10^1 copies/μL, 1: 10° copies/μL, 0: negative control.

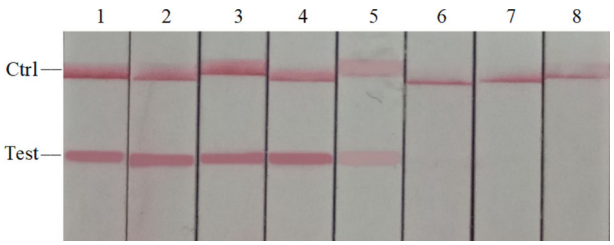

Figure 8. Sensitivity of RT-RAA-LFD assay. 1: 10^6 copies/μL, 2: 10^5 copies/μL, 3: 10^4 copies/μL, 4: 10^3 copies/μL, 5: 10^2 copies/μL, 6: 10^1 copies/μL, 7: 10° copies/μL, 8: negative control.

In this experiment, RT-RAA was combined with LFD to detect IBV. After screening the primers, the downstream primer was labeled with biotin, and the designed probe was modified with a FAM fluorophore at its 5' end. A single-base gap at a position 30 bp from the 5' end was modified with tetrahydrofuran residue, the 3' end was blocked by phosphorylation, and a compatible probe was helpful to improve the specificity of RT-RAA reaction. Notably, the RT-RAA kit completed reverse transcription and DNA amplification in 1 step, saving detection time and reagents. Finally, the double-labeled products amplified by RT-RAA could be detected directly by LFD and visually observed. Reaction could be completed by this technique at a constant temperature of 37°C, amplification did not need complex instruments, and amplification products could be visually detected by LFD. It was proved that the specificity of RT-RAA was good, and the sensitivity of this method was slightly lower than that of RFQ-PCR, but 100 times higher than that of conventional PCR.

CONCLUSION

The RT-RAA-LFD assay had good specificity for detection of IBV and there was no cross reaction with the nucleic acids of other similar avian pathogens. It was highly sensitive, and its LDL was 10^2 copies/μL, which was 100 times higher than that of conventional PCR (10^4 copies/μL). The operation was simple, and results could be visually observed and determined by LFD within 3 min after 20 min reaction under isothermal condition (37°C), so it is very suitable for clinical veterinary and grass-roots laboratories.

ACKNOWLEDGMENTS

This work was supported by Open Fund of State Key Laboratory of Veterinary Etiological Biology (No. Y2017PT44) from Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, and ShiJiazhuang Key Technology R&D Program grant (No. 2020) from Department of Science& Technology, ShiJiazhuang City, China.

REFERENCES

Cavanagh, D. 2007. Coronavirus avian infectious bronchitis virus. Vet. Res. 38:281.

Cheng, J. L., C. Y. Huo, J. Zhao, T. Liu, X. Li, S. H. Yan, Z. J. Wang, Y. X. Hu, and G. Z. Zhang. 2018. Pathogenicity differences between QX-like and Mass-type infectious bronchitis viruses. Vet. Microbiol. 213:129–135.

Cook, J., and H. V. D. Heijden. 2010. Infectious bronchitis virus in Asia, Africa, Australia and Latin America: history, current situation and control measures. Rev. Bras. Cienc. Avic. 12:97–106.

Cook, J. K. A., M. Jackwood, and R. C. Jones. 2012. The long view: 40 years of infectious bronchitis research. Avian Pathol. 41:239–250.

Daher, R. K., G. Stewart, M. Boissinot, and M. G. Bergeron. 2016. Recombinase polymerase amplification for diagnostic applications. Clin. Chem. 62:947–958.

Faruku, B., A. S. Suri, H. B. Mohd, M. Hassan, and O. A. Rahman. 2015. Progress and challenges toward the development of vaccines against avian infectious bronchitis. J. Immunol. Res. 2015:1–12.

Giovanni, F., M. Paola, T. C. Maria, B. Ilaria, T. Giovanni, F. Laura, M. Ciccozzi, A. Lavazza, M. Cecchinato, and A. Moreno. 2017. Think globally, act locally: phylodynamic reconstruction of infectious bronchitis virus (IBV) QX genotype (GI-19 lineage) reveals different population dynamics and spreading patterns when evaluated on different epidemiological scales. PLoS One 9:1–20.

Haji-Abdolvahab, H., A. Ghalyanchilangeroudi, A. Bahonar, S. A. Ghafouri, M. V. Marandi, M. H. F. Mehrabadi, and F. Tehrani. 2019. Prevalence of avian influenza, Newcastle disease, and infectious bronchitis viruses in broiler flocks infected with multifactorial respiratory diseases in Iran, 2015–2016. Trop. Anim. Health Prod. 51:689–695.

Kim, J., and C. J. Easley. 2011. Isothermal DNA amplification in bioanalysis: strategies and applications. Bioanalysis 3:227–239.

Leghari, R. A., B. Fan, H. Wang, J. Bai, L. Zhang, S. H. Abro, and P. Jiang. 2016. Full-length genome sequencing analysis of avian infectious bronchitis virus isolate associated with nephropathogenic infection. Poult. Sci. 95:2921–2929.

Lin, Z., A. Kato, Y. Kudou, and S. Ueda. 1991. A new typing method for the avian infectious bronchitis virus using polymerase chain reaction and restriction enzyme fragment length polymorphism. Arch. Virol 116:19.

Piepenburg, O., C. H. Williams, D. L. Stemple, and N. A. Armes. 2006. DNA detection using recombination proteins. PLoS Biol. 4:e204.

Wu, Z. Q., Q. W. Yang, C. Fu, X. Y. Zhao, and J. Ignjatovic. 1998. Antigenic and immunogenic characterization of infectious bronchitis virus strains isolated in China between 1986 and 1995. Avian Pathol. 27:578–585.

Zhang, T., J. Chen, C. G. Wang, X. H. Zhai, and S. L. Zhang. 2018. Establishment and application of real-time fluorescence-based quantitative PCR for detection of infectious laryngotracheitis virus using SYBR Green I. Poult. Sci. 97:3854–3859.

Zhao, Y., X. Y. Liu, J. L. Cheng, Y. P. Wu, and G. Z. Zhang. 2014. Molecular characterization of an infectious bronchitis virus strain isolated from northern China in 2012. Arch. Virol. 159:3457–3461.