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A duplex RT-PCR assay for detection of H9 subtype avian influenza viruses and infectious bronchitis viruses

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Abstract
H9 subtype avian influenza virus (AIV) and infectious bronchitis virus (IBV) are major pathogens circulating in poultry and have resulted in great economic losses due to respiratory disease and reduced egg production. As similar symptoms are elicited by the two pathogens, it is difficult for their differential diagnosis. So far, no reverse transcription-polymerase chain reaction (RT-PCR) assay has been found to differentiate between H9 AIV and IBV in one reaction. Therefore, developing a sensitive and specific method is of importance to simultaneously detect and differentiate H9 AIV and IBV. In this study, a duplex RT-PCR (dRT-PCR) was established. Two primer sets target the hemagglutinin (HA) gene of H9 AIV and the nucleocapsid (N) gene of IBV, respectively. Specific PCR products were obtained from all tested H9 AIVs and IBVs belonging to the major clades circulating in China, but not from AIVs of other subtypes or other infectious avian viruses. The sensitivity of the dRT-PCR assay corresponding to H9 AIV, IBV and mixture of H9 AIV and IBV were at a concentration of 1×10¹, 1.5×10¹ and 1.5×10¹ 50% egg infective doses (EID₅₀) mL⁻¹, respectively. The concordance rates between the dRT-PCR and virus isolation were 99.1 and 98.2%, respectively, for detection of samples from H9N2 AIV or IBV infected chickens, while the concordance rate was 99.1% for detection of samples from H9N2 AIV and IBV co-infected chickens. Thus, the dRT-PCR assay reported herein is specific and sensitive, and suitable for the differential diagnosis of clinical infections and surveillance of H9 AIVs and IBVs.

Keywords: avian influenza viruses, H9 subtype, infectious bronchitis viruses, duplex RT-PCR

1. Introduction
Avian influenza virus (AIV) and infectious bronchitis virus (IBV), belonging to family Orthomyxoviridae and family Coronaviridae, respectively, are major ribonucleic acid (RNA) pathogens causing respiratory disease in poultry (Mayo and Pringle 1998; Alexander and Brown 2000; Cavanagh 2007). The H9N2 subtype AIV is a notable member of influenza A genus as it can infect not only avian species but also, although sporadically, mammals such as pigs and humans (Peiris et al. 2001; Butt et al. 2005). H9N2 AIV has been
detected in bird populations in Asia, Europe and Africa (Chen et al. 1994; Jackwood and Stallknecht 2007; Lebarbenchon et al. 2007). In China, H9N2 AIV has become widespread in poultry since the first isolation in chickens in 1994 (Chen et al. 1994). Avian infectious bronchitis virus was first described in 1931, and now is prevalent in all countries with an intensive poultry industry (Zanella et al. 2003; Gelb et al. 2005; Bochkov et al. 2006; Ignjatovic et al. 2006; Worthington et al. 2008; Mase et al. 2010). In China, IBV, which was first isolated in the early 1980s, poses a constant threat to domestic poultry (Feng et al. 2012). Therefore, China is considered as important endemic area of the two viruses. Infections of the two viruses both lead to severe economic losses in poultry industry because of respiratory symptoms, poor weight gain and reduced egg production. Co-infection with the two pathogens is also found in poultry stocks (Nili and Asasi 2002; Seifi et al. 2010). Similar clinical signs and gross lesions lead to the difficulty in differentiating the two pathogens clinically. So far, no assay has been reported to differentiate between H9 AIV and IBV in one reaction. Therefore, it is necessary to develop a sensitive and specific diagnostic assay which is able to diagnose and differentiate the two viruses.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time RT-PCR are routine methods to detect RNA viruses, which is quicker and less labor-intensive than virus isolation (Bustin and Nolan 2004; Fu et al. 2010). However, real time RT-PCR needs expensive equipment and specific technical training which limit the use of it as routine laboratory tests. In contrast, RT-PCR, as an economically-sound method, is particularly suitable for farm-level laboratories instead of sending samples to higher level diagnostic centers, especially in the developing countries.

In the study, a duplex RT-PCR (dRT-PCR) for detection of H9 AIVs and IBVs was developed. To evaluate the diagnostic accuracy of the dRT-PCR assay, experimental samples from virus-inoculated chickens were tested, and the results of the dRT-PCR analysis were compared with those achieved from virus isolation method.

2. Materials and methods

2.1. Viruses

28 avian infectious viruses were used in this study, including 12 H9N2 AIVs and nine IBVs belonging to the main clades circulating in China, four AIVs of other subtypes and three other infectious avian viruses (Table 1). All AIVs and IBVs were preserved in our laboratory and three infectious avian viruses, including infectious bursal disease virus, Newcastle disease virus and avian reovirus, were kindly provided by Dr. Zhang Guozhong (College of Veterinary Medicine, China Agricultural University, Beijing). 12 H9N2 AIVs belonged to the phylogenetic clades 2, 6, 8 and 9 of hemagglutinin (HA) gene (Fig. 1-A) based on the clade classification (Pu et al. 2015), while nine IBVs belonged to the clades I, III, and IV of nucleocapsid (N) gene (Fig. 1-B) based on the phylogenetic analysis (Zhao et al. 2013). Among these tested viruses, one H9N2 AIV and four IBVs were identified in this study by using a reference RT-PCR method and DNA sequence analysis (Hoffmann et al. 2001; Mo et al. 2013). Other viruses were characterized in previous studies (Liu et al. 2009, 2011; Pu et al. 2009, 2015). H3N8 AIV, H4N6 AIV, H5N1 AIV, H6N1 AIV and three other infectious avian viruses were used for the specificity tests of the dRT-PCR assay. Virus propagation was performed in 9–11-day old embryonated specific pathogen free (SPF) chicken eggs and stored at –80°C until use. The 50% egg infective doses (EID50) for each of the viruses were determined in 5–7 days after inoculation with the highest dilution that produces visible gross lesions.

Table 1  Viruses used in this study

| Virus | Strain name | Clade |
|-------|-------------|-------|
| H9N2 AIV | A/chicken/Beijing/3/1999 | 2 |
| H9N2 AIV | A/chicken/Shandong/2B/2007 | 8 |
| H9N2 AIV | A/chicken/Hebei/06/2007 | 9 |
| H9N2 AIV | A/chicken/Shandong/22/2008 | 9 |
| H9N2 AIV | A/chicken/Shandong/zzc2/2009 | 9 |
| H9N2 AIV | A/chicken/Jiangsu/TS/2010 | 9 |
| H9N2 AIV | A/chicken/Hebei/YT/2010 | 9 |
| H9N2 AIV | A/chicken/Guangdong/01/2011 | 6 |
| H9N2 AIV | A/chicken/Jilin/05/2012 | 6 |
| H9N2 AIV | A/chicken/Shandong/ql1013/2012 | 9 |
| H9N2 AIV | A/chicken/Shaanxi/xa414/2013 | 9 |
| H9N2 AIV | A/chicken/XY/8/2014 | 9 |
| H3N8 AIV | A/duck/Beijing/33/2004 | – |
| H4N6 AIV | A/duck/Shandong/1/2010 | – |
| H5N1 AIV | A/chicken/Huabei/202/2010 | – |
| H6N1 AIV | A/duck/Beijing/1/2003 | – |
| IBV | CONN | I |
| IBV | H20 | I |
| IBV | H52 | I |
| IBV | M41 | I |
| IBV | HOLTE | I |
| IBV | HS-X5 | III |
| IBV | DFC-X2 | IV |
| IBV | XZ-X4 | IV |
| IBV | SD-X6 | IV |
| Newcastle disease viruses | Duck/Shandong/2010 | – |
| Infectious bursal disease virus | B87 | – |
| Avian reovirus | HC | – |

1 AIV, avian influenza virus; IBV, infectious bronchitis virus.
2 Viruses identified previously.
3 This virus was used as the reference strains in the development of the duplex reverse transcription (dRT)-PCR.
4 –, not shown.
calculated using the Reed and Muench formula (Reed and Muench 1938).

2.2. Samples

Experimental specimens were collected to evaluate the dRT-PCR in detecting field sample. 330 experimental samples were taken from 15 white Leghorn specific pathogen free chickens (six weeks old; Beijing Experimental Animal Center, Beijing) infected with H9 AIV (A/chicken/Jiangsu/TS/2010) alone, IBV (M41) alone, or co-infected with H9 AIV and IBV. Briefly, 10 chickens were divided randomly into two equal groups. Chickens from each group were inoculated intranasally with H9 AIV or IBV, respectively, at a dose of 10⁶ EID₅₀ for each stock virus; the other five chickens were co-infected intranasally with H9 AIV and IBV at a dose of 10⁶ EID₅₀ for each stock virus. Tracheal and cloacal swabs were collected from 1 to 11 days post-inoculation. To evaluate the diagnostic accuracy of the dRT-PCR assay, all of these samples were also inoculated into 9–11-day old embryonated chicken eggs for virus isolation. Hemagglutination-positive samples and samples induced dwarf embryo were selected and confirmed to be H9 AIV- or IBV-positive samples, respectively, by RT-PCR and DNA sequence analysis (Hoffmann et al.

Fig. 1 Phylogenetic analysis of H9 avian influenza virus (AIV) and infectious bronchitis viruses (IBVs). A, phylogenetic trees of hemagglutinin (HA) genes of representative H9 AIVs. B, phylogenetic trees of nucleocapsid (N) genes of representative IBVs. The unrooted phylogenetic trees were generated by the distance-based neighbor-joining method using MEGA 4.1 (DNAStar Inc., Madison, WI, USA). The reliability of the tree was assessed by bootstrap analysis with 1,000 replications. Virus strains used in the present study were underlined.
2001; Mo et al. 2013). Then the results obtained by virus isolation and dRT-PCR were compared in a time course.

2.3. Primer design

Sequence data for the HA genes from AIVs of different subtypes and the N genes from IBVs were obtained from GenBank (GenBank: http://www.ncbi.nlm.nih.gov/nuccore) and aligned using the MegAlign program (DNASTAR Inc., Madison, WI, USA). Primers specific for the HA genes of the H9 AIVs and the N genes of IBVs were designed using Primer3.0 primer design software (Premier Biosoft International, Palo Alto, CA, USA). The basic primer design rules were as follows: the primer could have only one target site in the template DNA; the optimal melting temperature for primers was in the range of 52–61°C; the two primers of a primer pair should have closely matched melting temperatures within a difference of 5°C; there should be an absence of primer secondary structures including hairpins, self-dimers, and cross dimers; few of adenine residues in the 3’ end.

2.4. Viral RNA extraction and dRT-PCR assays

Viral RNA was isolated from infectious allantoic fluid, tracheal or cloacal samples as described previously with some modifications (Wei et al. 2006). Briefly, 300 μL of allantoic fluid or specimens was mixed with 900 μL of Trizol LS reagent (Invitrogen, Carlsbad, CA, USA) and placed on ice for 10 min. Chloroform (200 μL) was added and the mixture placed on ice for a further 5 min. The resulting suspension was centrifuged for 15 min at 12,000×g. The RNA-containing aqueous phase was precipitated with an equal volume of isopropanol and centrifuged at 13,000×g for 15 min at 4°C. The RNA pellet was washed with 1 mL of 75% ethanol and centrifuged at 12,000×g for 5 min at 4°C. The supernatant was removed. Then the RNA was dried and resuspended in 12 μL of diethyl-pyrocarbonate-treated deionized water. RT reactions were performed using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) according to the manufacturer’s protocol. The dRT-PCR was performed in 25 μL reaction volumes containing 5 μL of 5× reaction buffer, 0.5 μL dNTP Mix (10 mmol L⁻¹ of each dNTP), 1 μL of 25 mmol L⁻¹ MgSO₄ (Promega, Madison, WI, USA), 2.5 U of Taq polymerase (Promega), 0.5 μL of each primer (20 μmol L⁻¹ of each primer) and 2 μL of cDNA template. RNase-free water was added to make the volume up to 25 μL.

The amplification conditions were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 45 s, and extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. The final amplified products (4 μL) were loaded onto 1% (w/v) agarose gels containing 0.5 μg mL⁻¹ ethidium bromide. Electrophoresis was conducted using 1× TAE buffer and PCR products were visualized under UV trans-illumination.

2.5 Optimization of PCR assays

The parameters optimized in the dRT-PCR included: the annealing temperature, the primers molar ratio, the number of cycles and the extension time. The PCR protocol was submitted to optimization using a univariate method. Optimal PCR conditions were determined by performing a gradient of alternatives as follows: 1) annealing temperature (50, 53, 56, 59, 62 and 65°C); 2) mole ratio of IBV primer pair and H9 subtype AIV primer pair (4:1, 2:1, 1:1, 1:2 and 1:4); 3) amplification cycles (30, 35, 40 and 45 cycles); 4) extension time (1, 2, and 3 min). The examination samples for optimization assays were mixed embryo allantoic fluid of H9 AIV (A/chicken/Jiangsu/TS/2010) and chicken infectious bronchitis virus (M41) at a low concentration of 1, 10⁻¹ and 10⁻² EID₅₀ of each virus mL⁻¹ to maximize the sensitivity of the assay. The appropriate PCR condition was that reached the highest sensitivity.

2.6. Specificity of the dRT-PCR

To test specificity of the dRT-PCR, RNA templates were extracted from H9 AIVs, IBVs, AIVs of other subtypes and other avian infectious viruses. 12 H9 AIVs, nine IBVs, four AIVs of other subtypes and three other types of avian infectious viruses were used in specificity assay (Table 1).

2.7. Sensitivity of the dRT-PCR

H9 AIV (A/chicken/Jiangsu/TS/2010) and IBV (M41) were selected as reference strains to evaluate the sensitivity of the dRT-PCR assay. The sensitivity of the dRT-PCR was determined by testing RNA extracted from reference viruses that had been diluted serially 10-fold in PBS. The detection limits of the dRT-PCR assay for the H9 AIV (A/chicken/Jiangsu/TS/2010), IBV (M41) and a mixture of the two viruses were determined. Individual H9 AIV and IBV were diluted serially 10-fold in PBS from 1×10⁴ to 1 EID₅₀ mL⁻¹, from 1.5×10⁴ to 1.5 EID₅₀ mL⁻¹, respectively. Mixtures of the two viruses described above were diluted serially 10-fold in PBS from 1.5×10⁴ to 1.5 EID₅₀ mL⁻¹. The sensitivity of the duplex RT-PCR was confirmed in five independent experiments.

2.8. Sequencing

Duplex RT-PCR products of the expected sizes were purified from agarose gels using an AxyPrep DNA Gel Extraction Kit
(Axygen Scientific Inc., CA, USA) according to the manufacturer’s instructions, and then sequenced in both directions at the Beijing Genomics Institute (China). Sequence alignments were done using the ClustalW multiple sequence alignment program to evaluate the specificity of the assay.

3. Results

3.1. Primer design and selection

Five sets of primers were designed targeting the conserved regions of HA sequences of H9 AIV strains or N gene of IBVs, respectively. Among different combinations of H9 AIV specific primers and IBV specific primers, based on their amplification efficiencies, specificities and the abilities to distinguish PCR products by sizes, two sets of primers were selected (Table 2). One primer set amplified a 473-bp fragment within the HA gene of H9 AIVs and the other primer set amplified a 654-bp fragment within the N gene of IBVs.

3.2. Optimization of dRT-PCR

The mole ratio of IBV primer pair and H9 AIV primer pair, annealing temperature, extension time and number of cycles were optimized during the development of dRT-PCR using mixed embryo allantoic fluid of H9 AIV (A/chicken/Jiangsu/TS/2010) and IBV (M41) at a low concentration of 1, 10 and 10² EID₅₀ mL⁻¹. The condition of dRT-PCR which achieved the highest sensitivity was selected.

Optimization assays show that optimal mole ratio of IBV primer pair and H9 AIV primer pair is 1:1, while the optimal annealing temperature, extension time and number of cycles were 59°C, 2 min, 30 cycles, respectively (data not shown).

3.3. Specificity of the dRT-PCR assay

In the dRT-PCR assay, the correct size amplification products (Fig. 2-A and B) were obtained with 12 H9 AIVs and nine IBVs belonging to the main clades circulating in China (Fig. 1-A and B). None of the other viruses, including H3N8 AIV, H4N6 AIV, H5N1 AIV, H6N1 AIV, infectious bursal disease virus, Newcastle disease viruses and avian reovirus yielded PCR products following amplification (Fig. 2-C). The obtained PCR products were sequenced to evaluate the specificity of the assay. Sequence analysis showed greater than 99% homology between the PCR products and the target gene sequence.

3.4. Sensitivity of the dRT-PCR assay

The sensitivity of the dRT-PCR was determined by testing RNA extracted from reference viruses that had been diluted serially 10-fold in PBS. The sensitivity of the dRT-PCR assay corresponding to H9 AIV, IBV and mixture of H9 AIV and IBV are at a concentration of 1×10⁻¹, 1.5×10⁻¹ and 1.5×10⁻¹ EID₅₀ mL⁻¹, respectively (Fig. 3).

3.5. Evaluation of the dRT-PCR assay using experimental specimens

To examine the ability of the dRT-PCR to detect target viruses in infected samples, 330 experimental tracheal and cloacal specimens were tested with the dRT-PCR. Among the 330 experimental specimens, 110 tracheal and cloacal swabs were collected from H9 AIV inoculated chickens, 110 swabs from IBV inoculated chickens and 110 swabs from H9 AIV and IBV co-inoculated chickens. The results were compared with those obtained using conventional virus isolation methods combined with sequencing (Hoffmann et al. 2001; Mo et al. 2013). The concordance rates between the dRT-PCR and virus isolation were 99.1 and 98.2%, respectively, for detection of samples from H9N2 AIV or IBV infected chickens, while the concordance rate was 99.1% for detection of samples from H9N2 AIV and IBV co-infected chickens (Table 3). The result implies that the developed dRT-PCR assay is capable of simultaneously detecting H9 AIVs and IBVs. Consistent with virus isolation result, dRT-PCR confirmed that chickens co-infected with H9N2 AIV and IBV showed more virus shedding (59/110 for AIV, and 74/110 for IBV) than the chickens infected with H9N2 AIV (50/110) or IBV (63/110) alone.

4. Discussion

As a time-saving and economical method, RT-PCR assays have been widely developed and used for detection of

| Specific primers¹ | Primer sequences (5’→3’) | Target gene | Tm (°C) | Positions (bp) | PCR products (bp) |
|------------------|--------------------------|-------------|---------|----------------|------------------|
| H9-HA-F          | CATCGGCTACCAATCAACAAC    | HA          | 59.8    | 99–120²       | 473              |
| H9-HA-R          | GATTATTTGTGTATTGGCGTC    | HA          | 56.9    | 550–571¹      |                  |
| IB-N-F           | CAGGTAAAGGCGGAGAAAC      | N           | 58.7    | 621–641       | 654              |
| IB-N-R           | TQAAGCCCATCTGGTTGAGAG    | N           | 58.3    | 1255–1274     |                  |

¹) HA, hemagglutinin; N, nucleocapsid; F, forward primer; R, reverse primer.
²) H9 numbering.
AIVs and IBVs. RT-PCR methods designed for subtyping H9N2 AIVs are sensitive in detection of H9 AIVs isolated in China and other Asia countries (Noroozian et al. 2007; Chen et al. 2009; Tang et al. 2012; Nguyen et al. 2013). Similarly, RT-PCR methods to detect IBVs isolates from Europe, America and other countries in Asia have also been established (Zwaagstra et al. 1992; Wang et al. 1999; Lee et al. 2003; Pohuang et al. 2009). However, the genetic
co-inoculated chickens

Samples collected from H9 AIV and IBV inoculated chickens

Samples collected from H9 AIV and IBV co-inoculated chickens

AIV and IBV were 1×10^1, 1.5×10^1, and 1.5×10^1 EID_50 mL^-1, respectively. Using three reference strains from the above pathogens in China (Xu and Yin 2014). However, the detecting target genes (HA gene of H9 AIVs and N gene of IBVs) circulating in China belong to multiple clades (Zhao et al. 2013; Pu et al. 2015). In the present study, 21 H9 AIV and IBV strains, covering almost all major clades circulating in China up until 2014 (Zhao et al. 2013; Pu et al. 2015), were used to develop and validate the assay. Our assay suggests that the established method is suitable for the detection of H9 AIVs and IBVs that are circulating in China.

The detection limits for the H9 AIV, IBV and mixture of H9 AIV and IBV were 1×10^3, 1.5×10^3, and 1.5×10^3 EID_50 mL^-1, respectively. Compared with the previously established multiplex nested RT-PCR assay for the detection of AIV, IBV, and Newcastle disease virus, our method is 5.2 times more sensitive for the detection of IBV and 7.9×10^3 times more sensitive for the detection of type A AIV, and the multiplex assay established previously can not further subtype influenza A viruses (Nguyen et al. 2013). Although real-time RT-PCR has characteristic of high sensitivity (Meir et al. 2010; Shabat et al. 2010), the detection limits of dRT-PCR methods established in our study were more sensitive than that (10^2.3 EID_50 mL^-1) of a real-time RT-PCR assay for the detection of IBV (Meir et al. 2010), and also slightly more sensitive than that (10^2 EID_50 mL^-1 for H9 AIV detection) of a real-time PCR assay for simultaneous detection of subtypes H5, H7, and H9 AIV (Monne et al. 2008). In our study, the concordance rates for the dRT-PCR and virus isolation in detection of H9 AIVs and IBVs are above 98.2%, demonstrating the sensitivity and specificity of this developed assay is suitable for detecting target virus from infected chickens. Furthermore, the dRT-PCR assay can be finished in almost 5–6 h by detection directly with tracheal or cloacal samples, while virus isolation method combined with hemagglutination inhibition assay or sequencing to detect H9 AIV and IBV usually cost 3–4 and 9–10 days, respectively. So the established dRT-PCR is an alternative rapid, sensitive and economical detection method in comparison to the standard virus isolation and real time RT-PCR.

In summary, this study reported the successful development of a rapid dRT-PCR assay capable of simultaneously detecting H9 AIVs and IBVs. The assay can serve as a rapid and useful tool for the diagnosis and monitoring of H9 AIVs and IBV infections.

5. Conclusion

The dRT-PCR established in this study is both specific and sensitive, and it can be used to detect and differentiate H9 AIVs and IBVs.

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