Vet Res Commun (2008) 32:491–498
DOI 10.1007/s11259-008-9052-z

ORIGINAL ARTICLE

Rapid discrimination of H5 and H9 subtypes of avian influenza viruses and Newcastle disease virus by multiplex RT-PCR

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Accepted: 2 April 2008 / Published online: 15 May 2008

Abstract Avian influenza and Newcastle disease are the highly contagious and most economically important diseases in poultry industry throughout the world. A multiplex reverse transcription polymerase chain reaction (mRT-PCR) assay was developed for the rapid and specific discrimination of H5 and H9 subtypes of avian influenza viruses (AIV) and Newcastle disease virus (NDV). Three sets of specific primers were applied in the assay based on the sequences of the hemagglutinin gene of H5-AIV, H9-AIV and fusion protein gene of NDV. 59 clinical samples including the throat washes, oral swabs, and cloacal scrapings were detected by mRT-PCR and single RT-PCR (sRT-PCR), respectively. The results indicated that the sensitivity and specifity of mRT-PCR were in accordance with sRT-PCR. The mRT-PCR developed in this study may therefore provide a new avenue to rapid detection of these important pathogens in one reaction.

Keywords Multiplex RT-PCR · Avian influenza · H5 subtype · H9 subtype · Newcastle disease

Abbreviations

AI	avian influenza
AIV	avian influenza virus

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F	fusion protein
HA	hemagglutinin
mRT-PCR	multiplex RT-PCR
ND	Newcastle disease
NDV	Newcastle disease virus
RT-PCR	reverse transcription polymerase chain reaction
sRT-PCR	single RT-PCR

Introduction

Avian influenza (AI) and Newcastle disease (ND) are two of the most devastating avian diseases in the world, which is caused by avian influenza virus (AIV) and Newcastle disease virus (NDV), respectively. AIV is responsible for major disease problem in birds as well as in humans (Alexander 2000; Cameron et al. 2000; Li et al. 2003; Tran et al. 2004; Normile 2005; Ng and To 2007), There are 16 hemagglutinin (HA) subtypes and 9 neuraminidase (NA) subtypes of AIV (Fouchier et al. 2005), but only a few subtypes have been recovered from mammals and humans (Xie et al. 2006). Recently, the repeated incidences of H5N1 subtype of AIV in commercial chickens (Webster 2006) and the high prevalence of H9N2 in chickens throughout Asia, and along with their potential important role in the emergence of avian influenza pandemic (Lin et al. 2000), have accelerated the development of detection of AIV subtypes. It is difficult to discriminate AI and ND according to the clinical signs because both diseases may cause loss of egg production, acute respiratory infection and serosal hemorrhages of the gastrointestinal tract of domestic avian species. (Capua and Alexander 2004; Hubalek 2004; de Leeuw et al. 2005; Sakai et al. 2006). Rapid differential diagnosis of H5-AIV, H9-AIV and NDV is therefore a matter of urgency in order to prevent and control the epidemics.

Although virus isolation in embryonating eggs is a routinely sensitive method for diagnosis of AIV and NDV (Alexander et al. 1999; Alexander 2000), it may be time-consuming and laborious. Other tests such as reverse transcription polymerase chain reaction (RT-PCR) and real time RT-PCR have been previously applied to the detection of AIV and NDV (Bruckner et al. 1996; Fouchier et al. 2000; Wise et al. 2004; Pham et al. 2005; Ng et al. 2006; Das et al. 2006), but the single RT-PCR (sRT-PCR) only recognizes one specific virus in one reaction and expensive equipment and specific technical training requirements limit usefulness of real-time RT-PCR as routine laboratory tests. No multiplex RT-PCR (mRT-PCR) has been reported so far to detect H5-AIV, H9-AIV and NDV. In this study, a specific and sensitive mRT-PCR was developed for simultaneous detection and differentiation of these viruses.

Materials and methods

Viruses and plasmids

The viruses including field isolates of H5-AIV (A/duck/HN/AH01/2007(H5N1)), H9-AIV (A/chicken/GD/GZ01/2007(H9N2)), and reference strain of NDV (Roakin/NDV) from Chinese Veterinary Microorganism Conservation Center were used to standardize the mRT-PCR. All of the strains were identified by sRT-PCR and sequencing.

Recombinant plasmids of pT-H9, pT-H5 and pT-F were constructed by ligation of pGEM-T easy vector (Promega, Shanghai, China) and HA genes of H5-AIV, H9-AIV and fusion (F) protein gene of NDV (Roakin/NDV) and served as standard for determining the specificity of the assays.

Clinical specimens

Clinical specimes include throat washes, oral swabs and cloacal scrapings. Throat washes were collected with 1 ml syringe filled with 0.05M pH7.4 phosphate buffer to rinse the throat of the bird one or two times. 49 specimens comprised of 15 throat washes, 13 oral swabs, and 21 cloacal scrapings were collected from 25 chickens and 24 ducks that were infected naturally after initial appearance of clinical syndrome. In addition, 10 throat washes from specific-pathogen-free (SPF) chickens were used as negative control. The clinical specimens were suspended immediately in viral transport medium, which was made up of 0.05 M phosphate buffered saline (PBS), pH 7.4, containing antibiotics of penicillin (2000 units/ml), streptomycin (2 mg/ml), gentamycin (50 μg/ml) and mycostatin (1000 units/ml) for throat washes and oral swabs, and the antibiotic concentration is five-fold higher for cloacal scraping samples. The details of clinical samples were listed in Table 1.

RNA extraction

The processing of specimens was performed under biosafety level 3 containment facilities. The clinical sample suspensions were centrifuged at 6,500 × g for 1 min and the

Table 1 Clinical specimens and amplification results with sRT-PCR and mRT-PCR

Specimen	Specimen no.[a]	sRT-PCR result			mRT-PCR result		
		H5-AIV	H9-AIV	NDV	H5-AIV	H9-AIV	NDV
throat washes	1, 2	−	−	+	−	−	+
	3–5	+	−	−	+	−	−
	6–12	−	+	−	−	+	−
	13	+	−	+	+	−	+
	14	−	+	+	−	+	+
	15	+	+	−	+	+	−
	16–25	−	−	−	−	−	−
oral swabs	26, 27	−	−	+	−	−	+
	28–30	+	−	−	+	−	−
	31–34	−	+	−	−	+	−
	35–37	+	−	+	+	−	+
	38	+	+	−	+	+	−
cloacal scrapings	39–41	−	−	+	−	−	+
	42–46	+	−	−	+	−	−
	47–54	−	+	−	−	+	−
	55–57	+	−	+	+	−	+
	58	−	+	+	−	+	+
	59	+	+	−	+	+	−

[a] specimen no. 1–12, 26–30, 47–54 were collected from infected chickens, no. 13–15, 31–38, 39–46, 55–59 were from infected ducks and no. 16–25 were from SPF chickens

supernatants were collected. Viral RNAs were extracted from 140 μl of the supernatants by using a QIAamp viral RNA mini kit (Qiagen). After lysis of the specimens, the mixture was applied to a spin column as described by the manufacturer's protocol. The extracted RNAs were eluted in a total volume of 60 μl of elution buffer and were stored at −70°C until further use.

sRT-PCR and sequencing

Three different sets of primers specific for amplifying conserved region of HA gene of H5-AIV, H9-AIV and F gene of NDV were designed for sRT-PCR and mRT-PCR and listed in Table 2. The sRT-PCR was performed using SuperScript one-step RT-PCR kit (Invitrogen). Primers of H5F/H5R, H9F/H9R of AIV and F1/F2 of NDV were employed in the reaction, respectively. The sRT-PCR was performed in a 50 μl total reaction volume with 50 pmol of each primer according to the manufacturer's protocol. The thermal profile for sRT-PCR was 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min and a final extension cycle at 72°C for 5 min.

All of the 59 clinical samples including negative control were identified by sRT-PCR and sequencing for H5-AIV, H9-AIV and NDV, respectively.

The purified sRT-PCR products for clinical samples were sent out to Takara Biotechnology Co., Ltd, Dalian, China for DNA sequencing with an automated ABI model 373A Stretch DNA sequencer. DNA sequences of the products were then analyzed using the DNAStar to confirm the amplified DNA sequence.

mRT-PCR

The mRT-PCR was performed using SuperScript one-step RT-PCR kit (Invitrogen). Three sets of primers were employed in one reaction. The mRT-PCR was carried out in a reaction volume of 50 μl containing 10 pmol of each primer, 1 μl of RT/Taqase Mix, 25 μl of 2× Reaction Mix and 5 μl of each viral RNA or plasmids of pT-F, pT-H5 and pT-H9. Reverse transcription reaction was carried out at 52°C for 30 min followed by the cycling protocol of an initial denaturing at 94°C for 2 min, then 30 cycles that each consisted of denaturing at 94°C 30 s, annealing at 52°C 30 s, and extension at 72°C for 1 min. The sample was then heated at 72°C for 2 min. The mRT-PCR products were separated in 1% agarose gels, run in 0.5× TAE buffer with 0.5 μg/ml ethidium bromide at 10 V/cm for 25 min, and visualized under UV light. These different mRT-PCR products were subjected to DNA sequencing.

Table 2 Specific primer pairs used for sRT-PCR and mRT-PCR

Virus	Primer name	Primer sequence	Expected Product (bp)
H5-AIV	H5F	5′-ACGTATGACTATCCACAATACTCAG -3′	229
	H5R	5′- AGACCAGCTACCATGATTGC-3′	
H9-AIV	H9F	5′- CTACTGTTGGGAGGAAGAGAATGGT-3′	266
	H9R	5′- TGGGCGTCTTGAATAGGGT-3′	
NDV	F1	5′- TCAATCATAGTCAAGTTGC-3′	136
	F2	5′- ACCCTTGTATCCTGCGGAT-3′	

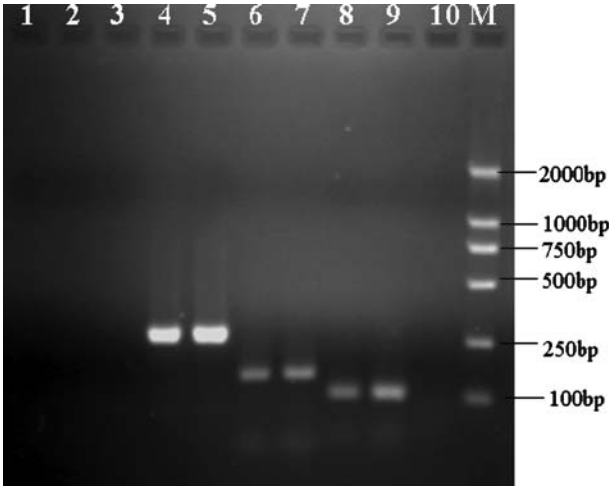

Fig. 1 Specificity of the mRT-PCR for the differentiation of H9-AIV, H5-AIV and NDV with different templates, Lane 1–3, SPF chicken throat washes; Lane 4, H9N2 strain; Lane 5, plasmid pT-H9; Lane 6, H5N1 strain; Lane 7, plasmid pT-H5; Lane 8, NDV strain; Lane 9, plasmid pT-F; Lane 10, PCR reagent buffer as the negative control; Lane M, DNA marker DL2000

Results

The specificity of mRT-PCR

The mRT-PCR products visualized by gel electrophoresis were 266 bp for H9-AIV, 229 bp for H5-AIV and 136 bp for NDV (Fig. 1). The mRT-PCR was found to be a specific assay for H5-AIV, H9-AIV and NDV, with no amplification of nucleic acid from SPF chicken throat washes. After DNA sequencing, DNAStar software analysis indicated that the mRT-PCR amplified DNA products were similar to sequences of HA of H5-AIV and H9-AIV, and F gene of NDV, respectively.

Evaluation of mRT-PCR with clinical samples

The applicability of mRT-PCR assay for detection of H5-AIV, H9-AIV and NDV in the diagnosis was validated by evaluating the assay system with the 59 clincal samples as

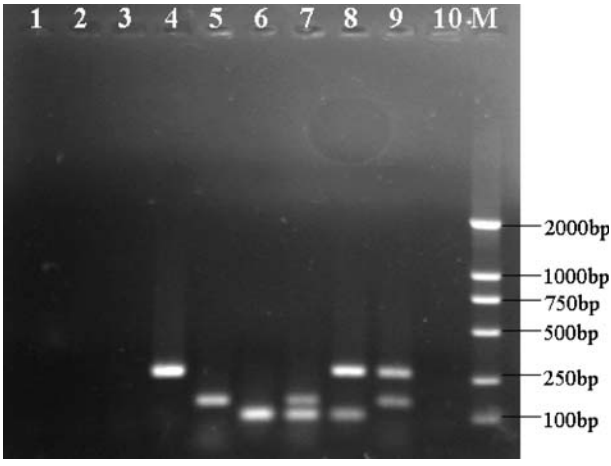

Fig. 2 Agarose gel electrophoresis analysis of mRT-PCR products of partial clinical samples for H5-AIV, H9-AIV and NDV. Lane 1–3, SPF chicken throat washes; Lane 4, H9N2 (specimen no. 7); Lane 5, H5N1 (specimen no. 3); Lane 6, NDV (specimen no. 1); Lane 7, H5N1 and NDV (specimen no. 36); Lane 8, H9N2 and NDV (specimen no. 58); Lane 9, H5N1 and H9N2 (specimen no. 38); Lane 10, PCR reagent buffer as the negative control; Lane M, DNA marker DL2000

mentioned above. Among 49 positive clinical samples, 37 birds were infected individually and 12 birds were co-infected. The assay indicated that 7 samples were positive for NDV, 19 samples were positive for H9-AIV, 11 samples were positive for H5-AIV, 7 samples were coinfected with both H5-AIV and NDV, 2 samples were coinfected with both H9-AIV and NDV, and 3 samples were coinfected with both H5-AIV and H9-AIV (Table 1). In addition, all the negative controls from the SPF chickens throat washes were shown to be free of these pathogens. All of these clinical samples were also tested separately by sRT-PCR. The results indicated that the sensitivity and specificity of mRT-PCR were in accordance with sRT-PCR. The mRT-PCR gel electrophoresis analysis of partial clinical samples was shown in Fig. 2.

Discussion

Upon typical conditions of intensive poultry production, several clinically similar viral diseases can occur which require laboratory differential diagnosis. Avian influenza is the most important viral zoonosis from avian sources (Antal et al. 2007; Tsuda et al. 2007; Wakamatsu et al. 2007) and Newcastle disease is also an economically important disease in poultry industries (Steel et al. 2008). Since 2002 the incidence of H5N1 in domestic poultry has increased substantially, while H9N2 has become endemic in Europe and Asia. Recent studies have shown the presence of multiple subtypes of AIV from samples collected from chickens and waterfowls (Panigrahy et al. 2002; Hanson et al. 2005; Nguyen et al. 2005). The mRT-PCR assay which can rapidly identify H5-AIV, H9-AIV and NDV will be therefore very important for the control of disease transmission from avian species to humans and reduction of the economic losses in poultry industries (Shu et al. 2006; Corbanie et al. 2007; Han et al. 2008).

In this study, mRT-PCR assay was demonstrated to be capable of rapid detection and differentiation of three important avian RNA viruses in clinical specimens. Precise primer design and the appropriate ration of each primer pair are crucial for a successful amplification in mRT-PCR (Bej et al. 1990). In general, the relative sensitivity of mRT-PCR was lower than that of sRT-PCR (Huang et al. 2004), but the similar sensitivity and specificity of mRT-PCR for clinical samples was obtained compared with that of sRT-PCR in the study. This may result from the optimization of primer designation or limited samples. Further studies have to be evaluated with a large-scale investigation in order to advance our understanding in this respect.

The throat washes, oral swabs, and cloacal scrapings are the samples of choice during the early stages of infection, which may have a higher predictive value of detecting H5-AIV, H9-AIV and NDV infection during disease surveillance screening. Importantly, the early detection suggests two potential uses in disease control for the assay: as a surveillance tool in areas free of the disease and as a screening assay for monitoring a disease outbreak.

In conclusion, the mRT-PCR method for rapid detection of H5-AIV, H9-AIV and NDV provides a more convenient and reliable means for routine diagnosis of bird disease and may be employed to screen for potential carriers in birds.

Acknowledgements This work was supported in part by grants from the National Key Technologies R&D Program of China (No.2006BAD06A03). This study was also supported by the National Natural Science Foundation of China (No.30671563 and No.30700597).

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