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Development of TaqMan real-time RT-PCR for detection of avian reoviruses

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Avian reoviruses (ARVs) are an important cause of economic losses in commercial poultry. A TaqMan real-time RT-PCR assay for detecting of ARVs was developed. The primer-probe set was from the conserved region of ARV S4 genome segment. Real-time RT-PCR detected ARV strains including CO8 and ss412 strains, which belonged to different serological subgroups, and the test had no cross-reaction with other avian viruses. The detection limit of this assay was 5 ARV genome copies per 5 μl and was 150 times more sensitive than traditional RT-PCR. Statistical analyses indicated excellent reproducibility. For ARV strain 2408, a titer of 50% embryo infection dose and 50% tissue culture infectious dose equivalent to 3.9 ± 0.8, and 2.9 ± 0.3 ARV genome copies, respectively. This test was rapid, specific, and sensitive for the detection of ARVs and will be useful in veterinary diagnostic laboratories and for the quantitation of vaccine viruses for pharmaceutical companies.

1. Introduction

Avian reoviruses (ARVs) cause tenosynovitis and are associated with viral enteritis, chronic respiratory disease, malabsorption syndrome, hepatitis, myocarditis, hydropericardium, etc. in chickens (Bains and MacKenzie, 1974; Fahey and Crawley, 1954; Kerr and Olson, 1969; Kibenge and Wilcox, 1983; McFerran et al., 1976; Page et al., 1982).

ARVs belong to the genus *Orthoreovirus* and the family *Reoviridae* (Joklik, 1983; Mertens, 2004; Nibert, 1998). They have a non-enveloped, double-layer concentric capsid 70–80 nm in diameter, enclosing the segmented double-stranded RNA (dsRNA) genome (Spandidos and Graham, 1976). The 10 dsRNA segments are categorized into 3 groups, designated L (large), M (medium), and S (small), based on their size and electrophoretic mobility. These segments code for at least 8 structural proteins (λA, λB, λC, μA, μB/μBC, σC, σA and σB) and 4 non-structural proteins (μNS, P10, P17, and σNS) (Benavente and Martinez-Costas, 2007; Schnitzer, 1985; Varela and Benavente, 1994). All genome segments of vaccine strains S1133 and 1733 have been sequenced. Recently the L2 genome segment of strain 176 was sequenced (GenBank: EU707936). ARV S4 genome segment codes for σNS protein. It is the smallest in size and is relatively conserved among all other genome segments, Therefore, it was selected as the target for real-time PCR detection.

Virus isolation, serological methods, e.g., virus neutralization, and histopathological assays are all used for the detection and differentiation of ARVs (Robertson and Wilcox, 1986). Viral propagation of the samples in chicken embryos or cell culture can take 3–5 days. Serological methods, such as agar-gel precipitin assay (Adair et al., 1987), fluorescent antibody assay (Menendez et al., 1975), and enzyme-linked immunosorbent assay (Slaght et al., 1978), although fast, lack sensitivity compared to real-RT-PCR. In addition, chickens are often vaccinated against ARVs so the presence of antibody alone cannot be used in diagnosis. Reverse transcriptase (RT)-PCR (Bruhn et al., 2005), nested PCR (Liu et al., 1997), multiplex PCR (Caterina et al., 2004), and PCR followed by restriction fragment length polymorphism (RFLP) (Lee et al., 1998; Liu et al., 2004) have been used to detect and differentiate ARVs.

Despite the use of multiple vaccines, outbreaks of ARV induced disease may still occur partially due to infection with different subtypes (BIOMUNE Inc., 2007; Rosenberger et al., 1989).

Real-time PCR allows detection and quantitation of target DNA molecules as they proceed through amplification cycles. By incorporating fluorescent reporters, real-time PCR can measure and monitor target DNA amplification during the exponential phase, resulting in more accurate readings compared to conventional PCR.

The current study developed the first TaqMan probe based real-time RT-PCR assay for detecting ARVs in different serologic subtypes. It was more rapid, specific, sensitive, and reproducible than other molecular tests. It can find use in veterinary diagnostic laboratories for more rapid viral detection and for pharmaceutical

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companies to develop more efficacious vaccines using a more efficient measure of vaccine genome copies.

2. Materials and methods

2.1. Virus propagation and preparation

Eight live ARV strains were used. All strains were propagated in chicken embryo kidney (CEK) cell cultures from specific pathogen free (SPF) embryos. Flasks were incubated at 70 °C for 48–72 h when 75–85% cytopathic effect (CPE) was observed. Cell cultures were frozen and thawed three times to rupture the cell membrane and release virus particles. Cell culture materials were transferred into 30 ml centrifugation tubes, which contained 3 ml of 40% sucrose. Centrifugation was at 100,000 × *g* using Type 30 rotor with Beckman® (Fullerton, CA) ultracentrifuge L8-70 for 1.5 h. The bottom phase, which contained concentrated virus and cell debris, was collected in cryopreservation vials and stored in −70 °C. Virus was concentrated to remove some of the RT-PCR inhibitors.

2.2. Viral RNA extraction

Viral RNAs were extracted using the Qiagen RNeasy® Mini Kit (Valencia, CA) with modifications. Briefly, 250 μl of concentrated virus-cell suspension were mixed with 3.5 volumes (875 μl) of lysis buffer RLT and 20 U Proteinase K (Sigma–Aldrich, St. Louis, MO) and incubated at 37 °C for 10 min. Two and half volumes (625 μl) of cold 100% ethanol were added and mixed. 700 μl of the mixture were transferred to an RNeasy column (Valencia, CA) and centrifuged at 9000 × *g* for 15 s, then the flow-through discarded. The remaining mixture was filtered through the same column and subsequently the column was washed once with 700 μl of buffer RW1 and twice with 500 μl RPE buffer. The column was transferred to a 2 ml tube and centrifuged at 14,000 × *g* for 2 min to remove excess reagents from the filter. Total RNA was eluted in 30 μl of nuclease-free water.

2.3. Primer and probe design

The S4 gene sequences of 9 ARV strains were obtained from GenBank nucleic acid database: 2408 (AF213468), 1733 (AF294772), 176 (AF059724), 601SI (AF294773), OS161 (AY573913), 750505 (AF213470), 919 (AY573912), T6 (AF213469) and S1133 (U95952). These sequences were aligned using AlignX® from Vector NTI™ Sequence analysis and data management software v10.3 (Invitrogen Corporation, Carlsbad, CA). One set of gene-specific primers and hydrolysis probe combination, which generates a 139-nucleotide PCR product, was designed from the conserved region of the S4 genome segment for use in real-time RT-PCR. This gene was used, because it codes for the antigenic and pathogenic protein of the virus. Another set of primers that encompassed the S4 genome segment was selected for obtaining the full-length S4 gene segment. All probes and primers (Table 1) were manufactured by Integrated DNA Technologies® Inc. (Coralville, IA).

2.4. Real-time RT-PCR

Real-time RT-PCR was performed on a LightCycler® (Roche Applied Science, Indianapolis, IN) with 20 μl, containing 5 μl viral RNA sample and 15 μl of reaction mixture. The Qiagen One-Step RT-PCR Kit R (Valencia, CA) was used for making reaction mixtures, which contain 4 μl of 5× buffer, 3.75 mM of $MgCl_2$, 325 μM of dNTPs (each), 0.5 μM R (1 μl) of each primer (R-S4F and R-S4R), 20 U of RNase inhibitor, 0.8 μl of Enzyme Mix, and 0.25 μM of probe S4-P. Reverse transcription was carried out at 45 °C for 30 min and the reaction heated at 95 °C for 15 min to inactivate the reverse transcriptase. The PCR stage was subjected to 40 cycles at 95 °C, denaturation for 5 s, 59 °C annealing for 20 s, and 72 °C extension for 10 s.

2.5. Construction of ARV σNS RNA standard

Full length (1104 bp) ChickVac® (Fort Dodge, Animal Health Inc., Overland Park, KS) was used. The S4 cDNA segment was obtained by RT-PCR using the primer set S4-FF/S4-FR. A two-step RT-PCR was conducted in GeneAmp® PCR System 9700 (Applied BioSystems, Foster City, CA) with The GeneAmp® RNA PCR Core Kit (Applied BioSystems, Foster City, CA) following the manufacture's recommendation with modifications. In the RT step, 1 μl of primer S4-FF (100 μM) was mixed with 2 μl of each RNA sample in a MicroAmp® tube (Applied BioSystems, Foster City, CA), and the mixture boiled at 99 °C for 5 min for denaturation. Reaction tubes were rapidly cooled on ice for 5 min to prevent re-annealing of denatured strands. For each reaction, 8 μl of RT reaction mixture with a final concentration of 4.55 mM $MgCl_2$, 1 μl 10× PCR buffer II, 0.9 mM each dNTP, 10 U RNase inhibitor, and 25 U MuLV reverse transcriptase were added. The RT reaction was performed at 42 °C for 60 min and 72 °C for 15 min. In the PCR step, 40 μl of reaction mixture containing the final concentrations of 2 mM $MgCl_2$, 4 μl 10× PCR buffer II, 2 μM primer S4-FR, and 2.5 U Amplitaq® DNA polymerase were dispensed into each tube. Thermal cycling was for 5 min at 95 °C, and 35 cycles of 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C, and followed by 7 min at 72 °C for final extension. PCR products were detected in 1.5% agarose gel electrophoresis and purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI).

The full length ChickVac™ S4 gene cDNA was cloned into NovaBlue Singles™ competent (*Escherichia coli*) cells via T7Blue-3 vectors, which was supplied by a blunt-end cloning kit (Novagen, Darmstadt, Germany). All procedures followed the manufacturers' instructions.

To verify the correct insertion, plasmids were extracted using the Wizard® Plus SV kit (Promega, Madison, WI). Plasmids were digested with restriction enzymes *SnabI* and *HindIII* (Promega, Madison, WI) to select plasmids with inserts of correct size, and with restriction enzymes *HindIII* and *MfeI* (Roche, Penzberg, Germany) to determine correct orientation.

Plasmids with the correct inserts were sequenced using the T7 primer to confirm the correct sequence. Plasmids were linearized

Table 1
Real-time and conventional RT-PCR primers and TaqMan probe sequences.

RT-PCR	Primers and probes	Length	Positions
Real-time	R-S4F: ATTATGGCTGGCTTTGTACCT	21	433–453
	S4-P: FAM[a]-CGTGAAGGTGATGACTTTGCTCC-TAMRA[b]	23	454–476
	R-S4R: ACAATCTGAGGACGACCATC	20	572–553
Full-length	S4-FF: ATGGACAACACCGTGCGT	18	1–18
	S4-FR: CTACGCCATCCTAGCTGGAGA	21	1104–1084

[a] FAM: 6-carboxyfluorescein.
[b] TAMRA: 6-carboxytetramethylrhodamine.