Chapter 14

PCR Amplification and Sequencing Analysis of Full-Length Turkey Coronavirus Spike Gene

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Abstract

Turkey coronaviral enteritis caused by turkey coronavirus (TCoV) continues to infect turkey flocks, resulting in significant economic loss. Determining and understanding genetic relationships among different TCoV isolates or strains is important for controlling the disease. Using two-step RT-PCR assays that amplify the full length of TCoV spike (S) gene, TCoV isolates can be sequenced, analyzed, and genotyped. Described in this chapter is the protocol on PCR amplification and sequencing analysis of full-length TCoV S gene. Such protocol is useful in molecular epidemiology for establishing an effective strategy to control the transmission of TCoV among turkey flocks.

Key words Turkey coronavirus, Spike gene, Sequencing, Genotype

1 Introduction

Turkey coronaviral enteritis caused by turkey coronavirus (TCoV) has been reported with varied severity in clinical signs in the affected turkey flocks from different states in the USA [1, 2], Canada [3], Brazil [4], and Europe [5]. The major clinical signs of TCoV infection include depression, ruffled feathers, watery diarrhea, decreased body weight gain, and uneven flock growth. The most striking gross lesions are markedly distended intestine with gaseous and watery content, especially in the ileum and ceca. The salient histopathologic findings include shortening of the intestinal villi, increase in crypt depth, and widening of intervillous spaces [1]. TCoV belongs to species *Avian coronavirus* of the genus *Gammacoronavirus* in the family *Coronaviridae*. The genome of TCoV is a linear positive-sense single-stranded RNA encoding three major structural proteins, including spike (S), membrane (M), and nucleocapsid (N) protein. While M and N genes are conserved, S gene is a more common target used for

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coronavirus (CoV) differentiation because S gene is highly variable among different CoVs. The S gene sequences among different TCoV isolates (93–99.7 %) are relatively conserved as compared to different infectious bronchitis virus (IBV) strains, although both TCoV and IBV belong to the same species *Avian coronavirus* [6–8]. Because the pair-wise comparison of S gene sequences revealed only 34 % of similarity between TCoV isolates and IBV strains while the remaining 3′-end encoding region shared over 80 % of similarity, it has been suggested that TCoV arose through a recombination of S gene from IBV [6, 9–11].

A protocol for PCR amplification, sequencing, and sequence analysis of TCoV S gene for genotyping of TCoV isolates based on TCoV S gene sequences is highlighted in this chapter. In **step** 1, intestine tissues are collected from TCoV-infected turkeys and TCoV is purified through ultracentrifugation on continuous sucrose gradient. In **step** 2, TCoV RNA is extracted from intestines and reverse transcribed to cDNA. In **step** 3, the full-length S gene of TCoV is amplified and sequenced. In **step** 4, the nucleotide sequence of the full-length S gene of TCoV is assembled from 12 sequence fragments flanking the entire length of S gene. The S gene sequences of TCoV isolates and those from other coronaviruses published in GenBank are further analyzed by alignment and phylogenetic tree and thus TCoV genotypes are determined.

2 Materials

2.1 Turkey Coronavirus Preparation

2.1.1 Reagent

1. Antiserum against TCoV/IN540/94 (GenBank accession number EU022525).
2. Phosphate-buffered saline (PBS) is composed of 1.44 g Na_2HPO_4, 8 g NaCl, 0.24 g KH_2PO_4, and 0.2 g KCl in 1 L ddH_2O. The solution is adjusted to pH 7.2 and autoclaved before use.
3. Whatman 0.45 and 0.22 μm syringe filter (Thermo Fisher Scientific, Waltham, MA, USA).
4. Thin-wall polypropylene ultracentrifuge tubes, open-top, transparent, 17 mL with 16×102 mm for rotor SW28 of Optima XL-100 K ultracentrifuge (Beckman Coulter, Fullerton, CA, USA).
5. RNApure™ reagent (GenHunter, Nashville, TN, USA).
6. Diethyl pyrocarbonate (DEPC) (Sigma-Aldrich, St. Louis, MO, USA): DEPC is very sensitive to moisture, so it needs to be stored at 2–8 °C to help reduce exposure to moisture.
7. Preparation of DEPC-treated water (DEPC-H_2O):
   (a) Prepare 0.1 % (v/v) DEPC in the water undergone reverse osmosis filtration and deionization. Add 0.1 mL DEPC

solution to 100 mL of water. DEPC appears as globules and needs continuous stirring until the globules disappear. It takes 12 h at 37 °C in a fume hood. DEPC can dissolve plastic, so it is better to store DEPC solution in a glass bottle.

(b) Autoclave 0.1 % DEPC solution at 121 °C for 15 min to remove any trace of DEPC. The autoclaved DEPC-H_2O can be stored at any temperature.

2.1.2 Equipment

1. Bio-Gen Pro200 homogenizer (Pro Scientific, Oxford, CT, USA).
2. Optima XL-100K ultracentrifuge (Beckman Coulter, Fullerton, CA, USA).
3. Bio-Rad Model 395 Gradient former (Bio-Rad, Hercules, CA, USA).
4. GeneQuant 1300 Spectrophotometer (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

2.2 RNA Extraction and cDNA Preparation

2.2.1 Reagent

1. SuperScript® III reverse transcriptase (Invitrogen™, Life Technologies, Grand Island, NY, USA).

2.3 PCR Amplification and Sequencing

2.3.1 Reagent

1. *Taq* DNA polymerase (Promega, Madison, WI, USA).
2. *Pfu* DNA polymerase (Stratagene™, Agilent Technologies, La Jolla, CA, USA).
3. Primers can be synthesized by Integrated DNA Technologies (IDT, San Jose, CA, USA) or other equivalent companies. The primers and sequence information are listed in Table 1. The lyophilized powders of products prepared by company are to be dissolved in DEPC-H_2O to 100 μM as stock solution (100×). The working solution of primers is 10 μM. Both stock and working solution of primers are stored at −20 °C.
4. Zymoclean™ gel DNA recovery kit (Zymo, Irvine, CA, USA).
5. TOPO® TA Cloning® Kit with pCR®II-TOPO® Vector and One Shot® TOP10F′ Chemically Competent E. coli (Invitrogen™, Life Technologies, Grand Island, NY, USA).
6. DNAstar Lasergene® software (DNASTAR, Madison, WI, USA).

2.3.2 Equipment

1. Applied Biosystems® GeneAmp® PCR System Thermal Cyclers (Life Technologies, Grand Island, NY, USA).

Table 1
Oligonucleotide primers and sequences used for turkey coronavirus spike gene polymerase chain reaction and sequencing

	Name	5′ → 3′ sequence	Position[a]	Orient
PCR primers	Sup	TGAAAACTGAACAAAAGACAGACT	−63 to −4	+
	Sdown3	TTTGTTGAATTATTTGCTGACCA	3813–3835	−
Sequencing primers	AS-cor	CCAAACATACCAAGGCCACTT	3392–3413	+
	S101F1	TAATTTAACATGGGGCAACT	410–429	+
	S101F2	TTCCCTAAAGTTAAAAGTGTT	459–479	−
	S101R1	CTGTCTAAATATGATGCCACTTCC	2985–3008	−
	S101R2	GGGCATTACACCATACTTTCCAGA	2955–2978	+
	S102F1	AAGTTGTGTATATTAGGGCTGAA	936–959	+
	S102F2	TTTATACGCAACATTCAT	982–999	−
	S102R1	TTACACTCTCAAAACCTCTA	2501–2520	−
	S102R2	ACCTCTAGCTACTTCAACAAATCC	2484–2507	+
	S103F1	ACCGTGCCAGACAGTTTCA	1439–1457	+
	S103F2	GAGTTTTGTAGGCTTGTTTCTTC	1481–1503	−
	S103R1	GTAGAACAAGCGACAAATCAAACC	2035–2058	−

[a]The positions of primers were relative to the start codon of nucleotide sequence of TCoV/IN/540/94 (EU022525)

3 Methods

3.1 Turkey Coronavirus Preparation

3.1.1 Clinical Sample Collection and Virus Propagation

1. Field cases of turkey coronaviral enteritis are diagnosed by clinical signs, gross lesions, histopathologic findings, immunofluorescence antibody (IFA) assay with antiserum against TCoV/IN540/94, electron microscopy, and reverse transcription polymerase chain reaction (RT-PCR) targeting partial fragment of N or S gene.
2. Homogenize the intestines from TCoV-infected turkeys with 5 volumes of chill sterile PBS on ice using Bio-Gen Pro200 homogenizer (*see* **Note 1**).
3. Centrifuge the homogenate at 1449 × *g* for 10 min at 4 °C.
4. Filter the supernatant without any tissue debris through Whatman 0.45 and 0.22 μm syringe filters, respectively.
5. Inoculate the filtrate into the amniotic cavity of 22-day-old embryonated turkey eggs.
6. Harvest turkey embryo intestines after 3 days of incubation at 99.3 °F with humidity of 56 %.
7. Repeat the procedures of virus propagation in 22-day-old embryonated turkey eggs up to five times serially, if needed.

3.1.2 Turkey Coronavirus Purification

1. Homogenate three harvested turkey embryonic intestines after TCoV propagation described in Sect. 3.1.1 with 15 mL of chill sterile PBS on ice using Bio-Gen Pro200 homogenizer.
2. Centrifuge the homogenate at 3000 rpm for 10 min at 4 °C.
3. Layer about 10 mL of the supernatant on top of 4 mL of 30 % sucrose (middle layer) and followed by 2 mL of 60 % sucrose (bottom layer) in a 17 mL ultracentrifuge tube for rotor SW28. The total volume needs to be about 80–90 % height of the ultracentrifuge tube or the tube may crush after ultracentrifugation.
4. Centrifuge the sample by ultracentrifugation in rotor SW28 at 103,679 × *g* for 3 h at 4 °C in Optima XL-100K ultracentrifuge.
5. Collect the interface between 30 % sucrose and 60 % sucrose with 10 mL pipet gently and carefully. Add PBS to the collected interface materials till the maximum volume of 5 mL and suspend the materials containing TCoV thoroughly.
6. Place 5 mL of interface materials on top of 10 mL of continuous 40–60 % sucrose gradient (*see* **Note 2**).
7. Centrifuge the sample with sucrose gradient by ultracentrifugation in rotor SW28 at 24,000 rpm at 4 °C for 20 h.
8. Collect the TCoV-containing band of buoyant density 1.16–1.24 g/mL as the viral stock and save it in the −80 °C freezer or liquid nitrogen until used.

3.2 RNA Extraction and cDNA Preparation

3.2.1 Total RNA Extraction

1. Take 200 μL of purified TCoV and mix with 1 mL of RNApure™ reagent in a 1.7 mL microcentrifuge tube.
2. Vortex briefly to dissolve any debris completely. Incubate at 4 °C for 10 min.
3. Add 180 μL of chloroform. Vortex the tube for 10 s. Incubate the tube at 4 °C for 10 min. Centrifuge the tube at 13,000 × *g* at 4 °C for 10 min.
4. Take the upper colorless aqueous phase to a new 1.7 mL microcentrifuge tube.
5. Add equal volume (about 0.5 mL) of cold isopropyl alcohol and vortex the tube for 10 s. Incubate the tube at 4 °C for 10 min.
6. Centrifuge the tube at 13,000 × *g* at 4 °C for 10 min. Discard the supernatant.
7. Wash the RNA pellet with 1 mL of 70 % ethanol by resuspending the pellet and centrifuge at 13,000 × *g* at 4 °C for 2 min. Discard the supernatant.
8. Air-dry the RNA pellet with the tube upside down for 1 min.
9. Dissolve the RNA pellet with 30 μL of DEPC-H_2O and measure the concentration of RNA using GeneQuant 1300 Spectrophotometer.

3.2.2 Reverse Transcription

1. Obtain cDNA from the extracted RNA by reverse transcription (RT) using SuperScript® III reverse transcriptase. In the first reaction, add 1 μL of random hexamer primer (50–100 ng/μL) into 9 μL of RNA (9–15 μg), incubate at 70 °C for 3 min, and quickly chill in ice. Briefly centrifuge the tube and proceed the second reaction. Add 4 μL of 5× reaction buffer, 2 μL of DTT (0.1 M), 2 μL of dNTPs (10 mM each dATP, dTTP, dCTP, dGTP), 1 μL of RNaseOUT™ (40 U), and 1 μL of SuperScript™ III reverse transcriptase (200 U) into the first reaction solution. Incubate the second reaction at room temperature for 5 min and 50 °C for 1 h and inactivate at 70 °C for 15 min.

3.3 PCR Amplification and Sequencing

1. Amplify the full-length TCoV S gene (about 3.9 kb) by PCR with the primers Sup and Sdown3 (Table 1) and the mixture of *Taq* DNA polymerase and *Pfu* DNA polymerase in a ratio of volume:volume = 64:1.
2. Prepare 50 μL of PCR reaction mixture (Table 2).
3. Perform PCR reaction in an Applied Biosystems® GeneAmp® PCR System Thermal Cycler 9700 according to the cycling program: 94 °C for 5 min and 40 cycles of 94 °C for 1 min, 55 °C for 4 min, and 72 °C for 10 min, and then stop at 4 °C for storage.
4. Electrophorese the PCR product on 1 % agarose gel to confirm the size (about 3.9 kb) and purity of the PCR product amplified by the primers Sup and Sdown3.
5. Purify Sup/Sdown3 PCR product from agarose gel by Zymoclean™ gel DNA recovery kit for sequencing.

Table 2
Reaction mixture for polymerase chain reaction amplifying the full length of turkey coronavirus spike gene

Components	Volume (μL)
cDNA	1
10× Reaction mix	5
dNTPs (10 mM each)	1
$MgCl_2$ (25 mM)	3
Taq:*Pfu* DNA polymerase (v:v = 64:1)	1
Forward primer Sup 10 μM	1
Reverse primer Sdown 10 μM	1
DEPC H_2O	37
Total volume	50

6. Obtain the nucleotide sequences of the purified Sup/Sdown3 PCR product by sequencing using the 12 sequencing primers listed in Table 1 at a certified genomics facility.
7. Clone the purified Sup/Sdown3 PCR product into pCR®II-TOPO® plasmid vector and transform it into *Escherichia coli* strain One Shot® TOP10F′ for storage purpose (*see* **Note 3**).

3.4 Sequence Analysis

1. Assemble 12 nucleotide sequences from 12 sequencing primers (Table 1) to obtain the full length of TCoV S gene by using DNAstar Lasergene® software.
2. Analyze the full-length S genes from different TCoV field isolates by Clustal W alignment method using DNAstar Lasergene® software or Web-based MEGA6 program (http://www.megasoftware.net). Sequence alignment can arrange the sequences of DNA and deduced amino acids to identify regions of similarity resulting from evolutionary relationships among the compared sequences.
   (a) Open MEGA6 program, click "Align" on the tool bar, choose "Edit/Build Alignment," check the option of "create a new alignment" in the first dialog box, push "OK," and then choose either "DNA" or "Protein" sequence alignment, and the Alignment Explorer window will open.
   (b) There are three ways to add sequences into alignment program. For the first two ways, go to "Edit" menu and click either "Insert Blank Sequence" to key in each sequence manually or "Insert Sequence From File" to import the selected sequences in Text (*.txt, *.seq) or FASTA file formats. For the third way, go to "Web" menu and click "Query GenBank" to import Web-based sequences by checking the "Add to Alignment" option for each interested sequence.
   (c) After all sequences for alignment are added, go to "Edit" menu in the Alignment Explorer window to select all sequences for alignment, and then go to "Alignment" menu to select "Align by Clustal W (Codons)" for the process of alignment. Choose Align Codons for DNA alignment to avoid introducing gaps into positions that would result in frame shifts in the real sequences. Use the default settings of Clustal W method for DNA alignment but change the Multiple Alignment Gap Opening penalty to 3 and the Multiple Alignment Gap Extension penalty to 1.8 for protein alignment. When the alignment is complete, go to "Data" menu and choose "Save Session" to save the alignment result as Aln session (*.mas) format. For further construct of phylogenetic tree, go to "Data":

menu and click "Export Alignment" to choose "MEGA Format" to save the alignment result as .meg extension.

3. A phylogenetic tree can be constructed by many methods used widely and maximum likelihood (ML) method is chosen based on previous coronaviral studies.
   (a) ML uses a variety of substitution models to correct for multiple changes at the same site during the evolutionary history of the sequences, like Tamura 3-parameter and Kimura 2-parameter models, and the best model to use for each analysis can be calculated by MEGA6 program. Go to MEGA6 main window and choose "Models" to select "Find Best DNA/Protein Models (ML)." Choose the alignment file saved before for calculation. Note the preferred model to construct a phylogenetic tree based on the list of models in order of preferences.
   (b) Go to MEGA6 main window and choose "Phylogeny" to select "Construct/Test Maximum Likelihood Tree." Fill in the parameters of "Model/Method" and "Rates among Sites" based on the preferred parameters calculated previously in the preference dialog appearing. Select "Partial Deletion" in "Gap/Missing Data Treatment" to avoid losing too much information. Set "No. of Bootstrap Replicates" to 1000 under "Phylogeny Test" to estimate the reliability of the tree.
   (c) After computing, a tree explorer window will open the tree. Save the tree as a (*.mts) file, choose "Export Current Tree (Newick)" from "File" menu for further modification in other tree drawing program, choose "Save as PDF File" from "Image" menu for graphic file format accepted for publishing, or choose "Save as PNG File" or "Save as Enhanced Meta File" for further process.

4 Notes

1. Put the turkey intestines containing TCoV in 50 mL tube. If the volume of intestines reaches 5 mL mark of 50 mL tube, add 25 mL (5 volumes) of chill sterile PBS for homogenization. It is recommended to homogenize the intestine tissue for 30 s and cool the tube in ice in cycles until the intestine tissue is homogenized thoroughly because the heat produced by the process of homogenization can damage the integrity of RNA in intestine tissues.
2. To prepare 40–60 % sucrose continuous gradient, Bio-Rad Model 395 Gradient former or equivalent gradient maker is used. Put 5 mL of 40 % sucrose and 5 mL of 60 % sucrose in

two connected compartments of Gradient former, respectively. Place a stirrer bar in the compartment with 40 % sucrose (lower extreme concentration of sucrose gradient). There is a valve to control the connection between two compartments and a plastic tube leading to an ultracentrifuge tube (Beckman Coulter) from the compartment with 40 % sucrose. Put the Gradient former on top of magnetic stirrer. Open the stirrer and wait till the stirrer bar stir steady and then open the valve. Collect the mixed sucrose in an ultracentrifuge tube to get 10 mL of 40–60 % sucrose continuous gradient.

3. To increase the successful rate of TA cloning, a 3′ A-overhangs post-amplification is performed. Add *Taq* polymerase buffer, dATP (10 mM), and 0.5 U of *Taq* polymerase to the purified PCR product. Incubate the reaction for 10–15 min at 72 °C. For further TA cloning, mix 1 μL of pCRII-TOPO vector, 1 μL of Salt Solution provided by the kit, and 4 μL of the purified PCR product gently and incubate the reaction at room temperature for 30 min. For further transformation, add 2 μL of TA cloning reaction to 50 μL of Top10F′ *E. coli* and incubate on ice for 30 min. Heat-shock the cells for 30 s at 42 °C without shaking and transfer the cells to ice immediately. Add 250 μL of room temperature S.O.C. medium provided by the kit. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37 °C for 1 h. Spread 10–50 μL from the transformation on a pre-warmed selective plate and incubate overnight at 37 °C. To ensure even spreading of small volumes, add 20 μL of super optimal broth with catabolite repression (S.O.C.) medium (Sigma-Aldrich). Pick about ten white or light blue colonies for analysis. Do not pick dark blue colonies.

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