

Chapter 3

Virus Neutralization Assay for Turkey Coronavirus Infection

Yi-Ning Chen, Ching Ching Wu, and Tsang Long Lin

Abstract

Turkey coronavirus (TCoV) infection induces the production of protective antibodies against the sequent exposure of TCoV. Serological tests to determine TCoV-specific antibodies are critical to evaluate previous exposure to TCoV in the turkey flocks and differentiate serotypes from different isolates or strains. A specific virus neutralization assay using embryonated turkey eggs and immunofluorescent antibody assay for determining TCoV-specific neutralizing antibodies is described in this chapter. Virus neutralization titer of turkey serum from turkeys infected with TCoV is the dilution of serum that can inhibit TCoV infection in 50 % of embryonated turkey eggs. Virus neutralization assay for TCoV is useful to monitor the immune status of turkey flocks infected with TCoV for the control of the disease.

Key words Turkey coronavirus, Virus neutralization assay, Turkey embryonated eggs, Immunofluorescence antibody assay

1 Introduction

Turkey coronavirus (TCoV) causes atrophic enteritis in turkeys and belongs to species *Avian coronavirus* of the genus *Gamma coronavirus* in the family *Coronaviridae*. The most closely related coronavirus is infectious bronchitis virus (IBV) causing respiratory disease in chickens. Turkey coronavirus infection induced protective antibody responses because turkeys that survived the infection did not develop clinical signs and TCoV was not detected in their intestines and feces after subsequent exposure to TCoV [1, 2]. Therefore, serological tests can be used to detect previous exposure to TCoV and differentiate the serotypes or strains from different isolates. The TCoV-specific antibodies can be detected by either enzyme-linked immunosorbent assay (ELISA) using TCoV S protein [3, 4], TCoV N protein [5, 6], or IBV virions [7] or immunofluorescent antibody (IFA) assay using intestine sections containing TCoV [8]. Among different serological tests, virus neutralization (VN) assay is the most specific test showing the inhibition of viral infection to target host tissues or cells by protective neutralizing antibodies.

Without cell culture system available for TCoV, embryonated turkey eggs are used for VN assay of TCoV. Because TCoV infection usually does not cause embryonic death, IFA assay [4, 8] or real-time RT-PCR [9] is used to detect TCoV in the intestines of turkey embryos to determine whether or not turkey embryos are infected by TCoV. In this chapter, a protocol for VN assay is described for determining the VN titer of serum from turkeys infected with TCoV or receiving experimental vaccines against TCoV infection. In **step 1**, TCoV stock and serum to be tested are diluted and incubated for neutralization reaction. In **step 2**, the mixture of TCoV and serum is inoculated into embryonated turkey eggs. In **step 3**, the infection of TCoV is determined by the detection of TCoV in the intestines of turkey embryos using IFA assay. In **step 4**, VN titer, the dilution of serum that can inhibit TCoV infection in 50 % of inoculated embryonated turkey eggs, is calculated according to the results of IFA assay [4, 10].

2 Materials

2.1 Neutralization Reaction

1. Turkey coronavirus stock, TCoV/IN/540/94 (GenBank accession number EU022525), is purified through 30–60 % sucrose gradient by ultracentrifugation at $103679\times g$ for 3 h at 4 °C.
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 ddH₂O. The solution is adjusted to pH 7.2 and autoclaved before use.

2.2 Egg Inoculation

1. Turkey eggs (British United Turkey of America, BUTA) are obtained from Perdue Farm (Washington, IN, USA).
2. Egg incubator (Jamesway, Cambridge, ON, Canada; Natureform, Jacksonville, FL, USA).
3. Egg candling device (Lyon Technologies, Inc. Chula Vista, CA, USA).

2.3 Immunofluorescence Antibody Assay

1. Minotome Plus™ Cryostat (Triangle Biomedical Systems (TBS), Durham, NC, USA).
2. Whirl-Pak bag (Thermo Fisher Scientific, Waltham, MA, USA).
3. Anti-TCoV antiserum to TCoV/IN/540/94.
4. FITC-conjugated goat anti-turkey IgG (H + L) antibody (KPL, Gaithersburg, MD, USA).
5. Vectashield® mounting medium (Vector Laboratories Inc., Burlingame, CA, USA).
6. Fluorescent microscope (Nikon, Melville, NY, USA).

3 Methods

3.1 Neutralization Reaction

3.1.1 TCoV Stock Preparation

1. Inoculate 0.2 mL of TCoV/IN/540/94 into 22-day-old turkey embryos with the procedures identical to those described in Sect. 3.2.
2. Harvest TCoV-containing intestines after 3 days of incubation.
3. Titrate TCoV-containing intestinal homogenate and store at -80°C freezer (*see Note 1*).

3.1.2 Neutralization Reaction

1. Inactivate the serum to be tested at 55°C in water bath for 30 min.
2. Dilute the serum with sterile PBS in two- or fourfold serially.
3. Take TCoV stock from -80°C freezer and place the vials directly in a 37°C water bath for fast thawing process. Right before the virus is completely thawed, remove the vials from the 37°C water bath and place them on ice (*see Note 2*).
4. Dilute TCoV stock to the final concentration of 200 EID₅₀/mL (50 % embryo infectious dose) with sterile PBS.
5. Mix the same volume of the diluted serum and TCoV together and incubate in a 37°C water bath for 1 h.

3.2 Egg Inoculation

1. Three or five 22-day-old embryonated turkey eggs are used for each dilution of serum to be tested. Each egg is inoculated with 100 μL of diluted serum mixed with 100 μL of 200 EID₅₀/mL TCoV. Embryonated eggs inoculated with serially diluted anti-TCoV serum (with a known titer) mixed with 100 μL of 200 EID₅₀/mL TCoV are used as the positive control for virus neutralization, embryonated eggs inoculated with anti-TCoV serum only are used as the negative control for no virus neutralization, and embryonated eggs inoculated with TCoV only are used as the inoculation control for virus infection.
2. Candle 22-day-old embryonated egg and mark the general location of the embryo at the base of the air cell.
3. Position eggs air cell up and disinfect the area directly at the top of the egg with 70 % ethanol spray. Label eggs.
4. Take eggs to a darkroom because this procedure requires illumination of the egg with an egg candling device while being inoculated.
5. Drill a small hole through the eggshell at the point near the back and head of embryo above the line that separates air cell and the rest of egg.

6. Use a 1 mL syringe with a 22-gauge needle in the length of 1 ½ in. (38 mm) and aim the needle toward the head or back shadow of the embryo. When the end of needle approaches the amniotic sac, give a quick stab toward the embryo to permit the needle to penetrate the amniotic membrane, and then inject 0.2 mL of inoculum (mixture of diluted serum and TCoV). To verify that the needle is in the amniotic sac, carefully move the needle sideways. If the needle has entered the amniotic sac, the embryo should reflect the same movement as the tip of the needle.
7. Seal the holes of eggs with glue and return the eggs to the incubator.
8. Incubate the eggs in the inoculator at 99.3 °F with humidity of 56 % for 3 days until embryonated turkey eggs are 25 days old, 3 days prior to hatching.

3.3 Immunofluorescence Antibody (IFA) Assay

1. After 3 days of incubation, the embryo intestines are harvested.
2. Open the shell via the air cell.
3. Pull the turkey embryo out, and then break the neck quickly.
4. Separate the yolk sac gently.
5. Open the abdominal cavity, and cut the connective tissue between stomach and intestine. Gently separate gall bladder and spleen from intestine.
6. Cut the cloaca and remove the whole intestine from abdomen.
7. Arrange the intestine loop together as a circle and place the intestine in the Whirl-Pak bag (Thermo Fisher Scientific). Snap freeze the intestines in dry ice and store at –80 °C freezer later.
8. Cut frozen intestine sections using the cryotome (TBS Minotome Plus™) and place the sections onto the glass slides.
9. Fix the sections in acetone for 10 min at room temperature (RT).
10. Add 50–100 µL of 2 % goat serum diluted with PBS to each section and incubate at RT for 30 min.
11. Dip slides into ddH₂O and wash slides with PBS with stirring for 10 min.
12. Dip slides into ddH₂O and get rid of excess water. Leave the slides to air-dry till almost dry.
13. Add 50–100 µL of 1:40 anti-TCoV serum diluted with PBS to each section and incubate at RT for 1 h.
14. Dip slides into ddH₂O and wash slides with PBS with stirring for 15 min.

15. Dip slides into ddH₂O and get rid of excess water. Leave the slides to air-dry till almost dry.
16. Add 50 to 100 µL of 1:100 FITC-conjugated goat anti-turkey IgG secondary antibody (KPL) diluted with PBS to each section and incubate at RT for 1 h in the dark by covering with aluminum foil or using a covered container.
17. Dip slides into ddH₂O and wash slides with PBS with stirring for 15 min.
18. Dip slides into ddH₂O and get rid of excess water. Leave the slides to air-dry till almost dry.
19. Place Vectashield® mounting medium (Vector Laboratories) over the sections and cover the slides with cover slips.
20. Observe the green fluorescent signals of TCoV in the intestine for positive IFA on a fluorescent microscope (Nikon). Keep the record of IFA results for further VN titer calculation.

3.4 Calculation of Virus Neutralization (VN) Titer

1. Calculate the number of TCoV-infected or non-infected eggs by IFA results.
2. Calculate the accumulated number of TCoV-infected or non-infected eggs.
3. The VN titer is the dilution of serum that can neutralize TCoV and inhibit TCoV infection in 50 % of embryonated eggs (*see Note 3*).

4 Notes

1. Titration of TCoV is to measure the concentration of infectious TCoV in the TCoV-containing intestine homogenate. The infectivity unit of TCoV is the 50 % embryo infectious dose or EID₅₀. One EID₅₀ unit is the amount of virus that will infect 50% of virus-inoculated embryonated eggs. Immunofluorescence antibody assay is used to determine the number of turkey embryos infected with TCoV. Example for calculating virus infectivity titer is illustrated below. According to the results of IFA assay, the number of TCoV-infected or non-infected eggs, the accumulated number of TCoV-infected, non-infected, or tested eggs, and the infection percentage of eggs inoculated with tenfold serially diluted TCoV can be calculated (Table 1). The index is calculated by the infection percentage:

$$\text{Index} = \frac{(\% \text{ Infected at dilution immediately above } 50 \%) - 50 \%}{[(\% \text{ Infected at dilution immediately above } 50 \%) - (\% \text{ Infected at dilution immediately below } 50 \%)]} \\ \% \text{ infected at dilution immediately above } 50 \%: 10^{-3} \rightarrow 62.5 \%$$

Table 1
Calculation of turkey coronavirus infectivity titer

Dilution	Infected eggs #	Non-infected eggs #	Accumulated #		Tested (A + B)	Infection percentage $A/(A + B) \times 100\%$
	IFA (+)	IFA (-)	Infected (A)	Non-infected (B)		
10^{-1}	5	0	14	0	14	100 %
10^{-2}	4	1	9	1	10	90 %
10^{-3}	3	2	5	3	8	62.5 %
10^{-4}	2	3	2	6	8	25 %
10^{-5}	0	5	0	11	11	0 %

% infected at dilution immediately below 50 %: $10^{-4} \rightarrow 25\%$
 Index = $(62.5\% - 50\%) / (62.5\% - 25\%) = 12.5\% / 37.5\% = 0.33$.

The index is then applied to the dilution that produced the percentage infected immediately above 50 %. In this example is the 10^{-3} dilution. The index of 0.33 is applied to this dilution. In this example, the dilution that provided the 50 % infection of eggs or 1 EID₅₀ is $10^{-3.33}$.

The reciprocal of this dilution is the amount of virus contained in the 0.2 mL of the original suspension = $10^{3.33}$ EID₅₀/0.2 mL = $5 \times 10^{3.33}$ EID₅₀/mL = 1.05×10^4 EID₅₀/mL. Thus, the infectivity titer of TCoV has been calculated to be 1.05×10^4 EID₅₀/mL.

2. Turkey coronavirus is an enveloped single-stranded RNA virus, so it is very fragile and sensitive to the process of freezing and thawing. It is recommended to use -80 or -20 °C pre-equilibrated CoolBox™ (VWR, Batavia, IL, USA) to transport virus vials from -80 °C freezer to laboratory before dilution and inoculation.
3. Example for calculating VN titer is illustrated below.

Serum sample A is used for VN titer calculation. Anti-TCoV serum is used as a positive control serum. Both serum A and anti-TCoV serum are diluted into 1:4, 1:16, and 1:64, respectively. Infection of TCoV in the embryonated eggs is determined by IFA assay. The number of TCoV-infected or non-infected eggs, the accumulated number of TCoV-infected, non-infected, or tested eggs, and the infection percentage of eggs inoculated with tenfold serially diluted TCoV can be calculated (Table 2). The index is calculated by the neutralization percentage:

Index = $[(\% \text{ Neutralized at dilution immediately above } 50\%) - 50\%] / [(\% \text{ Neutralized at dilution immediately above } 50\%) - (\% \text{ Neutralization at dilution immediately below } 50\%)]$.

Table 2
Calculation of turkey coronavirus virus neutralization titer

Titration	Infect #	Non-infect #	Accumulated #		Test (A+B)	Neutralization percentage
	IFA (+)	IFA (-)	Infect (A)	Non-infect (B)		$B/(A+B) \times 100\%$
NC	0	5	0	5	5	–
TCoV	5	0	5	0	5	0 %
Anti-TCoV serum						
1:4 (4^{-1})	0	5	0	11	11	100 %
1:16 (4^{-2})	1	4	1	6	7	85.7 %
1:64 (4^{-3})	3	2	4	2	6	33.3 %
Serum A						
1:4 (4^{-1})	2	3	2	5	7	71.4 %
1:16 (4^{-2})	3	2	5	2	7	28.6 %
1:64 (4^{-3})	5	0	10	0	10	0 %

1. For anti-TCoV serum, % neutralized at dilution immediately above 50 % is 85.7 % and the dilution is 4^{-2} ; % neutralized at dilution immediately below 50 % is 33.3 % and the dilution is 4^{-3} . Therefore, the index is $(85.7\% - 50\%) / (85.7\% - 33.3\%) = 0.68$.

The index is then applied to the dilution that produced the percentage neutralized immediately above 50 %, which is 4^{-2} dilution.

For anti-TCoV serum, the dilution that can provide 50 % neutralization of TCoV on the infection of turkey embryo is $4^{-2.68}$. Therefore, the VN titer of anti-TCoV serum is 1:41.

2. For serum A, % neutralized at dilution immediately above 50 % is 71.4 % and the dilution is 4^{-1} ; % neutralized at dilution immediately below 50 % is 28.6 % and the dilution is 4^{-2} . Therefore, the index is $(71.4\% - 50\%) / (71.4\% - 28.6\%) = 0.5$.

The index is then applied to the dilution that produced the percentage neutralized immediately above 50 %, which is 4^{-1} dilution.

For serum A, the dilution that can provide 50 % neutralization of TCoV on the infection of turkey embryo is $4^{-1.5}$. Therefore, the VN titer of anti-TCoV serum is 1:8.

3. In conclusion, anti-TCoV serum has higher VN titer of 1:41 than the VN titer of serum A, which is 1:8.

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