

PATHOGENICITY, IMMUNOGENICITY, PROTECTION EFFICACY, AND SPIKE PROTEIN GENE SEQUENCE OF A HIGH-PASSAGE TURKEY CORONAVIRUS SERIALLY PASSAGED IN EMBRYONATED TURKEY EGGS

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ABSTRACT

Experimental infection of a high-passage turkey coronavirus passaged serially in embryonated turkey eggs for 344 times (P344 TCoV 540) showed no enteritis-related clinical signs, decreased body weight gains, gross, and microscopic lesions. TCoV spike (S) protein specific antibodies appeared from 14 days post infection (dpi) and increased gradually. Virus neutralization (VN) titers of the serum from P344 TCoV 540-inoculated turkeys were 1:13 at 14 dpi, 1:16 at 28 dpi, and 1:36 at 56 dpi against P344 TCoV 540. P344 TCoV 540-inoculated turkeys were protected against the challenge by homologous P344 TCoV 540 completely or low passage P3 TCoV 540 partially as revealed by lack of histopathological alterations, absence of TCoV by immunofluorescent antibody assay in the intestines, and reduction in TCoV viral RNA loads in the intestines and feces. The serum from P344 TCoV 540-vaccinated turkeys had higher VN titers against P344 TCoV 540 than those against P3 TCoV 540. P344 TCoV 540 had 52 amino acid substitutions as compared to those of P3 TCoV in the S protein. The results indicated that a high passage TCoV can induce protective humoral and cellular immune response and have potentials to become an attenuated vaccine.

Keywords: Turkey coronavirus; Serial passage; Spike protein; Antibodies; Attenuated vaccine.

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INTRODUCTION

Turkey coronavirus (TCoV) belongs to the species Avian coronavirus with infectious bronchitis virus (IBV) in the genus *Gammacoronavirus* in the family *Coronaviridae*.¹ Turkeys infected with TCoV would suffer from acute atrophic enteritis resulting in increased mortality or decreased body weight gain with poor feed conversion. Clinical signs include depression, anorexia, watery diarrhea, and weight loss. Gross lesions are seen primarily in the intestinal tract. Contents of the duodenum, jejunum, and cecum are watery and gaseous. Microscopic lesions consist of a decrease in villous length, increase in crypt depth, and decreased intestinal diameter.² Significantly economic loss caused by turkey coronaviral enteritis in the turkey industry have occurred in Canada, the United States, Europe, and Brazil.²⁻⁶

Humoral and cellular immunities to TCoV are elicited in turkeys following infection with TCoV. Antibody titers to TCoV in turkey serum detected by immunofluorescent antibody assay (IFA) gradually increased from 7 to 63 days post infection (dpi) or enzyme-linked immunosorbent assay (ELISA) increased from 7 to 42 dpi.^{7,8} TCoV-specific lymphocyte proliferation responses and turkey interferon gamma (IFN γ) were induced in TCoV-infected turkeys.^{7,9} Turkeys have developed protective immune responses after the first exposure of TCoV and showed no clinical signs or shedding of TCoV in feces after the second exposure of virulent TCoV.⁸ No effective vaccines or treatments are applied to prevent and control TCoV infection in the field.

The genome of TCoV is a single-stranded positive sense linear RNA with a size of about 28 kb and has a cap at the 5' end and poly (A) tail at the 3' end. The TCoV polyprotein gene accounts for two-thirds of the genome at the 5' end and the remaining genome at the 3' end contains genes encoding four structural proteins, including spike (S) protein, envelope (E) protein, membrane (M) protein, and nucleocapsid (N) protein, and four accessory proteins designed as 3a, 3b, 5a, and 5b.^{10,11} The sequence of S protein is highly variable among different CoVs isolates.^{1,12} The S proteins of many CoVs are cleaved into S1 and S2 subunits by host cellular proteases after the C-terminal arginine (R) residue in consensus motif RXR(K)R/X (X is any amino acid, K is lysine, and slash [/] indicates the cleavage position).^{1,13} The C-terminal S1 subunit forms a globular structure outside the viral membrane and contains the regions that bind to cellular receptor, induce neutralizing antibodies, and determine host range, cell tropism and serotype specificity.¹⁴⁻¹⁷ The N-terminal S2

subunit constructs a stalk structure and includes two heptad repeats (HR), fusion peptides, and transmembrane domain (TM) responsible for the fusion between viral and cellular membranes and the subsequent viral assembly.¹⁸⁻²⁰

Serial passage has been used to attenuate virulent virus for the development of live viral vaccine that can trigger protective immunity against the target virus in the infected animals.²¹⁻²³ Serial passage of IBV in embryonated chicken eggs for 74 or 115 passages has been reported to render the virus attenuated.²¹ The attenuated IBV strains were not pathogenic, still capable of inducing virus-specific neutralizing antibody response, and conferred 90% protection against challenge by heterologous field IBV strain.²¹ Point mutations found in IBV and TCoV serially passaged in eggs are accumulated predominantly in the S protein.^{11,12,24-26}

The attenuation of IBV by high passage in embryonated chicken eggs suggests the possibility that the pathogenicity, replication efficiency, infectivity, and immunogenicity of TCoV can be altered in embryonated turkey eggs. Due to the lack of cell culture system and the relatively fast degradation of TCoV in storage, TCoV needs to be passaged in embryonated turkey eggs to maintain its infectivity. The purpose of the present study was to evaluate the pathogenicity and immunogenicity of a high-passage TCoV. In addition, the S gene sequences of TCoV before and after serial passages in embryonated turkey eggs were compared to identify substitutions.

MATERIAL AND METHODS

Virus Preparation

Turkey coronavirus Indiana isolate 540 (TCoV 540) was propagated by inoculation into 22-day-old embryonated turkey eggs via amniotic sac route for 3 passages (P3), 5 passages (P5), or 344 passages (P344). The intestines of turkey embryos inoculated with TCoV 540 were homogenized with 5-fold volume of sterile nutrient broth (1 g of beef extract, 2 g of yeast extract, 5 g of peptone, 5 g of sodium chloride in 1 L of distilled water) without antibiotics and clarified by centrifugation at 5000 \times g for 10 min at 4°C. The supernatant was served as virus stock for virus titration and experimental infection in turkeys. To determine the viral titer of virus stock P344 TCoV 540, 10-fold serially diluted virus stock from 10⁻³ to 10⁻⁷ were inoculated into five 22-day-old embryonated turkey eggs per dilution as previously described.⁹ After three days of inoculation, the embryonic intestines

were harvested and subjected to IFA assay with P5 TCoV 540 antiserum to determine the median effective infectious dose (EID₅₀), the dose that infected 50% of embryos. The EID₅₀ was calculated by the method of Reed and Muench described in the laboratory manual.²⁷ P5 TCoV 540 antiserum were collected from the turkeys orally inoculated with 10⁴ EID₅₀/mL of P5 TCoV 540 and tested positive by IFA on turkey embryos inoculated with P5 TCoV 540. The complete genome sequence of P5 TCoV 540 has been submitted to GenBank and was given accession number of EU022525.¹⁰

Turkey Eggs and Turkey Poults

Turkey eggs and one-day-old turkey poults were obtained from Perdue Farm (Thorntown, Indiana, USA). Turkey eggs were incubated in the incubator (Jamesway, Indian Trail, NC, USA) at 37.5°C with a humidity level of 55%. Turkey poults were housed in isolated floor pens. Feed and water were provided *ad libitum*. The protocol for care and use of turkey eggs and turkey poults in the present study was approved by Purdue University Animal Care and Use Committee (No. 1111000302). Sera collected from all poults one day before experimental inoculation of virus or sterile phosphate buffered saline (PBS) were negative for TCoV-specific antibody and showed the same background level as that in TCoV S protein-specific antibody-capture ELISA.

Experimental Infection with P344 TCoV 540

A total of 30 17-day-old turkey poults were inoculated orally with 100 EID₅₀/0.2 mL of P344 TCoV 540 (P344 group) and 24 turkey poults were fed with 200 μL of sterile PBS as the negative control (NC) group. Clinical signs, including depression, diarrhea, and ruffled feathers, were monitored for 56 days after the inoculation. Four turkeys from NC group and 5 turkeys from P344 group were weighed, bled, and necropsied at 3, 7, 14, 28, 41 and 56 days post-infection (dpi). Various tissues, including thymus, spleen, ileum, bursa of Fabricius, and cecal tonsil, were collected for histopathologic examination and TCoV antigen detection by IFA assay. The pathogenicity of P344 TCoV 540 was evaluated by clinical signs, body weight gain, gross lesions, and histopathological changes. Sera were obtained for TCoV S protein-specific antibody-capture ELISA and virus neutralization (VN) assay.

Protection Efficacy Study of P344 TCoV 540 as Vaccine

A schematic figure to illustrate the immunization, virus challenge, and sample collection for the protection efficacy study of P344 TCoV 540 is presented in Fig. 1. A total of 30 7-day-old turkey poults were inoculated orally with 10⁴ EID₅₀/mL of P344 TCoV 540 in P344 group or 15 7-day-old turkey poults were fed with PBS in NC group. At 3 dpi, 5 turkeys each from the P344 group and NC group were necropsied for the observation of gross lesions and collection of tissues including thymus, spleen, cecal tonsil, ileum, jejunum, and bursa for histopathological examination and IFA assay. At 21 dpi, half of the turkeys vaccinated with P344 TCoV 540 were challenged with 10⁴ EID₅₀/mL of P344 TCoV 540 orally (P344-P344 group) and another half of P344 TCoV 540 vaccinated-turkeys were challenged with 10⁴ EID₅₀/mL of P3 TCoV 540 orally (P344-P3). At 3 and 7 days post the viral challenge with P344 or P3 TCoV 540, 5 turkeys from NC group, P344-P344 group, and P344-P3 group were sacrificed for tissue collection. Cloacal swabs with feces were obtained at 3, 6, 14, and 20 dpi of the vaccination with P344 TCoV 540 (20 dpi is 1 day before the viral challenge) and 1, 2, 3, and 7 dpi of the challenge with P344 or P3 TCoV 540 for RT-PCR targeting TCoV S2 gene to monitor the shedding of TCoV. Sera were collected at 3 and 20 dpi of the vaccination and 3 and 7 dpi of the viral challenge for TCoV 540 S protein-specific ELISA. VN assay was performed on the serum samples collected at 1 day before the viral challenge.

Detection of TCoV Antigen by IFA

The antiserum to P5 TCoV 540 diluted in 1:40 as the primary antibody and fluorescein isothiocyanate (FITC)-conjugated goat anti-turkey IgG diluted in 1:100 as the secondary antibody (KPL, Gaithersburg, MD, USA) were used to stain the acetone-fixed sections of frozen embryonic ileum samples from turkey eggs used in viral titration and VN assay or frozen ileum samples from turkeys in NC and P344 groups. The infectivity of TCoV in turkeys was determined by IFA results and defined in the previous study as negative (-), weak (+), moderate (++), and strong (+++) responses.⁷

Quantitative RT-PCR (qRT-PCR) for TCoV RNA

The total RNA was extracted from the ileum samples stored in RNA *later*[®] stabilization reagent (Qiagen,

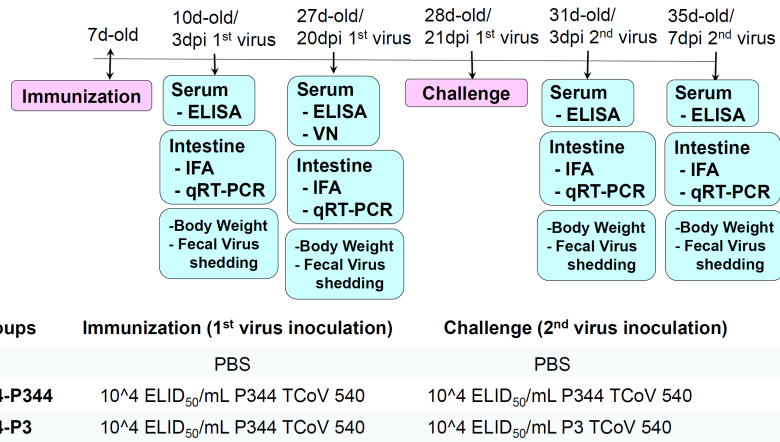


Fig. 1 A schematic figure to illustrate the immunization, virus challenge, and sample collection for the protection efficacy study of P344 TCoV 540.

Valencia, CA, USA) by using RNeasy mini kit (Qiagen) and reverse-transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen, San Diego, CA, USA) and random primer (Sigma Genosys, The Woodlands, TX, USA). The cDNA was subjected to TaqMan probe-based real-time PCR with forward primer QS1F (5'-TCGCAATCTATGCGATATG-3'), reverse primer QS1R (5'-CAGTCTTGGGCATTACAC-3'), and probe QS1P (5'-AbsoluteQuencher-TCTGTGGCAATGG-TAGCCATGTTTC-FAM-3') specific to TCoV S2 gene.²⁸ The cycle profile was 5 min of 94°C followed by 40 cycles of 94°C for 20 s and FAM acquiring at 61°C for 1 min in real-time PCR cycler, Rotor-Q (Qiagen). The concentration of TCoV was calculated by absolute quantitative standard curve developed by 10⁻³ to 10⁻⁸ of 10-fold serially diluted plasmid pTriEx3-6F/6R (nucleotide position 2490 to 3213 from start codon of S gene from P5 TCoV 540 with known concentration measured by GeneQuant pro RNA/DNA calculator (GE Healthcare Bioscience, Piscataway, NJ, USA)).

Antibody-capture ELISA for Antibody to TCoV

The procedures of TCoV S protein-specific ELISA described in a previous study were followed.¹⁵ Briefly, the purified recombinant 4F/4R S fragment, amino acid 482 to 678 from amino terminus of S protein of P5 TCoV 540 (EU022525), in PBS was coated onto NUNC Immuno 96 MaxiSorpTM plates (Thermo Fisher Scientific, Rochester, NY, USA) with 2 µg/100 µL/well overnight at 4°C. Bovine serum albumin in PBS (1% BSA) was added into the plates and incubated at 37°C for 1 h and washed with PBS containing 1% Tween 20

(PBST) 3 times prior to 1 h of the incubation with the serum diluted at 1:200 at 37°C. Bound antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-turkey IgG (KPL, Gaithersburg, MD, USA) diluted at 1:40,000 at 37°C for 1 h after washing 5 times with PBST. The color was developed by adding 100 µL/well of tetramethylbenzidine (TMB) substrate solution (Sigma-Aldrich, St. Louis, MO, USA) after washing 5 times with PBST, and the optical density at the wavelength of 450 nm (OD_{450nm}) was read with VMaxTM ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Virus Neutralization Assay for Antibody to TCoV

The serum samples collected at 14, 28, and 56 dpi from P344 TCoV 540-infected and non-infected turkeys were reacted to P5 TCoV 540 in VN assay. The neutralizing ability against P344 TCoV 540 or P5 TCoV 540 of the serum from P344 TCoV 540-infected turkeys collected at 56 dpi and the P5 TCoV 540 antiserum were also tested. The procedures of VN assay were modified slightly based on previous study.¹⁵ The serum samples were heat inactivated at 56°C for 30 min and then four-fold diluted serially. The diluted serum was incubated with 20 EID₅₀ of P344 or P5 TCoV 540 at 37°C for 1 h and the mixture was sequentially inoculated into five 22-day-old embryonated turkey eggs per dilution via amniotic sac route. Intestines of turkey embryos were harvested after three days of incubation in egg incubator and examined by IFA assay using the P5 TCoV 540 antiserum for the infection of P344 or P5 TCoV 540 in the intestine. The dilution of serum neutralizing the

infection of TCoV in 50% of eggs was calculated as VN titer by Reed and Muench method described in laboratory manual.²⁹

To determine the VN titers of serum samples with varied ELISA results, the serum samples collected from P344 TCoV 540-vaccinated turkeys at 1 day before the challenge with P344 or P3 TCoV 540 were distributed into 6 groups. First, the serum samples collected from the vaccinated turkeys scheduled for the challenge with P344 TCoV 540 were reacted with P344 TCoV 540 and those from the vaccinated turkeys scheduled for the challenge with P3 TCoV 540 were reacted with P3 TCoV 540 in VN assay using embryonated turkey eggs. Each group of serum samples from the two big groups was further divided into three smaller groups based on the OD_{450nm} values determined by ELISA, designated as low, medium, and high value group. Sera from each small group were pooled together for VN assay.

Spike Gene Sequencing of high-passage P344 TCoV 540

Viral RNA was extracted from P344 TCoV 540 by using QIAamp viral RNA mini kit (Qiagen) from 140 μ L of virus solution purified by continuous sucrose gradient from 40% to 60% in ultracentrifugation at 24,000 rpm at 4°C for 20 h. Reverse transcription was performed with SuperScript III reverse transcriptase (Invitrogen) and random primer to obtain viral cDNA for PCR. The full length of S gene from the transcription regulatory sequence (TRS) to the stop codon was amplified by primers, Sup (5'-TGAAAACCTGAACAAAAGACAGACT-3') and Sdown3 (5'-TTTGTTGAATTATTTGCTGACCA-3').³⁰ The PCR product was cloned into pCRII plasmid vector (Invitrogen) according to the manufacturer's instructions and followed by sequencing with 8 primers in the DNA sequencing laboratory at Purdue University genomic core facility (West Lafayette, IN, USA). The overlapping and continuous sequences covering the whole S gene were assembled and analyzed by Lasergene sequence analysis software (DNASTAR, Madison, WI, USA). The nucleotide and deduced amino acid sequence alignments of S genes from P344 TCoV 540 and P5 TCoV 540 (EU022525) were performed by CLUSTAL W program.³¹

Statistical Analysis

SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the data. Independent samples *t*-test was employed to compare the body weights and the results of

IFA, ELISA, and qRT-PCR from NC and P344 group in the pathogenicity study. Turkey IFN γ mRNA levels measured at different time points were compared by one-way analysis of variance (ANOVA). Statistical significance was set at $p < 0.05$.

RESULTS

Pathogenicity of P344 TCoV 540

No enteritis-related clinical signs, gross lesions, and histopathological alterations were seen in the negative control as well as P344 TCoV 540-infected turkeys throughout the experimental period. The length of intestinal villi and the distances between villi were similar between non-infected and infected turkeys (Fig. 2). The body weights of P344 TCoV 540-infected turkeys were comparable to those of non-infected turkeys at 3, 7, 14, 28, 41, and 56 dpi (Table 1). At 28 dpi, the infected turkeys were significantly heavier than non-infected turkeys ($p < 0.05$).

Infectivity of P344 TCoV 540

TCoV antigen-positive immunofluorescent enterocytes were detected at 3 and 7 dpi (Fig. 3). The number of TCoV antigen-positive cells and the intensity of fluorescence were decreased at 7 dpi. TCoV-specific immunofluorescence was not seen in the intestinal sections from the infected turkeys after 14 dpi (Table 2). The amount of TCoV RNA determined by qRT-PCR ranged from 96 to 2128 fg/ μ L in the ileum showing positive TCoV-specific immunofluorescence at 3 dpi. Corresponding to IFA results, the concentration of TCoV RNA in the ileum decreased and ranged from 1 to 464 fg/ μ L at 7 dpi. Only one out of five P344 TCoV 540-infected turkeys had detectable 2 fg/ μ L of TCoV RNA in the ileum while none of them showed positive TCoV-specific immunofluorescence at 14 dpi. No TCoV RNA was detected in the ilea of non-infected turkeys by qRT-PCR at all time points.

Antibody Responses to P344 TCoV 540

Serum antibodies specific to TCoV determined by TCoV 4F/4R S fragment-based ELISA appeared at 14 dpi and reached to the highest peak at 56 dpi in P344 TCoV 540-infected turkeys (Fig. 4). At 56 dpi, the level of antibody to TCoV S protein in P344 TCoV 540-infected turkeys was significantly higher than that in non-infected turkeys ($p < 0.05$). The VN titer of serum collected at 14, 28 and 56 dpi from P344 TCoV 540-infected turkeys

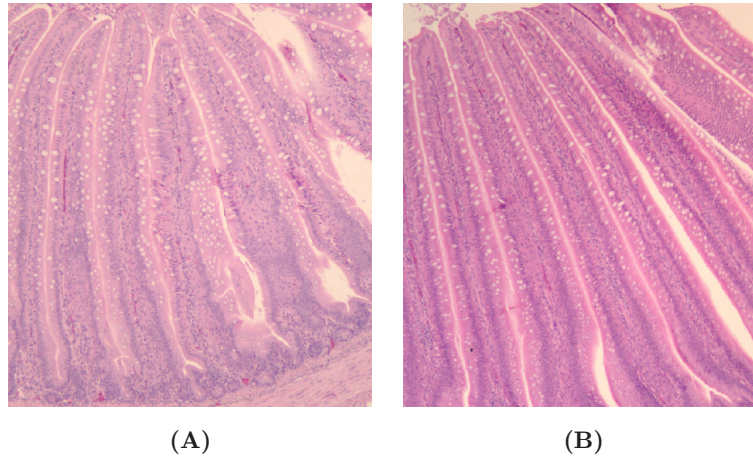


Fig. 2 Histopathological images of the intestinal mucosa stained with hematoxylin and eosin (H&E, 100 \times). (A) Negative control (NC) turkeys not infected with P344 TCoV 540; (B) Infected turkeys at three days after infection with P344 TCoV 540. No histopathological alternations were found.

Table 1. The Body Weights (g) of Infected (P344) and Non-infected Turkeys (NC) at Various Time Points after Experimental Infection with P344 TCoV 540.

Group	3 dpi ^a	7 dpi	14 dpi	28 dpi	41 dpi	56 dpi
NC	418 \pm 19	517 \pm 50	842 \pm 77	2063 \pm 170	4115 \pm 289	6490 \pm 380
P344	387 \pm 19	549 \pm 63	976 \pm 95	2780 \pm 166*	4432 \pm 64	6774 \pm 417

Notes: ^adpi = days post infection.

*Only at 28 dpi, the body weights of turkeys infected with P344 TCoV 540 (P344) are significantly higher than those of non-infected turkeys (NC) ($p < 0.05$) by independent t -test.

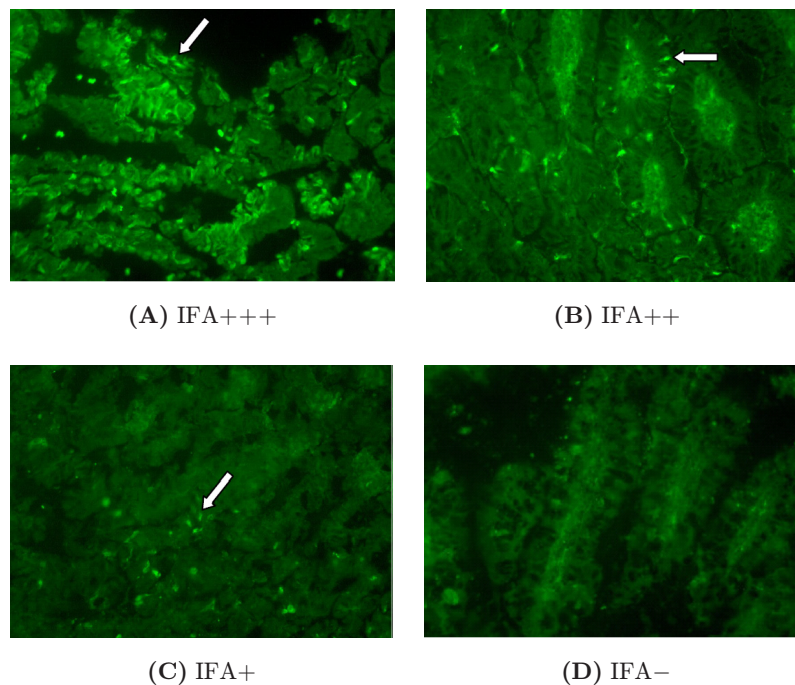


Fig. 3 Detection of TCoV antigen by IFA in the ileum of P344 TCoV 540-infected turkeys. (A) Strong IFA response (+++) found at 3 dpi; (B) Moderate IFA response (++) found at 7 dpi; (C) Weak response (+) found at 7 dpi; (D) No response (-) found at 14 dpi. Arrows in the pictures indicate TCoV-positive enterocytes 200 \times .

Table 2. Detection of TCoV Antigen by Immunofluorescence Antibody Assay (IFA) in the Infected and Non-Infected Turkey Intestines at Various Time Points after Infection with P344 TCoV 540.

dpi ^a	Negative Control				P344 TCoV 540-Infected			
	+++ ^b	++	+	-	+++	++	+	-
3	0	0	0	4	3	0	0	2
7	0	0	0	4	0	4	1	0
14	0	0	0	4	0	0	0	5
28	0	0	0	4	0	0	0	5
41	0	0	0	4	0	0	0	5
56	0	0	0	4	0	0	0	5

Notes: ^adpi = days post infection.

^bThe results of IFA were determined as follows: - (negative), + (weak), ++ (moderate), and +++ (strong).

was 13, 16, and 36, respectively. Positive correlation was shown between the TCoV-specific antibody level determined by TCoV S protein-based ELISA and the VN titer of serum collected from P344 TCoV 540-infected turkeys. The serum from non-infected turkeys did not neutralize the infection of TCoV in embryos. The VN titer of P5 TCoV 540 antiserum was 38 against P5 TCoV 540, similar to the VN titer of P5 TCoV 540 antiserum reacted with P344 TCoV 540, 33. The serum collected from P344 TCoV 540-infected turkeys at 56 dpi had VN titer of 36 against P5 TCoV 540 and VN titer of 33 against P344 TCoV 540. The similar VN

titers suggested that P344 TCoV 540 retains the same antigenicity as P5 TCoV 540 after serial passages and P344 TCoV 540 can induce neutralizing antibodies in the infected turkeys to inhibit the infection of both P5 and P344 passages of TCoV 540 in embryonated turkey eggs.

Protection Efficacy of P344 TCoV 540 as Vaccine

No enteritis-related clinical signs and gross lesions were observed after the vaccination with P344 TCoV 540 (P344) and the following challenge with P344 (P344-P344) or P3 (P344-P3) TCoV 540. The body weight gains of turkeys in NC, P344, P344-P344, and P344-P3 groups were comparable without significant differences at 3, 14, and 20 dpi of the vaccination and 3 dpi of the viral challenge (Table 3). At 7 dpi of the viral challenge, turkeys in group P344-P344 gained significantly more body weights than NC turkeys ($p < 0.05$).

Three days after the vaccination with P344 TCoV 540, TCoV antigen was detected by IFA in the ileum and jejunum of four out of five inoculated turkeys. All five turkeys, including the one showing negative IFA result, had detectable TCoV S2 gene in ileum and cloacal swabs. The concentration of TCoV ranged from 8 to 1906 fg/ μ L in ileum samples and 13 to 262 fg/ μ L in cloacal swabs. None of P344 TCoV 540-inoculated turkeys had detectable TCoV antigen by IFA in ileum and jejunum at 3 and 7 days after the challenge with P344 TCoV 540. Only two out of five turkeys in group P344-P344 had TCoV in ileum and the concentration was less than 1 fg/ μ L at both 3 and 7 days after the challenge with P344 TCoV 540. A very small amount of TCoV

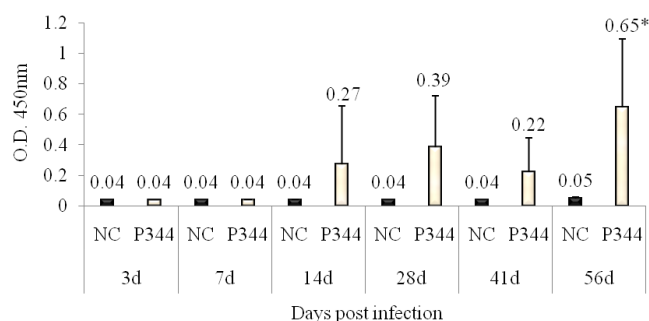


Fig. 4 Specific antibody response to S protein of TCoV 540 at various time points following experimental infection with P344 TCoV 540 as determined by TCoV 4F/4R (482-678) S fragment-based antibody-capture ELISA. The black solid bars are the data from non-infected turkeys (NC) and the light open bars are the data from P344 TCoV 540-infected turkeys (P344). The numbers on the top of bars are the average of OD450 nm values. The error bars with standard deviation are presented in each data bar. At 56 dpi, the level of antibody to TCoV S protein in P344 TCoV 540-infected turkeys is significantly higher than that in non-infected turkeys ($* = p < 0.05$).

Table 3. The Body Weight Gains (g) of Non-Infected Turkeys (NC) and Turkeys Inoculated with P344 TCoV 540 Followed by the Second Inoculation of P344 (P344-P344) or P3 (P344-P3) TCoV 540.

Age	10-Day Old	21-Day Old	27-Day Old
Group ^b	3 dpi ^a -1 st virus	14 dpi-1 st virus	20 dpi-1 st virus (1d pre-2 nd virus)
NC	109 ± 21 ^c	701 ± 94 ^c	1293 ± 196 ^c
P344	110 ± 15 ^c	695 ± 73 ^c	1134 ± 106 ^c
Age	31-Day Old	35-Day Old	
Group ^b	3 dpi-2 nd Virus	7 dpi-2 nd Virus	
NC	1217 ± 123 ^c	1673 ± 173 ^c	
P344-P344	1469 ± 372 ^c	1977 ± 78 ^d	
P344-P3	1401 ± 112 ^c	1757 ± 103 ^c	

Notes: ^adpi = days post-infection.

^bGroups: NC = negative control turkeys; P344 = turkeys inoculated with P344 TCoV 540 before the second viral challenge (1st virus); P344-P344 = P344 TCoV 540-infected turkeys challenged with P344 TCoV 540 (2nd virus); P344-P3 = P344 TCoV 540-infected turkeys challenged with P3 TCoV 540 (2nd virus).

^cThe same letter above the average with standard deviation indicates no significant difference between groups at the same time point by independent *t*-test or ANOVA.

^{c,d}At 7 dpi of the second viral challenge, the turkeys in group P344-P344 gained significantly higher body weight than the turkeys in group NC and P344-P3 ($p < 0.05$) by ANOVA.

(0.2 fg/ μ L) was found in one of five cloacal swabs at 3 dpi and no TCoV was found at 7 dpi from turkeys in group P344-P344. The amount of TCoV detected from turkeys in group P344-P344 was not significantly different from the negative results obtained in NC turkeys. However, TCoV antigen specific IFA signals were detected in the ileum of one turkey and in the jejunum of another turkey out of five turkeys in group P344-P3 at 3 dpi of the challenge with P3 TCoV 540. No IFA positive signals were detected in ileum and jejunum of turkeys in group P344-P3 at 7 dpi.

In the ileum of turkeys in group P344-P3, the concentrations of TCoV were from 3.9 to 7.9 fg/ μ L at 3 dpi and from 0 to 9.8 fg/ μ L at 7 dpi. In the cloacal swabs from turkeys in group P344-P3, the concentrations of TCoV were from 18 to 8402 fg/ μ L at 3 dpi and from 7 to 106 fg/ μ L at 7 dpi. The turkeys in group P344-P3 had significantly higher concentration of TCoV in ileum at 3 dpi than other turkeys in group NC and P344-P344 ($p < 0.05$). None of the turkeys in NC group showed IFA signals in ileum and jejunum and none had detectable TCoV S2 gene in ileum and cloacal swabs. According to the clinical signs and IFA results in

Table 4, the vaccination with P344 TCoV 540 provided 100% protection against the infection of homologous P344 TCoV 540 in ileum at 3 and 7 dpi, 60% protection against the infection of heterologous P3 TCoV 540 in ileum at 3 dpi, and 100% protection against P3 TCoV 540 at 7 dpi.

To monitor the shedding of TCoV, RT-PCR targeting the S2 gene of TCoV was performed on the RNA extracted from cloacal swabs collected at various time points through the experimental period (Fig. 5). All P344 TCoV 540-vaccinated turkeys (100%) released TCoV at 3 and 6 dpi but the number of TCoV-shedding turkeys decreased to 20% at 14 dpi and continually declined to 13% at 20 dpi. After the challenge with P344 TCoV 540, the number of turkeys in group P344-P344 shedding TCoV increased gradually from 18% to 50% till 7 dpi but the shedding was not as severe as the shedding status after the first inoculation of P344 TCoV 540. However, all cloacal swab samples from P344 TCoV 540-vaccinated turkeys challenged with P3 TCoV 540 were tested TCoV positive from 2 to 7 dpi. None of the cloacal swab samples from NC turkeys were detected TCoV positive.

Table 4. Detection of TCoV Antigen in Ileum and Jejunum by IFA and TCoV S2 Gene by TaqMan Real-time RT-PCR (qRT-PCR) in Ileums and Cloacal Swabs from Negative Control Turkeys (NC), Turkeys Vaccinated with P344 TCoV 540 (P344) (1st Viral Inoculation), and P344 TCoV 540-Vaccinated Turkeys Challenged with P344 (P344-P344) or P3 (P344-P3) TCoV 540 (2nd Viral Inoculation) at Various Time Points.

Time Groups	IFA+		IFA	qRT-PCR (fg/ μ L)	
	Ileum	Jejunum	Protection % ^b	Ileum	Cloacal swab
3 dpi ^a -1 st virus (vaccination)					
NC	0/5	4/5		0 ^c	811 \pm 992 ^c
P344	0/5	4/5		0 ^c 142	\pm 111 ^c
3 dpi-2 nd virus (challenge)					
NC	0/5	0/5		0 ^c	0 ^c
P344-P344	0/5	0/5	100	0.04 \pm 0.09 ^c	0.06 \pm 0.09 ^c
P344-P3	1/5	1/5	60	6.74 \pm 1.63 ^d	2,407 \pm 3,415 ^c
7 dpi-2 nd virus (challenge)					
NC	0/5	0/5		0 ^c	0 ^c
P344-P344	0/5	0/5	100	0 ^c	3.80 \pm 4.52 ^c
P344-P3	0/5	0/5	100	0.22 \pm 0.38 ^c	35 \pm 41 ^c

Notes: ^adpi = days post-infection.

^bProtection % is the ratio of IFA negative sample number to total sample number from each group at each time point. Negative IFA result indicates protection against the infection of P344 or P3 TCoV.

^cThe same letter above the average with standard deviation indicates no significant difference among groups at the same time point by ANOVA.

^{c,d}At three days after the challenge with P3 TCoV 540, significantly higher concentration of TCoV was detected in ileum from turkeys in group P344-P3 ($p < 0.05$) by ANOVA.

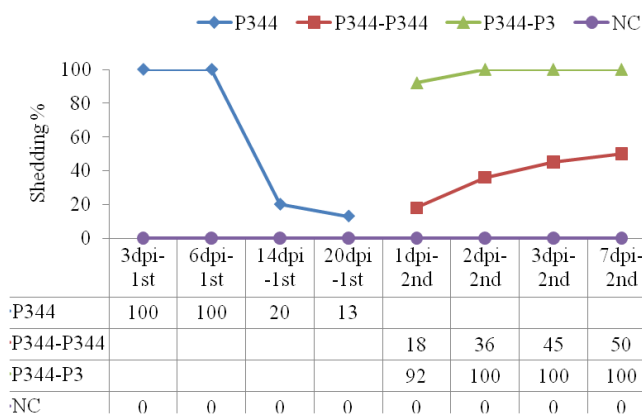


Fig. 5 Shedding of TCoV detected by RT-PCR targeting TCoV S2 gene in the cloacal fecal swabs collected from turkeys vaccinated with P344 TCoV 540 (1st viral inoculation) before the viral challenge (group P344), turkeys vaccinated with P344 TCoV 540 and challenged with P344 TCoV 540 (group P344-P344) or P3 TCoV 540 (group P344-P3) (2nd viral inoculation), and turkeys without any viral inoculation (group NC). The percentage of samples showing positive RT-PCR out of total samples at each time point is presented in this figure. Mark “◆” represents group P344, “■” P344-P344, “▲” P344-P3, and “●” NC.

Immune Responses Induced by P344 TCoV 540 as Vaccine

As shown in Fig. 6, P344 TCoV 540-vaccinated turkeys produced significantly higher level of serum IgG antibodies specific to TCoV S protein than non-vaccinated turkeys at one day before the challenge with P344 or P3 TCoV 540 ($p < 0.05$). At 7 dpi of the viral challenge, the antibody level produced by the turkeys in group P344-P3 is significantly higher than those in group NC ($p < 0.05$). No significant differences were found in the antibody levels produced by the turkeys in group P344-P344 and group P344-P3 at 3 and 7 dpi of the viral challenge ($p > 0.05$).

To determine the VN titers of serum samples with varied ELISA results, the serum samples collected from P344 TCoV 540-vaccinated turkeys one day before the challenge with P344 or P3 TCoV 540 were pooled together and distributed into six groups based on the OD450nm values determined by ELISA. The serum samples collected from the P344 TCoV 540 vaccinated turkeys scheduled for the challenge with P344 TCoV 540 were subjected to the VN with P344 TCoV 540 and

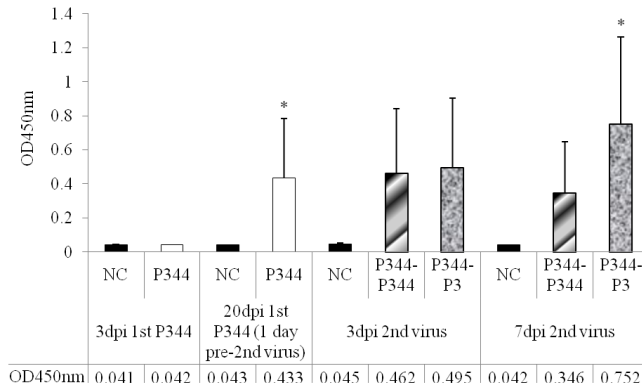


Fig. 6 TCoV-Specific antibody response were determined by TCoV S protein-based antibody-capture ELISA. The black solid bars are from negative control turkeys (NC). The white empty bars are from P344 TCoV 540-vaccinated turkeys (P344) at 3 and 20 dpi (1 day before the challenge of P344 TCoV 540). The bars with black and white lines are from P344 TCoV 540-vaccinated turkeys challenged with P344 TCoV 540 (P344-P344). The sandy bars are from P344 TCoV 540-vaccinated turkeys challenged with P3 TCoV 540 (P344-P3). One day before the viral challenge (20 dpi), P344 TCoV 540-vaccinated turkeys produced significantly higher level of TCoV S protein-specific antibody than non-vaccinated turkeys ($* = p < 0.05$). At 7 dpi of the viral challenge (2nd virus), the antibody level produced by the turkeys in group P344-P3 is significantly higher than those in group NC ($* = p < 0.05$).

those from the P344 TCoV 540-vaccinated turkeys scheduled for the challenge with P3 TCoV 540 were subjected to the VN with P3 TCoV 540. The pooled serum samples collected from the P344 TCoV 540-vaccinated turkeys had higher VN titers to homologous P344 TCoV 540 than those to heterologous P3 TCoV 540 in the respective groups of three different levels of O.D.540 nm values determined by TCoV 540 S protein-based ELISA (Table 5).

The level of IFN γ mRNA in the spleens of P344 TCoV 540-vaccinated turkeys was 3.53-fold higher than those in NC turkeys at 3 dpi of the vaccination. After the challenge, the turkeys in group P344-P344 had 4.12-fold higher IFN γ mRNA levels and the turkeys in group P344-P3 had 5.84-fold higher IFN γ mRNA levels compared to NC turkeys at 3 dpi the viral challenge.

Analysis of Spike Genes from P5 and P344 TCoV 540

The TRS, CTGAACAA, of S gene and the distance of 52 nucleotides between the 3' end of TRS and the ATG start codon for S gene of TCoV 540 remained the same after 344 passages in turkey embryonated eggs. The

Table 5. VN Titers of the Serum Samples Showing Low, Medium, and High Level of OD 450 nm by TCoV S Protein-Based ELISA were Determined by IFA. The Serum Samples were Collected from P344 TCoV 540-Vaccinated Turkeys One Day Before the Viral Challenge and then Incubated with P344 TCoV 540 or P3 TCoV 540.

Virus for VN	ELISA OD 450 nm		
	Low	Medium	High
P344 TCoV 540	0.06–0.21 46 ^a	0.27–0.44 105	0.49–0.93 126
P3 TCoV 540	0.04–0.11 4	0.39–0.60 38	0.97–1.05 105

Note: ^aThe VN titer is the dilution of turkey sera inhibiting the infection of P344 or P3 TCoV 540 in 50% of embryonated eggs.

coding region of S gene of P344 TCoV 540 contained 3612 nucleotides, 9 more nucleotides encoding 3 amino acids, compared to S gene of P5 TCoV 540. The CLUSTAL W alignment showed that S genes of P5 and P344 TCoV 540 shared 96.8% of nucleotide and 95.7% of deduced amino acid identity with 116 nucleotides and 52 amino acids changes after serial passages (Fig. 7). In the hyper-variable region at the N-terminal S1, P344 TCoV 540 had 5 amino acid substitutions and 1 amino acid insertion. Within the S fragment containing neutralizing epitopes, 4 amino acid substitutions were revealed in P344 TCoV 540. Six changes occurred at amino acids residues near the cleavage sequence (RRXR/S) where S protein is predicted to be cleaved into S1 and S2 protein. Three out of six substitutions above changed from polar threonine in P5 TCoV 540 to hydrophobic non-polar isoleucine in P344 TCoV 540. Within S2, 7 amino acids changes in heptad repeat (HR) region 1 and 1 amino acid substitution within transmembrane (TM) domain were observed in P344 TCoV 540. The ratio of synonymous to non-synonymous mutations was 26.8% and 128.6% in S1 and S2, respectively. There were more silent mutations than mutations causing amino acid changes in S2 gene.

Discussion

This is the first report with regard to the pathogenicity and immunogenicity of a high-passage TCoV serially passaged in embryonated eggs. TCoV 540 was first isolated from turkeys suffering coronaviral enteritis in

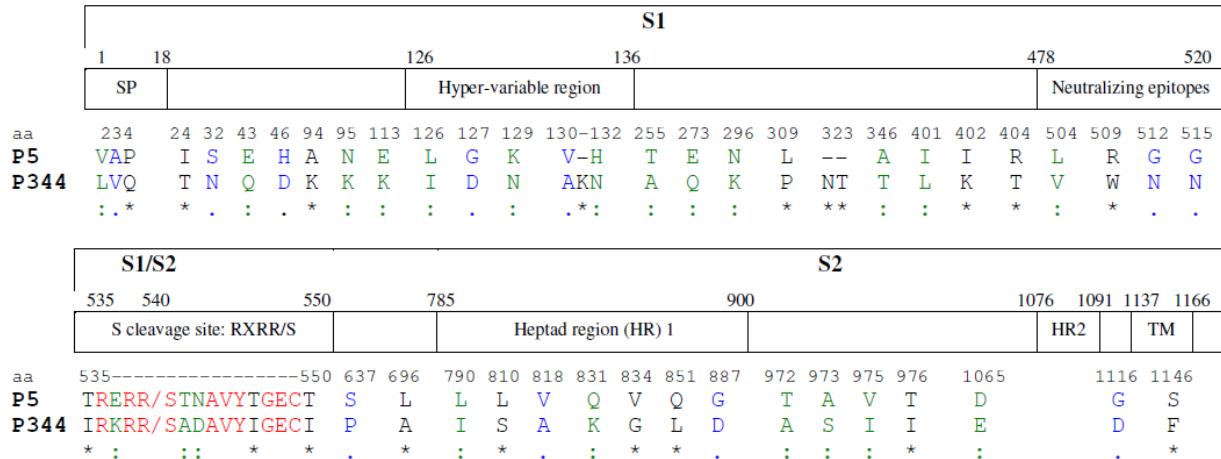


Fig. 7 Schematic representation of the turkey coronavirus (TCoV) spike (S) protein structure includes N-terminal signal peptide (SP), hyper-variable region, fragment containing neutralizing epitopes, predicted S cleavage site (RXRR/S), two heptad repeat (HR) regions (HR1 and HR2), transmembrane domain (TM), and C-terminal cytoplasmic tail. R is arginine, X is any amino acid, K is lysine, and slash [/] indicates the cleavage position. The numbers above and below two open bars indicate deduced amino acid position of S protein from P344 TCoV 540. Amino acids changed through serial passages are presented below the corresponding amino acid positions as original residues in P5 TCoV 540 and substituted residues in P344 TCoV 540. By comparing the biochemical characteristics of amino acids between P5 and P344 TCoV 540, two amino acid residues above the symbol “:” are strongly similar, “.” are weakly similar, and “*” are totally different.

Indiana, USA in 1994. Unusual low infectivity of high-passaged TCoV 540 was first noticed after the virus had been passaged in turkey embryonated eggs more than 300 times. The experimental infection of P344 TCoV 540 in 17-day-old turkeys showed a complete attenuation of P344 TCoV 540 due to no enteritis-related clinical signs, gross lesions, and histopathological changes. P344 TCoV 540-infected turkeys grew even heavier than NC turkeys when they were older. Compared to the results of experimental infection of P5 TCoV 540 in previous studies, IFA positive enterocytes were only found in P344 TCoV 540-infected turkeys at 3 and 7 days while positive IFA responses could be found in P5 TCoV 540-infected turkeys till 28 dpi.⁷ The concentration of TCoV detected by real-time RT-PCR in intestines from P5 TCoV 540-infected turkeys was much higher than those in ileum from P344 TCoV 540-infected turkeys.²⁸ This indicates that not only the pathogenicity but also the infectivity of P344 TCoV 540 have been reduced after serially passaged in turkey embryonated eggs.

P344 TCoV 540 elicited the production of TCoV S protein-specific antibodies in the infected turkeys. The serum of P344 TCoV 540-infected turkeys can neutralize the infection of P344 TCoV 540 and P5 TCoV 540 in embryonated turkey eggs. Positive correlation was shown between the OD450 nm values of serum IgG determined by TCoV 540 S protein-based ELISA and VN titer against P344 or P3 TCoV 540 in embryonated eggs.

Cellular immune response was also elicited after infection with P344 TCoV 540 because higher mRNA levels of IFN γ were detected in the spleens of P344 TCoV 540-infected turkeys than those in non-infected turkeys. IFN γ is primarily secreted by CD4⁺ Th1 cells and CD8⁺ cytotoxic T cells after antigen-specific immunity developments.^{7,32,33} This suggests that P344 TCoV 540 has the potential to be a live attenuated vaccine. Although serial passage of virus in embryonated eggs has been a common strategy to develop live attenuated viral vaccine in avian or poultry medicine, not all attenuated viruses retain their immunogenicity. Nonpathogenic IBV Beaudette strain was generated from more than 200 passages in chicken embryonated eggs but its immunogenicity was too low to be used as vaccine.³⁴ Another IBV strain, CK/CH/LHLJ/04V, also failed to induce immune responses after 110 passages.²⁶ However, attenuated passage 115 of IBV CK/CH/LDL/97I and nonpathogenic passage 74 of IBV 2575/98 stimulated antibody responses in infected chickens and the later provided 90% protection against challenge by virulent IBV field strain.^{12,21} Hence, the outcome of the attenuation process in the embryonated eggs is usually unpredictable and requires the comprehensive assessment. The changes of viruses after serial passages can come from mutations or the selection of a fit subpopulation adapted to a particular environment because a mixture of genetic mutants exists within an isolate of coronavirus.^{12,22,35} In a case of attenuated Ark

DPI-derived IBV vaccine, a rapid selection of virus population with similar S1 gene to the virulent parent strain occurred in only one back passage in chickens.³⁶ Therefore, the extensive evaluation of vaccine safety after performing back passages in the natural host is very important for the development of live attenuated virus vaccine.^{21,22}

Protection efficacy study of P344 TCoV 540 as attenuated live vaccine showed that P344 TCoV 540-vaccinated turkeys have acquired protection against the infection by homologous P344 TCoV 540 completely and partially against heterologous P3 TCoV 540 because no clinical signs, no gross and microscopic lesions, and comparable body weight gains were observed after the viral challenge and the results of IFA for TCoV antigen in intestines and qRT-PCR to measure TCoV in ileum and feces also indicated the complete and partial reduction of TCoV infection and shedding.

The higher sensitivity of qRT-PCR to IFA can explain the low amount of TCoV detected by qRT-PCR while the samples showed negative IFA results.²⁸ TCoV-infected turkeys can keep shedding TCoV after clinical signs resolve and no TCoV antigen is detected within intestinal epithelial cells by IFA. The observation is similar to the shedding patterns of P5 TCoV 540, TCoV ATCC VR-911, and TCoV-MG10 isolates, which can be detected up to 2 weeks post-inoculation, but much shorter than the shedding period up to 7 weeks of TCoV NC-95 isolate.^{10,28,37,38} After the challenge with P344 TCoV 540, the concentration of TCoV detected in the cloacal swabs from the turkeys in group P344-P344 is so low that it can be considered as remaining viral particles in the inoculums passing through the digestive tract but not from viral replication within intestinal epithelial cells tract. Similar protection was demonstrated by no virus shedding after the re-infection of homologous TCoV-MG10.¹⁰

Coronavirus genomic RNA accumulates point mutations during its replication due to the error-prone nature of RNA polymerase.³⁷ The identity percentage between P344 and P5 TCoV 540 within S protein was 96.8% at nucleotide level and 95.7% at amino acid level. It was lower than the amino acid sequence similarity of 99.3% observed between two TCoV isolates from Minnesota, TCoV ATCC in 1970s and TCoV 310 in 1996, but higher than those found among TCoV 540, ATCC, and 1440 from North Carolina in 1999 from 93% to 95%.³⁸ When TCoV/TX-1038/98 was passaged in 19-day-old turkey embryonated eggs, no sequence changes were observed for the first 20 passages. Between 30 and 52 passages, amino acid changes were only found in S1 subunit.¹¹ Most genetic changes occurred in the S1 during adaption to the different host systems; for

example, such genetic changes were seen in serial passages of virulent IBV strains in chicken embryonated eggs.^{25,26,39} Within six amino acid changes found nearby the predicted S cleavage site, P344 TCoV 540 had three hydrophobic non-polar amino acids substituted the original three polar amino acids of P5 TCoV 540. The shift to hydrophobic membrane-interacting residues also happened in passage 110 of IBV Ark DPI strain. The changes from polar charged residues to neutral residues were speculated to contribute to adaptation of field virus to embryonic tissue and subsequent attenuation of the virus.⁴⁰ However, when attenuated IBV strains have preserved immunogenicity shown in vaccine efficacy tests, no substitutions are found in the amino acids associated with antigenicity and/or neutralization inducing epitopes.^{21,25} Because P344 TCoV 540 remained highly immunogenic, the changes found in the amino acids might not relate to immunogenicity. Unlike most cases of attenuated IBV, more nucleotide changes were found in S2 than S1 in P344 TCoV 540 but 56% of them were silent mutations. The changes of S2 protein may contribute to the reduced infectivity of P344 TCoV 540 because S2 subunit contains highly conserved heptad repeat regions, fusion peptides, and transmembrane domains and minimal amino acid changes in S2 subunit are enough to alter the membrane fusion ability of the S protein and thereby the infectivity of virus.^{19,20,41,42} After the S gene of nonpathogenic IBV Beaudette strain with low immunogenicity was replaced with the S gene of pathogenic IBV M41 strain with high immunogenicity, the recombinant Beaudette strain restored immunogenicity but remained non-pathogenic.⁴³ Those results not only provided the information regarding the essential role of S protein for the immunogenicity of coronaviruses but also suggested that other proteins, in addition to S protein, may be involved in the infectivity and pathogenicity of coronaviruses.

In conclusion, serial passages of TCoV 540 in embryonated turkey eggs for 344 times caused attenuation of the virus but the high-passage virus still induced strong humoral immunity. Substitutions in S protein did not affect immunogenicity but reduced infectivity and pathogenicity of P344 TCoV 540. As a live attenuated virus vaccine, P344 TCoV 540 can provide complete protection against homologous challenge and partial protection against virulent parent TCoV.

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