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Recombinant nucleocapsid protein-based enzyme-linked immunosorbent assay for detection of antibody to turkey coronavirus

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A B S T R A C T

Nucleocapsid (N) protein gene of turkey coronavirus (TCoV) was expressed in a prokaryotic system and used to develop an enzyme-linked immunosorbent assay (ELISA) for detection of antibody to TCoV. Anti-TCoV hyperimmune turkey serum and normal turkey serum were used as positive or negative controls for optimization of the ELISA. Goat anti-turkey IgG (H+L) conjugated with horseradish peroxidase was used as detector antibody. Three hundred and twenty two turkey sera from the field were used to evaluate the performance of ELISA and determine the cut-off point of ELISA. The established ELISA was also examined with serum samples obtained from turkeys experimentally infected with TCoV. Those serum samples were collected at various time intervals from 1 to 63 days post-infection. The optimum conditions for differentiation between anti-TCoV hyperimmune serum and normal turkey serum were recombinant TCoV N protein concentration at 20 μg/ml, serum dilution at 1:800, and conjugate dilution at 1:10,000. Of the 322 sera from the field, 101 were positive for TCoV by immunofluorescent antibody assay (IFA). The sensitivity and specificity of the ELISA relative to IFA test were 86.0% and 96.8%, respectively, using the optimum cut-off point of 0.2 as determined by logistic regression method. Reactivity of anti-rotavirus, anti-reovirus, anti-adenovirus, or anti-enterovirus antibodies with the recombinant N protein coated on the ELISA plates was not detected. These results indicated that the established antibody-capture ELISA in conjunction with recombinant TCoV N protein as the coating protein can be utilized for detection of antibodies to TCoV in turkey flocks.

1. Introduction

Turkey coronavirus (TCoV) has been recognized as an important pathogen of young turkeys. TCoV infection causes significant economic losses in turkey industry due to uneven growth and poor feed conversion. The turkey coronaviral enteritis was the most costly disease of turkeys in Minnesota in the early 1960s (Panigrahy et al., 1973). Outbreaks of a similar enteric disease in turkey poults occurred in Indiana and North Carolina in the early and middle 1990s and remains as a threat to the turkey industry.

Immunofluorescent antibody assay (IFA) has been developed and successfully used in the serological diagnosis of turkey coronaviral enteritis (Patel and Pomeroy, 1976). The IFA assay is simple to perform and highly effective in detecting antibodies to TCoV in turkey flocks infected with TCoV or recovering from TCoV infection. However, the IFA procedures are time consuming and labor intensive for handling large numbers of samples. In addition, IFA requires well-trained personnel to prepare frozen sections of TCoV-infected turkey embryo intestines or slides with epithelial cells exfoliated from the infected bursa of Fabricuis as well as to perform fluorescent microscopy.

In order to have an early and rapid diagnosis of TCoV infection and effective control of TCoV-induced enteritis in turkey flocks, development of an antibody-capture enzyme linked immunosorbent assay (ELISA) for detecting antibodies to TCoV in serum is essential. ELISA has high specificity and sensitivity and can be applied to large numbers of samples simultaneously.

Development of ELISA for detection of TCoV infection requires large amounts of TCoV antigen. Attempts to propagate TCoV in cell culture in many laboratories have not been successful. When the viral antigen prepared from the intestines and intestinal contents

Abbreviations: BCoV, bovine coronavirus; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HRPO, horseradish peroxidase; IBV, infectious bronchitis virus; IFA, immunofluorescent antibody assay; Mab, monoclonal antibody; NC, negative control; PBS, phosphate buffered saline; PC, positive control; TCoV, turkey coronavirus; TGEV, transmissible gastroenteritis virus; TMB, tetramethyl benzidine.

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was used as the coating antigen, a strong non-specific reaction was observed in the ELISA (Loa et al., unpublished data).

Nucleocapsid (N) protein of TCoV appears to be the most abundant viral polypeptide in coronavirus infected cells during all stages of infection. The only known post-translational modification of coronavirus N proteins is phosphorylation (Garwes et al., 1984). The coronavirus N protein is immunodominant (Ignjatovic and McWaters, 1991). Carboxyl-terminal 119 amino acids of nucleocapsid protein of IBV expressed by Fowlpox Virus has been shown to induce cross-protective immunity against infections among different IBV strains (Yu et al., 2001). The N proteins from various RNA viruses, such as mumps, rabies, measles, and IBV have been used as coating antigens in antibody-capture ELISA for the diagnosis of infections (Reid-sanden et al., 1990; Hummel et al., 1992; Ndifuna et al., 1998). Subcloning of the N gene to a prokaryotic expression system has resulted in a high-level induction of recombinant protein in our laboratory (Loa et al., 2004). The recombinant TCoV N protein expressed from the prokaryotic system is expected to maintain its antigenic integrity because it is not glycosylated. The purpose of the present study was to develop a recombinant TCoV N protein-based ELISA for detection of antibodies to TCoV in turkey sera.

2. Materials and methods

2.1. Animal welfare statement

Turkey poults used for experimental infection with TCoV were obtained from a commercial operation (Perdue Farm, Thorntown, IN, USA). Turkeys were housed in the isolation units in the animal facility at Purdue University (West Lafayette, IN, USA). Food and water were supplied ad libitum. All turkeys were handled with the guidelines established by the United States Department of Agriculture (USDA) and the protocol for using turkeys for experimental infection with TCoV in the present study was approved by Purdue University Animal Care and Use Committee.

2.2. Preparation of N protein

The recombinant plasmid containing the entire N protein gene (pTri-N) was transformed to competent *Escherichia coli* strain Tuner (DE3) pLacI or Origami (DE3) pLacI (Novagen, Madison, WI, USA). Transformants were grown in LB medium containing 50 μg/ml ampicillin, 34 μg/ml chloramphenicol, and 1% glucose. A starter culture of bacteria cells containing recombinant plasmid pTri-N was prepared in 3 ml of LB medium. Cells were incubated at 37 °C with shaking at 250 rpm overnight. The entire 3 ml culture was added to 100 ml of fresh medium and incubated until the OD reached 0.5 at 600 nm wavelength. One milliliter of culture was separated as un-induced control. The remaining cultures were induced by addition of IPTG to a final concentration of 1 mM. The induced cultures were incubated for another 4 h. The bacterial cultures were harvested and the cell pellet was resuspended completely in 5 ml of BugBuster reagent (Novagen) per gram of wet cell weight. The nuclease reagent Benzonase (Novagen) was added with 1 μl (25 units) for every ml of BugBuster reagent used. After centrifugation at 16,000 × *g* for 20 min at 4 °C, the soluble supernatant was discarded and the insoluble fraction (inclusion bodies) was dissolved in 10 ml of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9) containing 6 M urea. The recombinant N protein in the dissolved inclusion bodies was purified by His-Bind column (Novagen) equilibrated with 10 ml of binding buffer. The column was washed sequentially with 10 ml of binding buffer and 10 ml of wash buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9) containing 6 M urea. The recombinant N protein was eluted from the column with 5 ml of elute buffer (1 M imidazle, 0.5 NaCl, 20 mM Tris–HCl, pH 7.9) containing 6 M urea. The concentration of purified N protein was determined by Protein Assay reagent (Bio-Rad, Hercules, CA, USA).

2.3. SDS-PAGE and Western blotting

Electrophoresis was performed with the discontinuous buffer system (Laemmli, 1970). For Western blotting, proteins on the acrylamide gel were transferred onto nitrocellulose membrane (Millipore, Bedford, MA, USA) with transfer buffer containing 50 mM Tris, 384 mM glycine, and 20% (v/v) methanol, pH 8.3. Membranes were incubated with turkey anti-TCoV antiserum at a dilution of 1:500 for 1 h at room temperature. Membranes were washed three times with PBS buffer containing 0.05% Tween-20 (PBS-T) and incubated with horseradish perioxidase-conjugated goat anti-turkey IgG (Kirkegard & Perry Laboratories, Gaithersburg, MD, USA) at a dilution of 1:500 for 1 h at room temperature. The membrane was washed with PBS-T and covered with 10 ml of perioxidase substrate, 0.05% diaminobenzidine (Kirkegard & Perry Laboratories) solution. The blot was developed and washed with distilled water to stop the reaction.

2.4. Serum samples and antibodies to different viruses

Positive control (PC) serum was the hyperimmune serum of turkeys experimentally infected with TCoV. Negative control (NC) serum was collected from normal healthy turkeys grown in the isolation rooms. Three hundred and twenty two turkey serum samples, positive or negative for TCoV by IFA, from the turkey flocks in the field were used to evaluate the performance of the recombinant N protein-based antibody-capture ELISA. Chicken antisera to IBV, avian reovirus, or rotavirus were obtained from SPAFAS (Storrs, CT, USA). Turkey antiserum to avian adenovirus was obtained from SPAFAS. Bovine antiserum to bovine coronavirus (BCoV) and porcine antiserum to transmissible gastroenteritis virus (TGEV) were obtained from National Veterinary Services Laboratory (Ames, IA, USA). Monoclonal antibody to enterovirus was obtained from Dr. J. S. Guy (North Carolina State University, Raleigh, NC, USA). Dilution factors and other information of the antibodies used in the present study are listed in Table 1.

2.5. Optimization of ELISA

The PC and NC serum samples were used to optimize the conditions of ELISA for detection of antibody to TCoV using recombinant TCoV N protein. The ELISA was optimized by checkerboard tests of recombinant TCoV N protein concentrations from 5 to 160 μg/ml, dilutions of serum from 1:200 to 1:1600, and dilutions of conjugate antibody from 1:5000 to 1:20,000. The ELISA plate coated with recombinant N protein was incubated at 4 °C for 16 h. The wells of the ELISA plate were washed with PBS-T for 3 times and 150 μl of PBS solution containing 1% bovine serum albumin was added to each well. Following incubation at 37 °C for 1 h, the plate was washed with PBS-T for 3 times as described above. The diluted serum samples for PC and NC were prepared and 100 μl was added to each respective well. The plate was incubated at 37 °C for 1 h and washed with PBS-T for 5 times. Goat anti-turkey IgG (H+L) conjugated with horseradish peroxidase was diluted and 100 μl was added to each well. Incubation at 37 °C for 1 h and wash with PBS-T for 5 times were followed by addition of substrate tetramethyl benzidine (Kirkegard & Perry Laboratories) at 100 μl/each well. After incubation in the dark for 30 min, 100 μl of 2 N HCl was added to each well. The absorbance of each well at 450 nm was determined.

Table 1
List of antibodies against avian viruses and different coronaviruses and their sources used in this study.

Antibody[a]	Conjugate	Source[b]	Dilution[c]
Chicken anti-IBV (Mass 41)	None	SPAFAS	1:800
Chicken anti-rotavirus	None	SPAFAS	1:800
Chicken anti-reovirus	None	SPAFAS	1:800
Turkey anti-TCoV-IN	None	C.C. Loa	1:800
Turkey anti-adenovirus	None	SPAFAS	1:800
Anti-enterovirus Mab	None	J.S. Guy	1:800
Bovine anti-BCoV	None	NVSL	1:800
Porcine anti-TGEV	None	NVSL	1:800
Goat anti-mouse IgG (H+L)	HRPO	KPL	1:10,000
Goat anti-turkey IgG (H+L)	HRPO	KPL	1:10,000
Rabbit anti-chicken IgG (H+L)	HRPO	Sigma	1:10,000
Rabbit anti-porcine IgG (H+L)	HRPO	Sigma	1:10,000
Rabbit anti-bovine IgG (H+L)	HRPO	Sigma	1:10,000

[a] BCoV, bovine coronavirus; IBV, infectious bronchitis virus; TCoV-IN, turkey coronavirus Indiana isolate; TGEV, transmissible gastroenteritis virus; Mab, monoclonal antibody; IgG (H+L), immunoglobulin G (heavy plus light chains); HRPO, horseradish peroxidase.
[b] SPAFAS, Storrs, CT; Dr. C. C. Loa, Purdue University, West Lafayette, IN; Dr. J. S. Guy. North Carolina State University, Raleigh, NC; NVSL, National Veterinary Services Laboratory, Ames, IA; KPL, Kirkegaard & Perry Laboratories, Gaithersburg, MD; Sigma, St. Louis, MO.
[c] Un-labeled antibodies were diluted at 1:800 and HRPO conjugated antibodies were diluted at 1:10,000. Those dilution factors were in line with the optimized conditions of serum sample and conjugate antibody for the ELISA.

The ratio of PC to NC was calculated for different combinations of recombinant TCoV N protein concentration, serum dilutions, and conjugate dilutions. The combination that consistently produced the highest ratio of PC to NC was selected as the optimal condition for detecting antibodies to TCoV by antibody-capture ELISA.

2.6. Evaluation of ELISA

Three hundred and twenty two turkey serum samples from turkey flocks in the field were used as the test serum samples to evaluate the ELISA. Two wells containing all the reagents except serum were left as blank references in each plate. The PC, NC, and test serum samples were analyzed in duplicate. The ELISA value (S/P ratio) of each test serum sample was calculated as (absorbance value of test serum sample minus absorbance value of NC) divided by (absorbance value of PC minus absorbance value of NC). For determination of the optimum cut-off point of the ELISA value, logistic regression analysis was used to distinguish serum samples positive or negative for TCoV by IFA (Shoukri and Pause, 1999). The relative sensitivity of the ELISA was calculated as the percentage of serum samples positive for TCoV by IFA, which were positive in ELISA. The relative specificity of the ELISA was calculated as the percentage of serum samples negative for TCoV by IFA, which were negative in ELISA. Statistical computations were performed by the SAS program (SAS institute, Inc., Cary, NC, USA).

The established ELISA were further evaluated with the serum samples collected from experimentally infected turkey poults. One group of 40 turkey poults were inoculated with turkey embryo-propagated TCoV. The other group of 40 turkey poults of the same age were inoculated with PBS and used as the non-infected control. Five turkeys were randomly selected from each group and sacrified at 1, 3, 7, 14, 21, 28, 42, and 63 days after inoculation. The collected serum samples were tested by ELISA using ELISA plates coated with recombinant TCoV N protein prepared from the present study.

For evaluation of cross-reactivity, the N protein coated on the ELISA plates was reacted with antibodies to IBV, BCoV, TGEV, avian rotavirus, reovirus, adenovirus, or monoclonal antibody to avian enterovirus.

3. Results

3.1. Expression and purification of recombinant N protein in E. coli

The recombinant N protein from TCoV was induced successfully as a fusion protein in *E. coli* strains Tuner or Origami cells as revealed by SDS-PAGE and Western blotting (Fig. 1). The size of induced N protein is approximately 57 kDa. The recombinant N protein was purified by Nickel-chelating affinity chromatography. The yield of purified N protein could be 10 mg from a 100-ml culture of Tuner cells. In contrast, 2 mg of recombinant N protein could be recovered from the same volume of Origami cell culture. Thus, the recombinant N protein expressed from Tuner cells was used for the development of antibody-capture ELISA for antibodies to TCoV due to the yield being higher than that from Origami cells.

3.2. ELISA development and optimization

When the dilution of conjugate was 1:5000, the maximum PC/NC ratio of 27 was obtained with TCoV N protein at 40 μg/ml and serum dilution at 1:1600. When the dilution of conjugate was 1:10,000, the maximum PC/NC ratio of 92 was obtained with coating concentration of TCoV N protein at 20 μg/ml and serum dilution at 1:800 (Fig. 2). When the dilution of conjugate was 1:20,000, the maximum PC/NC ratio of 65 was obtained with coating concentration of TCoV N protein at 20 μg/ml and serum dilution at 1:200. The combination of recombinant TCoV N protein at 20 μg/ml, serum dilution at 1:800 and conjugate dilution at 1:10,000 was chosen as the best and optimal condition for antibody-capture ELISA based on the maximum PC/NC ratio.

3.3. ELISA evaluation

Among the 322 serum samples collected from the field, 101 were positive and 221 were negative for TCoV by IFA. The ELISA values (S/P ratio) of IFA positive serum samples ranged from 1.0882 to 0.0132 and that of IFA negative serum samples ranged from 0.2762 to −0.004. The distribution of ELISA values (S/P ratio) of serum samples positive for TCoV by IFA and those negative for TCoV by IFA are shown in Fig. 3. The logistic regression model at the optimum cut-off point was $0 = (\beta 1)X + \beta 0$, where $\beta 1$ and $\beta 0$ were −20.1502 and 4.2067, respectively. The optimum cut-off point (X) is 0.2 (−4.2067/−20.1502). The sensitivity and specificity of the established ELISA relative to IFA were 86.0% and 96.8%, respectively. The agreement between ELISA and IFA assay was 95.6% (Table 2).

The ELISA results of serum samples collected from the turkey poults experimentally infected with TCoV showed that the antibody response was detected initially at 14 days after infection and increased gradually until 28 days after infection. The antibody response was detectable up to 63 days after infection when the experiment was terminated (Fig. 4). The ELISA responses of normal turkey sera were negative throughout the entire experimental period (Fig. 4).

Cross reactivity of recombinant TCoV N protein coated on the ELISA plate with bovine anti-BCoV or porcine anti-TGEV was not detected with species-specific conjugate antibodies. The cross reactivity with chicken anti-IBV antibodies was observed with chicken-specific conjugate antibody (Table 3). The reactivity of recombinant TCoV N protein on the ELISA plate with bovine anti-BCoV, pocine anti-TGEV, turkey anti-adenovirus, chicken anti-rotavirus, chicken anti-reovirus, or anti-enterovirus monoclonal antibody was not detectable with turkey-specific conjugate antibody (Table 4). The cross reactivity of recombinant TCoV N protein

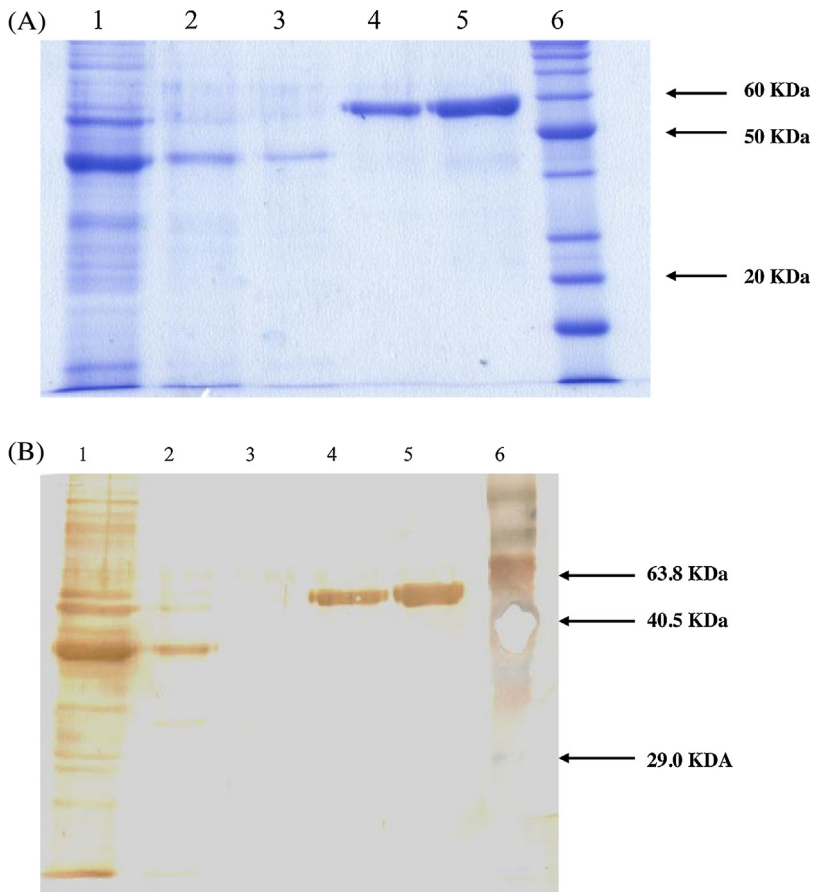

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (A) and Western blotting with antiserum to turkey coronavirus (B) of purified recombinant turkey coronavirus (TCoV) nucleocapsid (N) protein by His-Bind column chromatography. Lane 1, Insoluable fraction (inclusion bodies); lane 2, flow through of loading the inclusion bodies to a column; lane 3, the filtrate with 10 ml binding buffer; lane 4, the filtrate with wash buffer; lane 5, the eluted recombinant protein; lane 6, molecular weight marker.

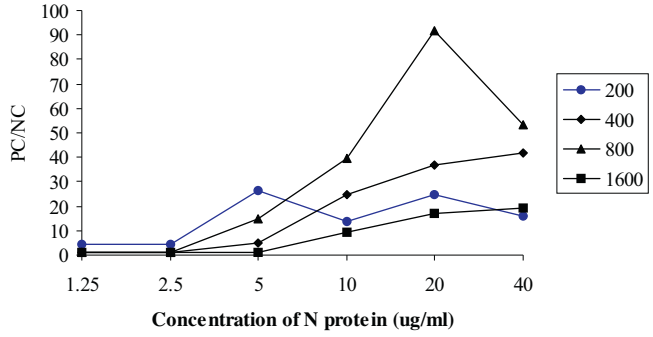

Fig. 2. Checkerboard tests for optimizing coating concentration of recombinant turkey coronavirus (TCoV) nucleocapsid (N) protein and serum dilution for antibody-capture enzyme-linked immunosorbent assay (ELISA). Each line represents a dilution factor of serum. The conjugate dilution was 1:10,000. The highest PC/NC ratio of 92 was obtained with coating concentration of N protein at 20 μg/ml and serum dilution at 1:800.

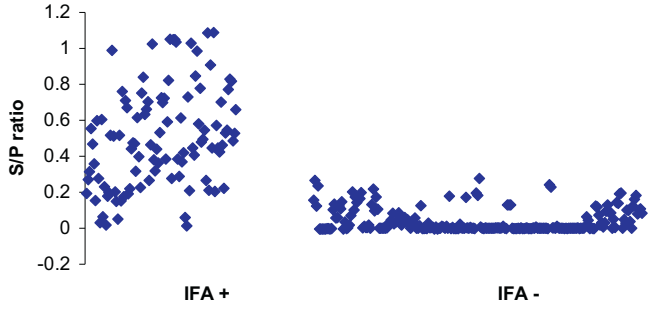

Fig. 3. Distribution of enzyme-linked immunosorbent assay (ELISA) values (S/P ratio) of serum samples that were positive (IFA+) or negative (IFA−) for turkey coronavirus (TCoV) by immunofluorecence antibody assay (IFA). The assay conditions were recombinant turkey coronavirus (TCoV) nucleocapsid (N) protein at 20 μg/ml, serum dilution at 1:800, and conjugate dilution at 1:10,000.

coated on the ELISA plate with chicken anti-IBV antibodies was still detected when turkey-specific conjugate antibody was used.

4. Discussion

According to the manufacturer, Tuner cells were used for regular expression of pTri-EX system while Origami cells were especially designed to enhance the formation of disulfide bonds in the recombinant protein. The results of the present study suggested that the construction of this particular function in Origami cells might affect their expression capacity, because the expression level of recombinant TCoV N protein was apparently higher in Tuner cells than that in Origami cells.

It was reported that a recombinant TCoV N protein from baculovirus-expression system was used for the development of a competitive antibody-capture ELISA. There were at least 2 proteins of different sizes in the purified N protein from the baculovirus expression system (Breslin et al., 2001). In comparison, the recombinant TCoV N protein expressed from the prokaryotic system could be purified to be a homogenous preparation containing only a

Table 2
Comparison of antibody-capture enzyme-linked immunosorbent assay (ELISA) utilizing recombinant turkey coronavirus (TCoV) nucleocapsid (N) protein as coating antigen with immunofluorescent antibody assay (IFA) for detection of antibody to TCoV.

Total no. samples	IFA		ELISA[c]		Relative Sensitivity[d]	Relative Specificity[e]	Agreement[f]
	+[a]	–[b]	+	–			
322	101		87	14	86.0%		95.6%
		221	7	214		96.8%	

[a] Serum sample positive for antibody to turkey coronavirus (TCoV) in IFA.
[b] Serum sample negative for antibody to TCoV in IFA.
[c] The optimium cutoff ELISA value (S/P ratio) of 0.20 was obtained by logistic regression method as described in materials and methods. Serum sample had ELISA value higher or lower than the cutoff value was positive (+) or negative (–), respectively, in ELISA.
[d] Relative sensitivity= 87/(87 + 14) × 100%.
[e] Relative specificity= 214/(7 + 214) × 100%.
[f] Agreement = (87 + 214)/(87 + 14 + 7 + 214) × 100%.

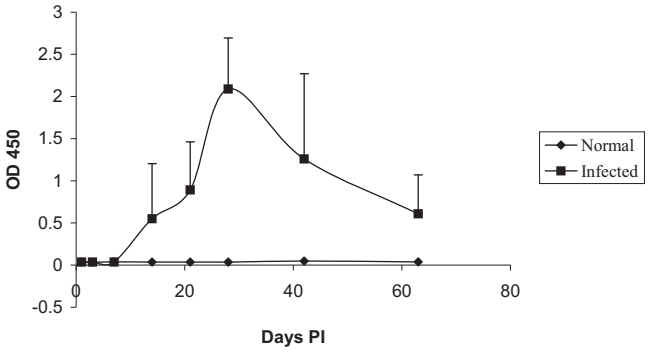

Fig. 4. Evaluation of antibody responses in serum samples of turkeys experimentally infected with turkey coronavirus (TCoV) by the established enzyme-linked immunosorbent assay utilizing recombinant turkey coronavirus (TCoV) nucleocapsid (N) protein as coating antigen. Turkey poults were infected with TCoV at 10 days of age. Five birds were randomly selected and sacrificed at 1, 3, 7, 14, 21, 28, 42 and 63 days after infection. Serum samples were collected, diluted at 1:800, and analyzed by the established ELISA method with recombinant TCoV N protein at 20 μg/ml and conjugate antibody dilution at 1:10,000.

Table 3
Reactivity of recombinant turkey coronavirus (TCoV) nucleocapsid (N) protein with antibodies specific for different coronaviruses as determined by enzyme-linked immunosorbent assay and using species-specific conjugate antibody.

Antibodies[a]	Absorbance value	S/N[b]	Result
Turkey anti-TCoV	3.986	90.590	+
Normal turkey serum	0.038		
Chicken anti-IBV	3.826	112.529	+
Normal chicken serum	0.034		
Bovine anti-BCoV	0.035	1.093	–
Normal bovine serum	0.032		
Porcine anti-TGEV	0.032	1.103	
Normal porcine serum	0.029		

[a] BCoV, bovine coronavirus; IBV, infectious bronchitis virus; TCoV, turkey coronavirus; TGEV, transmissible gastroenteritis virus.
[b] S/N was calculated as absorbance value of antibody tested divided by absorbance value of normal control serum.

single protein as shown in the present study. Although a protein band below 50 kDa with reactivity to a TCoV antiserum was noted in the inclusion body and flow through solutions. The protein was likely a degradation product of the recombinant N protein through dissolution and extraction steps of inclusion body pellets. Expression of two different forms (52 and 43 kDa) of TCoV N protein was observed in previous publications (Guy et al., 2002; Gomaa et al., 2008). The protein size as noted in the present study is approximately in the range of smaller form of those previous observations. This protein of smaller size was not found in the final elution solution of purified N protein and, subsequently, was not involved in the ELISA reactivity.

Table 4
Reactivity of recombinant turkey coronavirus (TCoV) nucleocapsid (N) protein with antibodies specific for different coronaviruses and avian viruses as determined by enzyme-linked immunosorbent assay and using goat anti-turkey horse radish peroxidase (HRPO) conjugate.

Antibodies	Absorbance value	S/P ratio[a]	Result
Chicken anti-IBV	3.799	1.002	+
Chicken anti-Rotavirus	0.449	0.108	–
Chichen anti-Reovirus	0.353	0.083	–
Normal chicken serum	0.038		
Bovine anti-BCV	0.038	0.0009	–
Normal bovine serum	0.034		
Porcine anti-TGEV	0.037	0.001	–
Normal porcine serum	0.035		
Turkey anti-adenovirus	0.048	0.001	–
Normal turkey serum	0.037		
Anti-enterovirus Mab	0.037	0.001	–
Cell culture medium	0.037		

[a] S/P ratio was calculated as absorbance value of serum sample minus absorbance value of negative control divided by absorbance value of positive control minus absorbance value of negative control.

Expression level of the cell culture-based baculovirus expression system is usually lower than that of prokaryotic expression system. It is cheaper and more convenient to prepare large amounts of pure recombinant TCoV N protein by the prokaryotic system than by the cell culture-based baculovirus system. The expression of prokaryotic system as demonstrated in the present study from preparation of starter for a 100-ml culture to the production of 10 mg of pure N protein could be finished in 20 h. For the expression of baculovirus system, it took 52–54 h for incubation of Sf9 cells after infection with the recombinant baculovirus. The yield of N protein by the baculovirus system in the final preparations was not reported (Guy et al., 2002).

Based on the optimum coating concentration of recombinant TCoV N protein at 20 μg/ml, the production of recombinant TCoV N protein from a 100-ml culture is enough for coating 50 ELISA plates. The optimum dilution of conjugate at 1:10,000 is higher than that (1:1600) in IBV-based antibody-capture ELISA as shown in our previous study (Loa et al., 2000). The results from the present study indicated that ELISA using recombinant TCoV N protein as coating antigen is more sensitive than that using IBV antigen and requires less amount of conjugate antibody in the reaction. Consequently, the cost of ELISA using recombinant TCoV N protein for every sample to be tested is reduced. The optimum dilution of testing serum samples at 1:800 indicated that the established ELISA is convenient to use and only requires a very small amount of serum for testing. Theoretically, 0.25 μl of serum sample is enough for testing in duplicate wells in the ELISA. In addition, in order to alleviate variation in the preparation of serum dilutions, 2-step sequential

dilutions at 1:100 and 1:8 were performed for the serum samples in the present study.

The relative specificity (96.8%) of the established ELISA using recombinant TCoV N protein from *E. coli* is similar to that (96.2%) using recombinant TCoV N protein from baculovirus-expression (Guy et al., 2002) or that (96.7%) using IBV antigen (Loa et al., 2000). The relative sensitivity seemed lower in the present study than that from different studies and different laboratories. It is possible that decay of specific antibodies in the serum samples during the time after IFA test and before ELISA may contribute, at least in part, to this finding. The relative sensitivity (86.0%) of the established ELISA in the present study is comparable to that (92.9 or 93.1%) from the other 2 studies. However, such slight variation might be due to different pools of serum samples in different studies. The results of lower sensitivity and higher specificity in these studies suggested that the predictive quality of positive results is more reliable than that of negative results in the ELISA. This is true to IFA test with the known low sensitive nature.

It should be noted that the ELISA using another recombinant N protein (Gomaa et al., 2008) was evaluated with the serum samples collected from experimentally infected and negative control turkeys. As demonstrated in the present study, the negative control group consistently got low background readings in ELISA throughout the entire experimental period. In contrast, the serum samples negative for TCoV by IFA from the fields got higher readings and variations in ELISA. Therefore, the cut-off point based on evaluation of ELISA performance with those two experimental groups tends to be lower. The cut-off value as OD reading was at 0.18 in that report. The cut-off point as ELISA value (S/P ratio) at 0.2 in the present study usually refers to OD readings above 0.5. Furthermore, the dilution factors of serum sample at 1:250 and conjugate at 1:2000 in that study were lower than those (serum sample at 1:800 and conjugate at 1:10,000) in the present study. Those differences in the ELISA suggested less amount of testing serum and lower cost of conjugate reagents are required by the ELISA for antibodies to TCoV established in the present study.

The observation of anti-TCoV antibody response that was induced in experimentally infected turkeys from 2 weeks after infection to the end of the experiment in the present study using recombinant TCoV N protein in ELISA was in line with that using IBV as coating antigen (Loa et al., 2000). The ELISA results from experimentally infected turkeys were comparable to those of IFA results as demonstrated previously (Loa et al., 2000).

The cross-reactivity between the recombinant TCoV N protein and antiserum to IBV was expected due to the high similarity (>90%) at the amino acid sequences between TCoV N protein and IBV N protein (Breslin et al., 1999; Akin et al., 2001). In addition, there is cross-antigenicity between turkey and chicken immunoglobulins (Stephensen et al., 1999). Therefore, both antibody to TCoV in turkey serum and antibody to IBV in chicken serum were detectable by the established ELISA as shown in the present study. The presence of antibody to IBV in turkey serum is not likely because infection of IBV to turkeys has never been established or reported. In the absence of IBV infection, antibody to IBV is not expected to be induced in turkey serum. If there were IBV infection in turkeys, it could be diagnosed by the established ELISA method.

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