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Expression and purification of turkey coronavirus nucleocapsid protein in *Escherichia coli*

C.C. Loa, T.L. Lin*, C.C. Wu, T.A. Bryan, T. Hooper, D. Schrader

Department of Veterinary Pathobiology and Animal Disease Diagnostic Laboratory, Purdue University, West Lafayette, IN 47907-1175, USA

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Abstract

Purification of turkey coronavirus (TCoV) nucleocapsid (N) protein, expressed in a prokaryotic expression system as histidine-tagged fusion protein is demonstrated in the present study. Turkey coronavirus was partially purified from infected intestine of turkey embryo by sucrose gradient ultracentrifugation and RNA was extracted. The N protein gene was amplified from the extracted RNA by reverse transcription-polymerase chain reaction and cloned. The recombinant expression construct (pTri-N) was identified by polymerase chain reaction and sequencing analysis. Expression of histidine-tagged fusion N protein with a molecular mass of 57 kd was determined by Western blotting analysis. By chromatography on nickel-agarose column, the expressed N protein was purified to near homogeneity as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. The protein recovery could be 2.5 mg from 100 ml of bacterial culture. The purified N protein was recognized by antibody to TCoV in Western blotting assay. The capability of the recombinant N protein to differentiate positive serum of turkey infected with TCoV from normal turkey serum was evident in enzyme-linked immunosorbent assays (ELISA). These results indicated that the expressed N protein is a superior source of TCoV antigen for development of antibody-capture ELISA for detection of antibodies to TCoV.

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1. Introduction

Turkey coronavirus (TCoV) causes an acute and highly infectious enteric disease. Turkey coronaviral enteritis is the most costly disease of turkeys encountered in Minnesota between 1951 and 1971. Coronavirus-associated outbreaks of poult enteritis remained as a major concern in the turkey industry. The clinical signs usually appear at 7–28 days of age and include inappetence, wet droppings, ruffled feathers, decreased weight gain, growth depression, and uneven flock growth. There is currently no specific treatment or vaccination available to control and prevent this disease. Rapid diagnosis and monitoring of immune status of a flock is critical for controlling outbreaks.

The immunofluorescent antibody (IFA) test is currently the most important serologic diagnosis of TCoV infection. The IFA procedures need antigen prepared from infected tissues, highly trained personnel, and expensive equipment.

When the test is applied to evaluate large number of clinical samples, it is labor-intensive and time-consuming. Development of an antibody-capture enzyme-linked immunosorbent assay (ELISA) for rapid diagnosis and effective control of turkey coronaviral enteritis is essential. However, large amount of highly purified viral antigen for coating ELISA plate requires propagation of TCoV in cell culture, which is not available at the present time. Alternatively, molecular cloning and expression of major structural proteins of TCoV was carried out for preparation of large quantities of highly purified viral proteins.

Coronavirus is enveloped and positive-stranded RNA virus that possesses three major structural proteins including a predominant phosphorylated nucleocapsid (N) protein, peplomeric glycoprotein (spike protein, S), that makes up the large surface projections of the virion, and membrane protein (M) (Dea and Tijssen, 1988; Saif, 1993). The N protein is abundantly produced in coronavirus-infected cells and is highly immunogenic. The N protein binds to the viral genomic RNA and composes the structural feature of helical nucleocapsid. The complete sequence of TCoV N gene was recently obtained in this laboratory (Akin et al.,

* Corresponding author. Tel.: +1-765-494-7927; fax: +1-765-494-9181.

E-mail address: tlin@purdue.edu (T.L. Lin).

2001). The nucleotide and deduced amino acid sequences of TCoV N gene shared high (>90%) similarity with those of infectious bronchitis coronavirus (IBV) N gene (Bournsnel et al., 1987).

The N protein is a preferred choice for developing a group-specific serologic assay in account of highly conserved sequence and antigenicity. The nucleocapsid proteins of various RNA viruses, such as mumps, rabies, vesicular stomatitis, measles, Newcastle disease, and IBV viruses, have been used as coating antigens in diagnostic ELISA (Linde et al., 1987; Reid-Sanden et al., 1990; Hummel et al., 1992; Ahmad et al., 1993; Errington et al., 1995; Ndifuna et al., 1998). The N protein gene of TCoV had been expressed in baculovirus system recently (Breslin et al., 2001). A complicate and competitive ELISA was demonstrated with this baculovirus-expressed N protein (Guy et al., 2002). However, the expression level of the cell culture-based baculovirus system is usually lower than that of prokaryotic system and the purity of this recombinant N protein was not clear. It is cheaper and more convenient to prepare large amount of pure recombinant protein in prokaryotic system. In addition, the antigenic integrity of N protein expressed in prokaryotic system is expected to be maintained because it is not glycosylated. The complete sequence of TCoV S and M genes has not been reported. The purpose of the present study was to express TCoV N gene with a prokaryotic expression system for preparation of large quantities of highly purified viral protein, which can be used as coating antigen for development of Ab-capture ELISA for serologic diagnosis of TCoV infection.

2. Materials and methods

2.1. Virus propagation and purification

The TCoV isolate was obtained originally from field outbreak in Southern Indiana. The agent was maintained in the laboratory by blind passages in turkey embryo as described previously (Loa et al., 2000).

2.2. Construction of N gene in the expression vector pTriEx

Total RNA was extracted from the partially purified TCoV by a modified method using guanidinium thiocyanate and acid-phenol (Chomczynski and Sacchi, 1987; Akin et al., 1999). Primers NF (TCTTTTGCCATGGCAAGC) and NR (TTGGGTACCTAAAAGTTCATTCTC) containing restriction sites Nco I and Kpn I, respectively, were designed according to nucleotide sequence of TCoV N gene as reported (Akin et al., 2001). Turkey coronavirus N protein gene was amplified by reverse transcription-polymerase chain reaction (RT-PCR) with these two primers NF and NR. The amplified product containing the entire open reading frame (1,230 bp) was digested with Nco I and Kpn I and analyzed by agarose

gel electrophoresis. The digested TCoV N gene fragment was purified and cloned to Nco I and Kpn I sites of plasmid pTriEx-1 (Novagen, Madison, WI). The pTriEx expression system allows the expression of recombinant N protein with a six histidine-tagged sequence on the C-terminal end. The construct was transformed to competent *Escherichia coli* strain Origami (DE3)pLacI (Novagen). Transformants were grown in LB medium containing 100 µg/ml ampicillin, 34 µg/ml chloramphenicol, and 1% glucose. Plasmids were purified by QIAquick mini-prep kit (Qiagen, Chatsworth, CA) and sequenced by DAVIS sequencing (Davis, CA) to confirm that the inserted TCoV N gene was in frame. The correct construct was referred as pTri-N

2.3. Expression of recombinant N protein in *E. coli*

For expression of the recombinant protein, Origami bacteria transformed with pTri-N plasmid DNA were inoculated in a tube containing 3 ml of LB broth supplemented with 100 µg/ml ampicillin, 34 µg/ml chloramphenicol, and 1% glucose and cultured overnight at 37 °C in a shaking incubator (225 rpm). The 3 ml culture was transferred to a 500 ml flask containing 100 ml of LB broth supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. The flask was shaken at 37 °C until the culture reached an O.D. 600 of 0.5. Protein expression was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Before the addition of IPTG and at 30 min, 1, 2, or 4 h after the addition of IPTG, 1 ml of the culture was collected and centrifuged. The bacteria pellet was resuspended in Laemmli sample buffer (Laemmli, 1970) and boiled for 5 min before analysis with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

2.4. Extraction of recombinant N protein from bacteria cell lysate

The bacteria were harvested by centrifugation at 10,000 × g for 10 min. The supernatant was discarded and the cell pellet was resuspended in Bugbuster reagent (Novagen) with a volume of 1 ml for every gram of pellet (wet weight). After complete resuspension of the pellet, a mixture of nuclease solution, Benzonase (Novagen), was added to remove the viscous nucleic acids at a volume of 1 µl for every 1 ml of Bugbuster reagent. The mixture was gently rotated at room temperature for 20 min. The lysate was then centrifuged at 16,000 × g for 20 min at 4 °C. The supernatant and inclusion body pellet were analyzed by SDS-PAGE and Western blotting for the presence of recombinant N protein.

2.5. Purification of recombinant N protein by chromatography with nickel-agarose column

The inclusion bodies containing the recombinant N protein were dissolved in Binding buffer containing 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, and 6 M urea at

pH 7.9. The dissolved inclusion bodies were filtered through a 0.45 µm syringe filter (Millipore, Bedford, MA) and loaded on a nickel chelating agarose column (10 mg protein/ml of gel) equilibrated in Binding buffer. The column was washed sequentially with 10 bed volumes of Binding buffer and Washing buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, and 6 M urea at pH 7.9). The recombinant N protein was eluted from the column with Eluting buffer containing 1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, and 6 M urea at pH 7.9. Fractions eluted from the column were analyzed by SDS-PAGE on a 10% polyacrylamide / bisacrylamide gel (Laemmli, 1970). Identity of the recombinant N protein was confirmed by SDS-PAGE of fractions eluted from the column and Western blotting analysis of electrotransferred protein on nitrocellulose membrane (Millipore) with reagent specific to histidine tag, horseradish peroxidase-conjugated nickel-NTA (Qiagen).

2.6. SDS-polyacrylamide gel electrophoresis and Western immunoblotting

The samples were solubilized in sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 0.001% bromophenol blue, and 1% 2-mercaptoethanol and boiled for 5 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out using the discontinuous buffer system (Laemmli, 1970). Polypeptide bands were revealed by staining the gel with Coomassie brilliant blue G-250. For immunoblotting, polypeptides separated by SDS-PAGE were electrotransferred onto nitrocellulose membrane (Millipore) with transfer buffer containing 50 mM Tris, 384 mM glycine, and 20% (v/v) methanol, pH 8.3. Electrotransfer was carried out at 65 V for 1 h. The nitrocellulose membrane was incubated for 1 h in PBS buffer containing 0.05% Tween 20 (PBS-T). After washing three times in PBS-T, membrane was incubated for 2 h at room temperature with turkey anti-TCoV antiserum or chicken anti-IBV antiserum (SPAFAS, Storrs, CT) at 1:500 of dilution in PBS-T. Three times of washing was followed by addition of horseradish peroxidase-conjugated goat anti-turkey or chicken IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD). After incubation of 2 h at room temperature, the membrane was washed three times and covered with the peroxidase substrate, 3,3'-diaminobenzidine (DAB). The blot was allowed to develop and the reaction was stopped by washing the membrane in distilled water.

2.7. Enzyme-linked immunosorbent assay

The purified recombinant N proteins were diluted with PBS buffer, coated on 96-well microtiter plates, and evaluated for capability to differentiate turkey anti-TCoV antiserum from normal turkey serum by ELISA. The coating concentrations of N protein were at 2-fold serial dilutions from 1.25 to 40 µg/ml. Serum positive (PC) for TCoV was the hyperimmune serum prepared from turkey experimen-

tally infected with TCoV. The negative control (NC) serum was collected from a 4-month-old normal healthy turkey raised in an isolation room in the laboratory. Serum samples were serially diluted at 2-fold from 1:200 to 1:1,600 in dilution buffer containing 150 mM phosphate buffer, 0.85% NaCl, 1% BSA, and 0.02% Tween-20. One hundred microliters of diluted serum sample was added to the well in duplicate and plates were incubated at 37 °C for 1 h. After incubation, wells were emptied, washed three times with PBS-T. Horseradish peroxidase-conjugated goat anti-turkey IgG (Kirkegaard & Perry Laboratories) diluted at 1:20,000 in dilution buffer was added to each well. Plates were incubated and washed as in the previous step, followed by the addition of 100 µl of enzyme substrate, tetramethyl benzidine (TMB) solution, to each well. After incubation at room temperature for 30 min, a 2 N HCl solution was added at 100 µl/well. The absorbance value of each well was measured at 450 nm using a spectrophotometer (Vmax™ kinetic microplate reader, Molecular Devices Corporation, Menlo Park, CA). The absorbance values and ratios of PC and NC serum samples were calculated.

3. Results

3.1. Construction and expression of N gene in the expression vector pTriEx

The entire open reading frame corresponding to TCoV N gene ligated to Nco I and Kpn I sites of plasmid pTriEx was confirmed by sequencing of both strands. The reading frame of N gene was in frame with the downstream six histidine-tagged sequence in the vector. Expression of the construct, pTri-N, in the host cell Origami (DE3) pLacI was induced with IPTG. Time course studies of induction of the recombinant fusion protein by IPTG indicated that the expression of N protein increased from 30 min to 4 h according to the analysis of SDS-PAGE and Western blotting with reagent specific to histidine tag (Fig. 1). The induction with IPTG for 4 h was selected in order to produce more N protein.

3.2. Extraction and purification of recombinant TCoV N protein

Soluble and pellet (inclusion body) fractions obtained by centrifugation in the extraction were examined by SDS-PAGE and Western blotting analysis (Fig. 1). The results indicated that recombinant N protein was not readily soluble in the buffer. Most of the protein was found in the inclusion body. The inclusion body was dissolved in the 6 M urea-containing buffer and further purified by chromatography on a nickel-agarose column. About 85% of the proteins loaded on the column passed through during the loading and washing steps (Table 1). Pure N protein was eluted with 1 M imidazole-containing buffer. As shown in Fig. 2, SDS-PAGE analysis indicated the presence of a single protein

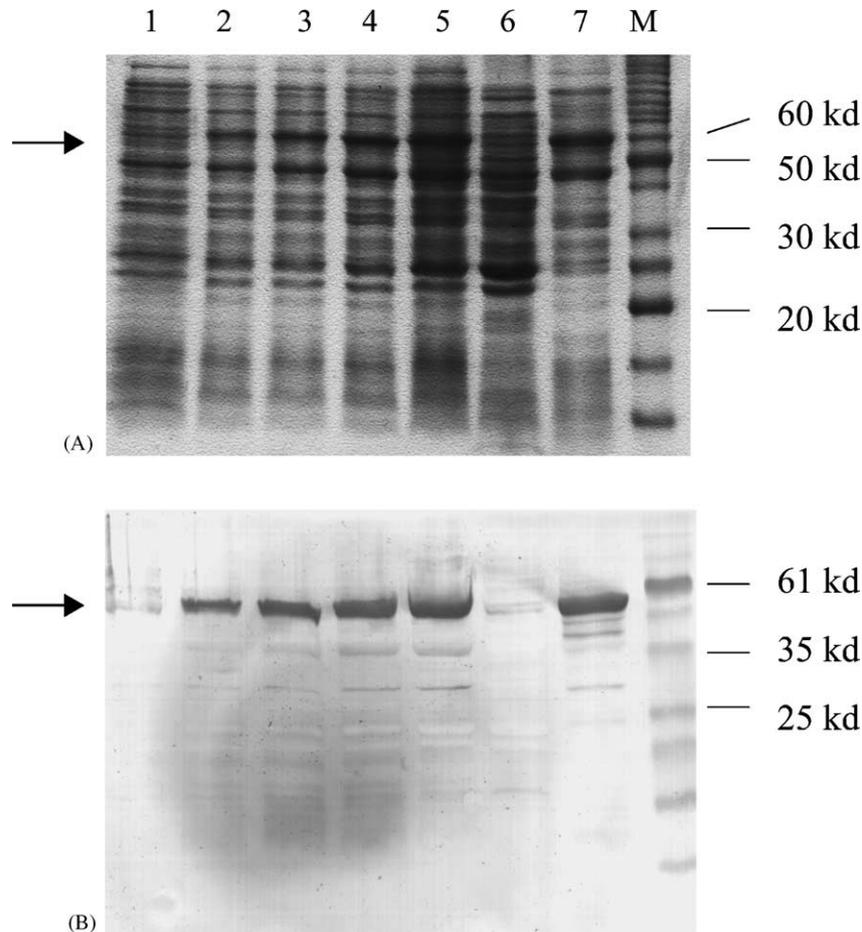


Fig. 1. Induction of nucleocapsid fusion protein expression by treatment of bacteria containing pTri-N with IPTG. Bacteria transformed with the recombinant construct pTri-N vector were cultured as described in Materials and Methods and 1 mM IPTG was added when the O.D. 600 of the culture reached 0.5. At time zero (lane 1), 30 min (lane 2), 1 h (lane 3), 2 h (lane 4), or 4 h (lane 5) after addition of IPTG, aliquots of the culture were collected and centrifuged. Bacteria pellets from the 4 h induction were further extracted with Bugbuster reagent and the supernatant (lane 6) and inclusion body (lane 7) fractions were separated. The cell pellets, supernatant, or inclusion bodies were reconstituted in Laemmli sample buffer and boiled for 5 min. Protein contents and presence of N protein were examined by SDS-PAGE (A) or Western blotting analysis using reagent specific to histidine-tag of the fusion N protein (B). The arrow indicates the expressed nucleocapsid protein. M: molecular mass markers.

band with a molecular mass about 57 kD, which is similar to the expected histidine-tagged fusion N protein. The pure N protein band was recognized by reagent specific to histidine tag in the Western blotting analysis (Fig. 2). Determination of protein recovery indicated that 2.5 mg of pure N protein could be purified by chromatography on nickel-agarose column from 100 ml of bacterial culture (Table 1).

3.3. Antigenic cross-reactivity of recombinant TCoV N protein with antibodies to different avian coronaviruses

As shown in Fig. 3, the purified N protein reacted with antibodies to TCoV or IBV in Western blotting. The normal turkey serum and chicken serum did not react with the N protein in Western blotting (data not shown).

Table 1

Purification of expressed nucleocapsid (N) protein from a representative 100 ml of *E. coli* culture by chromatography on nickel-agarose column

Step	Total volume (ml)	Total protein ^a (mg)	N protein ^b (mg)	Recovery total protein ^c (%)	Recovery N protein ^d (%)
Inclusion body ^e	6	16.9	6.0	100	100
Eluent ^f	5	2.5	2.5	14.8	41.7

^a Protein concentration was determined by the method of Lowry et al. (1951).

^b Estimated from band intensity on SDS-PAGE.

^c Total protein of e or f/total protein of e.

^d N protein of e or f/N protein of e.

^e The inclusion bodies were dissolved in Binding buffer containing 6 M urea.

^f The recombinant N protein was eluted from the column with Eluting buffer containing 1 M imidazole.

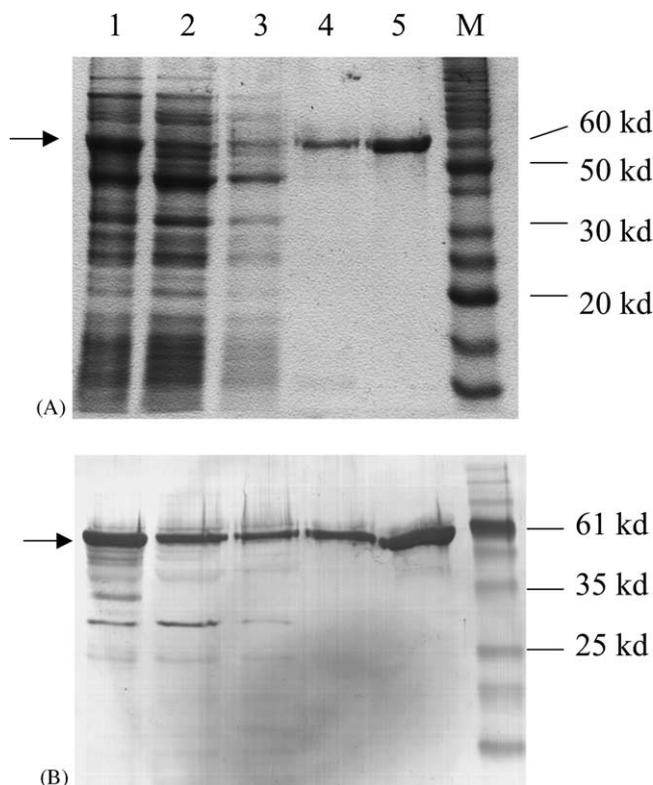


Fig. 2. Purification of recombinant nucleocapsid protein from Origami (DE3) pLacI bacteria expressing pTri-N by chromatography on nickel-agarose column. Inclusion bodies were dissolved in Binding buffer containing 6M urea (lane 1) and loaded on a nickel-agarose column (lane 2). The column was sequentially washed with Binding buffer (lane 3) and Washing buffer (lane 4). The recombinant N protein was eluted from the column with Eluting buffer containing 1M imidazole (lane 5). Fractions from each individual elution step were analyzed by SDS-PAGE (A) or Western blotting using reagent specific to histidine-tag of the fusion N protein (B). The arrow indicates the expressed nucleocapsid protein. M: molecular mass markers.

3.4. ELISA

The differentiation of PC from NC serum samples in the ELISA assay was observed at a coating concentration of N

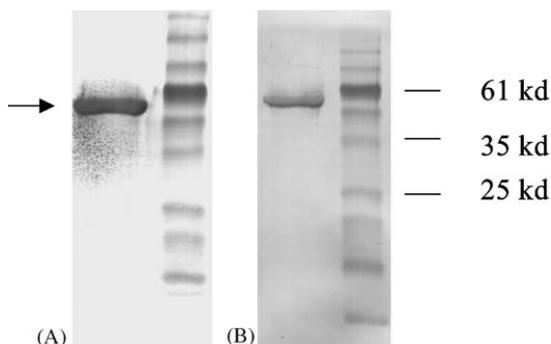


Fig. 3. Antigenic integrity of the recombinant nucleocapsid (N) protein as determined by Western blotting. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with antibodies specific to turkey coronavirus (A) or infectious bronchitis coronavirus (B).

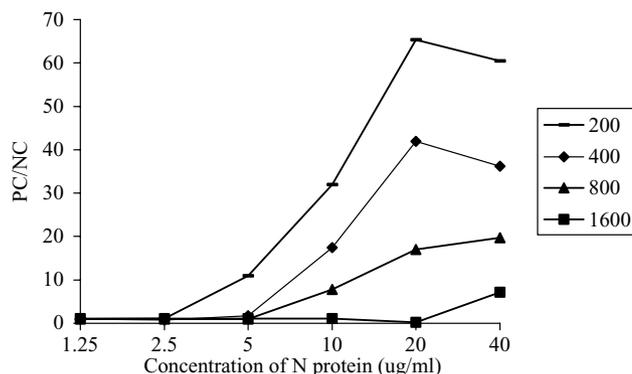


Fig. 4. Enzyme-linked immunosorbent assay of plates coated with the recombinant nucleocapsid protein for differentiation between turkey anti-TCoV antiserum (PC) and normal turkey serum (NC). The PC/NC is the ratio of anti-TCoV antiserum absorbance value to normal serum absorbance value. Each line represents a dilution factor of serum samples. The conjugate antibody was diluted at 1:20,000.

protein as low as 5 $\mu\text{g/ml}$ when serum dilution was 1:200 (Fig. 4). The capability of the recombinant N protein to differentiate PC from NC was markedly enhanced at higher coating concentrations from 5 to 40 $\mu\text{g/ml}$ with apparently higher ratios of PC/NC. The highest ratio of PC/NC was observed at 65 when coating concentration and serum dilution were 20 $\mu\text{g/ml}$ and 1:200, respectively.

4. Discussion

Cloning and expression of TCoV N protein as a histidine-tagged fusion protein in *E. coli* and the purification by chromatography on nickel chelating agarose column is demonstrated in the present study. Studies on the diagnosis, prevention, and control of TCoV infection have been hampered by the failure to propagate TCoV in cell culture. Without cell culture of the virus, molecular cloning and expression is the most important method for preparation of large quantities of highly purified viral antigens. The expression and purification procedures as described in the present study provide a simple and efficient method to obtain pure N protein of TCoV in large quantity. The yield from 100 ml of bacterial culture could be 2.5 mg of pure N protein after extraction and column chromatography.

The observed molecular mass at 57 kD of the expressed fusion N protein is within the expected range. There are 30 additional amino acids for the histidine tag in the C-terminal of the expressed fusion N protein. These extra amino acids increase the molecular mass of expressed target protein by approximately 3.3 kD. The molecular mass of IBV N protein has been reported to be from 51 to 54 kD (Saif, 1993). The size of N protein gene of TCoV and IBV is the same at 1,230 nucleotides. The molecular mass of N protein of TCoV and IBV is expected to be similar or the same on the basis of sequence information. The predicted molecular mass of the

expressed fusion N protein of TCoV was therefore from 54.3 to 57.3 kd. It was reported that two proteins with molecular mass at 52 and 43 kd were produced in the expression of TCoV N gene from baculovirus system (Breslin et al., 2001). The difference of molecular mass between this 52 kd protein from baculovirus expression and the fusion protein expressed from prokaryotic system in the present study is mainly caused by the histidine tag. In contrast, there is only one single polypeptide band in the purified N protein in the present study.

It has been reported that TCoV and IBV are antigenically related in the studies of IFA (Guy et al., 1997; Loa et al., 2000; Lin et al., 2002) or ELISA (Loa et al., 2000; Ismail et al., 2001). Sequence analysis of a conserved region of RNA polymerase gene (Stephensen et al., 1999), a segment spanning from 3' end of M gene to 5' end of N gene (Breslin et al., 1999a, Ismail et al., 2001; Lin et al., 2002), or N gene (Breslin et al., 1999b; Akin et al., 2001) indicated that TCoV and IBV are genetically related. The observations that the recombinant N protein of TCoV reacted with antibodies specific to TCoV or IBV in the present study extend these previous findings of close antigenic and genetic relationship between TCoV and IBV.

Based on the close antigenic relationship between TCoV and IBV, an antibody-capture ELISA for detection of antibodies to TCoV was established using commercially available ELISA plates coated with IBV antigen (Loa et al., 2000). However, an antibody-capture ELISA using TCoV antigens, instead of IBV antigens, should still be pursued in order to improve the sensitivity and specificity of the assay. Development of such ELISA system depends on readily available preparations of pure antigens. The recombinant N protein of TCoV as prepared in the present study was reactive with antibody to TCoV, suggesting intact antigenic integrity, and could be prepared inexpensively in large quantity. A preliminary ELISA method using the recombinant N protein as coating antigen could well differentiate the positive control serum from normal turkey serum. It is feasible to use the recombinant TCoV N protein for development of antibody-capture ELISA for serological diagnosis of TCoV infection.

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