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Short communication

Characterization of turkey coronavirus from turkey poults with acute enteritis

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Abstract

The present study was to characterize turkey coronavirus associated with turkey poult enteritis and mortality. Intestinal contents or intestines from affected turkey poults and inoculated turkey embryos contained coronaviruses as revealed by electron microscopy or were positive for turkey coronavirus by immunofluorescent antibody assay. Sucrose density gradient ultracentrifugation of the virus-containing intestinal homogenate yielded two opalescent bands corresponding to the buoyant densities of 1.14–1.15 and 1.18–1.20 g/ml, respectively. Coronaviral particles from intestinal contents or the sucrose density gradient preparation were mainly spherical in shape and had envelope and central depression. They were surrounded by a fringe of regularly spaced petal-shaped projections attached to the particles by a short stalk. Purified viruses hemagglutinated rabbit erythrocytes with a titer of 16. Major protein bands of purified viruses analyzed by SDS-PAGE were located at 200, 100–110, 50–60, and 30–35 kDa. The patterns of protein bands were consistent with those of Minnesota or Quebec turkey coronavirus isolates. A 568 bp nucleotide fragment of turkey coronavirus spike protein gene was amplified from RNA of inoculated turkey embryo intestine or purified virus. Sequence analysis of the 568 bp PCR product revealed high degree of identity with the corresponding spike protein gene sequence of human and bovine coronaviruses. The results indicated that turkey coronavirus was associated with turkey poults with acute enteritis.

Keywords: Coronavirus; Turkey; Enteritis; Characterization

1. Introduction

Coronaviruses are enveloped, positive-stranded RNA viruses that infect a wide range of mammalian and avian species. Coronaviral particle diameter ranges from 50 to 150 nm and

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bears the characteristic petal- or pear-shaped surface projections, giving it a morphologic appearance of a solar corona. The virus particle density ranges from 1.18 to 1.20 g/ml (Deshmukh and Pomeroy, 1974; Dea et al., 1985).

The major structural proteins of coronavirus include a predominant phosphorylated nucleocapsid (N) protein with molecular weight (Mr) ranging from 45 to 60 kDa, a peplomeric glycoprotein (spike protein, S) with Mr of 170–200 kDa, and a transmembrane glycoprotein (matrix protein, M) with an approximate Mr of 20–30 kDa. The S protein contributes to the distinctive peplomers on the viral surface. Some coronaviruses in the antigenic group II possess hemagglutinin (HE) that is a disulfide-linked dimer of 65 kDa subunits (Dea and Tijssen, 1988a; Saif, 1993).

Turkey coronavirus (TCV) is one of the major causative agents of infectious diarrhea in turkey poults (Dea and Tijssen, 1988b). Turkey coronaviral enteritis is characterized by anorexia, watery droppings, marked dehydration, and decreased body weight gain (Gonder et al., 1976). Infection with TCV causes rapid histopathologic changes in the mucosa of the intestinal tract of turkeys. The major changes are the granularity of the epithelial cells, loss of microvilli, margination of chromatin in the nucleus, and increased cellularity of the lamina propria. These altered physical conditions of the mucosal cells cause failure of absorption, loss of appetite, and accumulation of fluids and gases in the intestinal tract, resulting in diarrhea (Deshmukh et al., 1974; Gonder et al., 1976).

In the early 1990s, repeated outbreaks of acute enteritis in young turkey flocks occurred in Indiana, North Carolina, and other states and contributed to significant economic losses in the US turkey industry. Even at the present time, it remains a serious threat to the turkey producers in North Carolina. The estimated loss was reflected by mortality, decreased feed efficiency, stunting, and medication cost. The clinical signs of the turkey poult enteritis usually appear at 7–28 days of age and include inappetence, wet droppings, ruffled feathers, decreased weight gain, growth depression, and uneven flock growth. Analysis of intestinal contents from affected turkeys by electron microscopy (EM) at Animal Disease Diagnostic Laboratory, Purdue University, often revealed the presence of coronavirus. The clinical signs of turkey poults with acute enteritis and the continuing finding of coronavirus were very similar to TCV-induced bluecomb disease of turkeys encountered in Minnesota in 1960 and 1970s. The purpose of the present study was to identify TCV isolate from the intestinal contents of turkey flocks with outbreaks of acute enteritis and characterize its morphological, serological, and molecular properties.

2. Methods and results

Intestines and intestinal contents were obtained from 28-day-old turkey poults from flocks which had experienced outbreaks of acute enteritis in DuBois county, southern Indiana. The specimens were homogenized in 5 volumes of Tris-buffered saline (TBS) (pH 8.0) and clarified by centrifugation at 3000*g* for 10 min. The supernatant was filtered through 0.22 μm membrane filter (Millipore, Bedford, MA) and inoculated into the amniotic cavity of 22-day-old embryonated turkey eggs. Embryo intestines were harvested on day 3 post-inoculation. Harvested embryo intestines were propagated serially in embryonated turkey eggs for another four passages.

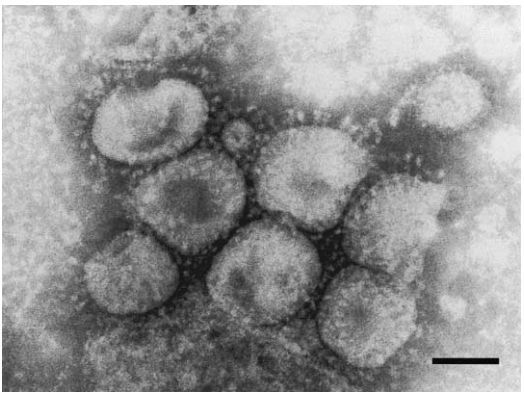

Fig. 1. Electron micrograph of coronaviral particles from the intestinal contents of a 4-week-old turkey poult with enteritis from southern Indiana. Variably sized coronaviral particles were largely round and have envelope and central depression. They were surrounded by a double fringe of regularly spaced peplomer spikes attached to viral particles by a short stalk. Bar = 100 nm.

Feces or cecal contents from the affected turkey poults or inoculated turkey embryos were clarified as described above. Clarified samples were ultracentrifuged at 100,000*g* for 2 h at 4 °C. Pellets were resuspended in distilled water and one drop of this suspension was placed on 200-mesh formvar carbon-coated grid. The grid was negatively stained with 2% of phosphotungstic acid, pH 6.5, and examined on an EM. Coronavirus particles were observed from the intestinal contents or feces of affected turkey poults from flocks which had experienced outbreaks of acute enteritis and mortality (Fig. 1). The viral particles were spherical, enveloped, and surrounded by regularly spaced petal- or pear-shaped projections. A double fringe of peplomers could be seen. They were 50–150 nm in diameter. Inoculation with the virus-containing filtrate from the intestinal homogenate did not kill turkey embryos. The intestinal tract of inoculated turkey embryos was more distended than that of normal turkey embryos. The intestinal contents were watery, gaseous, and greenish. Numerous intact coronaviral virion particles similar to those described previously were found in the intestinal contents from the inoculated embryos by EM.

Frozen intestines of affected turkey poults or inoculated turkey embryos were embedded in the embedding medium and frozen sectioned. Acetone-fixed tissue sections were incubated with turkey anti-TCV antiserum in a humidifying chamber at room temperature for 20 min. The turkey antiserum specific to the prototype TCV Minnesota isolate was obtained from Dr. Saif (The Ohio State University, Wooster, OH). After washing with phosphate-buffered saline (PBS) solution three times, intestinal sections were incubated with fluorescein isothiocyanate conjugated goat anti-turkey IgG (H + L) (Kirkegaard and Perry Laboratories, Gaithersburg, MD) in a humidifying chamber at room temperature for 30 min. Slides with intestinal sections were rinsed, air dried, and mounted. The slides were examined using an UV light microscope (Patel et al., 1975). The intestines of affected turkey poults and inoculated turkey embryos were positive for TCV using turkey anti-TCV antiserum in IFA. The infected epithelial cells had intense bright immunofluorescent staining.

For purification of virus, intestines of affected turkey poults or inoculated turkey embryos were homogenized with 5 volumes of TBS by mortar and pestle. After freeze and thaw for three times and sonication, the homogenate was centrifuged at 8000*g* for 20 min. The supernatant was ultracentrifuged at 100,000*g* for 3 h through a cushion of 15 ml of 30% sucrose solution. After resuspension of the pellet in TBS, the sample was layered on the top of 40–65% sucrose gradient and ultracentrifuged overnight at 100,000*g*. Fractions were collected from the bottom of the tubes and both absorbance and sucrose density of each fraction were determined.

Two absorbance peaks corresponding to densities of 1.18–1.20 and 1.14–1.15 g/ml, respectively, were obtained from the sucrose density gradient purification. Both peaks contained intact coronaviral particles with characterized petal- or pear-shaped surface projections revealed by EM. The size of viral particles varied. The peak corresponding to densities of 1.18–1.20 g/ml contained more intact coronaviral particles than that of 1.14–1.15 g/ml. The purified virus hemagglutinated rabbit erythrocytes with a titer of 16. Fractions of both peaks had infectivity to 22-day-old turkey embryos via amniotic cavity route of inoculation as evidenced by IFA and EM examination of intestines and intestinal contents from inoculated turkey embryos (data not shown).

The viruses purified by sucrose gradient 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 (SDS-PAGE) was carried out using the discontinuous buffer system (Laemmli, 1970). Polypeptide bands were revealed by staining the gel with Coomassie brilliant blue G-250 (Bio-Rad, Hercules, CA).

Protein contents of the sucrose gradient purified viral preparation are shown in Fig. 2. Lanes 2, 3, 4, and 5 were loaded with different amounts, 10, 20, 50, and 100 μg, respectively, of the sucrose gradient purified viral preparation. Numerous protein bands

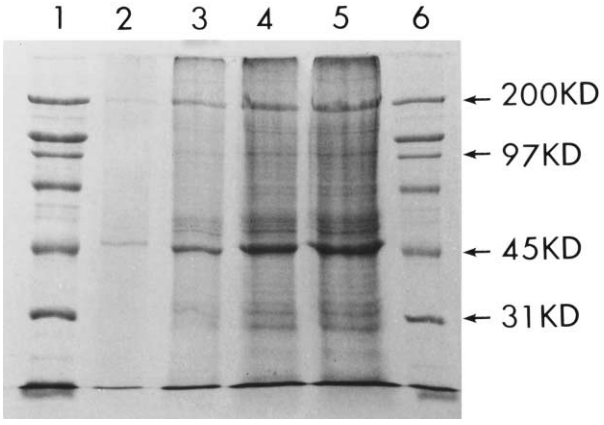

Fig. 2. SDS-PAGE analysis of turkey coronaviruses purified by sucrose density gradient ultracentrifugation. Purified coronaviral preparations were electrophoresed in 10% gel under reducing conditions. Lanes 1 and 6, molecular weight markers; lanes 2–5, corresponding to 10, 20, 50, and 100 μg of purified viral proteins. The major protein bands of 200, 100–110, 50–60, and 30–35 kDa were noted.

were seen when the loaded amount was more than 50 μg. The major protein bands could be grouped into 200, 100–110, 50–60, and 30–35 kDa. Only these major protein bands were detectable in lane 3 where 20 μg of purified virus was loaded. When 10 μg of purified virus was loaded in lane 2, only two bands with molecular weight of 200 and 50 kDa were detectable.

Total RNA was extracted from intestines of inoculated turkey embryos or sucrose density gradient purified viral preparations using acid guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi, 1987). Oligonucleotide primers for RT and PCR reactions were designed from the S1 region of spike (S) gene sequence of bovine coronavirus (BCV) (Rekik and Dea, 1994). The primers for first PCR reaction were S5 (5′TAATTTACCTGCTGCTAATG3′) corresponding to nucleotides from 1295 to 1314

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TCV TGTGTAGGTAATGGTCCTGGTATAGATGCTGGTTATAAAAATAGTGGTATAGGCACTTGTCCTGCAGGTA  70
BCV ..........................................................................  70
HCV ..........................................................................  70

TCV CTAATTATTTAACTTGCCATAATGCTGCCCAATGTGATTGTTTGTGCACTCCCGACCCCATTACATCTAA  140
BCV ..........................................................................  140
HCV ..........................................................................  140

TCV ATCTACAGGGCCTTACAAGTGCCCCCAAACTAAATACTTAGTTGGCATAGGTGAGCACTGTTCGGGTCTT  210
BCV ..........................................................................  210
HCV ..........................................................................  210

TCV GCTATTAAAAGTGATTATTGTGGAGGTAATCCTTGTACTTGCCAACCACAAGCATTTTTGGGTTGGTCTG  280
BCV ..........................................................................  280
HCV ..........................................................................  280

TCV TTGACTCTTGTTTACAAGGGGATAGGTGTAATATTTTTGCTAATTTTATTTTGCATGATGTTAATAGTGG  350
BCV ..........................................................................  350
HCV ..........................................................................  350

TCV TACTACTTGTTCTACTGATTTACAAAAATCAAACACAGACATAATTCTTGGTGTTTGTGTTAATTATGAT  420
BCV ..........................................................................  420
HCV ..........................................................................  420

TCV CTTTATGGTATTACAGGCCAAGGTATTTTTGTTGAGGTTAATGCGCCTTATTATAATAGTTAGCAGAACC  490
BCV ..........................................................G.........  490
HCV ..........................................................G.........  490

TCV TTTTATATGATTCTAATGGTAATCTCTATGGTTTTAGAGACTACTTAACAAACAGAACTTTTATGATTCG  560
BCV ..........................................................................  560
HCV ..........................................................................  560

TCV TAGTTGCT                                                                 568
BCV ........                                                                 568
HCV ........                                                                 568
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Fig. 3. Alignment of the nucleotide sequences of the 568-nucleotide PCR product from TCV with the corresponding S gene sequences of HCV-OC43 (Kuenkel and Herrler, 1996; accession number Z32768) and BCV-L9 (Zhang et al., 1991; accession number M64667). Nucleotides identical to the consensus sequence are shown as dots. There is a single nucleotide substitution from G to A at the position of 482 in the TCV genome.

of BCV S gene and S4 (5′TAGTCTTTTTGTAGAGTAATCCA C3′) corresponding to antisense of nucleotides from 2274 to 2297 of BCV S gene. The primers for nested-PCR reaction were S3 (5′ATGTGTGTAGGTAATGGTCCTGG3′) corresponding to nucleotides from 1488 to 1507 of BCV S gene and S6 (5′AGCAACTACGAATCATAAAA3′) corresponding to antisense of the nucleotides from 2036 to 2055 of BCV S gene. Reverse transcription was operated using primer S4 with Superscript RT kit (Gibco-BRL) according to the manufacturer's protocol. The complementary DNA (cDNA) product as prepared above was used as the template for PCR amplification. For the first PCR reaction, 5 μl of the cDNA was added to 45 μl of PCR reaction mixture containing 1 mM MgCl, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.1 mg/ml gelatin, 10 mM (each) dNTPs (Promega, Madison, WI), 1 μg primer S4, 1 μg primer S5, and 2.5 U of Taq DNA polymerase (Promega). The reaction mixture was amplified in a Perkin Elmer thermal cycler (Model 480, Perkin Elmer, Norwalk, CT) with an initial denaturation at 94 °C for 2 min and 35 cycles of denaturation (95 °C for 1 min), annealing (55 °C for 1 min), and extension (72 °C for 1 min). Nested PCR was carried out using primers S3 and S6 under identical conditions. The PCR product was recovered from the agarose gel, cloned into a pBK-CMV cloning vector (Stratagene, La Jolla, CA), and transformed into *E. coli* DH5a (Gibco BRL) according to the manufacturer's recommendations. Plasmids containing the PCR inserts were sequenced using the dsDNA cycle sequencing kit (Gibco BRL) according to the manufacturer's instruction.

A nested-PCR product of 568 bp nucleotide fragment was obtained. The 568-nucleotide sequence analyzed by the computer program Megalign (DNASTAR, Madison, WI) for matching sequences with BCV-L9 (Zhang et al., 1991; accession number M64667) or human coronavirus (HCV-OC43) (Kuenkel and Herrler, 1996; accession number Z32768) revealed high degree of nucleotide sequence homology among them. Pairwise comparisons of the nucleotide sequence to the corresponding S gene sequences of BCV or HCV-OC43 demonstrated 99% of similarity. There was a single nucleotide substitution from G to A at the position of 482 in the TCV sequence (Fig. 3).

3. Discussion

The ultrastructural features of the viruses characterized in the present study and the consistent findings of viruses with the same morphological properties in samples from affected turkeys, inoculated turkey embryos, and sucrose density gradient purified viral preparations provided morphological evidence that the virus is a member of the Coronaviridae family. The positive responses of the intestinal epithelial cells of inoculated turkey embryos to anti-TCV antiserum in the IFA assay further provided serological evidence that these agents were turkey coronavirus.

The observation of many polypeptide bands when the protein contents of sucrose gradient preparation were analyzed by SDS-PAGE is most likely due to contamination of host proteins in the preparation of coronaviruses from the infected intestines and intestinal contents. The same problem was also encountered in a previous study (Dea and Tijssen, 1988a). The major protein bands of the purified materials were grouped and compared to those reported for Minnesota or Quebec isolates of TCV (Dea and Tijssen, 1988a). The

clear band close to 50 kDa may represent the N phosphonucleoprotein and the 200 kDa and a faint 100 kDa band may be the non-split and split form of the S peplomeric glycoprotein. The triplets of bands around 26, 30, and 32 kDa correspond to M protein. A diffuse band can be deduced between 52 and 60 kDa which may represent the monomeric form of the HE glycoprotein. The HA activity of purified TCV with rabbit erythrocytes was comparable to a previous report (Dea et al., 1985).

The positive RT-PCR reaction of RNA extracted from the embryo intestines inoculated with TCV using primers designed from BCV S1 gene sequences and the high nucleotide identity obtained by comparative sequence analysis of the PCR product with the corresponding sequences of BCV or HCV-OC43 provided molecular evidence that the virus isolated is a coronavirus. Because there were no BCV or HCV-OC43 in the laboratory during the present study, the opportunity of contamination with either BCV or HCV-OC43 was very unlikely. The positive RT-PCR reaction and sequence of the PCR product should reflect the nature of the isolated TCV instead of contamination of BCV or HCV-OC43. Nevertheless, a single nucleotide substitution from G to A was seen. Spike protein of coronavirus has been known to be important for receptor binding and penetration of target cells during the infection process. The single nucleotide substitution from G to A at position 482 may be involved in the mechanisms of different host tropism. Further study on molecular analysis of full-length S gene of TCV is required to elucidate such information as well as the genetic relationships among TCV, BCV, and infectious bronchitis virus (IBV).

In summary, the present study has provided morphological, serological, and molecular evidences that the viral agent associated with the outbreak of turkey poult enteritis was a coronavirus, which has been successfully propagated in turkey embryos via amniotic inoculation.

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