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Research Note—

Antigenic Relationship of Turkey Coronavirus Isolates from Different Geographic Locations in the United States

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SUMMARY. The purpose of the present study was to examine the antigenicity of turkey coronavirus (TCV) isolates from various geographic areas with antibodies to different viruses. Seventeen isolates of TCV were recovered from intestinal samples submitted to Animal Disease Diagnostic Laboratory, Purdue University, from turkey farms located in different geographic areas. The prototype TCV Minnesota isolate (TCV-ATCC) was obtained from the American Type Culture Collection. Intestinal sections were prepared from turkey embryos infected with different TCV isolates and reacted with polyclonal or monoclonal antibodies to TCV, infectious bronchitis virus (IBV), bovine coronavirus (BCV), transmissible gastroenteritis virus (TGEV), reovirus, rotavirus, adenovirus, or enterovirus in immunofluorescent antibody staining. All 18 TCV isolates have the same antigenic reactivity pattern with the same panel of antibodies. Positive reactivity was seen with polyclonal antibodies to the TCV Indiana isolate, the TCV Virginia isolate, TCV-ATCC, and the IBV Massachusetts strain as well as monoclonal antibodies to the TCV North Carolina isolate or the membrane protein of IBV. Antibodies to BCV or TGEV were not reactive with any of the TCV isolates. Reactivity of antibodies to unrelated virus, rotavirus, reovirus, adenovirus, or enterovirus with different TCV isolates was all negative, except positive response was seen between enterovirus antibody and a TCV western North Carolina isolate, suggesting coinfection of turkeys with TCV and enterovirus in that particular case. The results indicated that the TCV isolates from these geographic locations in the U.S. shared close antigenicity and were antigenically related to IBV.

RESUMEN. *Nota de Investigación*—Relación antigénica entre aislados de coronavirus de los pavos obtenidos en diferentes localidades geográficas de los Estados Unidos.

Se estudió la antigenicidad de diferentes aislados de coronavirus de los pavos obtenidos de varias áreas geográficas que presentaban anticuerpos específicos contra varios virus. Diecisiete aislados del coronavirus de los pavos fueron obtenidos a partir de muestras de intestinos enviadas al Laboratorio de Diagnóstico de Enfermedades en Animales de la Universidad de Purdue, procedentes de parvadas de pavos de diferentes áreas geográficas. Se obtuvo el virus prototipo Minnesota de la American Type Culture Collection (TCV-ATCC). Se prepararon secciones de tejido intestinal a partir de embriones de pavo infectados con diferentes aislados del virus, los cuales fueron incubados con anticuerpos policlonales o monoclonales específicos contra el coronavirus de los pavos, virus de la bronquitis infecciosa, coronavirus bovino, gastroenteritis transmisible, reovirus, rotavirus, adenovirus o enterovirus, con el fin de detectar estos antígenos mediante la técnica de tinción con anticuerpos fluorescentes. Los 18 aislados del coronavirus de los pavos presentaron el mismo perfil antigénico con el mismo grupo de anticuerpos. Fue posible detectar reactores positivos con anticuerpos policlonales contra la cepa de coronavirus de los pavos de Indiana, Virginia, TCV-ATCC, el virus de bronquitis infecciosa de la cepa Massachussets, y con anticuerpos monoclonales específicos contra el aislado de coronavirus de los pavos de Carolina del Norte y la proteína de la membrana del virus de bronquitis infecciosa. No hubo reacción entre los anticuerpos específicos contra la

diarrea viral bovina y la gastroenteritis transmisible y los aislados del coronavirus de los pavos. Tampoco hubo reacción entre los anticuerpos específicos contra rotavirus, reovirus, adenovirus o enterovirus y los diferentes aislados de coronavirus de los pavos, excepto en un caso en el cual hubo reactividad entre los anticuerpos contra el enterovirus y un aislado de la región este de Carolina del Norte, indicando una infección concomitante del coronavirus de los pavos y el enterovirus en este caso particular. Estos resultados indican que los aislados del coronavirus de los pavos obtenidos de estas localidades en los Estados Unidos son similares antigénicamente entre sí y también están relacionados antigénicamente con el virus de bronquitis infecciosa.

Key words: antigenicity, infectious bronchitis virus, turkey coronaviral enteritis, turkey coronavirus

Abbreviations: ATCC = American type culture collection; BCV = bovine coronavirus; ELISA = enzyme-linked immunosorbent assay; FITC = fluorescein isothiocyanate; HCV = human coronavirus; HE = hemagglutinin; HEV = hemagglutinating encephalomyelitis virus; HI = hemagglutination inhibition; IBV = infectious bronchitis virus; IEM = immunoelectron microscopy; IFA = immunofluorescent antibody assay; M = membrane protein of coronavirus; Mab = monoclonal antibody; MHV = mouse hepatitis virus; N = nucleocapsid protein of coronavirus; PBS = phosphate-buffered saline; PEMS = poult enteritis and mortality syndrome; PRCV = porcine respiratory coronavirus; S = spike protein of coronavirus; TCV = turkey coronavirus; TGEV = transmissible gastroenteritis virus; TMB = tetramethyl benzidine; VN = virus neutralization

Coronaviruses are enveloped, positive-stranded RNA viruses that infect a wide range of mammalian and avian species. The diameter of a coronaviral particle varies from 50 to 150 nm. The virion bears the characteristic petal- or pear-shaped surface projections, giving it a morphologic appearance of a solar corona (13). The coronavirus particle contains three major structural proteins including the spike (S), membrane (M), and nucleocapsid (N) proteins. The spike protein contains neutralizing and/or group-specific epitopes and is highly variable among different coronaviruses. In contrast, the M and N proteins are more conserved among coronaviruses between different antigenic groups (19).

Turkey coronavirus (TCV) was identified in the early 1970s as the causative agent of the most costly disease of turkeys encountered in Minnesota between 1951 and 1971 (23). Outbreaks of turkey poult enteritis associated with TCV have caused serious economic losses in Indiana, North Carolina, and other states for the last several years (7,28). Although the economic importance of this disease has been recognized for decades, reports regarding the antigenic relationships of TCV with other coronaviruses remain controversial.

Turkey coronavirus did not cross-react with antibodies to transmissible gastroenteritis virus (TGEV) (antigenic group 1 of coronavirus), hemagglutinating encephalomyelitis virus (HEV), bovine coronavirus (BCV), mouse hepatitis virus (MHV) (group 2), and infectious bronchitis virus (IBV) (group 3) by immunoelectron microscopy (IEM) (24) and hemagglutination inhibition (HI) (9). Based on these observations, TCV was initially determined to be antigenically distinct from all other coronaviruses (17).

These findings were questioned when the close relationship between TCV and BCV was demonstrated in a series of studies with enzyme-linked immunosorbent assay (ELISA) (10), virus neutralization (VN) (12), immunoblotting (12), IEM (8), hybridization with specific probes (26), sequence analysis of TCV M and N genes (27), and cross-reactivity of monoclonal antibodies (Mab) to TCV or BCV (11,22). Therefore, TCV is currently placed along with BCV in group 2. However, recent antigenic (16,21) and genomic (1,3,4) studies demonstrated that the avian coronaviruses, TCV and IBV, are related. Nevertheless, antibodies to TCV were not reactive with IBV-infected chicken kidney cells, while antibodies to IBV were positive with TCV-infected turkey embryo intestines in immunofluorescent antibody assays (IFA) (16). Furthermore, genetic relatedness between TCV and IBV was revealed only by sequence comparison of the N gene (1,4) and sequence between the M and N genes

(3) in a few TCV isolates. The entire genomic sequences of TCV, BCV, and IBV were not completely compared and analyzed.

Given the high mutation rate of RNA viruses estimated at about 10^{-4} (1 out of 10,000 nucleotides changes per replication), there would be at least 1 to 3 nucleotide changes between any 2 viral genomes in a TCV population since the genomic RNA of coronavirus is around 30,000 nucleotides (17). It was speculated that TCV isolates from different geographical locations in the U.S. may be antigenically different and the discrepant results regarding the relationships between TCV and BCV and between TCV and IBV observed in different laboratories may be caused by different isolates of TCV in the reported studies (6,15,18). The purpose of the present study was to examine the cross antigenic reactivity of TCV isolates from different geographical locations in the U.S. with antibodies to various coronaviruses, including TCV, BCV, and IBV.

MATERIALS AND METHODS

Viruses. Field isolates of TCV were recovered from the intestines of turkey poult flocks experiencing an outbreak of acute enteritis. The intestine samples were submitted to Animal Disease Diagnostic Laboratory, Purdue University, from turkey farms in Arkansas, Indiana, Minnesota, Missouri, North Carolina, Pennsylvania, South Carolina, Texas, and Virginia between 1994 and 1999 (Table 1). Turkey coronavirus prototype, TCV-ATCC, was obtained from the American Type Culture Collection (Rockville, MD). The TCV isolates were propagated in embryonated turkey eggs via amniotic route as described previously (9,21). Briefly, infected turkey intestines were homogenized in five volume of phosphate-buffered saline (PBS) and clarified by centrifugation at 3,000 × *g* for 10 min. The supernatant was filtered through a 0.22-μm membrane filter (Millipore, Bedford, MA). The filtrate was inoculated into the amniotic cavity of 22-day-old embryonated turkey eggs. The embryo intestines were harvested in 3 days.

Immunobiochemicals. Antisera against TCV isolates 517 or 1002 were prepared in turkeys orally inoculated with filtered intestinal homogenate from turkey embryos infected with TCV-517 or TCV-1002 (16,21). The sources and dilutions of antibodies used in the present study are listed in Table 2.

Preparation of antigens. Intestines from turkey embryos inoculated with different TCV isolates were collected and frozen at −20 C immediately. The frozen intestines were embedded in embedding medium (Tissue-Tek O.C.T. compound, Miles Laboratories, Elkhart, IN) and frozen sectioned with a cryostat (IECMinotome, International Equipment Company, Needham Heights, MA). Sections of 6-μm thickness were obtained, air dried for 10 min, and fixed in absolute acetone at room temperature for 10 min. Intestinal sections obtained from uninfected turkey embryos were used as negative controls.

Table 1. List of turkey coronavirus (TCV) isolates and their corresponding geographic area sources and years when the case samples were submitted to Animal Disease Diagnostic Laboratory in Purdue University used in the present study.

Isolate[A]	Geographic location	Year
ATCC	Minnesota (prototype)	—
517	Indiana	1994
540	Indiana	1994
310	Minnesota	1996
1020	Western North Carolina	1996
100	Eastern North Carolina	1996
284	South Carolina	1996
428	Arkansas	1996
1425	Arkansas	1996
1001	Virginia	1997
1002	Virginia	1997
1010	Western North Carolina	1997
1038	Texas	1998
682	Pennsylvania	1998
168	Missouri	1999
2216	Missouri	1999
2580	Missouri	1999
1440	Eastern North Carolina	1999

[A]TCV isolates were recovered from samples submitted to Animal Disease Diagnostic Laboratory, Purdue University, from turkey farms in different geographic areas in different years. The sources of TCV isolates were pooled intestines of affected turkey flocks with outbreaks of turkey coronaviral enteritis. These turkeys were from a single farm within one commercial company. ATCC, the TCV prototype isolate obtained from American Type Culture Collection (Rockville, MD).

Immunofluorescent antibody staining. Direct and indirect IFA staining procedures were used to evaluate antigenic reactivity of TCV isolates to various antibodies as described previously (21). Direct IFA staining was carried out by incubation of intestinal sections with fluorescein isothiocyanate (FITC) labeled antisera specific for BCV or TGEV in a humidifying chamber at room temperature for 30 min. For indirect IFA staining, acetone-fixed tissue sections were incubated with primary antibodies in a humidifying chamber at room temperature for 30 min. After washing with PBS solution for 3 times, intestinal

Table 2. List of antibodies and sources used in the present study.

Antibody[A]	Conjugate[B]	Source[C]	Dilution
Bovine anti-BCV	FITC	VMRD	Undiluted
Chicken anti-IBV (Mass 41)	None	SPAFAS	1:50
Chicken antireovirus	None	SPAFAS	1:50
Chicken antirotavirus	None	SPAFAS	1:50
Porcine anti-TGEV	FITC	VMRD	Undiluted
Turkey anti-TCV (517)	None	C. C. Loa	1:40
Turkey anti-TCV (1002)	None	C. C. Loa	1:40
Turkey anti-TCV (ATCC)	None	Y. M. Saif	1:40
Turkey antiadenovirus	None	SPAFAS	1:40
Antienterovirus Mab	None	J. S. Guy	1:100
Anti-IBV Mab 919	None	S. Naqi	1:50
Anti-IBV Mab 94	None	S. Naqi	1:50
Anti-TCV Mab 4.24	None	J. S. Guy	1:50
Goat antimouse IgG(H+L)	FITC	KPL	1:40
Goat antiturkey IgG(H+L)	FITC	KPL	1:40
Rabbit antichicken IgG(H+L)	FITC	Sigma	1:40

[A]BCV, bovine coronavirus; IBV, infectious bronchitis virus; TCV, turkey coronavirus; TGEV, transmissible gastroenteritis virus; Mab 94 and 919 are monoclonal antibodies specific to spike and membrane protein of IBV, respectively; Mab 4.24, monoclonal antibody specific to TCV North Carolina isolate.

[B]FITC = fluorescein isothiocyanate.

[C]KPL = Kirkegaard & Perry Laboratories, Gaithersburg, MD; Sigma, St. Louis, MO; SPAFAS, Storrs, CT; VMRD, Pullman, WA; C. C. Loa, Purdue University, West Lafayette, IN; J. S. Guy, North Carolina State University, Raleigh, NC (2); S. Naqi, Cornell University, Ithaca, NY (13); Y. M. Saif, The Ohio State University, Wooster, OH (12).

sections were incubated with FITC-labeled secondary antibodies in a humidifying chamber at room temperature for 30 min. Slides with intestinal sections were washed, air dried, and mounted. The slides were examined in a fluorescent microscope. The results of IFA were recorded as — (no response), + (weak response), ++ (moderate response), and +++ (strong response).

RESULTS

All 18 TCV isolates from different geographical areas, including the prototype TCV-ATCC, have the same antigenic reactivities (Table 3). Positive immunoreactivity was seen in antibodies to TCV-ATCC, TCV-517, TCV-1002, IBV (Massachusetts), Mab 4.24 to TCV, or Mab 919 to M protein of IBV reacted with TCV isolates studied. Antibodies to BCV or TGEV did not recognize any of the TCV isolates examined. Reactivity of antibodies to unrelated viruses, rotavirus, reovirus, adenovirus, or enterovirus with different TCV isolates were all negative except for the positive response between enterovirus antibody and turkey embryo intestines infected with a western North Carolina isolate, TCV-1010. This suggested coinfection of turkeys with TCV and enterovirus in that particular case. Intestinal sections of noninfected turkey embryos were not reacted with all the antibodies used.

DISCUSSION

The TCV isolates recovered from clinically infected turkeys in different geographical areas shared close antigenicity among them based on the results in the present study. All TCV isolates examined were antigenically related to IBV, but not BCV.

It is difficult to explain the discrepant results of literatures demonstrating close relationships between TCV and IBV as well as between TCV and BCV. It was suggested that the discrepancies may be caused by different methods applied in individual studies (16). For example, HI and IEM can only detect viral structural proteins on the viral envelope surface, while the IFA method is able to detect all viral structural proteins within the infected cells. However, this cannot explain the discrepant results in the lit-

Table 3. Antigenic reactivity of turkey coronavirus (TCV) isolates with antibodies specific to different viruses as determined by immunofluorescent antibody staining (IFA).

Antibodies[A]	Intestines infected with TCV isolates[B]								
	ATCC	517	540	310	1001	1002	1010	1020	100
Anti-TCV (ATCC)	+++[C]	+++	+++	+++	+++	+++	+++	+++	+++
Anti-TCV (517)	+++	+++	+++	+++	+++	+++	+++	+++	+++
Anti-TCV (1002)	+++	++	+++	+++	++	++	+++	++	++
Mab 4.24	+++	++	++	++	+++	++	+++	++	++
Mab 919	+++	++	++	+++	+++	+++	+++	+++	+++
Mab 94	—	—	—	—	—	—	—	—	—
Anti-BCV	—	—	—	—	—	—	—	—	—
Anti-TGEV	—	—	—	—	—	—	—	—	—
Anti-IBV	+	+	+	+	+	+	+	+	+
Antirotavirus	—	—	—	—	—	—	—	—	—
Antireovirus	—	—	—	—	—	—	—	—	—
Antiadenovirus	—	—	—	—	—	—	—	—	—
Antienterovirus	—	—	—	—	—	—	++	—	—

[A]Anti-TCV (ATCC), turkey antiserum to TCV prototype isolate; anti-TCV (517), turkey antiserum to isolate 517; anti-TCV (1002), turkey antiserum to isolate 1002; Mab 4.24, monoclonal antibody to TCV North Carolina isolate; Mab 919, monoclonal antibody to infectious bronchitis virus (IBV) M protein; Mab 94, monoclonal antibody to IBV S protein; anti-BCV, bovine antiserum to bovine coronavirus; anti-TGEV, porcine antiserum to transmissible gastroenteritis virus; anti-IBV, chicken antiserum to IBV; antirotavirus, chicken antiserum to rotavirus; antireovirus, chicken antiserum to reovirus; antiadenovirus, chicken antiserum to adenovirus; antienterovirus, monoclonal antibody to enterovirus.

[B]ATCC, prototype TCV from American Type Culture Collection; 517 and 540, Indiana isolates; 310, Minnesota isolate; 1001 and 1002, Virginia isolates; 1010 and 1020, western North Carolina isolates; 100 and 1440, eastern North Carolina isolates; 284, South Carolina isolate; 168, 2216, and 2580, Missouri isolates; 428 and 1425, Arkansas isolates; 1038, Texas isolate; 682, Pennsylvania isolate.

[C]The results of IFA were recorded as — (no response), + (weak response), ++ (moderate response), and +++ (strong response).

erature using the same methods. Ritchie *et al.* (24) reported that the TCV antigen did not react with antibodies specific to IBV, BCV, HEV, or TGEV by IEM. However, Dea and Garzon (8) reported that antibodies specific to TCV or BCV cross-reacted to each other and also to HEV by IEM. In addition, the sequences of M and N genes of TCV were found to be more than 99% similar to that of the corresponding genes of BCV (27). However, the extent of sequence homology between TCV and BCV is not observed in a recent sequence analysis of the N gene (1,4) and sequence between the N and M genes (3). Instead, these studies indicated high sequence homology between TCV and IBV. The sources of TCV in most of the studies demonstrating a close relationship between TCV and BCV were cell-culture–propagated. The effect of adaptation to cell culture on the antigenic and genomic characteristics of TCV was not known and may contribute to the discrepancies. For example, adaptation of human coronavirus (HCV) OC43 to Madin-Darby Canine Kidney (MDCK) or Vero cells caused a decrease of receptor-binding activity (20). Nucleotide and amino acid changes of the S protein gene were also noted. Amino acid changes of the BCV S protein resulting from propagation on HRT-18 cells were also documented (14). Nevertheless, among the serial studies demonstrating a close relationship between TCV and BCV, the cross-antigenic reactivity between TCV and IBV was observed in low levels by ELISA (10) and immunoblotting (12).

The antigenic similarity with distinct tissue tropism between TCV and IBV in avian species resembles that between TGEV and porcine respiratory coronavirus (PRCV) in pigs. Porcine respiratory coronavirus is indistinguishable from TGEV by classical seroneutralization tests. The primary sites of TGEV or PRCV infection

Table 3. Continued.

Antibodies	Intestines infected with TCV isolates									
	284	168	2216	2580	428	1425	1440	1038	682	Normal
Anti-TCV (ATCC)	+++	+++	++	+++	+++	++	+++	++	+++	—
Anti-TCV (517)	++	+++	++	+++	+++	++	+++	++	+++	—
Anti-TCV (1002)	++	++	++	++	++	++	++	+++	++	—
Mab 4.24	++	++	++	++	+++	++	+++	++	+++	—
Mab 919	+++	++	++	+++	++	++	+++	++	+++	—
Mab 94	—	—	—	—	—	—	—	—	—	—
Anti-BCV	—	—	—	—	—	—	—	—	—	—
Anti-TGEV	—	—	—	—	—	—	—	—	—	—
Anti-IBV	+	+	+	+	+	+	+	+	+	—
Antirotavirus	—	—	—	—	—	—	—	—	—	—
Antireovirus	—	—	—	—	—	—	—	—	—	—
Antiadenovirus	—	—	—	—	—	—	—	—	—	—
Antienterovirus	—	—	—	—	—	—	—	—	—	—

are enteric or respiratory tracts, respectively. Inoculation of pigs with PRCV did not cause enteric disease. Studies of interactions between immune responses to TGEV and PRCV have demonstrated that inoculation with PRCV primed antiviral immune responses and provided partial protection for pigs against TGEV challenge (5,25). Similarly, TCV and IBV cause different diseases. Turkey coronavirus causes enteric disease in turkey while IBV causes respiratory disease in chicken, respectively. Diseases caused by TCV or IBV in heterologous species have not been identified. Further clarification of antigenic and genomic relatedness between TCV and IBV may lead to development of efficient strategies to control and prevent these diseases.

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