AVIAN DISEASES 44:498–506, 2000

Detection of Antibody to Turkey Coronavirus by Antibody-Capture Enzyme-Linked Immunosorbent Assay Utilizing Infectious Bronchitis Virus Antigen

Chien Chang Loa, Tsang Long Lin,[A] Ching Ching Wu, Thomas A. Bryan, H. Leon Thacker, Tom Hooper, and Donna Schrader

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

Received 11 June 1999

SUMMARY. An antibody-capture enzyme-linked immunosorbent assay (ELISA) for detection of antibody to turkey coronavirus (TCV) utilizing infectious bronchitis virus (IBV) antigen was developed. Anti-TCV hyperimmune turkey serum and normal turkey serum were used as positive or negative control serum for optimization of the ELISA system. Goat anti-turkey immunoglobulin G (light plus heavy chains) conjugated with horseradish peroxidase was used as detector antibody. The performance of the ELISA system was evaluated with 45 normal turkey sera and 325 turkey sera from the field and the cutoff point was determined. Serum samples of turkeys experimentally infected with TCV collected sequentially from 1 to 63 days postinfection were applied to the established antibody-capture ELISA using IBV antigens. The optimum conditions for differentiation between anti-TCV hyperimmune serum and normal turkey serum were serum dilution at 1:40 and conjugate dilution at 1:1600. Of the 325 sera from the field, 175 were positive for TCV by immunofluorescent antibody (IFA) assay. The sensitivity and specificity of the ELISA relative to IFA test were 93.1% and 96.7%, respectively, based on the results of serum samples from the field turkey flocks using the optimum cutoff point of 0.18 as determined by the logistic regression method. The ELISA values of all 45 normal turkey sera were completely separated from that of IFA-positive sera. The ELISA results of serum samples collected from turkeys experimentally infected with TCV were comparable to that of the IFA assay. Reactivity of anti-rotavirus, anti-reovirus, anti-adenovirus, or anti-enterovirus antibodies with the IBV antigens coated in the commercially available ELISA plates coated with IBV antigens could be utilized for detection of antibodies to TCV in antibody-capture ELISA.

RESUMEN. Detección de anticuerpos contra el coronavirus de pavos por medio del inmunoensayo con enzimas asociadas por captura del anticuerpo utilizando antígenos del virus de bronquitis infecciosa.

Se desarrolló un inmunoensayo con enzimas asociadas (ELISA) por captura del anticuerpo utilizando antígenos del virus de bronquitis infecciosa para la detección de anticuerpos contra el coronavirus de pavo. Para la optimización de la técnica, se utilizaron como controles positivo y negativo, suero hiperinmune contra el coronavirus de pavo y el suero de aves normales, respectivamente. La inmunoglobulina G anti-pavo obtenida en cabra (cadenas liviana y pesada) conjugada con la peroxidasa de rábano picante fue utilizada como anticuerpo detector. El comportamiento de este sistema ELISA fue evaluado con 45 muestras de suero de pavos normales y 325 muestras tomadas en el campo, determinándose el valor ó punto de corte. Muestras de suero de pavos infectados experimentalmente con el coronavirus de pavo tomadas secuencialmente de 1 a 63 días después de la infección fueron evaluadas por medio del sistema ELISA por captura del anticuerpo establecido utilizando antígenos de bronquitis. Las condiciones óptimas para la diferenciación de suero hiperinmune contra coronavirus de pavo y suero de pavos normales fueron las diluciones de suero 1:40 y las diluciones de conjugado 1:1600. De las 325 muestras de campo, 175 fueron positivas para el coronavirus de pavo mediante la prueba de inmunofluorescencia. Basados en los resultados

[A]Corresponding author.

obtenidos con las muestras de parvadas de pavo de campo y utilizando un valor ó punto de corte de 0.18 determinado por medio del método de regresión logística, la sensibilidad y la especificidad de la prueba ELISA comparada con la prueba de inmunofluorescencia fue de 93.1% y 96.7%, respectivamente. Los valores normales de todas las 45 muestras de pavos normales estuvieron completamente distantes de los de las muestras que habían sido positivas a la prueba de inmunofluorescencia. No se detectó ninguna reactividad de los anticuerpos contra rotavirus, reovirus, adenovirus y enterovirus con los antígenos de bronquitis que recubren las placas de los sistemas ELISA disponibles comercialmente. Estos resultados indican que las placas recubiertas con antígenos del virus de bronquitis del sistema ELISA disponible comercialmente pueden ser utilizadas para la detección de anticuerpos contra el coronavirus de pavos en la prueba ELISA por captura del anticuerpo.

Key words: enteritis, enzyme-linked immunosorbent assay, turkey, coronavirus, infectious bronchitis virus

Abbreviations: BCV = bovine coronavirus; ELISA = enzyme-linked immunosorbent assay; FITC = fluorescein isothiocyanate; H + L = heavy plus light chains; HRPO = horseradish peroxidase; IBV = infectious bronchitis virus; IFA = immunofluorescent antibody; IgG = immunoglobulin G; Mab = monoclonal antibody; NC = negative control; PC = positive control; PEMS = poult enteritis and mortality syndrome; SIPAC = Southern Indiana Purdue Agricultural Center; S/N = sample to negative control ratio; TCV = turkey coronavirus; TCV-IN = TCV Indiana isolate; TCV-MN = TCV Minnesota isolate; TCV-NC = TCV North Carolina isolate; TGEV = transmissible gastroenteritis virus

Turkey coronaviral enteritis, the most costly disease of turkeys encountered in Minnesota between 1951 and 1971, is an acute, highly infectious disease (8). Outbreaks of similar enteric disease in turkey poults (also referred as poult enteritis and mortality syndrome [PEMS]) occurred in southern Indiana in the early and middle 1990s and have remained as a major concern in the turkey industry in North Carolina. The clinical signs usually appear at 7–28 days old and include inappetence, wet droppings, ruffled feathers, decreased weight gain, growth depression, and uneven flock growth. The morbidity is usually high and the mortality varies. The disease was reported to be associated with detection of coronavirus in the intestinal contents of affected turkeys by direct electron microscopy (4). Coronavirus was identified in 63% of PEMS-affected flocks in North Carolina (Guy and Barnes, unpubl. data). The clinical signs of turkey poults with acute enteritis and the continuing finding of coronavirus from the intestinal content or fecal material of affected poults are similar to turkey coronaviral enteritis.

Coronaviruses are in the family *Coronaviridae,* which are enveloped, positive-stranded RNA viruses that infect a wide range of mammalian and avian species. Coronaviruses are classified into three antigenic groups based on studies of virus neutralization, hemagglutination inhibition, and immunoelectron microscopy. Turkey coronavirus (TCV) and infectious bronchitis virus (IBV) belong to antigenic groups II and III, respectively (6). Coronaviral particles range from 50 to 150 nm and bear characteristic petal- or pear-shaped surface projections, giving a morphologic appearance of a solar corona (3). The coronavirus particle contains three major structural proteins, the spike, membrane, and nucleocapsid proteins. The spike protein contains neutralizing or group-specific epitopes and is highly variable among different coronaviruses. In contrast, membrane and nucleocapsid proteins are more conserved among coronaviruses between different antigenic groups (7). Some coronaviruses in antigenic group II, such as TCV, possess hemagglutinin protein on the virion surface.

The immunofluorescent antibody (IFA) test has been established in the branch laboratory of the Animal Disease Diagnostic Laboratory in southern Indiana (Southern Indiana Purdue Agricultural Center [SIPAC]) and successfully applied for the diagnosis of TCV-induced turkey poult enteritis. The IFA test is also one of the important control measures for PEMS in the turkey industry. However, the IFA test is labor-intensive and time-consuming when the test is applied to evaluate large numbers of clinical samples. In order to rapidly diagnose as well as effectively control turkey poult enteritis,

development of an antibody-capture enzyme-linked immunosorbent assay (ELISA) for serologic evaluation of TCV infection in turkey flocks is essential. Large numbers of clinical samples can be handled by ELISA with rapidity and precision. In addition, greater specificity, sensitivity, and reproducibility can be achieved by ELISA.

For the development of antibody-capture ELISA, a large amount of pure TCV antigen is required. However, attempts to propagate TCV in cell culture in many laboratories, including ours, have not been successful (1,5). In preliminary studies, intestines and intestinal contents obtained from infected turkey embryos were used for preparation of a large amount of purified TCV antigen in the laboratory. Because of the presence of intestinal constituents in the purified TCV preparations, a strong nonspecific reaction was observed in the antibody-capture ELISA. In the present report, antigenic cross-reactivities of TCV with other coronaviruses were investigated and alternative coronaviral antigen was used for the development of antibody-capture ELISA for antibody to TCV.

MATERIALS AND METHODS

Immunobiochemicals. Antiserum against TCV Indiana isolate (TCV-IN) was prepared in turkeys orally inoculated with filtered intestinal homogenate from a turkey embryo infected with TCV-IN. All antibodies applied in the present study are listed in Table 1. The dilutions of antibodies for IFA assay and ELISA were determined by checkerboard tests for high-positive response and low nonspecific reaction.

Preliminary studies on antigenic reactivity. The IFA assay was used to evaluate antigenic reactivity of TCV to various antibodies as described previously (9). Intestinal sections were obtained from turkey embryos that were inoculated with TCV-IN or TCV North Carolina isolate (TCV-NC) and incubated with various primary antibodies. Turkey antisera specific for TCV-IN or TCV Minnesota isolate (TCV-MN) were used at a dilution of 1:40. Chicken antiserum specific for IBV (Massachusetts, Mass 41) was used at a dilution of 1:100. Fluorescein isothiocyanate (FITC)-conjugated antibodies specific for bovine coronavirus (BCV) and transmissible gastroenteritis virus (TGEV) were used undiluted according to manufacturer's recommendations. Monoclonal antibodies specific for IBV were used at a dilution of 1:50. (FITC)-conjugated secondary antibodies were used at a dilution of 1:40. The results of IFA assay were recorded as − (no response), + (moderate response), and ++ (strong response).

The ELISA method for antigenic reactivity of IBV antigen with various antibodies was as described previously (2). The commercially available ELISA plates (IDEXX, Westbrook, ME) coated with a pool of IBV strains including Massachusetts, JMK, Arkansas, Connecticut, Clone 30, D274, and D1466 were used in the ELISA. Turkey antisera specific for TCV-IN or TCV-MN were used at a dilution of 1:40. Chicken antiserum specific for IBV (Massachusetts, Mass 41) was used at a dilution of 1:500. Bovine antiserum specific for BCV and porcine antiserum specific for TGEV were used at a dilution of 1:20. Monoclonal antibodies specific for IBV were used at a dilution of 1:50. Corresponding normal control sera from each animal species were also included in the assay. Primary antibodies at the appropriate dilution were added to the IBV-coated ELISA plate in quadruplicate and incubated at 37 C for 1 hr. Goat anti-turkey immunoglobulin G (IgG) (heavy plus light chains [H + L]) conjugated with horseradish peroxidase (HRPO) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was used at a dilution of 1:1600. Conjugate antibodies specific for chicken, bovine, porcine, or mouse were used at a dilution of 1:2000. Secondary conjugate antibodies were incubated at 37 C for 1 hr and followed by the addition of tetramethyl benzidine solution. 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 sample to negative control (S/N) ratio of each antibody sample was calculated as absorbance value of antibody sample divided by that of normal control serum or culture medium. Any antibody sample that had the S/N ratio above 3 was considered positive.

Optimization of ELISA. Sera positive or negative for TCV were used to optimize the antibody-capture ELISA for detection of antibody to TCV. Positive control (PC) serum was the hyperimmune serum prepared from turkeys as described above. Negative control (NC) serum was collected from 4-mo-old normal healthy turkeys grown in the isolation room.

Ninety-six–well microtiter plates coated with IBV antigens were obtained from IDEXX and used for the development of ELISA. Checkerboard tests of serum sample in twofold dilutions (1:10 to 1:320) and HRPO-labeled goat anti-turkey IgG (H + L) conjugate (Kirkegaard & Perry Laboratories) in twofold dilutions (1:100 to 1:3200) were performed to optimize the assay. The absorbance values and PC/NC ratios were calculated. The combination of each serum and conjugate dilution that gave the best discrimination between PC and NC serum samples was considered the optimal condition for the ELISA.

Evaluation of ELISA. Three hundred twenty-five turkey serum samples from the field turkey flocks

Table 1. List of antibodies and sources used in the present study.[A]

Antibody	Conjugate	Source[B]
Bovine anti-BCV	None	National Veterinary Services Laboratory
Bovine anti-BCV	FITC	VMRD
Chicken anti-IBV (Mass 41)	None	SPAFAS
Chicken anti-reovirus	None	SPAFAS
Chicken anti-rotavirus	None	SPAFAS
Porcine anti-TGEV	None	National Veterinary Services Laboratory
Porcine anti-TGEV	FITC	VMRD
Turkey anti-TCV-IN	None	C. C. Loa
Turkey anti-TCV-MN	None	Y. M. Saif
Turkey anti-adenovirus	None	SPAFAS
Anti-enterovirus Mab	None	J. S. Guy
Anti-IBV Mab 919	None	S. Naqi
Anti-IBV Mab 94	None	S. Naqi
Goat anti-mouse IgG (H + L)	HRPO	Boehringer Mannheim
Goat anti-mouse IgG (H + L)	FITC	Kirkegaard & Perry Laboratories
Goat anti-turkey IgG (H + L)	HRPO	Kirkegaard & Perry Laboratories
Goat anti-turkey IgG (H + L)	FITC	Kirkegaard & Perry Laboratories
Rabbit anti-bovine IgG (H + L)	HRPO	Sigma
Rabbit anti-chicken IgG (H + L)	HRPO	Sigma
Rabbit anti-chicken IgG (H + L)	FITC	Sigma
Rabbit anti-porcine IgG (H + L)	HRPO	Sigma

[A]BCV = bovine coronavirus; IBV = infectious bronchitis virus; TGEV = transmissible gastroenteritis virus; TCV-IN = turkey coronavirus Indiana isolate; TCV-MN = TCV Minnesota isolate; Mab = monoclonal antibody (Mab 94 and 919 are specific for spike and membrane protein of IBV, respectively) IgG (H + L) = immunoglobulin G (heavy plus light chains); FITC = fluorescein isothiocyanate; HRPO = horseradish peroxidase.

[B]National Veterinary Services Laboratory, Ames, IA; VMRD, Pullman, WA; SPAFAS, Storrs, CT; C. C. Loa, Purdue University, West Lafayette, IN; Dr. Y. M. Saif, The Ohio State University, Wooster, OH; Dr. J. S. Guy, North Carolina State University, Raleigh, NC; Dr. S. Naqi, Cornell University, Ithaca, NY; Boehringer Mannheim, Indianapolis, IN; Kirkegaard & Perry Laboratories, Gaithersburg, MD; Sigma, St. Louis, MO.

sent to SIPAC and 45 serum samples collected from the 4-wk-old normal healthy turkeys grown in the isolation room in the laboratory were processed for IFA assay using the procedures as described previously (9). The same serum samples were also used to evaluate the performance of ELISA. Two reference wells that contained all reagents except serum samples were included in each plate. PC and NC sera as well as test sera were tested in duplicate. The ELISA value or S/P ratio of each test serum was calculated as (absorbance value of sample serum minus absorbance value of negative control serum) divided by (absorbance value of positive control serum minus absorbance value of negative control serum).

Logistic regression analysis (11) was used to determine the optimum cutoff point of the ELISA value that distinguished serum samples from the field turkey flocks as positive or negative for TCV by IFA. The cutoff point was calculated from the logistic equation $\ln[P/(1 - P)] = \beta_1 X + \beta_0$, where X was the ELISA value. At the optimum cutoff point of the ELISA value, the probability of the sample being negative (P) would be the same as the probability of the sample being positive ($1 - P$), that is $P = 1 - P = 0.5$ or 50%. This value was found by solving $0 = \beta_1 X + \beta_0$ for X ($\ln[0.5/(1 - 0.5)] = \ln(1) = 0$). Logistic regression was used to estimate the coefficients β_1 and β_0. Statistical computations were performed using the SAS program (10). The relative sensitivity of the ELISA was calculated as the percentage of positive serum samples for TCV by IFA that were positive in ELISA. The relative specificity of the ELISA was calculated as the percentage of negative serum samples for TCV by IFA assay that were negative in ELISA.

Serum samples from experimentally infected turkey poults were obtained and evaluated by the ELISA. A group of 40 10-day-old turkey poults were orally inoculated with turkey embryo–propagated TCV-IN. Another group of 40 turkey poults of the same age was inoculated with phosphate-buffered saline buffer and served as the noninfected control. At 1, 3, 7, 14, 21, 28, 42, and 63 days after inoculation, five turkeys were randomly selected from each group and sacrificed. Sera collected from the turkeys were

Table 2. Turkey coronavirus Indiana isolate (TCV-IN) or North Carolina isolate (TCV-NC) from the intestines of 25-day-old turkey embryos inoculated with the respective isolate reacted with antibodies specific for TCV-IN, TCV Minnesota isolate (TCV-MN), infectious bronchitis virus-Massachusetts (IBV), bovine coronavirus (BCV), or transmissible gastroenteritis virus (TGEV) as determined by immunofluorescent antibody (IFA) assay.[A]

Intestines infected with	Antisera specific for					Anti-IBV Mab[B]	
	TCV-IN	TCV-MN	IBV	BCV	TGEV	94	919
TCV-IN	++[b]	+	+	−	−	−	+
TCV-NC	++	+	+	−	−	−	+

[A]The results of IFA assay were determined as − (no response), + (moderate response), and ++ (strong response).

[B]Mab = monoclonal antibody (Mab 94 and 919 were specific for spike and membrane protein of IBV, respectively).

examined by the IFA assay and ELISA and the results were compared.

In addition, IBV antigens coated in the ELISA plates (IDEXX) were reacted with antisera against avian rotavirus, reovirus, adenovirus, and monoclonal antibody specific for enterovirus for evaluation of cross-reactivity.

RESULTS

Preliminary studies on antigenic reactivity. In the IFA assay, positive immunoreactivity was seen in the embryo intestines infected with TCV-IN or TCV-NC isolate and reacted with antibodies specific for TCV-IN, TCV-MN, IBV (Massachusetts), and Mab 919 specific for membrane protein of IBV (Table 2). In the ELISA, IBV antigens coated in the commercially available ELISA plate was positively reacted with antibodies specific for TCV-IN, TCV-MN, IBV (Massachusetts), and Mab 919 and Mab 94 (Table 3). Antisera specific for BCV and TGEV did not react with IBV antigens coated on the ELISA plate.

ELISA development and optimization. Based on the positive antigenic cross-reactivity between TCV-IN and IBV, commercially available IBV-coated ELISA plates by IDEXX were used for detection of anti-TCV antibodies. The results of checkerboard tests for optimizing dilutions of control serum sample positive or negative for TCV and HRPO-labeled goat anti-turkey IgG (H+L) conjugate are shown in Figs. 1, 2. The maximum PC/NC ratio was 96 and was obtained with combination of serum dilution at 1:40 and conjugate dilution at 1:1,600. The PC/NC ratio with combination of serum dilution at 1:20 and conjugate dilution at 1:3200 was also high at 87. The PC/NC ratio was markedly decreased to 16 when serum was diluted to 1:10 and conjugate diluted at 1:3200 (Fig. 1). The combination of serum dilution at 1:40 and conjugate dilution at 1:1600 was chosen to be used in antibody-capture ELISA for detection of antibody to TCV.

ELISA evaluation. Of the 325 serum samples collected from the field, 175 were positive for TCV by IFA. The ELISA values (S/P ratio) of the IFA-positive, IFA-negative, and normal turkey serum samples ranged from 0.015125 to 1.065223, −0.000840 to 0.704872, and −0.000800 to 0.001446, respectively. The distribution of ELISA values (S/P ratio) of serum samples from these three groups is shown in Fig. 3.

The ELISA values of 325 serum samples from field turkey flocks compared with their IFA results are shown in Table 4. The logistic regression model at the optimum cutoff point was $0 = (-17.56)X + 3.17$, where β_1 and β_0 were −17.56 and 3.17, respectively. The optimum cutoff point (X) was 0.18 (−3.17/−17.56). The relative sensitivity and relative specificity of the ELISA using this cutoff value were 93.1% and 96.7%, respectively. The agreement between the results of ELISA and IFA assay was 94.8%.

The IFA-negative sera were suspect cases from field turkey flocks submitted to SIPAC for diagnosis. If the low sensitivity nature of the IFA assay was taking into consideration, IFA-negative sera might be false-negative for TCV. To further evaluate the ELISA method, a group

Table 3. Infectious bronchitis virus (IBV) antigens coated in commercially available enzyme-linked immunosorbent assay (ELISA) plate reacted with antibodies specific for various coronavirus as determined by ELISA.

Antibodies	Absorbance value[A]	S/N[B]	Result
Turkey anti-TCV-IN	3.022 ± 0.244	86.34	+
Turkey anti-TCV-MN	2.918 ± 0.028	83.37	+
Normal turkey serum	0.035 ± 0.001		
Chicken anti-IBV (Mass 41)	0.904 ± 0.009	24.43	+
Normal chicken serum	0.037 ± 0.002		
Bovine anti-BCV	0.037 ± 0.001	1.00	−
Normal bovine serum	0.037 ± 0.003		
Porcine anti-TGEV	0.036 ± 0.001	0.97	−
Normal porcine serum	0.037 ± 0.003		
Anti-IBV Mab 919[C]	1.745 ± 0.166	49.86	+
Anti-IBV Mab 94[D]	0.520 ± 0.013	14.86	+
Cell culture medium	0.035 ± 0.002		

[A]Each data point was presented as mean ± SD of four optical density 450-nm readings obtained from quadruplicate wells.

[B]S/N was calculated as absorbance value of antibody tested divided by absorbance value of normal control serum.

[C]Monoclonal antibody to membrane protein of IBV.

[D]Monoclonal antibody to spike protein of IBV.

of 45 serum samples collected from normal healthy turkeys grown in the isolation room in the laboratory was used as known negative serum samples for TCV. The logistic regression analysis was not applicable for determination of optimum cutoff point for distinguishing normal turkey sera from IFA-positive sera because the ELISA values of these two groups of serum samples were completely separated. When the ELISA value of 0.01 was selected as the cutoff point, all 175 IFA-positive sera had an ELISA value above the cutoff point, but all 45 normal turkey sera had an ELISA value below the cutoff point of 0.01 and were negative for TCV

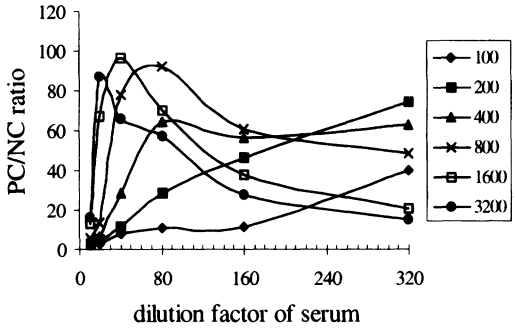

Fig. 1. Optimization of serum dilution for antibody-capture enzyme-linked immunosorbent assay (ELISA) for detection of anti-turkey coronavirus (TCV) antibodies. Each line represents a dilution factor of conjugate. The PC/NC is the ratio of positive control to negative control absorbance values using commercially available infectious bronchitis virus-coated plates. Serum dilution at 1:40 was selected in ELISA for anti-TCV antibodies.

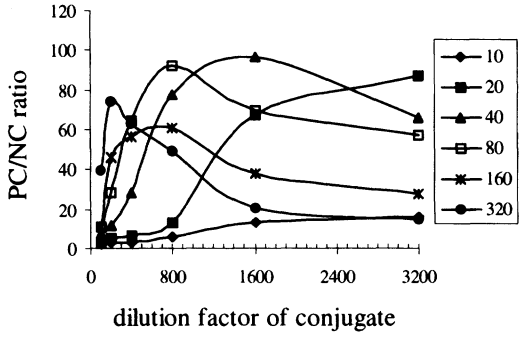

Fig. 2. Optimization of conjugate dilution for antibody-capture enzyme-linked immunosorbent assay (ELISA) for detection of anti-turkey coronavirus (TCV) antibodies. Each line represents a dilution factor of serum. The PC/NC is the ratio of positive control to negative control absorbance values using commercially available infectious bronchitis virus–coated plates. Conjugate dilution at 1:1600 was selected in ELISA for anti-TCV antibodies.

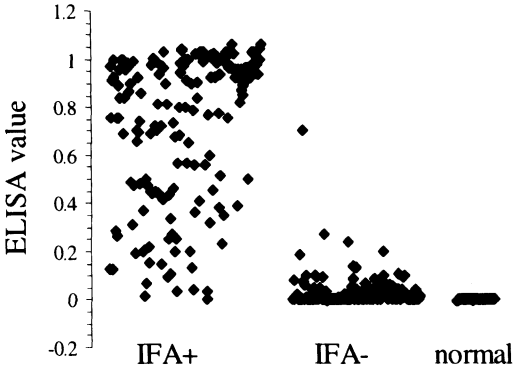

Fig. 3. Distribution of enzyme-linked immunosorbent assay (ELISA) values obtained with field turkey sera that were positive for turkey coronavirus (TCV) by immunofluorescent antibody assay (IFA+) or negative for TCV by IFA assay (IFA−) and sera collected from normal healthy turkeys (normal). The ELISA was done by utilizing commercially available infectious bronchitis virus–coated plates.

(Table 5). Sixty-five out of the 150 IFA-negative sera (43.3%) had ELISA values above 0.01. At a cutoff point of 0.30, only 1 of the 150 IFA-negative sera was above the cutoff point of 0.30 and was positive for TCV by ELISA. The percentage of positivity in IFA-positive sera became 86.3%.

The ELISA results of turkey sera sequentially taken from the turkey poults experimentally infected with TCV were found to be in line with those of the IFA assay. The anti-TCV antibody response of experimentally infected turkeys appeared at 2 wk after infection. The ELISA values of normal turkey sera were negative for TCV throughout the entire experimental period, so were the IFA results of normal turkey sera.

Reactivity of IBV antigen coated on the commercially available ELISA plate with antibodies specific for avian rotavirus, reovirus, adenovirus, or enterovirus was not detected.

DISCUSSION

The observation of antigenic cross-reactivity between IBV and TCV by IFA assay or ELISA in the present study was consistent with the previous reports (2,5). Dea and Tijssen studied antigenic reactivities of IBV (Beaudette, Connecticut, or Holland) antigens coated on ELISA plates with antisera specific for IBV (Beaudette), TCV, BCV, TGEV, murine hepatitis virus, rabbit enteric coronavirus, human coronavirus-229E, or hemagglutinating encephalomyelitis virus and found that positive reactions were seen only with antisera against IBV (Beaudette) or TCV (2). In the present study, the commercially available IBV-coated ELISA plate by IDEXX showed positive reactions with antisera specific for TCV or IBV (Massachusetts) but not with antisera specific for BCV or TGEV. Recently, Guy et al. reported that sections of TCV-infected turkey embryo intestine were positive with antisera specific for TCV or IBV (Massachusetts) but not with antisera specific for BCV or TGEV in IFA assays (5). However, they demonstrated that IBV (Arkansas)-infected chicken kidney cells were not reactive to antisera specific for TCV in IFA assay, whereas

Table 4. Comparison of antibody-capture enzyme-linked immunosorbent assay (ELISA) utilizing commercially available infectious bronchitis virus–coated ELISA plates with immunofluorescent antibody (IFA) assay for detection of antibody to turkey coronavirus (TCV).

Total no. samples	IFA		ELISA[C]		Relative sensitivity[D]	Relative specificity[E]	Agreement[F]
	+[A]	−[B]	+	−			
325	175		163	12	93.1%		94.8%
		150	5	145		96.7%	

[A]Serum sample positive for antibody to TCV in IFA assay.
[B]Serum sample negative for antibody to TCV in IFA assay.
[C]The optimum cutoff ELISA value (S/P ratio) of 0.18 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 = 163/(163 + 12).
[E]Relative specificity = 145/(5 + 145).
[F]Agreement = (163 + 145)/(163 + 12 + 5 + 145).

Table 5. Percentages of positivity and negativity of IFA-positive or IFA-negative field turkey sera and normal turkey sera at different cutoff points by antibody-capture enzyme-linked immunosorbent assay (ELISA) for detection of antibody to turkey coronavirus (TCV) utilizing commercially available infectious bronchitis virus–coated ELISA plates.

Cutoff[A] (S/P ratio)	Percentage of positivity in IFA-positive sera for TCV	Percentage of negativity in normal sera	Percentage of positivity in IFA-negative sera for TCV
0.01	100% (175/175)	100% (45/45)	43.3% (65/150)
0.3	86.3% (151/175)	100% (45/45)	0.7% (1/150)

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

strong positive response was observed using antisera specific for IBV (Massachusetts). The discrepancy may be due to differences in anti-TCV antibody titers of antisera or because different strains of IBV were used in different studies.

In searching for optimum conditions for the ELISA system, higher dilution of serum was required when the dilution of conjugate was lower and vice versa. The optimum combination of serum dilution at 1:40 and conjugate dilution at 1:1600 was selected based on the ratio of PC/NC. The combination that produced a higher PC/NC ratio was more feasible for establishment of the ELISA. The PC/NC ratio of 96 was the highest among all the combinations of serum sample and conjugate dilutions tested. Stability of the selected condition was also important in choosing the optimum combination. For example, the PC/NC ratio the combination of serum dilution at 1:20 and conjugate dilution at 1:3200 was high at 87. However, the ratio decreased dramatically to 16 when the serum dilution was 1:10 (Fig. 1). This suggested that little variation in the dilution of serum could adversely affect the PC/NC ratio and, therefore, reduce the capability of differentiation between serum samples positive or negative for TCV by the ELISA system.

Selection of cutoff point(s) was one of the most important factors in the development and interpretation of ELISA. In the present study, the optimum cutoff value of 0.18 for distinguishing serum samples from the field turkey flocks as positive or negative for TCV by IFA was determined by the logistic regression method. However, the IFA-negative sera were suspect cases from field turkey flocks submitted to SIPAC for diagnosis. If the low sensitivity nature of IFA was taken into consideration, IFA-negative sera might be false-negative for TCV. In contrast, the ELISA values of normal turkey sera were completely separated from that of IFA-positive sera. Those IFA-negative sera with an ELISA value above that of normal turkey sera possibly were actually positive for TCV. Thus, an ELISA value of 0.01 may be a good cutoff point and both the percentage of positivity in IFA-positive sera and percentage of negativity in normal turkey sera were 100%. On the other hand, if the IFA-negative sera were considered truly negative for TCV, an ELISA value of 0.30 as cutoff point was needed to prevent IFA-negative sera from being positive. Therefore, selection of an appropriate cutoff point is dependent on the purpose of the assay. If a lower probability of a false-positive is desired, a higher cutoff point should be applied. If a lower probability of a false-negative is desired, a lower cutoff point should be used. Selection of two cutoff points to interpret the ELISA results is also applicable. Any ELISA value below the lower cutoff point (0.01) was considered negative and that above the higher cutoff point (0.30) was positive. The serum samples with ELISA values in between the high and low cutoff points could be considered as suspect samples, which could be further confirmed by IFA assay or repeated ELISA on the follow-up serum samples from the original birds or farm.

One out of the 150 IFA-negative serum samples had a high ELISA value at 0.70. This sample had been repeatedly analyzed and had the same negative results in IFA assay with high

ELISA values obtained. Because intestinal or fecal samples from the same turkey were not available for confirmation of TCV infection by detection of virus with virus isolation, electron microscopy, or IFA assay, whether this serum sample was positive or negative for antibody to TCV could not be determined. However, the combination with high sensitivity and specificity of the ELISA method discussed above and low sensitivity of the IFA assay method suggested that this serum sample was probably false-negative for TCV in IFA assay and was actually positive for TCV as shown by the high ELISA value.

For the detection of antibody responses in sequential serum samples taken from turkeys experimentally infected with TCV, the established antibody-capture ELISA was well correlated with the IFA assay. The antibody response was initially detected at the same time point, 2 wk after infection, by the two different methods. The kinetics of antibody responses (increased from 2 to 4 wk after infection and remained on the plateau until 9 wk after infection when the experiment was terminated) was the same in both methods. All the normal control turkey sera were negative for TCV throughout the entire experimental period in both methods. These observations demonstrated that the established ELISA method was as effective as the IFA assay for detection of anti-TCV antibodies. Anti-TCV antibody was initially detected at 2 wk after infection; thus, at least 2 wk of separation between the first and the second serum samples is necessary for paired serum conversion test for the diagnosis of turkey coronaviral infection in turkey flocks.

The results of the present study indicated that the commercially available IBV-coated ELISA plate could be utilized for detection of anti-TCV antibodies in turkey serum by antibody-capture ELISA. Because of antigenic cross-reactivity, the IBV antigen instead of TCV antigen was used to establish the ELISA system for detection of anti-TCV antibodies. Both sensitivity and specificity of the established antibody-capture ELISA for anti-TCV antibodies were very high. The results of the ELISA were consistent with those of the IFA assay for evaluation of antibody responses in turkeys naturally or experimentally infected with TCV. Furthermore, the IBV antigens coated in the commercially available ELISA plate did not react with antibodies specific for unrelated avian viruses (rotavirus, reovirus, adenovirus, or enterovirus).

REFERENCES

1. Ali, A., and D. L. Reynolds. The in vitro propagation of stunting syndrome agent. Avian Dis. 42:657–666. 1998.

2. Dea, S., and P. Tijssen. Detection of turkey enteric coronavirus by enzyme-linked immunosorbent assay and differentiation from other coronaviruses. Am. J. Vet. Res. 50(2):226–231. 1989.

3. Deshmukh, D. R., and B. S. Pomeroy. Physicochemical characterization of a bluecomb coronavirus of turkeys. Am. J. Vet. Res. 35(12):1549–1552. 1974.

4. Goodwin, M. A., J. Brown, E. C. Player, W. L. Steffens, D. Hermes, and M. A. Dekich. Fringed membranous particles and viruses in faeces from healthy turkey poults and from poults with putative poult enteritis complex/spiking mortality. Avian Pathol. 24:497–505. 1995.

5. Guy, J. S., H. J. Barnes, L. G. Smith, and J. Breslin. Antigenic characterization of a turkey coronavirus identified in poult enteritis- and mortality syndrome-affected turkeys. Avian Dis. 41:583–590. 1997.

6. Holmes, K. V., and M. M. C. Lai. Coronaviridae: the viruses and their replication. In: Fields virology, 3rd ed. B. N. Fields, D. M. Knipe, and P. M. Howley, eds. Lippincott-Raven Publishers, Philadelphia, PA. pp. 1075–1093. 1996.

7. Karaca, K., S. Naqi, and J. Gelb, Jr. Production and characterization of monoclonal antibodies to three infectious bronchitis virus serotypes. Avian Dis. 36:903–915. 1992.

8. Nagaraja, K. V., and B. S. Pomeroy. Coronaviral enteritis of turkeys (bluecomb disease). In: Diseases of poultry, 10th ed. B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald, and Y. M. Saif, eds. Iowa State University Press, Ames, IA. pp. 686–692. 1997.

9. Patel, B. L., D. R. Deshmukh, and B. S. Pomeroy. Fluorescent antibody test for rapid diagnosis of coronaviral enteritis of turkeys (bluecomb). Am. J. Vet. Res. 36(8):1265–1267. 1975.

10. SAS institute, Inc. SAS user's guide: statistics, version 6.11 ed. SAS institute, Inc., Cary, NC. 1995.

11. Shoukri, M. M., and C. A. Pause. Statistical methods for health sciences, 2nd ed. CRC Press LLC, Boca Raton, FL. pp. 141–203. 1999.

ACKNOWLEDGMENTS

We would like to thank the following for providing support: the Commission of Agriculture, State of Indiana; Pfizer Animal Health; and North Carolina Turkey Spiking Mortality Task Force.