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Author(s): James S. Guy, Lynda G. Smith, Jamie J. Breslin, and Somsak Pakpinyo
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James S. Guy, Lynda G. Smith, Jamie J. Breslin, and Somsak Pakpinyo

Department of Microbiology, Pathology, and Parasitology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606

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SUMMARY. A competitive enzyme-linked immunosorbent assay (cELISA) was developed for detection of turkey coronavirus (TCV) antibodies. The cELISA utilized a recombinant baculovirus (*Autographa californica* nuclear polyhedrosis virus)-expressed TCV nucleocapsid (N) protein and biotin-labeled TCV N protein-specific monoclonal antibody. Sensitivity and specificity of the cELISA for detection of TCV antibodies were determined by comparison with the indirect fluorescent antibody test (IFAT) with 1269 reference, experimentally derived, and field-origin sera. Sera with discordant cELISA and IFAT results were further evaluated by western immunoblot analyses.

The cELISA detected antibodies specific for TCV and infectious bronchitis virus, a closely related coronavirus, but did not detect antibodies specific for other avian viruses. A high degree of concordance was observed between the cELISA and IFAT; sensitivity and specificity of the cELISA relative to IFAT were 92.9% and 96.2%, respectively. Western immunoblot analyses provided additional evidence of cELISA specificity. The findings indicate that the cELISA is a rapid, sensitive, and specific serologic test for detection of TCV antibodies in turkeys.

RESUMEN. Desarrollo de un inmunoensayo competitivo ligado a enzimas, para la detección de anticuerpos contra coronavirus de los pavos.

Se desarrolló un inmunoensayo competitivo ligado a enzimas (de las siglas en inglés c-ELISA) para la detección de anticuerpos contra coronavirus de los pavos. La prueba de cELISA utilizó un baculovirus recombinante (virus de la polihedrosis nuclear de *Autographa californica*) que expresaba la proteína (N) de la nucleocápside del coronavirus de los pavos, además de un anticuerpo monoclonal contra la misma proteína, marcado con biotina. La sensibilidad y la especificidad de la prueba de cELISA para la detección de anticuerpos fue comparada con la prueba indirecta de inmunofluorescencia en 1269 sueros de referencia provenientes de aves infectadas experimentalmente y de aves mantenidas bajo condiciones de campo. Los sueros que mostraron resultados discordantes con cELISA y la técnica de inmunofluorescencia se evaluaron posteriormente por análisis de inmunoelectrotransferencia puntual western. La prueba de cELISA detectó anticuerpos específicos para el coronavirus de pavo y el virus de la bronquitis infecciosa, que es un coronavirus cercanamente relacionado, sin embargo, la prueba no detectó anticuerpos específicos contra otros virus aviares. Se observó un alto grado de concordancia entre cELISA y la prueba de inmunofluorescencia, la sensibilidad y especificidad de cELISA con relación a inmunofluorescencia fueron de 92.9% y 96.2%, respectivamente. El análisis de inmunotransferencia aportó evidencia adicional acerca de la especificidad de cELISA. Los resultados indican que la técnica de cELISA es una prueba serológica rápida, sensible y específica para la detección de anticuerpos contra coronavirus de los pavos.

Key words: turkey coronavirus, baculovirus, enzyme-linked immunosorbent assay

Abbreviations: cELISA = competitive enzyme-linked immunosorbent assay; ELISA = enzyme-linked immunosorbent assay; IBV = infectious bronchitis virus; IFAT = indirect fluorescent antibody test; IgG = immunoglobulin G; M = membrane; MAb = monoclonal

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antibody; N = nucleocapsid; PBS = phosphate-buffered saline; PBST = phosphate-buffered saline containing 0.05% Tween 20; PE = postexposure; rBTCV/N = recombinant baculovirus containing TCV N gene; SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SF-9 = *Spodoptera frugiperda*; SPF = specific-pathogen free; TCV = turkey coronavirus

Turkey coronavirus (TCV) is the cause of an acute, highly contagious enteric disease of turkeys that initially was referred to as bluecomb disease (13). Bluecomb disease was first identified in turkeys in 1951, and a coronavirus was determined to be the cause of the disease in 1973 (13). In recent years, TCV has been increasingly recognized as an important cause of enteric disease in turkeys, resulting in economic loss due to impaired growth and poor feed conversion.

TCV is a member of the family *Coronaviridae*. The *Coronaviridae* comprise a large group of RNA viruses that infect a wide variety of avian and mammalian species (16,19). Coronaviruses have a distinctive morphology; they are pleomorphic, enveloped particles, 80–220 nm in diameter, with long club-shaped surface projections approximately 20 nm in length (16,19). The coronavirus genome consists of a positive-sense, single-stranded RNA molecule, 27–30 kb in size. Coronavirus virions are composed of three major structural proteins: surface glycoprotein (90–180 kD), integral membrane (M) protein (20–35 kD), and nucleocapsid (N) protein (50–60 kD). Additionally, some coronaviruses also contain a fourth major structural protein, the hemagglutinin esterase protein (120–140 kD) (16).

The coronavirus N protein binds to virion RNA and provides the structural basis for the helical nucleocapsid (12). It is the most abundant viral polypeptide in coronavirus-infected cells and it is immunodominant (11,12). Recently, the amino acid sequences of TCV M and N proteins were determined to be very similar (>90% identity) to infectious bronchitis virus (IBV) M and N proteins (1,2). Additionally, the TCV N protein was determined to have a molecular weight of approximately 52 kD (3). Sequence analysis of other TCV proteins has not been reported.

Serologic diagnosis of TCV infection currently is accomplished by indirect fluorescent antibody (IFAT) procedures (8,14). These IFAT procedures are labor intensive and time consuming. In addition, they require expensive equipment, highly trained personnel, and an antigen obtained from frozen sections of TCV-infected turkey embryo intestines or epithelial cells exfoliated from bursae of Fabricius of TCV-infected turkeys (8,14). A TCV-specific enzyme-linked immunosorbent assay (ELISA) would be an improved method for serologic diagnosis; however, the production of large quantities of high-quality antigen for this procedure has been hampered by the inability to propagate TCV in cell culture.

Recently, the TCV N protein was cloned and expressed in a baculovirus expression system (3). The present paper describes the development of a competitive ELISA (cELISA) with recombinant baculovirus-expressed TCV N protein.

MATERIALS AND METHODS

Virus. TCV (NC95) was isolated from enteritis-affected turkeys and propagated by amniotic inoculation of embryonated turkey eggs (9).

Recombinant antigen. Recombinant baculovirus (*Autographa californica* nuclear polyhedrosis virus) expressing the TCV N protein (rBTCV/N) (3) was propagated in suspension cultures of serum-free-adapted *Spodoptera frugiperda* (SF-9) insect cells (Gibco BRL, Grand Island, NY). SF-9 cells were grown in serum-free medium (SF-900 SFM medium; Gibco BRL) supplemented with penicillin (100 units/ml), streptomycin sulfate (100 μg/ml), and amphotericin B (0.25 μg/ml). Cells were grown in orbital shaker flasks with stirring at approximately 150 revolutions per minute, 28 C, in a non-CO_2 ambient-air incubator. SF-9 cells were grown to a density of approximately 2×10^6 cells/ml and infected with rBTCV/N at a multiplicity of infection of approximately 1. After incubation for 52–54 hr, cells were pelleted by centrifugation (800 × *g* for 10 min at 4 C) and resuspended in lysis buffer consisting of 0.1% Triton X-100 in phosphate-buffered saline (PBS), with protease inhibitors (phenylmethyl-sulfonylfluoride [100 μg/ml], leupeptin [0.5 μg/ml], pepstatin A [1 μg/ml]). Cell debris was removed by centrifugation (800 × *g* for 10 min at 4 C) and supernatant was stored at −75 C. Western immunoblotting was done to confirm expression of TCV N protein.

Monoclonal antibody (MAb). MAbs specific for TCV N protein were prepared by the procedure described by Carter *et al.* (5). Briefly, recombinant TCV N protein for immunization of mice was harvested from rBTCV/N-infected SF9 cells as described above and concentrated by ultrafiltration with a filter with a molecular weight cutoff of 10,000. Splenocytes were collected from immunized BALB/c mice and fused with murine myeloma cells. Hybridoma colonies secreting antibodies specific for TCV were detected by assay of culture supernatant fluids by IFAT (3). Each positive hybridoma colony was cloned twice by limiting dilution, and ascites fluid was produced by intraperitoneal injection of approximately 10^7 hybridoma cells into pristane-primed mice.

Specificity of MAbs for TCV proteins was determined by western immunoblot analysis. The immunoglobulin subclass of TCV-specific MAbs was determined by a commercial ELISA test system (MonoAb ID EIA kit; Zymed Laboratories, San Francisco, CA) used according to the manufacturer's instructions.

Biotin labeling of TCV-specific MAb. MAb 4.23 was purified from mouse ascites fluid and conjugated to biotin by Kirkegaard and Perry Laboratories, Gaithersburg, MD (10).

Reference, experimentally derived, and field-origin sera. Reference antisera against TCV strains (NC95, Minnesota) were prepared in 4-wk-old specific-pathogen-free (SPF) chickens (SPAFAS, Inc., Norwich, CT) as previously described (9). Antisera prepared in SPF chickens against IBV (Massachusetts, Arkansas, Connecticut, JMK), avian reovirus, avian influenza, avian adenovirus 1, avian paramyxovirus 3, Newcastle disease virus, and avian encephalomyelitis virus were obtained from SPAFAS, Inc. Negative control serum from unimmunized SPF chickens was obtained from SPAFAS, Inc.

Sera ($n = 67$) were collected from turkeys experimentally infected with TCV (NC95) at designated times postexposure (PE). Briefly, 10 2-wk-old turkeys were orally inoculated with approximately 2.8×10^4 embryo infectious doses of TCV (NC95). Serum samples were collected from each turkey on days 0, 4, 7, 10, 14, 21, and 28 PE.

A total of 1189 field-origin sera were obtained from turkeys in North Carolina, South Carolina, and Virginia. Field-origin sera originally were received for serologic evaluation by the IFAT. All sera were stored at −20 C until tested.

cELISA. Optimal concentrations of antigen, biotin-labeled MAb 4.23, and streptavidin–horseradish peroxidase were determined by checkerboard titration as described (4). The rBTCV/N antigen was diluted 1:1280 in 0.2 M carbonate/0.2 M bicarbonate buffer, pH 9.6; 75 μl was added to each well in 96-well ELISA plates (Pro-Bind™ Assay Plate, Falcon®; Becton Dickinson and Co., Lincoln Park, NJ) and incubated overnight at 4 C. Antigen-coated plates were washed three times with 0.01 M PBS, pH 7.2, containing 0.05% Tween 20 (PBST), then 200 μl of block buffer (PBST containing 1% nonfat dry milk) was added to each well and incubated for 1 hr at 25 C. Plates were washed three times with PBST. Positive and negative control sera and test sera were diluted 1:10 in block buffer and 50 μl samples of each were placed in duplicate wells; a diluent control consisting of block buffer (50 μl) also was placed in duplicate wells. Plates were incubated for 60 min at 25 C with gentle shaking, then washed three times with PBST. Biotin-labeled MAb 4.23 (50 μl, diluted 1:160 in block buffer) was added to each well except diluent wells and incubated for 60 min at 25 C with gentle shaking. Plates were washed three times with PBST, and 75 μl streptavidin–horseradish peroxidase (Kirkegaard and Perry Laboratories, Inc.) diluted 1:200 in block buffer was added to each well. Plates were incubated for 30 min at 37 C, then washed three times with PBST. ABTS (2, 2′-azino-bis [3-ethylbenzthiazoline-6-sulfonic acid]) substrate (Kirkegaard and Perry Laboratories, Inc.), 100 μl, was added to each well; color development was stopped after 20 min with 1% sodium dodecyl sulfate (w/v) in water. Optical densities of wells were read on an ELISA reader (BT 2000 MicroKinetics Reader; Fisher Scientific, Norcross, GA) at 405 nm. Optical densities of duplicate wells, including positive and negative control sera wells, and diluent wells were averaged. Percentage of inhibition of optical densities of test serum wells relative to negative control serum was calculated after subtracting the diluent control, which was subtracted from all test and control well averages, to yield a corrected value. Percentage of inhibition was calculated as $100 - (100 \times [\text{test serum} - \text{diluent/negative control} - \text{diluent}])$. Sera were considered to be positive if inhibition ≥45% was observed and negative if inhibition <45% was observed.

IFAT. The IFAT was performed as previously described with epithelial cells exfoliated from bursae of Fabricius of TCV-infected turkeys (3).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western immunoblot assay. TCV was partially purified from infected turkey embryo intestines as described (1). Proteins were analyzed on a 4% stacking, 10% resolving gel by a discontinuous SDS-PAGE system (15). Electrophoresis was performed with the Mini-Protean® II Dual Slab Cell apparatus (BioRad Laboratories, Richmond, CA). Electrophoretic separation of proteins was performed for 1 hr at 200 volts. After SDS-PAGE, proteins were transferred onto a 0.45-μm Trans-Blot® nitrocellulose membrane (BioRad Laboratories) with a Mini Trans-Blot® Electrophoretic Transfer Cell (BioRad Laboratories). Transfer was accomplished in 1 hr at 100 volts in transfer buffer (25 mM Tris, pH 7.5, 192 mM glycine, 20%

methanol). Membranes were air dried and incubated for 3 hr at room temperature in block solution consisting of 1% nonfat dried milk and 2% fetal bovine serum in PBS. Block solution was decanted and membranes were incubated for 2 hr at room temperature with MAb, chicken serum, or turkey serum diluted 1:25 in block solution. Membranes were washed for 15 min with four changes of TNT buffer (10 mM Tris-HCl, pH 7.5; 0.5 M NaCl; and 0.05% Tween 20), then incubated for 1.5 hr at room temperature with horseradish peroxidase–labeled goat anti-mouse immunoglobulin G (IgG) or horseradish peroxidase–labeled goat anti-chicken IgG (Kirkegaard and Perry Laboratories) diluted 1:2000 in block solution. Membranes were washed as above and reacted with a solution of 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA) for 2–5 min. The reaction was stopped by washing membranes in dH_2O for 5 min.

Statistics. Sensitivity and specificity were calculated with standard formulae (6).

RESULTS

MAb production and characterization. Two hybridoma cell lines were identified that secreted antibodies (MAb 1.01, MAb 4.23) specific for TCV. These cell lines were selected on the basis of a strong reaction of antibody to TCV antigens as determined by IFAT and absence of specific reaction when IFAT was performed with uninfected cells.

Western immunoblot analysis demonstrated specificity of MAbs 1.01 and 4.23 for TCV N protein (Fig. 1). MAbs 1.01 and 4.23 specifically reacted with TCV proteins approximately 52 and 46 kD in size. Both MAbs 1.01 and 4.23 were determined to be IgG1 isotypes. MAb 4.23 was arbitrarily chosen for conjugation to biotin and use in the cELISA.

Comparison of cELISA and IFAT test results. All cELISA and IFAT test results for reference antisera were in agreement (Table 1). The cELISA and IFAT detected antibodies in all antisera prepared against TCV strains (NC95, Minnesota) and IBV strains (Connecticut, Massachusetts, Arkansas, JMK). No antibodies (inhibition <45%) were detected in antisera prepared against avian reovirus, avian influenza virus, avian adenovirus 1, avian paramyxovirus 3, Newcastle disease virus, avian encephalomyelitis virus, and negative control serum.

The cELISA, IFAT, and western immunoblot test results for sera collected from turkeys experimentally infected with TCV are shown in Table 2. Sera were collected from experimentally infected turkeys on days 0, 4, 7, 10, 14, 21, and 28 PE. No antibodies to TCV were detected in turkeys on days 0, 4, and 7 PE by cELISA and IFAT. Seroconversion to TCV was detectable by both cELISA and IFAT beginning on day 10 PE; however, cELISA detected more antibody-positive birds at this time (10/10, 100%) than did IFAT (5/10, 50%). All birds tested by cELISA and IFAT at subsequent time intervals (days 14, 21, and 28 PE) were positive by both tests.

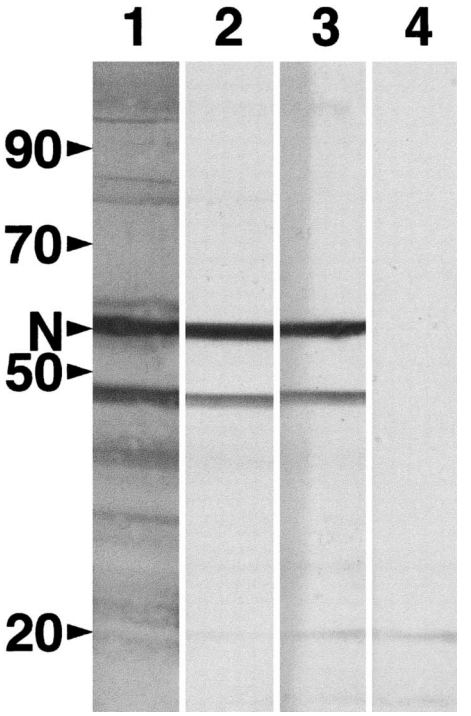

Fig. 1. Western immunoblot analyses showing specificity of MAbs 1.01 and 4.23 for TCV proteins. TCV proteins were separated by SDS-PAGE and analyzed by immunoblotting. Lane 1, chicken anti-TCV hyperimmune serum; lane 2, MAb 1.01; lane 3, MAb 4.23; lane 4, infectious laryngotracheitis virus–specific MAb (negative control). Molecular weight scale (kilodaltons) shown at left. TCV N protein is indicated by arrowhead.

Western immunoblot analysis detected seroconversion to TCV in 3 of 10 (30%) experimentally infected turkeys beginning on day 7 PE (Table 2; Fig. 2). All birds tested at subsequent time intervals were positive by western immunoblot analysis (Table 2).

Table 1. cELISA and IFAT results for reference antisera prepared in SPF chickens against TCV, IBV, and other avian viruses.

Antiserum	cELISA (% inhibition)[A]	IFAT
TCV (NC95)	+ (84%)	+
TCV (Minnesota)	+ (98%)	+
IBV (Massachusetts)	+ (99%)	+
IBV (Connecticut)	+ (86%)	+
IBV (Arkansas)	+ (94%)	+
IBV (JMK)	+ (96%)	+
Reovirus	− (−3%)	−
Adenovirus-1	− (19%)	−
Paramyxovirus-3	− (2%)	−
Avian influenza virus	− (−2%)	−
Newcastle disease virus	− (8%)	−
Avian encephalomyelitis	− (−7%)	−
Negative serum	− (−4%)	−

[A]− = negative, <45% inhibition; + = positive, ≥45% inhibition.

On the basis of cELISA, IFAT, and western immunoblot results for sera collected from experimentally infected turkeys, sensitivity of the cELISA was higher (92.5%) than that of IFAT (80%) when compared with immunoblotting. Specificity of cELISA and IFAT were both 100% when compared with immunoblotting.

Of the 1189 field-origin sera that were tested by cELISA and IFAT, 1133 (95.3%) were in agreement (Table 3). Fifty-six sera (4.7%) yielded discordant cELISA and IFAT results. On the basis of cELISA and IFAT results for field-origin sera, the cELISA was determined to have a sensitivity of 92.9% and a specificity of 96.2% when compared with IFAT (Table 3).

Western immunoblot analyses of sera with discordant cELISA and IFAT test results. Of 56 field-origin sera with discordant cELISA and IFAT results, 53 sera were of sufficient quantity for western immunoblot analysis. Western immunoblot results for the 53 discordant field-origin sera are shown in Fig. 3. Nineteen (63%) of the 30 sera that were positive by cELISA and negative by IFAT were positive by immunoblot analysis. Eighteen (78%) of the 23 sera that were cELISA negative and IFAT positive were positive by immunoblot analysis.

DISCUSSION

In the present study, a cELISA was developed for detection of TCV-specific antibodies with baculovirus-expressed TCV N protein and biotin-labeled TCV N protein-specific MAb. Specificity of the recombinant TCV N protein-based cELISA was indicated by analyses of reference antisera. The cELISA detected antibod-

Table 2. Detection of TCV-specific antibodies in sera of experimentally infected turkeys by cELISA, IFAT, and western immunoblotting.[A]

Days post-exposure	No. positive/no. tested (% positive)		
	cELISA	IFAT	Immunoblotting
0	0/10 (0)	0/10 (0)	0/10 (0)
4	0/10 (0)	0/10 (0)	0/10 (0)
7	0/10 (0)	0/10 (0)	3/10 (30)
10	10/10 (100)	5/10 (50)	10/10 (100)
14	10/10 (100)	10/10 (100)	10/10 (100)
21	10/10 (100)	10/10 (100)	10/10 (100)
28	7/7 (100)	7/7 (100)	7/7 (100)

[A]Two-week-old turkeys were orally inoculated with TCV (NC95). Serum was collected at intervals from day 0 to day 28 postexposure.

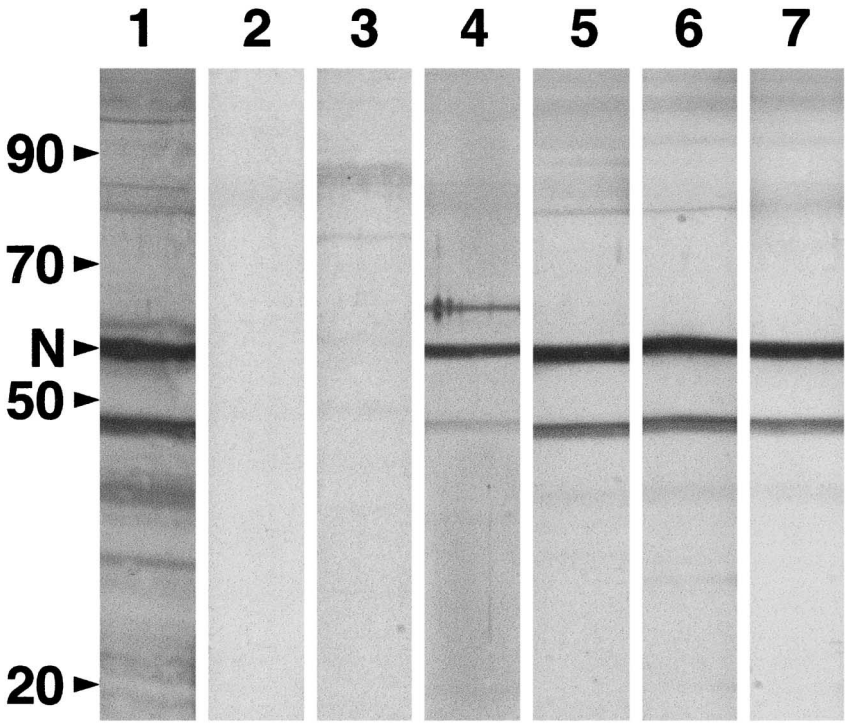

Fig. 2. Western immunoblot showing antibody response of experimentally infected turkey to TCV proteins. TCV proteins were separated by SDS-PAGE and analyzed by immunoblotting with sera collected on selected days postexposure. Lane 1, chicken anti-TCV hyperimmune serum; lanes 2, 3, 4, 5, 6, and 7, sera collected on days 0, 4, 7, 10, 14, and 28 postexposure, respectively. Molecular weight scale (kilodaltons) shown at left. TCV N protein is indicated by arrowhead.

ies in reference antisera prepared against TCV strains (NC95, Minnesota) as well as IBV strains (Connecticut, Massachusetts, Arkansas, JMK). The cELISA did not detect antibodies in reference antisera prepared against other avian viruses (reovirus, avian adenovirus 1, avian influenza virus, avian paramyxovirus 3, Newcastle disease virus, and avian encephalomyelitis virus). Previous studies have demonstrated close antigenic and genomic relatedness between TCV and IBV (1,2,3,9,18). Included in these studies was the demonstration that TCV N protein was identical in size and had greater than 90% amino acid identity with IBV N protein (2). The detection of IBV-specific antibodies by the cELISA provides additional support for these findings.

Both cELISA and IFAT detected TCV-specific antibodies in experimentally infected turkeys beginning on day 10 PE. All birds tested at 10 days PE were positive by cELISA, but only 50% were positive by IFAT. Western immunoblot analysis detected TCV-specific antibodies in experimentally infected turkeys prior to their detection by cELISA and IFAT, thus demonstrating the relative sensitivity of this serologic procedure. On the basis of analyses of sera collected from experimentally infected turkeys, sensitivity of cELISA (92.5%) was higher than that of IFAT (80%) when compared with immunoblotting; specificity of both cELISA and IFAT was 100%.

A high degree of concordance (95.3%) was observed between cELISA and IFAT when field sera (n = 1189) were analyzed by these two tests. On the basis of analyses of field sera and comparison with IFAT, the cELISA proved to be a sensitive and specific test.

Western immunoblot analysis was a useful method for verifying cELISA sensitivity and specificity; however, cELISA results were confirmed for only 24 of 53 discordant results by immunoblot analysis. Immunoblotting failed to fully resolve issues of specificity where cELISA

Table 3. Detection of TCV-specific antibodies in field-origin sera: comparison of cELISA and IFAT results.

No. samples	IFAT		cELISA[C]				
	+[A]	−[B]	+	−	Agreement[D]	Sensitivity[E]	Specificity[F]
1189	324		301	23	95.3%	92.9%	
		865	33	832			96.2%

[A]Serum samples positive for TCV antibody by IFAT.
[B]Serum samples negative for TCV antibody by IFAT.
[C]Serum samples tested by cELISA: − = <45% inhibition; + = ≥45% inhibition.
[D]Agreement = 301 + 832/1189.
[E]Sensitivity = 301/301 + 23.
[F]Specificity = 832/832 + 33.

appeared to be more sensitive than IFAT (11/53 discordant sera). Additionally, cELISA failed to detect antibody in 18 sera that were positive by IFAT and immunoblotting; the reason for this apparent insensitivity has not been determined.

Antibody to TCV N protein was the first and predominant antibody detected by immunoblot analysis (Fig. 2). This finding is consistent with previous reports indicating that this coronavirus protein is immunodominant (11,12).

The detection of both IBV- and TCV-specific antibodies by the cELISA would appear to be an obstacle to specific detection of TCV infection in turkeys in that the cELISA would not discriminate between TCV and IBV infection. However, on the basis of our current understanding of the host range of IBV, this is not an impediment because turkeys are not susceptible to this virus. IBV, like TCV and other coronaviruses, has a limited host range. Chickens and pheasants are the only known natural hosts for IBV; experimental attempts to infect a variety of other avian species, including turkeys, have not been successful (7,17).

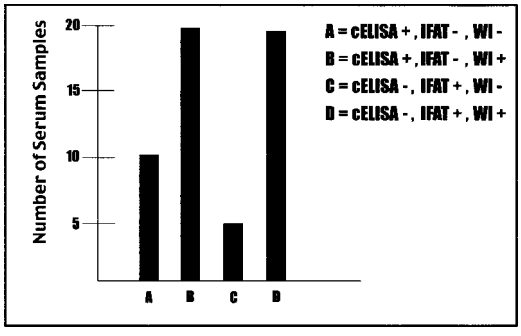

Fig. 3. Western immunoblot results for field-origin sera with discordant cELISA and IFAT results.

The findings of the present study indicate that the recombinant TCV N protein-based cELISA is a rapid, sensitive, and specific method for detection of TCV-specific antibodies in turkeys. The cELISA represents an alternative to the more cumbersome IFAT for screening large numbers of turkey sera for TCV-specific antibodies.

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