Seroprevalence of Turkey Coronavirus in North American Turkeys Determined by a Newly Developed Enzyme-Linked Immunosorbent Assay Based on Recombinant Antigen

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Turkey coronavirus (TCoV) causes diarrhea in young turkey poults, but little is known about its prevalence in the field. To address this, the complete nucleocapsid gene was cloned and expressed in Escherichia coli. Expressed nucleocapsid gene produced two distinct proteins (52 and 43 kDa); their specificity was confirmed by Western blotting using two different monoclonal antibodies. Recombinant N protein was purified and used as an antigen to develop an enzyme-linked immunosorbent assay (ELISA) for the serological detection of TCoV that was then validated using experimentally derived turkey serum. The N-based ELISA showed (97%) sensitivity and (93%) specificity for TCoV, which was significantly higher than an infectious bronchitis coronavirus-based commercial test for TCoV. To assess the utility of this recombinant ELISA, 360 serum samples from turkey farms in Ontario, Canada, and 81 serum samples from farms in Arkansas were tested for TCoV-specific antibodies. A high seroprevalence of TCoV was found in turkeys from the Ontario farms with 73.9% of breeders and 60.0% of meat turkeys testing seropositive using the N-based ELISA. Similarly, a high field prevalence was found in the turkeys from Arkansas, for which 64.2% of the serum samples tested seropositive.

Turkey coronavirus (TCoV) is a group III CoV closely related to infectious bronchitis virus (IBV) of chickens (10). TCoV is one of the most important causative agents of diarrhea in turkey poults. The infection of immunologically naive poult's with TCoV produces diarrhea and, as a consequence, negatively impacts their growth rates, causing significant economic losses. Although TCoV was identified as the causative agent of Bluecomb disease of turkey poult's over 50 years ago (28), vaccines are not available to control the disease. CoVs possess four major structural proteins: spike (S) glycoprotein, which consists of two subunits (S1 and S2); membrane (M) protein; small envelope (E) protein; and nucleocapsid (N) protein. The N protein is highly conserved among group III CoVs, and different strains of IBV and TCoV share 97% or greater identity at the amino acid sequence level (10). During IBV infections in chickens, the IBV N protein is expressed at high levels and produces antibodies that react with a variety of IBV serotypes (25). The B-cell epitopes have been mapped in the carboxy-terminal region of the N protein (3, 26). The N protein is also involved in the cell-mediated immunity and protection of chickens from IBV infection. As with IBV (19), enzyme-linked immunosorbent assay (ELISA) tests have been developed using recombinant N protein for various viruses such as measles virus (16), vesicular stomatitis virus (1), and Newcastle disease virus (8) and severe acute respiratory syndrome CoV (24).

Recently, the full-length genomic sequence of TCoV was completed (10) and provided the genetic information necessary to produce recombinant antigen for the development of diagnostic tests for TCoV in turkeys. The objective of this study was to develop and validate an ELISA for TCoV based on N recombinant antigen. This ELISA was compared with a commercial whole IBV-based ELISA for their ability to determine the seroprevalence of TCoV in breeder and meat turkey flocks in Ontario, Canada, and Arkansas.

MATERIALS AND METHODS

Virus. TCoV was isolated from an Ontario turkey suffering from acute enteritis and diarrhea. The intestines from affected birds were homogenized in phosphate-buffered saline (PBS) and then clarified by centrifugation at 4,000 × g for 15 min. The supernatant was filtered through a 0.22-μm membrane filter (Millipore, Bedford, MA). This isolate was named TCoV-MG10 (10) and used for this study.

Experimental turkeys. Day-old turkey poult's were obtained from a commercial turkey breeder (Hybrid Turkeys; Kitchener, Ontario, Canada). The birds were housed in the campus animal facility's isolation unit of the University of Guelph where they were fed and watered ad libitum. All animals were housed in the campus animal facility's isolation unit of the University of Guelph.

Control positive and negative turkey serum samples. Forty day-old birds were separated into two groups, 20 birds per group, and birds in the same group were maintained in the same rooms. TCoV-MG10 was inoculated orally into 20 birds, while 20 were kept as a negative control. Serum samples were obtained from both groups of birds at days 7, 14, 20, 35, and 42. All sera were heat-inactivated at 56°C for 30 min before use. Fifteen specific-pathogen-free (SPF) turkey serum samples were kindly provided by Billy Hargis (University of Arkansas, Fayetteville, AR), and they were used as additional negative controls.

Cloning of the TCoV N-protein genes. The total RNA was extracted from the intestinal tissue homogenates of infected turkey poult's using the Qiagen RNeasy...
Expression and purification of N-glutathione S-transferase (GST) fusion protein. 

Sensitivity, specificity, and cutoff values for the recombinant antigen ELISA.

Cross-reactivity of turkey sera with IBV assessed using a commercial IBV whole-virus ELISA.

Determination of TCoV seroprevalence in commercially reared turkeys.

RESULTS

Cloning and expression of the TCoV N recombinant protein.

Optimization and cutoff value of the TCoV N-based ELISA.

Expression and purification of N-glutathione S-transferase (GST) fusion protein.

Serum samples were obtained from Ontario turkey farms. Thirty samples were collected from each of six commercial meat turkey and six breeder turkey farms for a total of 360 field serum samples from Ontario. A further 81 field samples were obtained from various turkey farms in Arkansas. All samples were heat inactivated at 56°C for 30 min before being used in the TCoV N-based recombinant ELISA.

Statistical analyses. Differences in TCoV seroprevalence between breeder and meat turkey farms were tested with one-tailed tests assuming unequal variances. In all cases, differences were considered significant at P < 0.05. Spearman rank order correlation calculated using the Free Statistics software (version 1.23.3; P. Wessa, Office for Research Development and Education [http://www.wessa.net/]) was used to assess the correlation of the OD405 values, and the seropositive or seronegative status was determined by the cutoff OD405 value established for the TCoV N-based and whole IBV antigen-based ELISAs. The OD405 values from 168 paired field serum samples from the Ontario commercial turkeys were included in this correlation analysis.

RESULTS

Cloning and expression of the TCoV N recombinant protein.

Optimization and cutoff value of the TCoV N-based ELISA.

Using the described checkerboard titrations, the optimized ELISA conditions were established as follows: the recombinant antigen concentration for plate coating was 65 ng/µL TCoV N protein, primary antibodies (turkey serum samples) were diluted at 1:250, and the secondary goat anti-turkey IgG incubation at room temperature. The plates were washed three times with PBS-T again before the addition of the secondary antibody (goat anti-turkey IgG [H + L]) diluted 1:2,000. The plates were washed again three times with PBS-T followed by the addition of substrate (pNPP Microwell substrate system; Kirkegaard and Perry Laboratories, Gaithersburg, MD). The plates were incubated for 15 min in the dark, and the reaction was terminated using 2 M H2SO4. The plates were read at 405 nm using a BioTek Powerwave XS microplate ELISA reader (BioTek Instruments, Inc., Winooski, VT).

Cross-reactivity of turkey sera with IBV assessed using a commercial IBV whole-virus ELISA.

The cross-reactivity of turkey sera against IBV antigens was assessed using commercially available IBV whole-virus-coated ELISA plates (FlockChek infectious bronchitis virus antibody test kit; IDEXX, Westbrook, ME). The ELISA was carried out according to the manufacturer’s instructions with the minor modification that the turkey sera were only diluted 1:250 rather than 1:500 as is normally the case when testing chicken sera. A subsample of 168 of the 360 serum samples obtained to assess the seroprevalence of TCoV in Ontario commercial turkey flocks were tested using the IDEXX kit, and the results were compared with the readings obtained from the recombinant N-based ELISA described above. Seropositive or seronegative status was determined using a cutoff OD405 of 0.1 for the whole IBV antigen-based ELISA.

Seroprevalence in Ontario commercial turkeys.
(H+L)-AP conjugate was diluted at 1:2,000. Substrate reactions were stopped after 15 min.

Using a two-graph ROC analysis (11, 12) based on 73 known negative-control serum samples and 86 serum samples from experimentally infected turkeys at 14, 21, 28, 35, and 42 days postinfection as positive-control sera, we established the cutoff value for the TCoV N-based ELISA to be an OD$_{405}$ of 0.180; serum samples testing above this OD value were considered positive, while values at or below this value were considered negative. This cutoff value resulted in 97% sensitivity and 93% specificity for the TCoV N-based ELISA (Fig. 3).

Seroprevalence of TCoV in Ontario, Canada. The TCoV N-based ELISA was conducted on 360 random serum samples collected from 12 turkey farms (30 samples per farm). For the six breeder turkey farms, a mean prevalence of 73.9% (133/180 had OD$_{405}$ values of >0.180) was observed (Table 1). In serum samples obtained from six meat turkey farms, a mean seroprevalence of 60.0% ± 1.18% (108/180 had OD$_{405}$ values of >0.180) was observed (Table 2). There was a significantly lower (P = 0.042) overall positive seroprevalence for meat turkey farm serum samples compared with samples obtained from breeder turkey farms.

Seroprevalence of TCoV in turkeys from Arkansas. Eighty-one turkey serum samples were obtained from Arkansas, and 15 serum samples from SPF turkeys were tested using the TCoV N-based ELISA developed herein. A relatively high seroprevalence of 64.2% (51/81 had OD$_{405}$ values of >0.180) for TCoV was detected in the Arkansas field samples. No seroconversion (0/15 had OD$_{405}$ values of >0.180) was detected in the sera from the SPF turkeys.

Comparison of the recombinant TCoV N-based ELISA to a commercial IBV whole-virus ELISA for detecting seroconversion against TCoV. The ability of a commercial IBV whole-virus-coated ELISA to detect antibodies recognizing TCoV was compared to the developed TCoV N-based ELISA. From the 168 turkey serum samples tested, the ELISAs produced TCoV seroprevalence estimates that differed dramatically (Table 3). The IBV whole-virus-coated ELISA revealed only 20.24% (17/84) positive samples from breeder turkey farms and 13.10% (11/84) positive samples from commercial turkey farms. In contrast, the recombinant antigen TCoV ELISA reported much higher TCoV seroprevalence in both breeder turkey (78.57% [66/84]) and meat turkey (60.71% [51/84]) flocks (Fig. 4). The TCoV N-based ELISA reported the overall seroprevalence of TCoV as 70.24%; this was approximately fourfold higher than the 16.67% overall seroprevalence reported by the commercial IBV whole-virus ELISA. There was only a modest correlation between the OD readings obtained.
using the TCoV N-based and IBV whole-virus-based ELISAs ($R = 0.2623$) (Fig. 5).

**DISCUSSION**

TCoV is one of the most important etiological agents of diarrhea in young turkey poult (5) and is believed to be involved in poult enteritis and mortality syndrome. First efforts to determine the seroprevalence of TCoV were based on virus neutralization (23) and fluorescent antibody tests (22). Subsequently, ELISA-based detection methods were attempted; however, determining the seroprevalence of TCoV in commercial turkey populations remained difficult because TCoV cannot be propagated readily in cell culture (2, 4, 13; unpublished data) although at least some isolates can be propagated in turkey embryos (23). Thus, a variety of antigens other than whole TCoV have been used to determine the seroprevalence of TCoV in turkey flocks. These include the use of IBV whole-virus antigen in an antibody-capture ELISA (this study and reference 17). Recombinant TCoV N protein derived from a prokaryotic expression system was suggested as a potential antigen for an antibody-capture ELISA specific for TCoV (18), and N protein expressed in a baculovirus system (4) has been used to develop a competitive ELISA test in concert with MAbs raised against TCoV N protein (14).

In the present study, a recombinant antigen ELISA was developed based on the recently completed genomic sequence of a recent field isolate of TCoV (isolate MG10) (10). The utility of this recombinant ELISA was then assessed using field turkey serum samples from Ontario, Canada, and Arkansas, and the efficacy of this recombinant ELISA was compared to the use of a commercially available whole-virus IBV ELISA for detecting TCoV infections.

**Development of a TCoV N-based ELISA.** The nucleocapsid protein of most CoVs is produced at a high level as early as 12-h postinfection, making it a good candidate for the early detection of different CoVs (15, 20). The N protein of IBV is immunogenic and contains domains conserved among most IBV strains (29). The conserved nature of the N protein has permitted its use as a recombinant antigen in various ELISA systems to test serum samples for antibodies against IBV (7, 9, 21) as well as antibodies against TCoV (17). The TCoV N protein has been used in a competitive ELISA to detect antibodies against TCoV (14); not surprisingly, this ELISA was able to detect antibodies against IBV as well.

Our clone of TCoV N protein was expressed in two forms (52 and 43 kDa after thrombin cleavage). A similar pair of expressed forms (52 and 43 kDa) was observed when the competitive ELISA was used to detect TCoV infections in turkeys. These include the use of IBV whole-virus antigen in an antibody-capture ELISA (this study and reference 17). Recombinant TCoV N protein derived from a prokaryotic expression system was suggested as a potential antigen for an antibody-capture ELISA specific for TCoV (18), and N protein expressed in a baculovirus system (4) has been used to develop a competitive ELISA test in concert with MAbs raised against TCoV N protein (14).

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**TABLE 2. Seroprevalence of TCoV at Ontario meat turkey farms using a recombinant TCoV N-based ELISA**

<table>
<thead>
<tr>
<th>Ontario meat turkey farm</th>
<th>Mean turkey age (wk)</th>
<th>Total no. of samples</th>
<th>No. of positive samples</th>
<th>No. of negative samples</th>
<th>Prevalence (%)</th>
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<tr>
<td>1</td>
<td>10</td>
<td>30</td>
<td>21</td>
<td>9</td>
<td>70.00</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>30</td>
<td>14</td>
<td>17</td>
<td>46.67</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>30</td>
<td>22</td>
<td>10</td>
<td>73.33</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>30</td>
<td>20</td>
<td>10</td>
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<tr>
<td>5</td>
<td>15</td>
<td>30</td>
<td>11</td>
<td>19</td>
<td>36.67</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>30</td>
<td>20</td>
<td>10</td>
<td>66.67</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>180</strong></td>
<td><strong>108</strong></td>
<td><strong>72</strong></td>
<td></td>
<td><strong>60.00</strong></td>
</tr>
</tbody>
</table>

**TABLE 3. Comparison of a commercial whole-virus IBV ELISA with a recombinant-antigen TCoV N-based ELISA for detecting TCoV infections in turkeys using paired serum samples**

<table>
<thead>
<tr>
<th>ELISA</th>
<th>Primary serum dilution</th>
<th>Cutoff OD value</th>
<th>No. of positive samples</th>
<th>Total no. samples</th>
<th>Prevalence (% infected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCoV N</td>
<td>1:250</td>
<td>0.180</td>
<td>118</td>
<td>168</td>
<td>70.24</td>
</tr>
<tr>
<td>IDEXX IBV</td>
<td>1:250</td>
<td>0.100</td>
<td>17</td>
<td>168</td>
<td>16.67</td>
</tr>
</tbody>
</table>

**FIG. 4.** Comparison of the detection of TCoV in turkey serum samples obtained from breeder turkey farms (84 samples) and meat turkey farms (84 samples) in Ontario, Canada, examined in duplicate using TCoV N-based (gray bars) and IBV whole-virus-based (black bars) ELISAs. The recombinant TCoV N-based ELISA detected an overall seroprevalence of TCoV of 70.24% that was about fourfold higher than the 16.67% overall seroprevalence reported by the commercial IBV whole-virus-based ELISA.

**FIG. 5.** Scatter plot of the OD 405 values obtained from 168 paired turkey serum samples from Ontario turkey farms using TCoV N- and a whole-virus IBV-based commercial ELISA. Only a modest correlation ($R = 0.2623$) was observed between the recombinant antigen TCoV N-based ELISA and the commercial IBV whole-virus-based ELISA.
plete TCoV N protein was expressed using a baculovirus expression system (4). Finding a doublet protein product is not unusual for some CoVs such as IBV and MHV that each produce two forms of their nucleocapsid proteins (6, 19, 27). It has been shown that a whole-length IBV N-protein clone in E. coli expressed two protein bands (50 and 46 kDa) detectable using an MAb specific for the IBV N protein (29). Similar phenomena of having different forms of the nucleocapsid protein have been reported for Newcastle disease virus, where this pattern was explained as the proteolytic cleavage of the translation product to yield different forms of the same protein (20).

The recombinant TCoV antigen ELISA developed herein had high specificity and sensitivity as determined using a two-graph ROC analysis. Assuming a turkey flock seroprevalence of TCoV of about 65% in line with our observations and others (14), the TCoV N-based ELISA would typically have a positive predictive value of 96.26% and a negative predictive value of 94.35%. These strong predictive values would be more than adequate to justify the use of this recombinant antigen ELISA to accurately and economically determine the presence of antibodies against TCoV in turkey sera.

Comparison between the TCoV ELISA and IBV ELISA for TCoV antibodies. Using experimentally derived positive and negative turkey sera and a commercial IBV whole-virus antigen plate, TCoV-positive sera showed reactivity with the IBV antigens but at much lower OD readings. Thus, relatively few samples had OD readings above the cutoff values, and therefore, the inferred seroprevalence was much lower for this ELISA. For example, using a subset of 168 field serum samples, only 28 had OD_{650} readings above a cutoff value of 0.100 (established using a two-graph ROC analysis and experimental turkey sera) using the IDEXX FlockChek IBV whole-virus ELISA performed according to the manufacturer’s instructions and with the kit reagents (conjugated secondary antibody and enzyme substrate). In contrast, the TCoV-specific ELISA based on recombinant N protein found that 70.24% of these serum samples were positive for antibodies recognizing TCoV.

There was a positive, albeit weak, correlation of this commercial IBV-based ELISA with the recombinant TCoV ELISA (Fig. 5), presumably because the N protein is relatively conserved between IBV and TCoV (4, 14, 18).

Seroprevalence of TCoV in Ontario, Canada, and Arkansas. Despite its cosmopolitan presence in turkey flocks and potential importance in disease pathogenesis, few epidemiological surveys have been conducted to determine TCoV seroprevalence. Using an indirect fluorescent-antibody assay or a commercially available IBV ELISA (IDEXX), it was determined that in Indiana, 175/325 (53.84%) or 163/325 (50.15%) of the field samples had antibodies for TCoV, respectively (17). In a second study using a competitive ELISA and a recombinant TCoV N protein, 63% of the sampled turkey pouls were found to possess antibodies to TCoV (14). Our recombinant TCoV N-based ELISA detected 73.9% and 60.0% positive sera from breeder turkey farms and meat turkey farms in Ontario, Canada, respectively. Field serum samples obtained in Arkansas had similarly high seroprevalence. The higher observed prevalence of antibodies recognizing TCoV using our recombinant ELISA compared to results using the commercially prepared IBV whole-virus ELISA plate (17) and our results using the IDEXX IBV antigen plates suggest that TCoV-specific antibodies cross-react with IBV antigens but to a lower level than the homologous TCoV antigen. The prevalence of TCoV antibodies was high in both breeder turkey and meat turkey flocks as demonstrated by our data, but there was a significantly higher prevalence in the turkey breeder flocks, perhaps because the birds in the latter group are older and more likely to have had time to be exposed to the virus and mount a detectable serum antibody response.

In summary, a recombinant TCoV antigen-based ELISA was developed based on the complete nucleocapsid protein of TCoV. This ELISA performed significantly better at detecting antibodies against TCoV in turkey sera than a commercially available IBV ELISA kit. The recombinant antigen ELISA was instrumental in detecting the high seroprevalence of TCoV in breeder turkey farms in Ontario and meat turkey farms in Ontario and Arkansas.

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