Virus shedding and serum antibody responses during experimental turkey coronavirus infections in young turkey poults

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The course of turkey coronavirus (TCoV) infection in young turkey poults was examined using a field isolate (TCoV-MG10) from a diarrhoeal disease outbreak on a commercial turkey farm in Ontario, Canada. Two-day-old and 28-day-old poults were inoculated orally with TCoV-MG10 to examine the effect of age on viral shedding and serum antibody responses to the virus. The presence of coronavirus particles measuring 105.8 ± 21.8 nm in the cloacal contents was confirmed using transmission electron microscopy. The pattern of cloacal TCoV shedding was examined by reverse-transcription polymerase chain reaction amplification of the nucleocapsid gene fragment. TCoV serum antibody responses were assessed with two recently developed TCoV enzyme-linked immunosorbent assays that used TCoV nucleocapsid and S1 polypeptides as coating antigens. Poults were found equally susceptible to TCoV infection at 2 days of age and at 4 weeks of age, and turkeys of either age shed virus in their faeces starting as early as 1 day post-inoculation and up to 17 days post-inoculation. Poults infected at 2 days of age were immunologically protected against subsequent challenge at 20 days post-inoculation. The protection was associated with measurable serum antibody responses to both the nucleocapsid and S1 structural proteins of TCoV that were detectable as early as 1 week post-infection.

Introduction

Turkey coronavirus (TCoV) (Order Nidovirales, Family Coronaviridae) is a member of the group III coronaviruses. Other group III coronaviruses include infectious bronchitis virus (IBV) and several recently identified viruses affecting various avian species (e.g. pigeon coronavirus, quail coronavirus, duck coronavirus, goose coronavirus and pheasant coronavirus). Some avian coronaviruses, such as IBV or pheasant coronavirus, tend to have respiratory and renal tissue tropisms, while others, such as TCoV, quail coronavirus, duck coronavirus and goose coronavirus, are primarily enteric pathogens (Pennycott, 2000, Cavanagh, 2001, 2005, Jonassen et al., 2005; Circella et al., 2007; Gomaa et al., 2008a).

A clinical enteritis syndrome in turkeys associated with “filterable” agents was at first called “mud fever” or later “Blue comb disease” (Peterson & Hymas, 1951; Tumlin et al., 1957). The identification of TCoV as the causative agent was made by 1973 (Panigraphy et al, 1973; Ritchie et al. 1973). TCoV can infect turkeys at different ages, resulting in high morbidity with various mortality rates. Affected turkeys usually have diarrhoea, ruffled feathers, and decreased feed and water consumption in addition to poor growth rate (Barnes & Guy, 1997; Yu et al., 2000; Ismail et al., 2003). The disease can spread rapidly from farm to farm via fomites. There is no evidence of vertical transmission and the faecal-oral route appears to be the main route of transmission (Naqi et al., 1972). Infected birds can shed the virus in their droppings for up to 7 weeks post infection, even after cessation of the clinical signs (Larsen, 1979; Breslin et al., 2000). The economic losses in the case of TCoV infection usually resulted from poor feed conversion rates and, in the case of laying hens, production of poor-quality eggs known as “chalky eggs” (Nagaraja & Pomeroy, 1997).

The goal of this study was to explore the pattern of TCoV shedding in experimentally infected turkey poults by means of nucleocapsid (N)-gene reverse-transcription polymerase chain reaction (RT-PCR) and serum antibody responses using two recently developed TCoV enzyme-linked immunosorbent assays (ELISAs) with recombinant N and S1 proteins as coating antigens.

Material and Methods

Virus. TCoV-MG10 was isolated from birds on an Ontario turkey farm suffering from acute enteritis and diarrhoea. The collected intestines were homogenized in phosphate-buffered saline (PBS) with a mortar and pestle, and then clarified by centrifugation at 1000 x g for 15 min. The supernatant was filtered through a 0.22 µm membrane filter.
Turkeys and experimental design. Thirty 1-day-old turkey pouls received from Hybrid Turkeys (Kitchener, Ontario, Canada) were housed, fed and handled in the Campus Animal Facility’s Isolation Unit at the University of Guelph according to the University of Guelph’s Animal Care Committee rules and the Canadian Council on Animal Care’s Guide to the Care and Use of Experimental Animals. The pouls came from a high-biosecurity facility and had not received vaccination against TCoV nor was the exposure history of the parent birds to this agent known. Birds were wing-tagged and divided into two separately housed groups, 10 infected and 20 control birds. Two-day-old pouls were inoculated orally with 100 μl clarified virus-containing supernatant.

Collection of serum samples and cloacal swabs. Blood samples were collected by wing-vein puncture from all pouls pre-infection and at 7, 14, 28, 35 and 42 days of age. Blood was allowed to clot; serum was separated by centrifugation and then heat-inactivated at 56°C for 30 min.

Cloacal swabs were collected 1, 3, 5, 7, 14, and 21 days post infection (d.p.i.) for birds challenged at 2 days of age, and 1, 3, 5, 7, 14 and 17 d.p.i. for pouls challenged at 28 days of age. For birds re-challenged at 21 days of age, cloacal swabs were taken at 1, 3, 5, 7 and 14 days after challenge. Swabs were placed in 5 ml poly styrene test tubes (Sarstedt; Numbrecht, Germany) containing 1.5 ml PBS and the swab contents were suspended by pressing the swab repeatedly against the wall of the tube. The swabs were then removed and the suspension was centrifuged at 1000 x g for 10 min at 4°C. The supernatant was collected and then centrifuged at 2000 x g at 4°C. The supernatant from the second centrifugation was collected and passed sequentially through 0.45 μm and then 0.2 μm Millipore filters. The resulting filtrate was stored at −70°C until use for detection of virus by RT-PCR.

Results

Monitoring virus shedding with RT-PCR. All 10 birds inoculated with TCoV at 2 days of age developed diarrhoea 3 days after being inoculated with TCoV-MG10 orally; diarrhoea persisted for 2 days and then began to subside so that no birds were showing diarrhoea by 11 d.p.i. Diarrhoea was initially seen as loose faecal consistency that progressed rapidly to frothy, liquid diarrhoea, sometimes containing urates, which soiled the vent and surrounding areas. Two of the most profoundly affected turkey pouls were depressed, huddled and dehydrated with heavily soiled vents; both pouls died at 3 d.p.i.

RT-PCR for TCoV, turkey astrovirus and avian reovirus was performed on cloacal swabs containing faecal material collected from 10 pouls before inoculation (2 days of age) and at 1, 3, 5, 7, 11, 14, 21 d.p.i. (Figure 1 and Table 1). TCoV-specific amplicons were detected in three out of 10 birds at 1 d.p.i., in eight out of 10 birds at 3 d.p.i. and in all birds by 5 d.p.i. Shedding of TCoV, as determined by TCoV-specific RT-PCR, continued in four birds until 14 d.p.i., but had ceased by 21 d.p.i. in all turkeys infected at 2 days of age. Poults that were re inoculated (challenged orally and via intramuscular injection) at 21 days of age did not develop diarrhoea. Faint TCoV-specific amplicons were detected using TCoV-specific RT-PCR in cloacal swabs in two out of eight of these challenged birds at 1 d.p.i. and in one out of eight pouls at 2 d.p.i. At 3 d.p.i. and thereafter, none of the pouls challenged with TCoV-MG10 shed virus detectable using RT-PCR.

Electron microscopy. To confirm the physical presence of coronavirus particles in samples positive for TCoV by RT-PCR, filtrates of cloacal swab samples or faecal samples collected at 7 d.p.i. (9 days of age) were examined using transmission electron microscopy. One drop of sample was placed on the top of the carbon-coated Formvar grid, stained with 2% phosphotungstic acid and dried before examination in a Leo 912ab electron microscope within the University of Guelph Advanced Analysis Center.

Enzyme-linked immunosorbent assay. The serum samples collected from infected and control turkeys were heat-inactivated at 56°C for 30 min prior to testing using serum antibody ELISAs based on recombinant TCoV-N (Gomaa et al., 2008b) or TCoV-S1 (Gomaa, unpublished data) coating antigens. Briefly, recombinant antigens diluted in 0.1 M carbonate buffer were used to coat wells of ELISA plates (Nunc, Måssisort) overnight at 4°C. Plates were washed three times with PBS with 0.05% Tween-20 and were then blocked with 5% (w/v) bovine serum albumin in PBS with 0.05% Tween-20 for 1 h. After washing, heat-inactivated serum samples at 1:250 dilution were added for 1 h at room temperature. After washing, alkaline phosphatase-conjugated secondary antibody (goat anti-turkey IgG (H + L) diluted 1:2000) was used to detect bound antibody after adding substrate (pNPP Microwell substrate system; Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA) following additional washing. Finally, plates were read at 405 nm using a BioTek Powerwave XS microplate ELISA reader (BioTek Instruments Inc., Winooski, Vermont, USA). The N-based ELISA has demonstrated sensitivity of 97% and specificity of 93% for TCoV (Gomaa et al., 2008b), and the S1-based ELISA has demonstrated sensitivity of 95% and specificity of 92% (Gomaa, unpublished data). Each serum sample was tested using both ELISAs.

Statistical analysis. Mean optical density (OD) values obtained by either ELISA from turkey sera were tested for significant difference from age-matched control turkey pouls that had not been exposed to TCoV-MG10 using a two-tailed t test assuming unequal variance. Differences were considered statistically significant at P < 0.05.
For poults infected at 28 days of age, six out of eight developed diarrhoea by 3 d.p.i. that was resolved by 9 d.p.i. Cloacal swabs and faecal material were collected at 1, 3, 5, 7, 11, 14 and 17 d.p.i. TCoV was detected in two out of eight birds at 1 d.p.i., in four out of eight birds at 3 d.p.i. and in all birds by 5 d.p.i. Shedding continued in three birds until 14 d.p.i. and one poult was still shedding TCoV at 16 d.p.i. when the experiment was terminated (Table 1).

Turkey astrovirus and avian reovirus were not detected in the virus preparation used to inoculate the birds or in cloacal swabs or faeces from any birds throughout the study.

Confirmation of virus particles using electron microscopy. Viral particles measuring 105.8\(\pm\)21.8 nm (range 85 to 157 nm, \(n=9\)), morphologically similar to coronaviruses, were observed by electron microscopy in cloacal swabs and clarified faecal samples collected at 7 d.p.i. from birds infected at 2 days of age (Figure 2).

TCoV serum antibody response. Serum samples collected throughout the course of the trial were tested for the presence of the TCoV-specific antibodies with serum ELISAs based on N protein (Gomaa et al., 2008b) and S1 protein (Gomaa, unpublished data). The resulting median OD\(_{405}\) values for the N-based and S-based ELISAs over the course of the study are illustrated in Figures 3 and 4, respectively. Using both ELISAs, median OD\(_{405}\) values from 1-day-old turkey poult sera were 0.185\(\pm\)0.0264 and 0.166\(\pm\)0.0341 using the N-based and S1-based ELISAs, respectively. In the uninfected control poults, the median OD values of both ELISAs decreased steadily over the course of the first 2 to 3 weeks and then remained at a constantly low level for the remainder of the experiment (Figures 3 and 4).

Sera from poults inoculated at 2 days of age had significantly increased median OD values (\(P<0.05\)) relative to age-matched control poults at 7 d.p.i. for both ELISAs, and the median OD values remained significantly higher than in age-matched controls for all subsequent sample days. In response to administration of a second dose of TCoV by both the oral and intramuscular routes 3 weeks after their initial exposure to TCoV, poults produced TCoV-specific serum antibodies generating substantially increased OD values that lasted until the termination of the experiment at 42 days after the first infection (Figures 3 and 4).

Table 1. Shedding of TCoV as detected by RT-PCR of cloacal swabs from turkey poults inoculated orally with TCoV-MG10

<table>
<thead>
<tr>
<th>Time of challenge with TCoV</th>
<th>Age of poults (days)</th>
<th>Days post-inoculation</th>
<th>Incidence of TCoV shedding</th>
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<tr>
<td></td>
<td>Control poults</td>
<td>Infected poults</td>
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<tr>
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*Number shedding virus/number in group.
Discussion

The goals of the current experiment were to study the course of TCoV infections in young turkey poults using an isolate (TCoV-MG10) from a diarrhoeal disease outbreak on a commercial turkey farm in Ontario. Turkey coronavirus could be detected using TCoV-specific RT-PCR in cloacal swabs as early as 1 d.p.i. By 3 d.p.i. 50% or more of inoculated birds began to shed the virus, and by 5 d.p.i. all birds were shedding TCoV. Viral shedding persisted in about 50% of the birds at 14 d.p.i. and had ceased by 20 d.p.i. These observations are consistent with an earlier study in which shedding was observed for up to 2 weeks post-inoculation for the TCoV-ATCC VR-911 isolate (Ismail et al., 2003), but shorter than shedding of up to 7 weeks demonstrated for the TCoV-NC95 isolate (Breslin et al., 2000). Using indirect fluorescent antibody methods, TCoV antigen could be detected within intestinal epithelial cells of the intestinal tract for up to 28 days in young turkey poults (Patel et al., 1975). The duration and course of TCoV shedding did not differ between poults infected for the first time at 2 or 28 days of age. The relatively brief period of viral shedding and the presence of clinical signs in most infected poults may explain why TCoV was not detected in any faecal samples obtained from commercial turkey barns that contained turkeys considered healthy or normal by field personnel (Pantin-Jackwood et al., 2007, 2008).

Attempts to reinfect poults previously exposed to TCoV resulted in no resumption of clinical signs. There was no evidence of TCoV shedding resulting from endogenous viral replication. Although TCoV RNA was detected in cloacal swabs from a few birds on the first and second days after oral challenge, it is probable that the faint RT-PCR bands observed resulted from viral particles in the inoculum that passed through the digestive tract. This suggests that protective immunity was acquired after a single infection with TCoV and as early as 20 days after a primary infection. This observation was consistent with reports that birds that have recovered from TCoV infection showed resistance to further infection with the same virus (Pomeroy et al., 1975; Nagaraja & Pomeroy, 1997). TCoV-specific antibodies detected as early as 7 d.p.i. in poults challenged at 2 or 28 days of age are likely to contribute to the resistance of turkeys to reinfection with TCoV.

In newly hatched poults, TCoV-specific maternal antibodies were detected using both ELISAs. In the...
uninfected control birds, these maternal antibodies decreased steadily from day 1 until 2 to 3 weeks of age, after which no further reduction was detected. At 1 day of age, more than one-half of the poult's had OD$_{405}$ values above the seropositive cutoff values established for these ELISAs, but by 14 days of age only 10% of birds had OD values still above the cutoff level. After 21 days of age, no bird had an OD value for either ELISA above the tests’ cutoff values and all control birds of this age would be considered seronegative.

Maternal antibodies against both the N and S1 proteins of TCoV were likewise present in poult's experimentally infected at 2 days of age, but the presence of these serum antibodies did not prevent infection, clinical signs or virus shedding. What is not known from our data is whether maternal antibodies may partially ameliorate the TCoV infection. With IBV in chickens, newly hatched chicks with high levels of maternal antibody against IBV, specifically against the S1 glycoprotein, are protected against homologous IBV challenge at 1 day old; however, this protection is correlated with local respiratory antibodies and not serum antibodies (Mondal & Naqi, 2001). Post challenge with TCoV-MG10, both ELISAs demonstrated significantly increased mean OD$_{405}$ values compared with age-matched control poults, indicating the presence of newly produced TCoV-specific serum antibodies by 7 d.p.i. Both mean and median OD$_{405}$ values at 7 d.p.i. (for birds infected at 2 days of age) and at 10 d.p.i. (for birds infected at 28 days of age) were above the cutoff values established for both ELISAs (Gomaa et al., 2008b; Gomaa, unpublished data). Concordant results were reported when the serological response to TCoV was measured with an antibody capture ELISA using a mixture of seven IBV strains as coating antigen (Loa et al., 2001). In 28-day-old birds, a significant serological response was detected in eight out of eight challenged birds at 10 d.p.i. by both ELISAs, interestingly, even when TCoV was still detectable in cloacal swabs. This observation suggests that serum antibody levels do not correlate directly with protection against infection, as was observed in a study on IBV in chicks (Mondal & Naqi, 2001).

In summary, these results suggest that turkey poult's are equally susceptible to TCoV infections at 2 days and at 4 weeks of age and that poult's of either age shed virus in their faeces for a similar duration. Low levels of maternal antibodies did not completely protect poult's against infection as measured by clinical signs and virus shedding. Significant serum antibody responses to both the N and S1 structural proteins of TCoV were detected by 1 week post-infection in the present study. When given a virulent TCoV challenge at 20 d.p.i., poult's exposed to TCoV at 2 days of age did not demonstrate any clinical signs nor did they demonstrate any evidence of endogenous replication of TCoV. Perhaps locally available antibody in the intestinal tract and known cellular immune responses to TCoV (Loa et al., 2001) were able to protect poult's against the effects of avirulent challenge with TCoV at 20 d.p.i., even if poult's were infected at 2 days of age.

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References


