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# Enhancement of Enteropathogenic Escherichia coli Pathogenicity in Young Turkeys by Concurrent Turkey Coronavirus Infection

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SUMMARY. In a previous study, turkey coronavirus (TCV) and enteropathogenic *Escherichia coli* (EPEC) were shown to synergistically interact in young turkeys coinfected with these agents. In that study, inapparent or mild disease was observed in turkeys inoculated with only TCV or EPEC, whereas severe growth depression and high mortality were observed in dually inoculated turkeys. The purpose of the present study was to further evaluate the pathogenesis of combined TCV/EPEC infection in young turkeys and determine the role of these agents in the observed synergistic interaction. Experiments were conducted to determine 1) effect of EPEC dose, with and without concurrent TCV infection, and 2) effect of TCV exposure, before and after EPEC exposure, on development of clinical disease. Additionally, the effect of combined infection on TCV and EPEC shedding was determined.

No clinical sign of disease and no attaching and effacing (AE) lesions characteristic of EPEC were observed in turkeys inoculated with only EPEC isolate R98/5, even when turkeys were inoculated with $10^{10}$ colony forming units (CFU) EPEC (high dose exposure). Only mild growth depression was observed in turkeys inoculated with only TCV; however, turkeys inoculated with both TCV and $10^4$ CFU EPEC (low dose exposure) developed severe disease characterized by high mortality, marked growth depression, and AE lesions. Inoculation of turkeys with TCV 7 days prior to EPEC inoculation produced more severe disease (numerically greater mortality, significantly lower survival probability [$P < 0.05$], increased frequency of AE lesions) than that observed in turkeys inoculated with EPEC prior to TCV or simultaneously inoculated with these agents. Coinfection of turkeys with TCV and EPEC resulted in significantly increased ($P < 0.05$) shedding of EPEC, but not TCV, in intestinal contents of turkeys. These findings indicate that TCV infection predisposes young turkeys to secondary EPEC infection and potentiates the expression of EPEC pathogenicity in young turkeys.

RESUMEN. Aumento de la patogenicidad del Escherichia coli enteropatógeno en pavipollos por infecciones simultáneas con el coronavirus de los pavos.

En un estudio anterior se demostró la interacción sinérgica entre el coronavirus de los pavos y el *Escherichia coli* enteropatógeno en pavipollos al ser infectados simultáneamente con estos dos agentes. En dicho estudio se observó una enfermedad que varió de suave a inaparente en pavos inoculados únicamente con el coronavirus del pavo ó con el *E. coli* enteropatógeno. Sin embargo, en pavos inoculados con los dos agentes, se observó una depresión severa en el crecimiento y una mortalidad elevada. Se evaluó con mayor profundidad la patogenicidad de la infección combinada del coronavirus del pavo y el *E. coli* enteropatógeno en pavipollos y se determinó el papel de estos agentes en la interacción sinérgica observada. Se realizaron experimentos para determinar 1) el efecto de la dosis de *E. coli* enteropatógeno, con o sin la infección simultánea con el coronavirus del pavo, y 2) el efecto de la exposición al coronavirus del pavo, antes y después de la exposición al *E. coli* enteropatógeno, en el desarrollo de la enfermedad clinica. Además, se determinó el efecto de la

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infección combinada en la excreción del coronavirus del pavo y el *E. coli* enteropatógeno. No se observaron signos clínicos de la enfermedad ni lesiones características del *E. coli* enteropatógeno en pavos inoculados únicamente con el aislamiento R98/5 de *E. coli* enteropatógeno, aún al ser inoculados con $10^{10}$ unidades formadoras de colonias (dosis de exposición alta). En los pavos inoculados únicamente con el coronavirus del pavo solo se observó una depresión suave en el crecimiento, sin embargo, lo pavos inoculados con el coronavirus del pavo y con $10^4$ unidades formadoras de colonias de *E. coli* enteropatógeno (dosis de exposición baja) desarrollaron una enfermedad severa, caracterizada por una mortalidad alta, depresión marcada en el crecimiento y adhesiones. Se observó una enfermedad más severa con mayor mortalidad numérica, probabilidad de supervivencia significativamente menor ($P < 0.05$) e incremento en la frecuencia de adherencias en pavos inoculados con el coronavirus del pavo 7 días antes de ser inoculados con el *E. coli* enteropatógeno al ser comparada con la enfermedad observada en pavos inoculados con el *E. coli* enteropatógeno antes de la inoculación con el coronavirus del pavo o en pavos inoculados con los dos agentes al mismo tiempo. La infección simultánea de los pavos con el coronavirus del pavo y el *E. coli* enteropatógeno resultó en un incremento significativo ($P < 0.05$) de la excreción el *E. coli* enteropatógeno mas no del coronavirus del pavo en los contenidos intestinales de los pavos. Estos hallazgos indican que la infección por el coronavirus del pavo predispone a los pavipollos a infecciones secundarias por *E. coli* enteropatógeno e incrementan la patogenicidad del *E. coli* enteropatógeno en los pavipollos.

Key words: turkey coronavirus, poult enteritis–mortality syndrome, *Escherichia coli*

Abbreviations: AE = attaching and effacing; CFU = colony-forming units; DMEM = Dulbecco minimal essential medium; EAE = *Escherichia coli* attaching and effacing; $EID_{50}$ = 50% embryo infectious dose; EPEC = enteropathogenic *Escherichia coli*; ETEC = enterotoxigenic *Escherichia coli*; PCR = polymerase chain reaction; PE = postexposure; PEMS = poult enteritis–mortality syndrome; TCV = turkey coronavirus

Turkey coronavirus (TCV) and enteropathogenic *Escherichia coli* (EPEC) previously were associated as causes of poult enteritis–mortality syndrome (PEMS), a severe intestinal disease of young turkeys of unknown etiology (8,9). PEMS primarily affects turkeys during the brooding period and is characterized by diarrhea, dehydration, increased mortality, and growth depression (1).

TCV is a well-known cause of intestinal disease in turkeys (15). The virus causes an acute, highly contagious enteric disease of turkeys that initially was referred to as bluecomb disease (15). Bluecomb disease was first identified in turkeys in 1951, and a coronavirus was determined to be the cause of the disease in 1973 (15). EPEC have been identified as causes of intestinal disease in several different mammalian species including human beings, calves, pigs, lambs, goats, rabbits, dogs, and cats (5,13,14, 17,24). Recently, EPEC have been identified as causes of intestinal disease in chickens and turkeys (6,9,22).

EPEC represent one of four principal categories of diarrheagenic *E. coli,* the other three categories being enterotoxigenic, enteroinvasive, and enterohemorrhagic (17). Enterotoxigenic *E. coli* (ETEC) elaborate heat-labile or heat-stable toxins that induce diarrhea due to potentiation of intestinal secretion. Enteroinvasive *E. coli* invade intestinal cells and produce diarrhea in a manner similar to *Shigella* spp. Enterohemorrhagic *E. coli* produce intestinal disease by intimate adherence to intestinal epithelium and elaboration of shiga-like toxins. EPEC also adhere intimately to intestinal epithelial cell membranes; however, they produce intestinal disease without elaboration of shiga-like toxins or heat-labile or heat-stable toxins and they are not invasive.

A hallmark of EPEC strains is the production of characteristic "attaching and effacing" (AE) intestinal lesions (13,17). AE lesions are characterized by intimate attachment of bacteria to intestinal epithelial surfaces, epithelial degeneration and necrosis, microcolony formation, and effacement of microvilli (13,17). The *E. coli* attaching and effacing (EAE) gene, a chromosomal gene, is necessary for development of AE lesions and is a useful genetic marker for identifying EPEC (7,11,17).

Enteric infections involving more than one pathogen are commonly encountered in avian

and mammalian species; however, only a few of the enteric pathogens identified in these infections have been shown to synergistically interact in disease development. Rotavirus and ETEC, two extensively studied enteric pathogens, have been shown to synergistically interact in calves, pigs, foals, lambs, and mice (10,12,18,23,25, 26). In these species, coinfection with rotavirus and ETEC resulted in increased severity of disease compared with that observed in animals infected with either agent by itself. In experimental studies, primary rotavirus infection was shown to predispose animals to secondary ETEC infection. For example, Leece *et al.* (12) showed that rotavirus and ETEC, by themselves, caused subclinical infections in weanling pigs, whereas severe diarrhea was observed in pigs concurrently infected with both rotavirus and ETEC.

Recent studies demonstrated an apparent synergistic interaction between TCV and EPEC in turkeys (9). Severe intestinal disease clinically indistinguishable from PEMS was experimentally produced by coinfection of young turkeys with TCV and EPEC isolated from PEMS-affected turkeys (9). No clinical signs, no weight gain disturbance, and mild AE lesions were observed in turkeys infected with only EPEC, and only mild weight gain depression was observed in turkeys infected with only TCV. However, high mortality, marked weight gain depression, and extensive AE lesions were observed in turkeys coinfected with both EPEC and TCV (9). The purpose of the present study was to further evaluate the pathogenesis of combined TCV/EPEC infection in young turkeys and to determine the function of TCV and EPEC in the observed synergistic interaction.

## MATERIALS AND METHODS

**Bacteria.** EPEC strain R98/5, a gentamicin-resistant, lactose-nonfermenter strain was isolated from PEMS-affected turkeys (9). Inocula were prepared by growth of EPEC (R98/5) in Luria broth at 37 C with aeration. Bacteria were grown to optical densities at 600 μM that provided appropriate numbers of colony-forming units (CFU)/ml for inocula, as determined by growth curve experiments. Inocula were used immediately after preparation, then an exact titer was determined by preparing 10-fold dilutions in Luria broth and plating 0.1-ml volume of each dilution on Luria agar plates. Inoculated agar plates were incubated overnight at 37 C, and colonies were counted to determine CFU/ml.

**Virus.** TCV (NC95) was isolated from PEMS-affected turkeys and propagated in embryonated turkey eggs as described (8). An inoculum was prepared to contain approximately 4000 50% embryo infectious doses ($EID_{50}$)/0.1 ml and stored at −70 C.

**Turkeys.** Commercial medium white turkeys were obtained at 1 day of age from a primary breeder company (British United Turkeys of America, Lewisburg, WV). These turkeys were derived from a breeder flock that was free of *Mycoplasma gallisepticum, Mycoplasma synoviae, Mycoplasma meleagridis, Mycoplasma iowae, Salmonella pullorum, Salmonella typhimurium, Salmonella enteritidis, Salmonella arizona,* avian influenza virus, TCV, and reticuloendotheliosis virus; turkeys were not examined for other extraneous infectious agents. Turkeys were housed in wire-floored, electrically heated brooders in an isolation room with controlled access until inoculation. Nonmedicated game bird starter and water were provided *ad libitum.*

**Experimental design.** *Expt. 1. Effect of EPEC dose, with or without TCV, on disease development.* At 6 days of age, 96 turkeys were individually identified by wing bands, weighed, and randomly allocated to eight groups having approximately the same mean weight. Each group was distributed to separate Horsfall–Bauer isolation units. Turkeys were inoculated with a no. 10 French catheter (Monoject, St. Louis, MO) into the crop with a total inoculum volume of 1 ml as follows (Table 1): group 1 (sham-inoculated controls), Dulbecco minimal essential medium (DMEM); group 2, $1 \times 10^6$ CFU EPEC; group 3, $1 \times 10^8$ CFU EPEC; group 4, $1 \times 10^{10}$ CFU EPEC; group 5, 4000 $EID_{50}$ TCV; group 6, 4000 $EID_{50}$ TCV + $1 \times 10^4$ CFU EPEC; group 7, 4000 $EID_{50}$ TCV + $1 \times 10^6$ CFU EPEC; group 8, 4000 $EID_{50}$ TCV + $1 \times 10^8$ CFU EPEC.

Turkeys were observed twice daily for clinical signs and mortality and were weighed on days 7 and 14 postexposure (PE). Three birds in each group were randomly chosen and necropsied on day 4 PE. Duodenum, jejunum, ileum, and cecum were collected for histopathology. Ileum and cecum were collected, placed in a commercially available cryogenic compound (Tissue-Tek, O.C.T. Compound; Miles Laboratories, Elkhart, IN) and immediately frozen for immunohistochemical detection of TCV. Intestinal contents were collected and immediately placed on ice (4 C); these were examined for presence of EPEC.

*Expt. 2. Effect of TCV exposure, before or after EPEC exposure, on disease development.* At 4 days of age, 68 turkeys were individually identified by wing bands, weighed, and separated into six groups containing 11–13 birds, such that each group had approximately equal mean weight. Each group was distributed to separate Horsfall–Bauer isolation units. Turkeys were

Table 1. Effect of EPEC dose, with and without concurrent TCV infection, on mortality and weight gain.[A]

| Group | Inoculum: TCV[B] | Inoculum: EPEC (CFU) | Mortality (%) | Mean weight gain 0–7 days PE (g) | Average daily gain 0–7 days PE (g) |
|---|---|---|---|---|---|
| 1 | — | — | 0/10 (0%)[a] | 166.4[a] | 23.8[a] |
| 2 | + | — | 0/11 (0%)[a] | 102.0[b] | 14.6[b] |
| 3 | — | + ($10^6$) | 0/12 (0%)[a] | 162.9[a] | 23.1[a] |
| 4 | — | + ($10^8$) | 0/12 (0%)[a] | 152.9[a] | 21.8[a] |
| 5 | — | + ($10^{10}$) | 0/12 (0%)[a] | 162.8[a] | 23.3[a] |
| 6 | + | + ($10^4$) | 11/12 (91.7%)[b] | 62.2[c] | 8.9[c] |
| 7 | + | + ($10^6$) | 12/12 (100%)[b] | 72.3[c] | 10.3[c] |
| 8 | + | + ($10^8$) | 12/12 (100%)[b] | 23.0[d] | 3.3[d] |

[A]Total mortality is shown for the duration of the experiment (0–14 days PE); weight gain and average daily gain are shown for the 0–7 days PE. Values within a column followed by the same lowercase superscript letter are not significantly different ($P > 0.050$).
[B]Turkeys were inoculated with a standard dose of TCV (4000 $EID_{50}$).

inoculated with a no. 10 French catheter (Monojet) into the crop with a total inoculum volume of 1 ml as follows (Table 2): group 1, uninoculated controls; group 2, 4000 $EID_{50}$ TCV at 11 days of age; group 3, $2 \times 10^4$ CFU EPEC at 11 days of age; group 4, 4000 $EID_{50}$ TCV + $2 \times 10^4$ CFU EPEC at 11 days of age; group 5, $2 \times 10^4$ CFU EPEC at 4 days of age, then 4000 $EID_{50}$ TCV at 11 days of age; group 6, 4000 $EID_{50}$ TCV at 4 days of age, then $2 \times 10^4$ CFU EPEC at 11 days of age.

Turkeys were observed daily for clinical signs and mortality and were weighed on days 7 and 14 PE. Three birds were randomly selected from each group on day 4 PE and necropsied. Intestinal tissues and contents were collected and processed as described for Expt. 1.

*Expt. 3. Effect of dual infection on TCV and EPEC shedding.* At 5 days of age, 200 turkeys were individually identified by wing bands, randomly separated into four groups, and inoculated. Turkeys were inoculated with a no. 10 French catheter (Monojet) into the crop with a total inoculum volume of 1 ml as follows: group 1 (sham-inoculated controls), DMEM; group 2, 2000 $EID_{50}$ TCV; group 3, $4 \times 10^4$ CFU EPEC; group 4, 2000 $EID_{50}$ TCV + $4 \times 10^4$ CFU EPEC.

On days 0, 2, 4, 7, 10, and 14 PE, five birds were randomly selected from each group and necropsied. At necropsy, approximately 0.1 g intestinal contents was collected from the rectum of each bird and immediately placed on ice.

**Immunohistochemistry.** TCV infection in all TCV-inoculated groups and absence in non-TCV-inoculated groups (Expts. 1, 2) was confirmed by indirect fluorescent antibody staining of frozen sections prepared from ileum and cecum as described (3).

**EPEC detection and identification.** EPEC R98/5 infection in all EPEC-inoculated groups, and absence in non-EPEC-inoculated groups (Expts. 1, 2), was confirmed by culture of intestinal contents on MacConkey agar containing 10 μg/ml gentamicin (gentamicin-resistance and lactose-negative phenotype). One to three colonies from each sample were picked and subcultured; bacteria were examined for presence of EAE gene by polymerase chain reaction (PCR) procedure as described (19).

**Histopathology.** Tissues were placed in 10% neutral buffered formalin and processed routinely for histopathology; tissue sections were stained by the Giemsa method. The presence of AE lesions in the intestines of infected turkeys was evaluated. AE lesions were characterized by microcolony formation, intimate adherence of bacteria to intestinal epithelium, and degeneration and/or necrosis of epithelium at sites of bacterial adherence (9,13,17).

**EPEC titrations.** Intestinal contents were weighed and prepared as 10% (w/v) suspensions in DMEM. Suspensions were prepared as 10-fold dilutions in

Table 2. Effect of EPEC dose, with and without concurrent TCV infection, on development and distribution of attaching and effacing (AE) lesions in intestines of inoculated turkeys.

| Group | Inoculum | | AE lesions detected | | | |
|---|---|---|---|---|---|---|
| | TCV[A] | R985 (CFU) | Duodenum | Jejunum | Ileum | Cecum |
| 1 | — | — | 0/3[B] | 0/3 | 0/3 | 0/3 |
| 2 | + | — | 0/3 | 0/3 | 0/3 | 0/3 |
| 3 | — | + ($10^6$) | 0/3 | 0/3 | 0/3 | 0/3 |
| 4 | — | + ($10^8$) | 0/3 | 0/3 | 0/3 | 0/3 |
| 5 | — | + ($10^{10}$) | 0/3 | 0/3 | 0/3 | 0/3 |
| 6 | + | + ($10^4$) | 0/3 | 0/3 | 0/3 | 2/3 |
| 7 | + | + ($10^6$) | 0/3 | 1/3 | 1/3 | 3/3 |
| 8 | + | + ($10^8$) | 2/3 | 2/3 | 2/3 | 3/3 |

[A]Turkeys were inoculated with a standard dose of TCV (4000 $EID_{50}$).
[B]Number with lesions/number examined.

DMEM, and 0.1 ml of each dilution was plated onto MacConkey agar containing 10 μg/ml of gentamicin; the remaining 10% (w/v) suspensions were stored at −70 C for TCV titrations. Inoculated agar plates were incubated overnight at 37 C, and white (lactose-negative) colonies were enumerated to determine CFU/g feces. One to three selected colonies from each sample were confirmed to be EPEC on the basis of presence of EAE gene as determined by PCR (19).

**TCV titrations.** Intestinal contents (10% [w/v] suspensions) were removed from −70 C storage, thawed at 37 C, and clarified by centrifugation (3000 × *g* for 10 min). Tenfold dilutions were prepared in DMEM supplemented with antibiotics (penicillin [5000 U/ml], gentamicin [0.50 mg/ml], amphotericin B [10 μg/ml]), and each dilution was inoculated into each of three 20-to-23-day-old embryonated turkey eggs. Two days PE, embryo intestines were collected and examined for TCV antigen by indirect fluorescent antibody staining as described (3). TCV titers (50% $EID_{50}$) in intestinal contents were determined by the method of Reed and Muench (21).

**Statistical evaluation.** Weight gain and mortality were evaluated in inoculated groups by one-way analysis of variance (ANOVA) and chi square, respectively, with analytical software (Statistix 7.0; Analytical Software, Tallahassee, FL). EPEC and TCV titrations were evaluated by the Wilcoxon rank sum test for two independent samples and Student *t*-test, respectively. Survival probability was evaluated by the Kaplan–Meire method and chi square (2).

## RESULTS

**Expt. 1. Effect of EPEC dose, with and without concurrent TCV infection.** At 3 days PE, depression and inappetance were observed in turkeys inoculated with TCV + EPEC (groups 7, 8; $10^6$ and $10^8$ CFU EPEC, respectively). These same clinical signs were observed beginning on day 5 PE in turkeys inoculated with TCV + EPEC (group 6; $10^4$ CFU EPEC). No clinical signs were observed in sham-inoculated turkeys, turkeys inoculated with only TCV, or turkeys inoculated with only EPEC (groups 3, 4, 5; EPEC doses ranging from $10^6$ to $10^{10}$, respectively).

Mortality in turkeys inoculated with TCV + EPEC during the 0-to-14-day PE period ranged from 91.7% (group 6; $10^4$ CFU EPEC) to 100% (groups 7, 8; $10^6$ and $10^8$ CFU EPEC, respectively) (Table 1). No mortality was observed in sham-inoculated turkeys, turkeys inoculated with only TCV, or turkeys inoculated with only EPEC (doses ranging from $10^6$ to $10^{10}$ CFU EPEC).

Table 1 shows the mean body weight gains and average daily gains observed during the 0-to-7-day PE period. Excessive mortality in birds inoculated with TCV + EPEC prevented assessment of weight gains at times later than day 7 PE. Weight gains of turkeys inoculated with only EPEC (groups 3, 4, 5; EPEC doses ranging from $10^6$ to $10^{10}$ CFU) were not significantly different ($P > 0.05$) from those of sham-inoculated controls. Weight gains of turkeys inoculated with TCV + EPEC (groups 6, 7, 8; doses ranging from $10^4$ to $10^8$ CFU EPEC) were significantly depressed ($P < 0.05$) compared with sham-inoculated turkeys, turkeys inoculated with only TCV, or turkeys inoculated with only EPEC (groups 3, 4, 5; EPEC doses ranging from $10^6$ to $10^{10}$) (Table 1). Weight gains of turkeys inoculated with only TCV (group 2) were significantly depressed ($P <$

Table 3. Effect of TCV exposure, before or after EPEC exposure, on mortality and weight gain.[A]

| Group | Inoculation regimen: 4 days of age | Inoculation regimen: 11 days of age | Mortality (%) 11–18 days of age | Weight gain (g) 4–18 days of age | Survival probability at 5 days of age |
|---|---|---|---|---|---|
| 1 | — | — | 0/11 (0%) | 340[a] | 11/11 (100%)[a] |
| 2 | — | TCV | 0/10 (0%) | 281[ab] | 10/10 (100%)[a] |
| 3 | — | EPEC | 0/11 (0%) | 328[a] | 11/11 (100%)[a] |
| 4 | — | TCV + EPEC | 6/11 (64%) | 231[b] | 11/11 (100%)[a] |
| 5 | EPEC | TCV | 7/11 (73%) | 193[b] | 6/11 (55%)[b] |
| 6 | TCV | EPEC | 13/13 (100%) | ND | 0/13 (0%)[c] |

[A]Turkeys were inoculated at 11 days of age with either TCV, EPEC, or TCV + EPEC; clinical responses of these turkeys were compared with sequential inoculations at 4 days of age with TCV or EPEC and at 11 days of age with TCV or EPEC. Mortality is recorded for the period 11–18 days (7-day period after inoculation at 11 days of age); body weight gain is recorded for the period 4–18 days of age. Values within a column followed by the same lowercase superscript letter are not significantly different ($P > 0.05$).

0.05) compared with sham-inoculated controls (group 1); however, weight gains of turkeys inoculated with TCV + EPEC were significantly depressed ($P < 0.05$) compared with both sham-inoculated and TCV-inoculated turkeys.

No AE lesions were detected in sham-inoculated turkeys, turkeys inoculated with only TCV, or turkeys inoculated with only EPEC (groups 3, 4, 5; doses ranging from $10^6$ to $10^{10}$ CFU) (Table 2). AE lesions were detected only in turkeys inoculated with TCV + EPEC (groups 6, 7, 8; EPEC doses ranging from $10^4$ to $10^8$ CFU). AE lesions were characterized by 1) intimate attachment of bacteria to intestinal epithelium, 2) microcolony formation, 3) infiltration of the lamina propria with lymphocytes and heterophils, 4) degeneration and necrosis of epithelium, and 4) focal erosion of epithelium. In turkeys inoculated with both TCV and graded doses of EPEC, an EPEC dose response was evident; AE lesions were more extensively distributed in intestinal tissues in turkeys inoculated with highest doses of EPEC ($10^6$ and $10^8$ CFU). Turkeys inoculated with TCV and a low dose of EPEC ($10^4$ CFU) had detectable AE lesions only in ceca, whereas AE lesions were detected in small intestines and ceca of turkeys inoculated with TCV and higher doses of EPEC ($10^6$ and $10^8$ CFU).

**Expt. 2. Effect of TCV exposure, before or after EPEC exposure.** No clinical sign or mortality was observed in uninoculated controls (group 1) or in turkeys inoculated with only TCV or EPEC at 11 days of age (groups 2, 3, respectively). However, severe depression, inappetance, and high mortality (63%–100%; Table 3) were observed in turkeys inoculated with both TCV and EPEC, regardless of the sequence of inoculation (TCV + EPEC inoculation simultaneously at 11 days of age [group 4]; or TCV inoculation 7 days before [group 6] or 7 days after EPEC [group 5]). Mortality in turkeys inoculated with TCV 7 days prior to EPEC (group 6, [100%]) was numerically greater than but not significantly different ($P > 0.05$) from mortality observed in group 4 (TCV + EPEC, simultaneously) and group 5 (EPEC inoculation prior to TCV), 63% and 72%, respectively. However, survival probability on day 5 PE in group 6 birds (0) was significantly lower ($P < 0.05$) than that observed in groups 4 and 5 (100% and 55%, respectively). Excessive mortality in group 6 birds prevented assessment of survival probability at times later than day 5 PE.

Table 3 shows the mean body weight gains during the period 11–18 days of age (7-day period after inoculation at 11 days of age). Weight gains of turkeys inoculated at 11 days of age with only TCV (group 2) or only EPEC (group 3) were not significantly different ($P > 0.05$) from those of uninoculated controls (group 1). Weight gains of birds inoculated with only TCV at 11 days of age (group 2) were not significantly different ($P > 0.05$) from those in birds inoculated with both TCV and EPEC, either simultaneously at 11 days of age (group 4) or when EPEC inoculation preceded TCV inoculation (group 5). Excessive mortality in group 6 birds (TCV inoculation 7 days prior

Table 4. Effect of TCV exposure, before or after EPEC exposure, on development and distribution of attaching and effacing (AE) lesions in intestines of inoculated turkeys.[A]

| Group | Inoculation regimen | | AE lesions detected at 15 days of age | | | |
|---|---|---|---|---|---|---|
| | 4 days of age | 11 days of age | Duodenum | Jejunum | Ileum | Cecum |
| 1 | — | — | 0/3 | 0/3 | 0/3 | 0/3 |
| 2 | — | TCV | 0/3 | 0/3 | 0/3 | 0/3 |
| 3 | — | EPEC | 0/3 | 0/3 | 0/3 | 0/3 |
| 4 | — | TCV + EPEC | 0/3 | 0/3 | 1/3 | 2/3 |
| 5 | EPEC | TCV | 0/3 | 1/3 | 1/3 | 2/3 |
| 6 | TCV | EPEC | 3/3 | 3/3 | 3/3 | 3/3 |

[A]Turkeys were inoculated at 11 days of age with TCV, EPEC, or TCV + EPEC; at 4 days of age with TCV or EPEC; and at 11 days of age with TCV or EPEC. Turkeys were examined for presence of AE lesions in intestines at 15 days of age (4 days after inoculation at 11 days of age).

to EPEC) prevented assessment of weight gain during the period 11–18 days of age (7-day period after EPEC inoculation at 11 days of age).

AE lesions were identified only in turkeys inoculated with both TCV and EPEC (groups 4, 5, 6). AE lesions were more extensive in turkeys inoculated with TCV prior to EPEC (group 6) than in turkeys inoculated with TCV and EPEC simultaneously (group 4) or inoculated with EPEC prior to TCV (group 5). In turkeys inoculated with TCV prior to EPEC (group 6), AE lesions were detected in all intestinal tissues (duodenum, jejunum, ileum, cecum) of all birds examined. In birds inoculated with TCV and EPEC simultaneously (group 4) or with EPEC prior to TCV (group 5), AE lesions were less consistently detected. AE lesions in group 4 turkeys were identified only in ileum (one of three birds) and cecum (two of three birds), and in group 5 birds, in jejunum (one of three birds), ileum (one of three birds), and cecum (two of three birds).

Table 5. Detection and quantitation of EPEC (R98/5) shedding in intestinal contents of turkeys inoculated with only EPEC or TCV + EPEC.[A]

| Days PE | Inoculum[B] | |
|---|---|---|
| | EPEC only | TCV + EPEC |
| 4 | 2/5 ($8.6 \times 10^5$)[a] | 2/5 ($3.8 \times 10^5$)[a] |
| 7 | 1/5 ($4.0 \times 10^6$)[a] | 5/5 ($3.1 \times 10^8$)[b] |
| 10 | 2/5 ($1.8 \times 10^6$)[a] | 5/5 ($2.4 \times 10^8$)[b] |
| 14 | 1/5 ($9.4 \times 10^4$)[a] | 4/5 ($6.9 \times 10^6$)[b] |

[A]No EPEC were detected in sham-inoculated turkeys or turkeys inoculated with only TCV. No EPEC were detected on days 0 and 2 PE in turkeys inoculated with only EPEC or TCV + EPEC. EPEC (R98/5) was detected and quantitated on the basis of gentamicin resistance (growth on MacConkey agar containing 10 μg/ml gentamicin) and lactose-nonfermenter phenotype.

[B]Number of positive samples/number tested (mean CFU/0.1 g). Numbers within a row followed by the same lowercase superscript letter are not significantly different ($P > 0.05$).

**Expt. 3. Effect of combined infection on TCV and EPEC shedding.** EPEC strain R98/5, a gentamicin-resistant, lactose-nonfermenting strain of *E. coli*, was identified in intestinal contents of inoculated turkeys on the basis of these properties and presence of EAE genes as determined by PCR. EPEC were detected on days 4, 7, 10, and 14 in intestinal contents of turkeys inoculated with only EPEC and turkeys inoculated with TCV + EPEC. EPEC were not detected in sham-inoculated turkeys or turkeys inoculated with only TCV (Table 5). EPEC were shed in significantly higher ($P < 0.05$) numbers on days 7, 10, and 14 PE in intestinal contents of turkeys inoculated with TCV + EPEC than in turkeys inoculated with only EPEC. EPEC shedding on days 4, 7, 10, and 14 was detectable in more TCV + EPEC–inoculated turkeys (16/20) than in turkeys inoculated with only EPEC (6/20). Additionally, EPEC shedding occurred for an extended period in TCV + EPEC–inoculated turkeys; on day 14 PE, EPEC were detectable in one of five turkeys inoculated with only EPEC compared with four of five turkeys inoculated with TCV + EPEC. Shedding on day 4 PE was not sig-

nificantly different in turkeys inoculated with only EPEC or with TCV + EPEC.

TCV was detected on days 2–14 PE in intestinal contents of turkeys inoculated with only TCV and turkeys inoculated with TCV + EPEC (data not shown). TCV was not detected in intestinal contents of sham-inoculated turkeys or turkeys inoculated with only EPEC. TCV shedding was not significantly different ($P > 0.05$) at any time PE in birds inoculated with only TCV or birds inoculated with TCV + EPEC (data not shown).

## DISCUSSION

The findings of the present study indicate that TCV infection predisposes young turkeys to secondary EPEC infection. Turkeys coinfected with TCV and EPEC had significantly greater ($P < 0.05$) mortality and weight gain depression and increased frequency of AE lesions in intestines compared with turkeys inoculated with only TCV or only EPEC. Additionally, EPEC were shed in significantly greater ($P < 0.05$) numbers in intestinal contents of EPEC + TCV–infected turkeys as compared with turkeys infected with only EPEC. Increased severity of disease (numerically greater mortality, significantly lower [$P < 0.05$] survival probability, increased frequency of AE lesions) was observed in turkeys infected with TCV prior to EPEC compared with that observed in turkeys infected with EPEC prior to TCV or simultaneously infected with these agents. In addition, the expression of EPEC pathogenicity in young turkeys required TCV coinfection: turkeys infected with only EPEC did not develop clinically apparent disease or AE lesions, even when inoculated with high doses of EPEC ($10^{10}$ CFU).

Previous investigations demonstrated synergistic interactions between rotavirus and ETEC in pigs, calves, lambs, and mice (10,13,20,25,27). Snodgrass *et al.* (23) demonstrated that rotavirus infection markedly enhanced intestinal colonization by ETEC in 6-day-old conventional calves. Hess *et al.* (10), with 1-day-old specific-pathogen-free calves, demonstrated that inoculation with only ETEC did not produce clinical disease, and calves inoculated with only rotavirus exhibited mild diarrhea; however, severe disease was produced in calves dually inoculated with both rotavirus and ETEC. Additionally, Hess *et al.* (10) showed that severe diarrhea occurred either when calves were inoculated simultaneously with rotavirus and ETEC or rotavirus was inoculated prior to ETEC; no exacerbation of disease was observed if calves were inoculated with ETEC prior to rotavirus. These studies have provided experimental evidence that rotavirus enhances intestinal colonization by ETEC; however, the mechanism has not been determined. Whether rotavirus enhances ETEC colonization by altering intestinal epithelial surfaces and exposing sites for bacterial attachment, altering the intestinal lumenal environment such that bacterial proliferation is enhanced, or by impairing host immune responses is not known. Whether TCV promotes EPEC in a manner similar to that observed in rotavirus/ETEC infections remains to be determined; however, similar mechanisms likely are operative.

The results of the present study indicate that TCV infection promoted EPEC colonization of intestinal epithelium. Enhanced colonization of turkey intestines by EPEC in TCV + EPEC–infected turkeys was evident by increased frequency and distribution of AE lesions and increased EPEC shedding in intestinal contents. TCV infection of intestinal enterocytes possibly resulted in changes in cell surfaces that favored EPEC adherence and colonization. *In vitro* studies have shown that influenza virus infection of human pharyngeal epithelial cells increases adherence of several different bacterial species to these infected cells (4). However, this explanation seems unlikely because EPEC replication in intestines, as measured by AE lesions, would be expected to have the same distribution as TCV infection in TCV + EPEC–inoculated turkeys. Previous studies have shown that TCV replicates primarily within enterocytes in jejunum and ileum (20), whereas, in TCV + EPEC–inoculated turkeys, AE lesions were most consistently identified in cecum. TCV infection could result in changes in the intestinal lumenal environment as a result of physiologic changes engendered by enterocyte damage, malabsorption, and maldigestion, and these changes could lead to increased proliferation of EPEC. This explanation is supported by previous studies by Naqi *et al.* (16) in which TCV infection of turkeys resulted in increased proliferation of a variety of different intestinal bacteria. Alternatively, TCV infection could result in alteration of immune responses to

EPEC. TCV has been shown to infect epithelium of the bursa of Fabricius, and this infection has been associated with bursal lymphocyte depletion and atrophy; however, whether or not antibody formation is impeded by TCV infection has not been determined (9). TCV infection also could promote EPEC infection by impeding nonspecific immunity such as mucus secretion or intestinal motility. Additional studies aimed at determining the mechanism by which TCV infection potentiates EPEC infection are warranted.

The findings of the present study indicate that TCV infection potentiated EPEC infection, but not vice-versa; TCV shedding in turkeys infected with both TCV and EPEC did not differ from that observed in turkeys infected with only TCV. Thus, enhanced clinical disease in dually infected turkeys is most likely attributable to EPEC, not TCV. The mechanism by which EPEC induce diarrhea is poorly understood; however, malabsorption due to effacement of microvilli, signal transduction events leading to increased secretion, and increased epithelial permeability are believed to be important mechanisms (17). On the basis of the findings of the present study, TCV infection enhanced intestinal colonization by EPEC and, thus, potentiated its diarrheagenic effects. In dually infected turkeys, enhanced EPEC colonization of intestines was evident by increased distribution of AE lesions within intestines, increased EPEC shedding, and increased duration of shedding. Severe disease and mortality in dually infected turkeys likely are the result of TCV-enhanced EPEC colonization that results in prolonged diarrhea with dehydration and electrolyte imbalance.

The findings of the present study indicate that TCV infection enhances the ability of EPEC to colonize the intestines of young turkeys and potentiates the expression of EPEC pathogenicity in young turkeys. Expression of EPEC pathogenicity in turkeys was shown to require concurrent TCV infection; however, other enteric pathogens such as turkey astrovirus and *Cryptosporidium* spp. also may potentiate EPEC infection. Additional studies are needed to examine the interaction of EPEC and other enteric pathogens in the pathogenesis of enteric diseases of turkeys.

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