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# Pathogenicity of Turkey Astroviruses in Turkey Embryos and Poults

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SUMMARY. The pathogenicity of turkey astrovirus 2001 (TAstV2001) and turkey astrovirus 1987 (TAstV1987) in specific-pathogen-free (SPF) turkey embryos and commercial poults was investigated. The virus shedding in poults was monitored using electron microscopy (EM) and reverse transcription–polymerase chain reaction (RT-PCR) during the 14-day experimental period. Both viruses caused enteritis and growth depression in SPF turkey embryos and poults. The TAstV2001 did not induce macroscopic or microscopic lesions in thymuses and bursas of embryos or poults. No macroscopic changes were observed in thymuses and bursas of embryos and poults inoculated with TAstV1987, and no statistically significant differences in bursa weight/body weight ratios ($P > 0.05$) were detected. However, TAstV1987 infection resulted in microscopic lesions in bursas but not in thymuses of infected embryos and poults. Both TAstV2001 and TAstV1987 were shed during the whole 14-day experimental period as detected by EM and RT-PCR. These findings indicated that both TAstV1987 and TAstV2001 are etiologic agents of turkey enteritis. In addition, TAstV1987 might cause impairment of the immune system of infected poults. The pathogenicity of TAstV1987 is somewhat different from TAstV2001.

RESUMEN. Patogenicidad de los astrovirus de pavos para embriones de pavo y pavitos.

Se investigó la patogenicidad para embriones de pavo y pavitos comerciales libres de patógenos específicos, de dos cepas de astrovirus de pavo identificadas como TAstV2001 y TAstV1987. Mediante la microscopía electrónica y la prueba de reacción en cadena por la polimerasa-transcriptasa reversa, se evaluó la diseminación del virus en los pavitos durante los 14 días del período experimental. Ambos virus causaron enteritis y disminución del crecimiento tanto en los embriones como en los pavitos libres de patógenos específicos. La cepa TAstV2001 no indujo lesiones macro o microscópicas en el timo o en la bolsa de Fabricio de los embriones o de los pavitos. A su vez, no se observaron cambios macroscópicos en los timos o bolsas de los embriones o pavitos inoculados con la cepa TAstV1987, y no se detectaron diferencias significativas ($P > 0.05$) en el índice peso de la bolsa / peso corporal. Sin embargo, la infección con la cepa TAstV1987 resultó en lesiones microscópicas en la bolsa, pero no en los timos de los embriones o pavitos infectados. Ambas cepas se diseminaron durante todo el periodo experimental de 14 días, según pudo ser comprobado mediante microscopía electrónica y la prueba de reacción en cadena por la polimerasa-transcriptasa reversa. Estos hallazgos indican que tanto la cepa TAstV1987 como la cepa TAstV2001 son agentes etiológicos de la enteritis del pavo. Adicionalmente, la cepa TAstV1987 puede comprometer el sistema inmune en los pavitos infectados. La patogenícidad de la cepa TAstV1987 es un tanto distinta a la de la cepa TAstV2001.

Key words: pathogenicity, turkey astroviruses, embryos, poults, gastroenteritis, lesions

Abbreviations: BW = body weight; DPI = days postinoculation; $EID_{50}$ = mean embryo infective dose; EM = electron microscopy; GI = gastrointestinal; PEMS = poult enteritis and mortality syndrome; RT-PCR = reverse transcription–polymerase chain reaction; SPF = specific pathogen free; SRV = small round virus; TAstV = turkey astrovirus

Astrovirus was first described by Appleton and Higgins (1) in 1975. They observed small round virus (SRV) particles of 29 to 30 nm in diameter in the stools of infants hospitalized with mild diarrhea and vomiting. In the same year, Madeley and Cosgrove (8) observed SRVs with a five- or six-pointed star-like appearance in the samples of infantile viral gastroenteritis by direct electron microscopy (EM). They coined the name astrovirus based on the distinctive star-like surface features of these viruses. Subsequently, astroviruses were identified in association with gastroenteritis in a wide range of species, such as lambs, calves, piglets, dogs, turkey poults, red deer, cats, chickens, mice, and mink (2,9). In addition to causing intestinal illness, extraintestinal illness was also noted in ducklings and chicks (4,5).

Turkey astrovirus (TAstV) was first detected by McNulty *et al.* in Great Britain (1980) (10) in the feces of poults experiencing diarrhea and increased mortality. Subsequently, Saif *et al.* and Reynolds *et al.* identified this virus in the United States (14,16,17,18). Later, astroviruses were shown to be widely distributed in commercial turkey poults. In one survey, Reynolds *et al.* (15) found astroviruses in 78% of diseased turkey flocks with diarrhea; it was the most frequently detected enteric virus. In the 1980s, all detected astroviruses were strictly confined to the gastrointestinal (GI) tract of turkey poults, and there was no evidence that astroviruses could cause a systemic disease. The co-infection of astrovirus and group D rotavirus was the most frequently observed in naturally affected poults (16). In the 1990s, TAstV was found not only in the intestines, but also in thymus and bursa, suggesting a possible effect on the immune system (12). TAstV is one of the etiologic agents of turkey poult enteritis and mortality syndrome (PEMS), a devastating multisystem disease (12,19,26,27). PEMS is usually caused by multiple agents. Studies over the past two decades have primarily focused on identifying causative agents or combined infections, whereas studies on the role of each individual enteric virus in PEMS were limited (3,6,7,10,12,14,18,19,23,24,27). There are few studies on the pathogenicity of TAstV infection alone (12,14,23,24). Exposure to TAstVs can result in not only intestinal, but also lymphoid organ, infections (12,14,23,24). The gross and micro-

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Table 1. Treatment groups for trials 2 and 3.

| Group | Inoculum | Route of exposure | Bird age at exposure (wk) | Trial 2[A] | | Trial 3[B] | |
|---|---|---|---|---|---|---|---|
| | | | | No. of birds | Dose | No. of birds | Dose |
| 1 | None (control) | N/A | 2 | 15 | None | 25 | None |
| 2 | TAstV1987 | Oral | 2 | 15 | 100 $EID_{50}$ | 25 | $10^5$ $EID_{50}$ |
| 3 | Contact with group 2 | Contact | 2 | 15 | Unknown | 25 | Contact |
| 4 | TAstV2001 | Oral | 2 | 15 | 100 $EID_{50}$ | 25 | $10^5EID_{50}$ |
| 5 | Contact with group 4 | Contact | 2 | 15 | Unknown | 25 | Contact |

[A]In trial 2, clinical signs were monitored every day during the experimental period. At 2, 3, and 4 DPI, five poults from each group were euthanatized. Body weight and bursa weight for each bird were obtained, and the intestines, thymuses, and bursas of birds from inoculated groups and controls were observed for gross lesions. Thymuses and bursas were collected for histopathologic examination.

[B]In trial 3, as indicated in the table, virus dose for inoculated group birds was different from that in trial 2. Samples were collected at 2, 4, 7, 10, and 14 DPI, and the rest of the treatments were the same as in trial 2.

scopic lesions of the small intestines of poults infected with astroviruses are well documented (23,24). There is only one brief communication and a case report on histopathology of the thymus and bursa of infected birds with PEMS (3). Astrovirus is a positive sense single-stranded RNA virus. The polymerase of astrovirus has no proofreading function during the course of replication, so replication of the virus is error prone, and the virus has very high mutation rates. Currently, eight serotypes of human astroviruses have been identified. This raises the question about the possible existence of multiple TAstV strains with variable pathogenicity. To date, the diversity in pathogenicity of turkey astroviruses has not been documented.

In the present study, we have examined and compared the pathogenicity of two turkey astrovirus isolates, TAstV1987 and TAstV2001, in 26-day-old specific-pathogen-free (SPF) turkey embryonated eggs and 14-to-28-day-old turkey poults. Virus shedding in intestinal contents of infected/contact poults was monitored by EM and reverse transcription–polymerase chain reaction (RT-PCR).

## MATERIALS AND METHODS

**Embryonated eggs and turkey poults.** All eggs were obtained from our SPF turkey flocks maintained at the Ohio Agricultural Research and Development Center of Ohio State University (Wooster, OH) and were incubated at our facilities. The SPF turkey flock is free of known turkey pathogens including enteric viruses. Two-week-old conventional turkey poults were generously provided by the Animal Sciences Department, Ohio Agricultural Research and Development Center (Wooster, OH). Before use, all the poults were tested for presence of astrovirus by RT-PCR (27) and for antibodies by enzyme-linked immunosorbent assay. Poults were housed in a disease containment building that has rooms with high-efficiency particulate air–filtered intake and exhaust air. Birds had *ad libitum* access to feed and water.

**Viruses.** TAstV1987 and TAstV2001 used in this study were isolated in our laboratory from the feces of commercial poults with diarrhea. Originally, both TAstV1987 and TAstV2001 were mixed with other large viruses, such as rotavirus and coronavirus. To separate astrovirus, each mixture was filtered serially through 0.8-μm, 0.45-μm, 0.2-μm, 0.1-μm, and 0.05-μm filters. Subsequently, filtrates were examined by EM or immune EM to make sure the astroviruses were successfully purified. The filtrates containing only astrovirus were used as inocula for virus adaptation and propagation. Twenty 2-day-old SPF turkey embryonated eggs were first inoculated with 0.2 ml of the filtrate via the amniotic cavity route as described earlier (20). At 4 days postinoculation (DPI), the intestines of the infected embryos were harvested, diluted 1:5 or 1:10 (w/v) in 0.05 M Tris-HCl buffer containing 0.15 M NaCl and 15 mM $CaCl_2$, pH 7.5 (TNC), and homogenized. After freezing and thawing three times, the homogenates were clarified by centrifugation at 3000 × *g*, 4 C for 30 min. The supernatants were filtered through a 0.45-μm syringe filter. The filtrates were then examined by EM and used as inoculum for subsequent virus propagation in turkey embryos. Both TAstV1987 and TAstV2001 were passaged eight times in embryos. The original and different passages of viruses were stored at −70 C. The last filtrate from passage eight intestinal homogenates of infected embryos was used in this study.

The titrations for both viruses in turkey embryonated eggs were made using the method described by Villegas (25). Lack of embryonic lesions was considered the endpoint. The titers were expressed as mean embryo infective dose ($EID_{50}$), calculated by the method of Reed and Muench (13).

**Experimental design.** *Trial 1.* Forty 22-day-old SPF turkey embryonated eggs were randomly allotted into four groups of 10 eggs each and inoculated via the amniotic cavity route. Group 1 was inoculated with 100 $EID_{50}$ of TAstV1987 in 0.2 ml; group 2 was inoculated with 0.2 ml of SPF turkey embryo intestinal homogenate mock, which was negative control; group 3 was inoculated with 100 $EID_{50}$ of TAstV2001 in 0.2 ml; and group 4 was treated as group 2, a negative control. After 96 hr incubation, all eggs were chilled overnight at 4 C. The intestines, thymuses, and bursas from the embryos were examined for gross lesions. Thymuses and bursas were collected for histopathologic analysis.

*Trial 2.* Seventy-five (pretested astrovirus antigen and antibody free) 2-wk-old naive poults were randomly assigned to five groups. Each group had 15 birds that were tagged and weighed (day 0). The treatments for each group are summarized in Table 1. Clinical signs were monitored every day during the experimental period. At 2, 3, and 4 DPI, five poults from each group were euthanatized. Body weight and bursa weight for each bird were determined, and the intestines, thymuses, and bursas of birds from inoculated and control groups were observed for gross lesions. Thymuses and bursas were collected for histopathologic analysis.

*Trial 3.* Trial 3 is similar to trial 2. As indicated in Table 1, virus dose for inoculated group birds was different from that in trial 2; samples were collected at 2, 4, 7, 10, and 14 DPI; and the rest of the treatments were the same as in trial 2.

**Histopathology.** Thymuses and bursas from control and infected embryos and birds were fixed in 10% Prefer fixative solution (Antech, Ltd., Detroit, MI, 490T5), embedded in paraffin, and sectioned at 5 μm. Sections of thymuses and bursaes were stained with hematoxylin and eosin and examined by light microscopy with a digital camera equipment (Leica DMlRB, Leica Microsystems, Wetzlar, Germany), and photographs of the sections were taken using the same microscope.

**Statistics analysis.** The mean body weight (BW) and the average bursa/BW ratios were analyzed by one-way analysis of variance and the Fisher pairwise multiple comparisons (the student edition of MINITAB, release 12, Minitab Inc., State College, PA). The significance level was defined at $P \leq 0.05$.

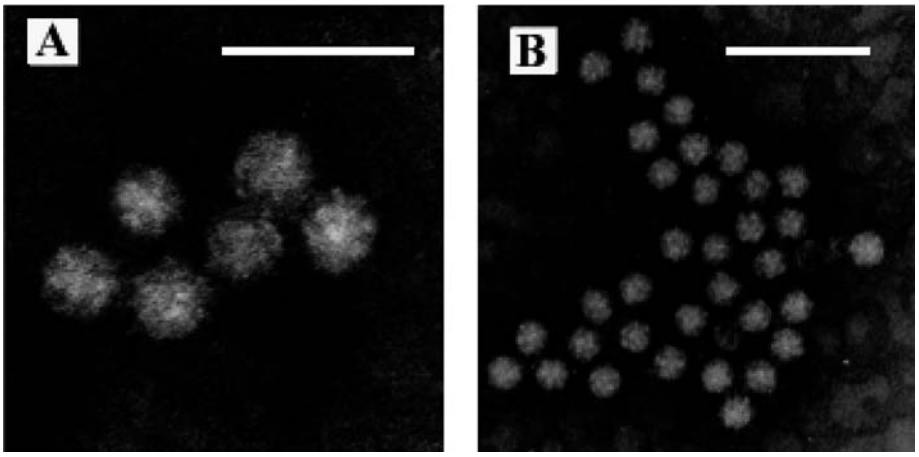


Fig. 1. Electron photomicrographs of turkey astrovirus particles in intestinal homogenate preparations from 26-day-old SPF turkey embryos inoculated with TAstV1987 or TAstV2001, respectively. (A) TAstV1987. (B) TAstV2001. Bar = 100 nm.

**EM and RT-PCR for monitoring virus shedding.** The intestinal contents of infected and contact poults in trial 3 were sampled to monitor virus shedding. Sampling time is summarized in Table 1. The samples from the same day and treatment were pooled and then tested by EM and RT-PCR. The procedures for detection of TAstV using EM and RT-PCR were described previously (21).

## RESULTS

**Virus isolation and propagation.** Both TAstV1987 and TAstV2001 were successfully propagated and isolated in turkey embryos via the amniotic cavity route inoculation. TAstV1987 and TAstV2001 of each passage were examined by EM and RT-PCR to confirm the virus presence. Both viruses were shown to be morphologically similar with average size of 30–32 nm in diameter (Fig. 1). Around 10 % of TAstV1987 particles exhibited star-shaped appearances (Fig. 1 A). However, TAstV2001 viral particles did not display very distinguishing surface features (Fig. 1 B). The eighth passage of each virus in embryos with titers of $5 \times 10^5$ to $5 \times 10^6$ $EID_{50}$/ml was used as inoculum in this study.

**Pathogenicity.** *Trial 1.* Both groups of turkey embryos inoculated with either TAstV1987 or TAstV2001 were stunted and had distinct intestinal gross lesions, but no mortality resulted. The gizzards were enlarged and filled with yellowish frothy contents; the intestines were very thin, and fragile, distended with greenish or yellowish gaseous fluid. No obvious lesions were observed in the thymus or bursa. Histopathologic examination using light microscopy showed no obvious lesions in the thymuses or bursas of embryos inoculated with TAstV2001 compared to those from non-inoculated SPF controls. Similarly, no microscopic lesions were found in the thymuses from embryos infected with TAstV1987. However, mild lesions were noted in bursas of this group of embryos (Fig. 2B) . The lesions of infected bursas were characterized primarily by lymphocytic depletion and enlarged follicles. On low magnification, there was a decrease in the density of lymphocytes in the medullar region. This was more apparent because of the normally lower lymphocyte density in this zone. There were no gross and microscopic lesions in the nonexposed control embryos (Fig. 2A).

*Trials 2 and 3.* All groups of inoculated poults and their contacts in both trials 2 and 3 developed severe diarrhea. The onset of clinical signs was at 2 DPI and persisted throughout the experimental period. The diarrhea was characterized by watery to frothy, yellow or brown droppings, anorexia, depression, and mild dehydration. At necropsy, gross lesions were first noted at 2 DPI as well. The ceca were severely dilated and filled with yellow or green foamy contents. The duodenum, jejunum, and ileum were also filled with watery gaseous fluid. Lesions of GI tract induced by TAstV1987 and TAstV2001 infection were similar. Body weights were reduced in the infected and contacted groups as compared with the controls (Fig. 3). Although differences of bursa weight to body weight ratios were observed, there was statistically no significant difference ($P > 0.05$) between infected/contacted groups and normal control group. Histopathologically, thymuses from birds inoculated with TAstV1987 and TAstV2001 and their contacts exhibited no changes in comparison with the negative control group. Microscopically, no lesions were noticed in TAstV2001 affected bursas in comparison with the negative controls (Fig. 2C). However, TAstV1987 did induce mild lesions in the bursa at 4 DPI (Fig. 2D), including mild lymphocyte depletion from follicle medullar zones with increased prominence in medullary stroma and increased stroma beneath the epithelial lining with hypercellularity.

**Virus shedding.** The virus shedding of TAst1987 and TAstV2001 in both inoculated and contact groups was detected during the whole 14-day experimental period when tested by EM and RT-PCR.

## DISCUSSION

In this study we have propagated and isolated TAstV2001. TAstV1987 was isolated by Reynolds *et al.* at our laboratory in 1987, which was the first turkey astrovirus isolate identified in the United States (14,15,16). We experimentally demonstrated that both TAstV1987 and TAstV2001 isolates induced enteritis in SPF turkey embryos and turkey poults. In addition to causing intestinal illness, TAstV1987 also caused bursa, but not thymus, lesions in embryos and poults. In early reports, the poults challenged with TAstVs alone or combined with other enteric viruses, such as turkey coronavirus, exhibited not only diarrhea, weight loss, and gross lesions (3,11,12,14,15,16,17,18,23,26), but also lymphoid tissue atrophy including spleen, thymus, and bursa (12). Yu *et al.* (26) reported that an SRV, which was later determined to be a turkey astrovirus, could cause hemorrhage in the infected thymus, and speculated that the SRV might induce dysfunction of the immune system, which would increase the susceptibility to other opportunistic pathogens. The results from our experiments were consistent with those of earlier reports in terms of clinical signs and gross

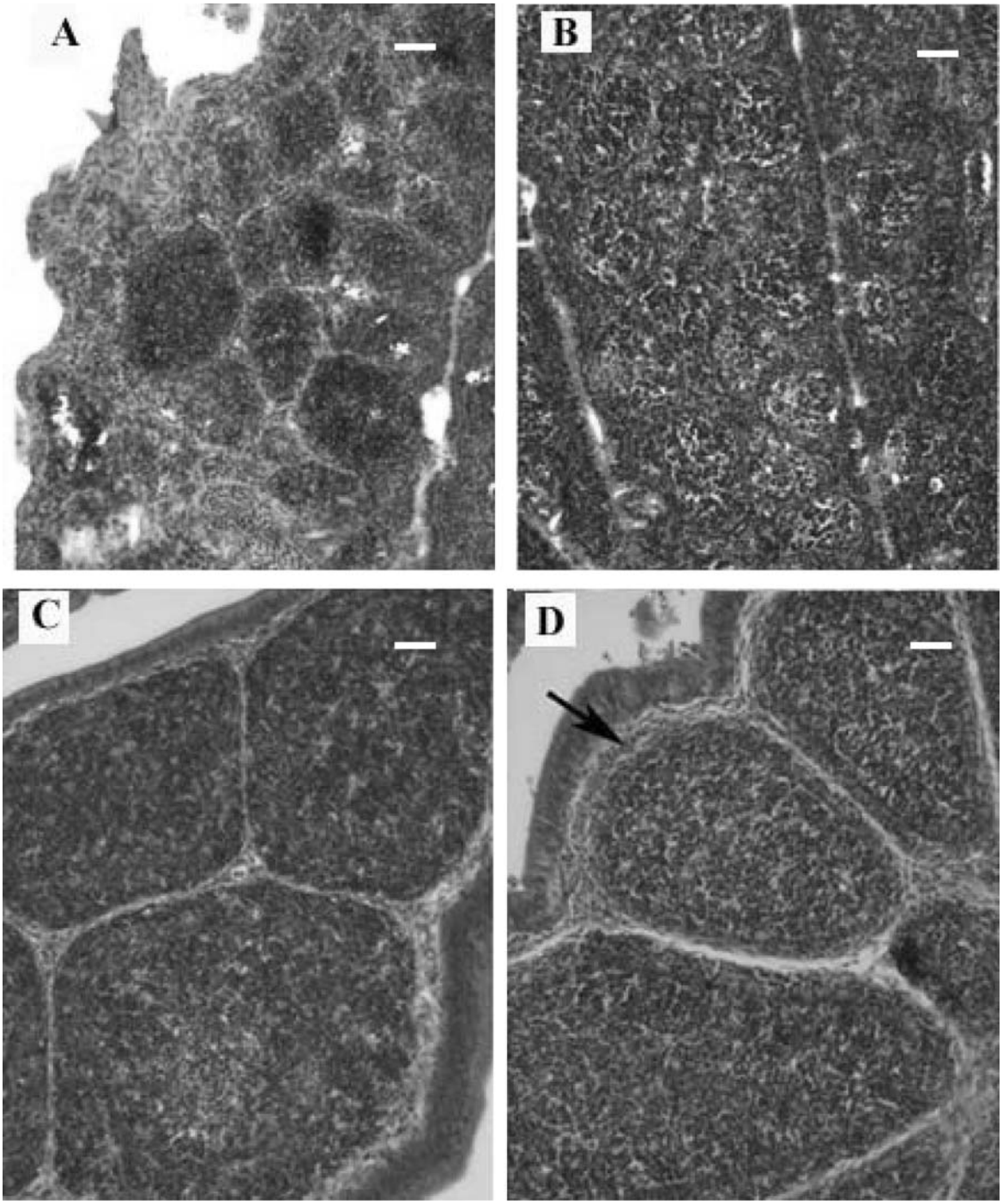


Fig. 2. Photomicrograph of turkey embryo and poult lymphoid tissue sections. (A) Normal control embryo bursa. (B) Bursa from an embryo inoculated with TAstV1987 with lymphocytic depletion. (C) Normal control poult bursa collected at 4 DPI in trial 2. (D) Four DPI bursa from a poult infected with TAstV1987 with mild lymphocytic depletion and increased stroma beneath the epithelial lining (indicated by arrowhead). Bar = 100 μm.

lesions in intestines. However, lymphoid organ atrophy was not found in our study either at the macroscopic or microscopic level. No gross or microscopic lesions were detected in the thymuses of poults and embryos infected with either TAstV1987 or TAstV2001. The only microscopic lesions detected were from TAstV1987-infected bursas, which consisted of mild lymphocyte depletion and stroma increase. Our findings in infected bursas and thymuses of challenged embryos and poults were similar to those of Behling-Kelly *et al.* (3). On the contrary, the results of statistical analysis of bursa weight to body weight ratios in infected poults were not in agreement with the above report. Virus strains, dose inoculated, passage number, host for virus propagation, sampling time, and strain of turkeys may contribute to the difference between our observations and the previous report. In an earlier study, it was shown that turkey astrovirus infection alone resulted in mortality as high as 11% in challenged poults. In this study, no mortality occurred in the poults affected with TAstV1987 or TAstV2001. The pathogenicity of TAstV1987 is somewhat different from

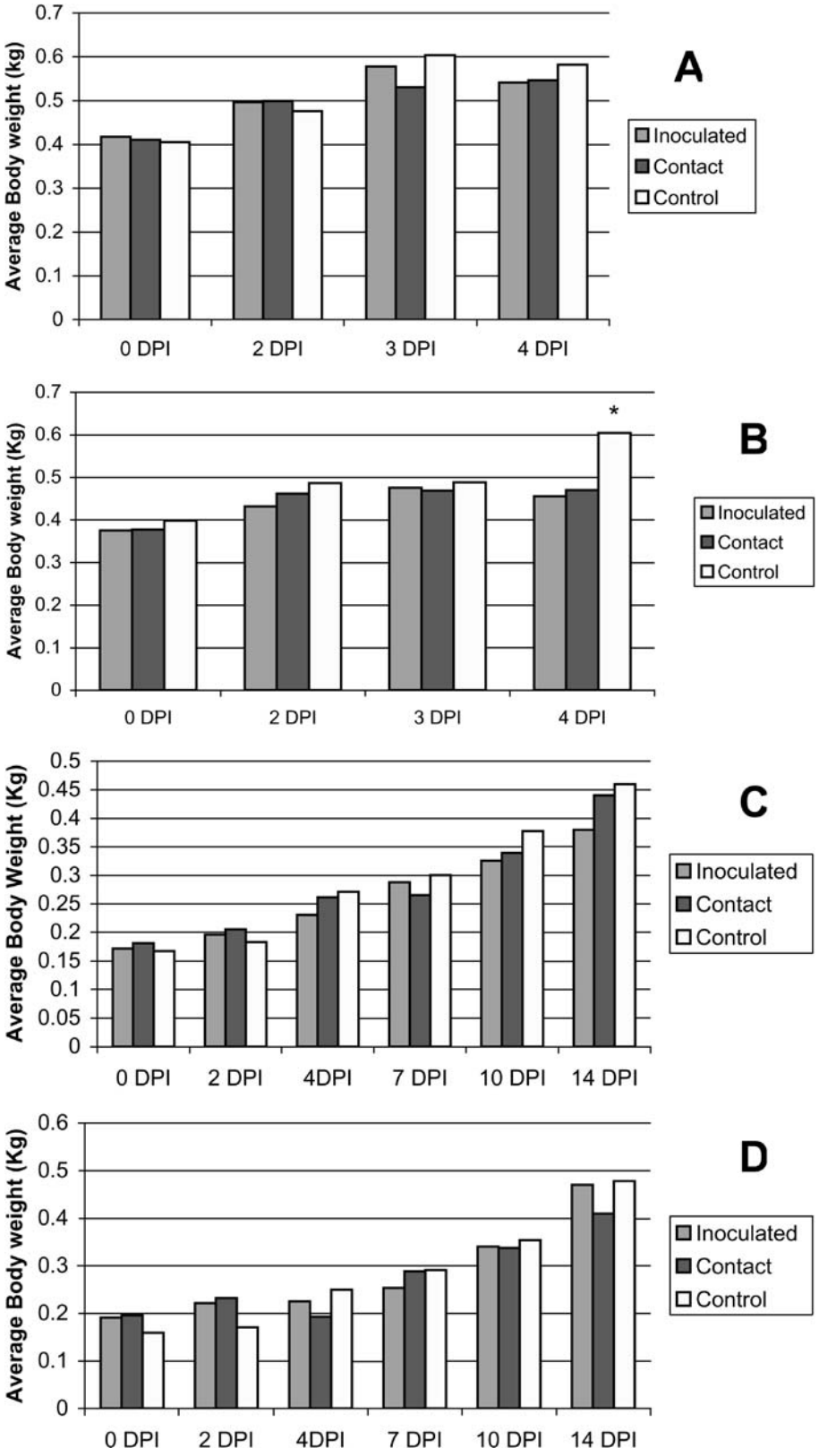


Fig. 3. Average body weight in experimentally treated and control birds in trial 2 (A and B) and trial 3 (C and D). (A) poults were inoculated/exposed to TAstV1987 on day 0 (2 wk old). There was growth depression in treated birds when compared with normal controls. However, there was no statistically significant difference ($P > 0.05$) among groups. (B) Poults were inoculated/exposed to TAstV2001 on day 0 (2 wk old). There was a significant difference in average body weight among treated and control groups on day 4 ($P \leq 0.05$) as indicated by asterisk (*). (C) Poults were inoculated/exposed to TAstV1987 on day 0 (2 wk old). There was growth depression in treated birds when compared with normal controls. However, there was no statistically significant difference ($P > 0.05$) among groups. (D) Poults were inoculated/exposed to TAstV2001 on day 0 (2 wk old). There was growth depression in treated birds in both treatments when compared with normal controls. However, there was no statistically significant difference ($P > 0.05$) among groups.

TAstV2001. The former caused both intestinal and extraintestinal lesions, and the latter only caused intestinal illness.

Morphologically, characteristic astrovirus star-shaped appearance was more prominent in TAstV1987 than in TAstV2001. Genetic analyses of the complete capsid gene of these two viruses revealed that they belong to different genotypes; sodium dodecyl sulfate–polyacrylamide gel electrophoresis results indicated those two viruses had different capsid protein profiles (22). All of these might contribute to the differences in morphology and pathogenicity of the two viruses.

Several attempts for propagation of turkey astrovirus in continuous cell lines were made by Yu *et al.* (27). Unfortunately none was successful. To date, the only laboratory hosts for TAstV isolation and propagation are turkey embryos or poults. Based on our observation, SPF turkey embryos were better hosts for TAstV propagation than SPF or commercial poults, and higher virus titers could be achieved by using embryos for virus propagation. Inoculation routes, either yolk sac or amniotic cavity, did not make much difference for virus growth, which was determined by monitoring the virus titer. We have also found that the amniotic sac route inoculation for turkey astrovirus propagation may help separate turkey astrovirus from other viruses, such as rotavirus, when an inoculum containing a mixture of viruses was used. This finding provided a valuable alternative for separating astrovirus from rotavirus, since they coexist with very high frequency in commercial poults (data not shown).

Previous reports indicated that TAstV was the most frequently detected enteric viruses in diseased turkey poults as well as in healthy poults (15,21). Yu *et al.* (27) found this virus was highly resistant to many physical and chemical environments. The fact that the virus was shed for up to 14 days (it could be longer but our experiment lasted 14 days only) and its reported resistance might provide a partial explanation for the widespread prevalence of astroviruses in turkeys.

In conclusion, both TAstV1987 and TAstV2001 are etiologic agents of turkey enteritis. TAstV1987 infection induced mild microscopic lesions in bursas, suggesting it might cause some impairment of the immune system of hosts. There are some differences in pathogenicity between TAstV1987 and TAstV2001.

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