Myocarditis Associated with Reovirus in Turkey Poults


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SUMMARY. Myocarditis associated with reovirus was diagnosed in 17-day-old, male turkey poults, based on virus isolation, reverse transcriptase-polymerase chain reaction (RT-PCR), demonstration of reovirus antigen in the cytoplasm of mononuclear inflammatory cells and myocytes in the heart by immunohistochemistry (IHC), and reovirus particles in the endoplasmic reticulum of myocytes by transmission electron microscopy (TEM). Clinical signs in the poults included anorexia, growth depression, and increased mortality. Gross lesions in the six poults examined were increased pericardial fluid, mild-to-moderate dilation of right ventricles, pale-yellow myocardium, and ascites. Other lesions in a few birds included mild pulmonary edema, congestion, and pale serosa of the small intestine that had watery contents in their lumens. Microscopically, in the heart, there was mild-to-severe necrosis of myocytes and infiltration of primarily lymphocytes mixed with a few heterophils, macrophages, and occasionally, plasma cells and multinucleated giant cells. There was mild-to-moderate lymphoid depletion in the bursa of Fabricius. Reovirus was isolated from the heart of the turkey poults in chicken-embryo liver cells and was confirmed by RT-PCR, IHC, and TEM. A retrospective search of the laboratory database for cases of myocarditis associated with reovirus in turkeys revealed that this condition has occurred sporadically in California turkey flocks since 1991. This is the first documentation of myocarditis in turkey poults associated with reovirus.

RESUMEN. Miocarditis asociada con reovirus en pavipollos.

La miocarditis asociada con reovirus fue diagnosticada en pavipollos machos de 17 días de edad, mediante el aislamiento viral, la transcripción reversa y reacción en cadena de la polimerasa (RT-PCR), por la demostración mediante inmunohistoquímica del antígeno de reovirus en el citoplasma de células inflamatorias mononucleares y de miocitos en el corazón y también por la observación de partículas de reovirus en el retículo endoplasmático de miocitos por microscopia electrónica de transmisión. Los signos clínicos en los pavipollos incluyeron anorexia, depresión en el crecimiento e incremento en la mortalidad. Las lesiones macroscópicas en los seis pavipollos examinados fueron aumento en el fluido pericárdico, dilatación leve a moderada de los ventrículos cardíacos derechos, presencia de miocardios amarillo pálido y ascitis. Otras lesiones incluyeron edema pulmonar leve, congestión, y palidez de la serosa del intestino delgado que contenía contenido acuoso en el lumen. Microscópicamente, en el corazón se observó necrosis leve a severa de los miocitos mezclada con algunos heterófilos, macrófagos y ocasionalmente células plasmáticas y células gigantes multinucleadas. Se observó despoblación linfocítica leve a moderada en la bolsa de Fabricio. Se aisló reovirus del corazón de los pavipollos en células hepáticas de embriones de pollo y fue confirmada por RT-PCR, inmunohistoquímica y microscopía electrónica de transmisión. Una búsqueda retrospectiva en las bases de datos del laboratorio con el fin de detectar los casos de miocarditis asociados con reovirus en pavos, reveló que esta condición ha ocurrido de manera esporádica en las pavadas de pavos en California desde 1991. Este es el primer reporte de miocarditis en pavipollos asociada con reovirus.

Key words: reovirus, myocarditis, turkeys, immunohistochemistry, polymerase chain reaction

Abbreviations: AGID = agar gel immunodiffusion; AIV = avian influenza virus; APMV = avian paramyxovirus; CAHES = California Animal Health and Food Safety Laboratory System; CEL = chicken embryo liver; CPE = cytopathic effect; ELISA = enzyme-linked immunosorbent assay; FA = fluorescent antibody; HI = hemagglutination inhibition; IHC = immunohistochemistry; MG = Mycoplasma gallisepticum; MM = Mycoplasma meleagridis; MPS = mononuclear phagocytic system; MS = Mycoplasma synoviae; NVSL = National Veterinary Services Laboratory; PAS = periodic acid Schiff; RT-PCR = reverse transcriptase-polymerase chain reaction; SEPRL = Southeast Poultry Research Laboratory; TEM = transmission electron microscopy

Diseases or syndromes associated with avian reoviruses in chickens include viral arthritis or tenosynovitis, malabsorption syndrome or running-stunting syndrome, immunosuppression, and occasionally respiratory disease and pericarditis, myocarditis, nephritis, and hepatitis (2,12,14,19,32,34). Lesions associated with reovirus in the joints, heart, kidney, and liver are primarily due to infiltration of lymphocytes and plasma cells (14). Immunosuppression is due to lymphoid depletion in the thymus and bursa of Fabricius (14). Central nervous system lesions have also been described in chickens because of reovirus (34).

However, the number of studies on reovirus in turkeys is limited. These include arthritis, synovitis, immune dysfunction, and poult enteritis caused by turkey reovirus distinct from chicken reovirus (6,7,10,15,21,31). Poult enteritis is a disease of complex etiologies, such as viruses, bacteria, and protozoa. Among various viruses, rotavirus, coronavirus, astrovirus, and turkey reovirus have been associated with it (3,23,35). Poult enteritis is of great economic significance to the turkey industry and has been well described (3). It is a common disease in California turkey flocks, and it is second only to the diagnosis of colibacillosis in relative frequency among the diseases diagnosed between 1989 and 2001 (29). The disease in pouls is characterized by anorexia, diarrhea, dehydration, weight loss, high morbidity, and variable mortality. It is also known that turkey reovirus can induce enteritis and immunosuppression in turkeys but not in chickens (31). However, myocarditis associated with turkey reovirus has, to our knowledge, not been described.

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Two live and four dead, 17-day-old, male turkey poults were euthanatized by use of carbon dioxide for 10 min. An aliquot of the heart was mixed with buffered saline containing 100–200 μl of 0.8% phosphotungstic acid; a drop was then placed on to a 200-mesh formvar-coated grid for 5 to 10 min. Unabsorbed material was wicked away with filter paper. Grids were examined on a Zeiss EM 10A electron microscope for virus particles.

The intestine and its contents and hearts were also examined for infections by negative-stain electron microscopy. The RNA from the samples, and PCR was used to detect enteritis virus, rotavirus, and coronavirus, as described. DNA was also subjected to RT-PCR using primers targeting the S4 genome segment of the virus, designated as TK/CA/SEP-N605/08, and the RNA extracted from the virus isolated and from frozen hearts and it was subjected to sequencing according to the method described (23). The antibody used in IHC was a polyclone antibody raised against turkey reovirus (23). Similarly, IHC was performed on the hearts using a chicken antireovirus antibody supplied by National Veterinary Services Laboratory (NVSL, Ames, IA).

**Virology.** Pools of hearts were mixed with buffered saline containing antibiotics, triturated with sterile silica with a pestle and mortar, and clarified by centrifugation at 1780 × g for 10 min. An aliquot of the supernatant was passed through a 0.25-μm membrane filter. The filtrate was inoculated on to monolayers of primary chicken embryo liver (CEL) cells and incubated at 37 C in an atmosphere containing 5% CO₂ for 5 days, at which time a cytopathic effect (CPE) was apparent. The cells were harvested and subjected for examination by negative-stain electron microscopy.

**Negative-stain electron microscopy.** Harvested CEL cells were clarified by centrifugation at 1780 × g and centrifuged at 140,000 × g for 75 min to pellet the virus. Pellets were resuspended in 0.3 to 1.0 ml deionized water, and 2–3 μl was mixed with 100–200 μl of 0.8% phosphotungstic acid; a drop was then placed on to a 200-mesh formvar-coated grid for 3 to 5 min. Unabsorbed material was wicked away with filter paper. Grids were examined on a Zeiss EM 10A electron microscope for virus particles. The antibody used in IHC was a polyclone antibody raised against turkey reovirus (23). Similarly, IHC was performed on the hearts using a chicken antireovirus antibody supplied by National Veterinary Services Laboratory (NVSL, Ames, IA).

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Laboratory (SEPRL) enteric reovirus δNS sequences are EU400274 through EU400299.

Serology. Sera from two turkeys submitted live were tested for reovirus by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s (IDEXX, Westbrook, ME) instructions. In addition, six sera from 30-day-old and five sera from 44-day-old turkeys of the same flock were tested for reovirus by ELISA. The reovirus antigen used in the ELISA was of chicken origin.

The two sera from the birds submitted were tested by standard methods for antibodies to Avian paramyxovirus (APMV) type I by hemagglutination inhibition (HI) test, Avian influenza virus (AIV) by agar gel immunodiffusion (AGID), and for Salmonella Pullorum, Salmonella Typhimurium, Mycoplasma gallisepticum (MG), Mycoplasma meleagridis (MM), and Mycoplasma synoviae (MS) by plate agglutination test, using the reagents from the NVSL.

Bacteriology. Swabs from the air sac, lungs, and livers were plated on 5% sheep blood agar and MacConkey agar (Remel, Lenexa, KS) and incubated at 37 °C and 7.5% carbon dioxide for 24 to 48 hr. For Salmonella spp. intestinal contents were inoculated in Selenite broth (Remel), incubated aerobically at 36 °C for 18 to 24 hr, and then plated onto brilliant-green agar with 20 μg/ml novobiocin and xylose-lysine-tergitol agar (Remel). These plates were incubated aerobically at 36 °C and examined at 24 and 48 hr for colonies of Salmonella spp. Swabs of air sacs were also plated onto modified Frey’s agar and inoculated into Frey’s broth and incubated at 37 °C in 7.5% carbon dioxide and 98% humidity for the isolation of Mycoplasma spp. The plates were observed for 14 days for typical Mycoplasma colonies. Broth cultures were plated on to Frey’s agar after 7 days of incubation, and the plates were observed for 7 days.

Retrospective search. To determine the prevalence of myocarditis in turkey poults, a search of data on turkey submissions diagnosed with myocarditis submitted to CAHFS between 1991 and 2009 was carried out.

RESULTS

Postmortem examination of the birds revealed mild-to-moderate increase in pericardial fluid and mild-to-moderate dilation of right ventricles in four birds. All birds had pale epicardium, which extended in to the myocardium, and the condition was most prominent in the left ventricles; however, the right ventricles were involved in two birds (Fig. 1). Lungs were mildly to moderately congested and edematous in three birds. There was 4–5 ml of serous fluid in the abdominal cavity in five birds. The small intestine was mildly distended with watery contents in the lumen, and the serosa was pale in three birds. Kidneys were moderately pale in five birds. Spleens were pale in two birds, and grossly, the bursa of Fabricius appeared normal in all birds.

Microscopically, the myocardium of the auricles and ventricles in most of the birds had multifocal infiltration of lymphocytes, mixed with macrophages and plasma cells randomly scattered throughout (Fig. 2). Heterophils were also present within the myocardium but were infrequent. There were also foci of necrosis of the myofibers accompanied by similar inflammation and the presence of multinuclear cells (Fig. 3). The inflammation was multifocal in...
the myocardium in most sections, but it was diffuse in a few sections. There were scattered lymphoid nodules within a few sections of the myocardium. Most of the livers had acute centrilobular degeneration, characterized by vacuolation of hepatocytes and necrosis without any evidence of inflammation. Liver from one bird had multifocal necrosis of the hepatocytes with infiltration of lymphocytes, plasma cells, and macrophages randomly scattered throughout. There was mild-to-moderate lymphoid depletion in the bursa of Fabricius of most birds, and interstitial edema was observed in two birds (Fig. 4).

Other changes included increased numbers of mononuclear phagocytic system cells in the spleen, mild increased cellularity in the lamina propria of the intestine, infiltration of a few mononuclear inflammatory cells in the interstitium of a few lungs, and mild lymphocytic infiltration and fibrinoheterophilic inflammation in the air sacs of a few birds.

Immunohistochemistry. IHC of the various organs with the antibody for turkey reovirus revealed multifocal-to-diffuse staining for reovirus antigen, most commonly in the heart, bursa of Fabricius, and spleen, followed by intestine, lung, and liver. In the heart, the cytoplasm of myofibers and mononuclear inflammatory cells, most likely macrophages, were positive (Fig. 5a). The staining was intense in some sections of the heart and faint in others. In the bursa of Fabricius, most of the lymphocytes in the follicles, a few mononuclear inflammatory cells in the interstitium, and occasional epithelial cells lining the bursal plicae exhibited strong positive response to the reovirus antigen in their cytoplasm (Fig. 5b). Similarly, many of the mononuclear phagocytic system cells in the spleen were positive for the reovirus antigen in their cytoplasm (Fig. 5c). Other organs in which positive reovirus antigen was found in the cytoplasm included enterocytes and mononuclear inflammatory cells in the lamina propria of the intestine (Fig. 5d), cells lining the atria of the lungs including a few mononuclear inflammatory cells in the interstitium (Fig. 5e), and occasional hepatocytes and a few Kupffer cells in the sinusoids of the liver.

Transmission electron microscopy. Ultrastructural changes in the heart included degeneration and necrosis of myofibers characterized by irregular Z bands, enlarged and vacuolated sarcoplasmic reticulum, and moderately to severely enlarged mitochondria with disorganized cristae and loss in some. Some of the sarcoplasmic reticulum contained numerous viral particles in different stages of development (Fig. 6a). Some of these viruses appeared only as complete or incomplete ring forms. In some sarcoplasmic reticulum, there were fully mature, round, non-enveloped virions with inner core (Fig. 6b). There was occasional budding of the virions from the membrane of what appeared to be the sarcoplasmic reticum (Fig. 6b). The mature virions were icosahedral and measured about 85–88 nm in diameter, consistent with the morphology of avian reoviruses.

Virology. The filtrate of heart that was inoculated on to monolayers of primary chicken embryo liver cells revealed CPE 4–5 days later. CPE, typical of reovirus, was characterized by degeneration, separation, and rounding of cells.

PCR and sequencing. RNA extracted from the virus isolate from hearts designated TK/CA/SEP-N605/08 was positive for turkey reovirus by RT-PCR using primers of turkey-origin reovirus (23). The frozen heart was weakly positive for turkey reovirus by RT-PCR. RNA from the reovirus isolated from the hearts and the frozen hearts were negative for astrovirus, rotavirus, coronavirus, HEV, and FAV by RT-PCR and by PCR using primers for each virus, respectively.

Sequences of the segment of the CNS gene of the reovirus isolate from the field outbreak aligned with the sequences obtained from
enteric reovirus isolates at the SEPRL (Fig. 7). The GenBank accession number for the isolate TK/CA/SEP-N605/08 is FJ969425.

**Negative-stain electron microscopy.** The chicken embryo liver cells harvested revealed 75-nm, spherical virus particles consistent with the size and morphology of reoviruses. No viruses were detected in the homogenate prepared directly from heart and intestine.

**Serology.** Sera from the two live birds at 17 days of age had an ELISA titer of group 1 for reovirus. Sera from six birds from the same flock at 30 days of age had ELISA titers as follows: one bird at group 0, three birds at group 1, and two birds at group 2, respectively, for reovirus. Sera from five birds from the same flock at 44 days of age had ELISA titers as follows: one bird at group 1, two birds at group 2, one bird at group 3, and one bird at group 4, respectively for reovirus. All these sera were negative for *Salmonella* Pullorum and *Salmonella* Typhimurium.

Two sera from the live birds submitted for necropsy were negative for AI, APMV-1, MG, MS, and MM.

**Bacteriology.** No significant bacteria were isolated from the air sacs, lungs, and livers. No *Mycoplasma* spp. were isolated from the air sac, and the intestinal contents were negative for *Salmonella*.

**Retrospective search.** The retrospective search of turkey submissions diagnosed with myocarditis submitted to CAHFS between 1991 and 2009 revealed 50 cases. The age of the turkeys with myocarditis ranged from 10 to 45 days, with an average age of 20 days. Reovirus was isolated from the heart, liver, pancreas, and intestine in some of the turkeys (data not shown).

**DISCUSSION**

All six turkey poults in this study had myocarditis. In addition, four poults also had concurrent round hearts. These lesions are probably quite significant and caused the clinical signs and mortality of the poults in the flock. Lesions in the liver, spleen, intestines, and to an extent, in the lung also contributed to the

Fig. 4. Photomicrograph of bursa of Fabricius showing lymphoid depletion in the follicles and interstitial edema. H&E.

200 μm
problem. Most of the birds also had mild-to-moderate lymphoid depletion with interstitial edema in the bursa of Fabricius. Based on the demonstration of the reovirus antigen in the cytoplasm of myocytes of the heart and in the inflammatory cells, most likely macrophages and lymphocytes, myocarditis was probably caused by reovirus. In addition, reovirus was isolated from the heart and confirmed by PCR. Further, reovirus particles were demonstrated in the endoplasmic reticulum of myocytes by TEM. Reovirus antigen was also demonstrated in the cytoplasm of mononuclear inflammatory cells of the spleen, bursa of Fabricius, lamina propria of the intestine, lung, and liver, suggesting disseminated infection.

Similar myocarditis has been described in humans and in mice because of reoviruses and coxsackie B viruses (picornaviruses) (11,17,24). Even giant cells have been demonstrated with myocarditis because coxsackie B virus in humans and mice (17). It is interesting that multinucleated cells were seen in a few heart sections of the turkeys in the present study. The mechanism of formation of these multinucleated cells is not known, but multinucleated giant cells are common in inflammatory exudate in avian species. However, it is well known that one of the characteristics of avian reoviruses is the tendency to induce fusion of cells in cell culture; it has not been demonstrated in tissues (3). The possibility that these multinucleate cell formations were induced by reovirus should be considered.

It is interesting that most of the birds with myocarditis also had dilatation of the right ventricles with increased pericardial fluid and ascites. It is unlikely that myocarditis caused the right heart dilatation, but it might have contributed to the condition to a degree. Dilated cardiomyopathy, also called round heart disease of turkeys, is a common condition seen mostly in male turkeys at around 3 wk of age (5). It is probably not a coincidence that the turkeys examined in the present study were 17-day-old males. The cause of round heart disease in turkeys is not known, but evidence suggests that it has a genetic basis (5). It is probable that these turkeys had spontaneous dilated cardiomyopathy and happened to coincidentally contract a reovirus infection. One of four 10-day-old turkey poult submitted from the same breeder source (data not shown) also had dilatation of the right heart suggesting a genetic basis.

Except for the myocardium, the distribution of reoviral antigen in various organs in the turkeys in this study was similar to what has been described in experimentally infected young turkeys (23). But in one study, reovirus antigen was demonstrated by fluorescent antibody (FA) in the heart also in addition to other organs (18). In the present study, reoviral antigen was also demonstrated in the bursa of Fabricius, spleen, intestine, and the liver. Compared with the experimental study (23), there were a few differences in the distribution of viral antigen between the natural outbreak and the experimental study. In the natural outbreak, reovirus antigen was strongly positive in the lymphocytes of the bursal follicles and mononuclear cells of the lamina propria of the intestine. In addition, some of the cells lining the atria and some of the mononuclear inflammatory cells in the interstitium of the lungs were also positive in the present study, but this was not reported in the experimental study (23).

Except for myocarditis, the rest of the lesions described in these birds have been reproduced in turkeys with turkey reovirus (23). A possible explanation for the lack of myocarditis in the turkeys of the experimental study is that there are probably differences in the pathogenicity of turkey reovirus isolates at the genetic level, even though, sequencing and comparing of the reovirus isolated from the turkeys in this study to other reoviruses suggested that it was similar to the isolates associated with poult enteritis. The sequence data on the reovirus isolate from the field outbreak are based on the analysis of the eNS gene, located on the reovirus S4 genome segment (23). However, in one study involving reovirus in mice, it has been suggested that the M1 gene is probably the determinant of myocarditis (16,26,27). It will be interesting to analyze the M1 gene of the field reovirus isolate and compare it with the M1 gene of other corresponding reovirus isolates. It has also been demonstrated that by manipulating the genes in the reovirus through reassortment, it is possible to derive an efficient myocarditic reovirus variant in mice (16,26). Another possibility of lack of myocarditis in the experimental was the use of specific-pathogen-free turkeys, which are genetically different from commercial turkeys.

Myocarditis and arthritis/synovitis have been described in chickens because of the chicken reovirus (19,32). Myocarditis in addition to arthritis/synovitis, has also been demonstrated in chickens inoculated with reovirus isolated from turkeys (33). But arthritis/synovitis was not seen in the turkeys in the present study. This is probably due to the differences in the pathogenicity between reoviruses of chicken and turkey origins (13,18,23,25,31).

Myocarditis associated with other viruses has also been described in turkey poult. In one study, myocardial degeneration, inflammation, and pericarditis were reproduced in 6-day-old turkey poult by inoculating them with picorna-like virus (1). Picorna viruses measure about 25–30 nm in diameter. The virus particles found in the heart of turkeys by TEM in our study were around 87 nm in diameter consistent with the size and morphology of reoviruses (4). In another study in turkeys of myocarditis and round heart disease because of dilatation of the left heart, virus-like particles 60–90 nm in diameter were demonstrated in the endoplasmic reticulum of cardiac myocytes (20). The authors suggested that these virus-like particles resemble viruses of the avian leukosis group. However, the authors in that study did not characterize the virus particles. Myocarditis has also been demonstrated in turkey poult inoculated with eastern equine encephalomyelitis and Highland J viruses (9). Both viruses belong to alphavirus group of togaviruses and are endemic to eastern part of United States. Among bacteria, Salmonella Pullorum and Salmonella Gallinarum can cause similar lesions (28), but the birds were negative both by isolation and by serology.

Serologic titer for reovirus were very low in birds 4 wk after the outbreak of myocarditis. The reovirus antigen used in the ELISA system was that of chicken origin, suggesting that there is little cross-reaction to antibodies between the reovirus of chicken and turkeys.

The retrospective search revealed that myocarditis is of sporadic occurrence in California turkey flocks, about 50 cases in 17 yr, but this condition has not been reported in other places where turkeys are raised. The reason for this is not known. One possibility is that there are many types of reoviruses circulating in turkeys, and the type

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Fig. 5. Photomicrographs showing reovirus antigen in the (a) cardiac myocytes (arrows) and mononuclear inflammatory cells of the heart, (b) in the epithelial cells (arrows) and lymphocytes of bursa of Fabricius, (c) in the mononuclear cells of the spleen, (d) in the enterocytes and mononuclear cells of the lamina propria in the intestine, and (e) in the mononuclear cells lining the atria and interstitium of the lung. Immunoperoxidase labeling, hematoxylin counterstain.
of reovirus that causes myocarditis in addition to enteritis, is endemic to California turkey flocks.

It is not known how myocarditis was induced by reovirus in these turkey poults, nor is the pathogenesis of the reovirus in turkeys known. It is probable that reoviruses are transmitted orally in the turkeys, and the virus multiplies in the intestine and in the bursa of Fabricius, enters the circulation, becomes viremic, and affects various organs, including the heart, the spleen, the lungs, and other organs. It is also known that reovirus can be transmitted vertically. This may explain why we are seeing myocarditis in some birds as young as 10 days of age (data not shown).

To study the pathogenesis and prove that the reovirus isolated from this outbreak can cause myocarditis and other lesions, a well-designed experiment is essential to reproduce the disease in specific-pathogen-free turkey poults.

REFERENCES


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