Canine coronavirus-associated puppy mortality without evidence of concurrent canine parvovirus infection

J. F. Evermann, J. R. Abbott, S. Han

Abstract. This report presents 2 cases in which puppy fatalities were associated with canine coronavirus (CCV), but no evidence of concurrent canine parvovirus (CPV-2) disease was observed. Case 1 involved a 7-week-old, male short-haired Chihuahua, which had become lethargic 24 hours after purchase from a pet store. Within 72 hours, the puppy began to vomit, had diarrhea, and was admitted to the veterinary clinic, where it was placed on IV fluids. The parvovirus Cite® test was negative. The puppy died within 12 hours of admission and was submitted for diagnostic workup. Gross pathology revealed an enteritis suggestive of CPV-2. Histopathology on intestines showed scattered dilated crypts with necrotic cellular debris and neutrophils. There was moderate depletion and necrosis of lymphoid follicles. Electron microscopy (EM) on intestinal contents was positive for coronavirus and negative for parvovirus. Immunohistochemistry (IHC) on gut sections was positive for CCV and negative for CPV-2. Case 2 was an 8-week-old, male Shih Tzu, which was admitted to the veterinary clinic exhibiting symptoms of severe gastroenteritis with abdominal pain. The referring veterinarian euthanized the puppy, and the entire body was submitted for diagnostic evaluation. Necropsy revealed a severe ileo-cecal intussusception and segmental necrotic enteritis of the small intestine. Electron microscopy of the intestinal contents was positive for coronavirus and negative for parvovirus. Immunohistochemistry on sections of affected gut were positive for CCV and negative for CPV-2. These cases emphasize the importance of pursuing a diagnosis of CCV in young puppies when CPV-2 disease has been ruled out by IHC.

Key words: Canine coronavirus; canine enteritis; canine parvovirus; immunohistochemistry.

Canine Coronavirus (CCV) was initially reported as the causal agent of acute enteritis in dogs housed in a military canine unit in 1974.[8] The morbidity was high, but the mortality rate was low.[8] Over the next 10 years, the epidemiology of CCV was investigated further, and it was generally regarded as a mild, usually sublethal, highly contagious viral infection of young dogs, especially puppies less than 12 weeks of age.[5,8,19,25] In 1978, another canine enteritis virus was reported, canine parvovirus-2 (CVP-2).[3,5] This virus had a high morbidity and mortality in a high percentage of the dog population world-wide.[3,5] Due to the severity of the disease caused by CPV-2, a rapid diagnostic assay was developed, and both inactive and modified live vaccines were utilized. However, experimental pathogenesis studies using CVP-2 in specific-pathogen-free dogs showed some viral replication and pathogenesis, but the mortality rate was much less severe than observed in street (conventional) dogs.[1,3] The importance of concurrent viral infections as a cause of the severe fatal enteritis in dogs was initially speculated based on case investigations.[3,9,14,17,18,24] Wherein multiple infectious agents were reported, such as CPV-2 and CCV, CPV-2 and canine rotavirus, and even all 3 viruses simultaneously.[3] This led to experimental studies that demonstrated the severity of clinical signs were more pronounced in dogs that had both CPV-2 and CCV infections compared with CPV-2 alone.[1]

Over the past 26 years, the etiology of canine enteritis has changed as variants of CPV-2 have evolved.[4,20] The CPV-2 variants have become more adapted to the gastrointestinal tract, are less virulent, and are carried in a higher percentage of dogs and cats as subclinical infections.[4] In contrast with CPV-2, the virulence of CCV was not observed to change as rapidly.[22,23] However, beginning in the late 1990s and into 2000, reports from Japan,[2] Australia,[11] and Italy[12,15] have indicated that CCV appeared to be more virulent than was previously recognized. This report summarizes 2 cases in which dogs were reported to have severe gastrointestinal symptoms, and CPV-2 was regarded as the primary differential. After diagnostic testing was performed, it was noted that both dogs were positive for CCV and negative for CPV-2. The implications of these findings will be discussed in light of reports of newer strains of CCV in the dog population.

The first case involved a 7-week-old, male, short-haired Chihuahua. The puppy was acquired from a pet store and vaccination history was not available. Four days after arrival at the residence, the puppy was lethargic and anorexic. At the veterinarian's office, a parvovirus Cite® test was negative. Within a day, the puppy began vomiting and developed diarrhea, and on the third day, the puppy died. The whole body was submitted to the diagnostic laboratory for analysis. Selected tissue samples were collected at necropsy and submitted for histopathology and electron microscopy (EM). The second case was an 8-week-old, male Shih Tzu. The puppy was admitted to the referring veterinarian with severe gastroenteritis. History of the litter revealed other littermates had similar symptoms and had died. The puppy was eutha-

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nized and a necropsy performed at the clinic. The puppy had an intussusception. Selected tissue samples were collected and fixed in buffered formalin for histopathology. Selected fresh tissues (lung, liver, kidney, spleen, small intestine) were also submitted for bacteriology. Gastrointestinal contents were submitted for EM.

Tissues collected for histopathology were fixed in 10% buffered formalin for at least 24 hours. The tissues were trimmed, embedded in paraffin, and 5-µm sections were stained with hematoxylin and eosin (HE). Formalin-fixed sections (3 µm thick) were cut from paraffin blocks and placed on glass slides for immunohistochemistry (IHC). Sections were deparaffinized in a clearant and placed in absolute alcohol, and then brought to water through graded alcohols. Endogenous peroxidase on the CPV-2 sections were inhibited by a 5-minute incubation in 3% hydrogen peroxide in methanol. Sections were enzyme digested in 0.1% trypsin and 0.1% calcium chloride in 0.05 M Tris, pH 7.6, for 30 minutes at 37°C for CPV-2. For CCV, sections were placed in Zymed citrate buffer for steaming for 30 minutes and cooling at room temperature (RT) for 20 minutes. Sections were blocked for nonspecific binding of secondary antibody in 5% normal goat serum for 5 minutes at RT and incubated in diluted primary antibody for 30 minutes at RT. For CPV, sections were incubated with anti-CPV-2 (murine monoclonal),[a] and for CCV, sections were incubated with anticoronavirus (murine monoclonal).[b] Murine ultra streptavidin detection system horseradish peroxidase[c] was used as the detection system for both primaries. After the biotinylated linking antibody (30 minutes at RT), the CCV sections were treated 5 minutes with 3% H_2O_2 in water at RT to block endogenous peroxidase. Sections were incubated 30 minutes at RT in the ultra streptavidin enzyme complex, labeling reagent, and subsequently in AEC (3-amino-9-ethyl-carbazole in N,N-dimethylformamide)[d] for 2 × 4 minutes at RT. Following rinse, sections were counterstained 4 minutes with Mayer hematoxylin and mounted. Known positive intestine controls were used for both CPV-2 and CCV staining. Negative controls consisted of negative isotype (IgG2a) controls at the same protein concentration as the positive primary monoclonal antibodies.

Gastrointestinal contents from both cases were processed for EM. The samples were placed in 1 ml of sterile water, vortexed, and centrifuged for 20 minutes at 31,000 × g at 4°C. The supernatant was removed and 1.5 ml added to a microcentrifuge tube. The tubes were centrifuged for 30 minutes at 13,000 × g. The pelleted material in each tube was resuspended with a 1-ml mixture of distilled water, 4% phosphotungstic acid, and 100 µl of 2% bovine serum albumin. The resuspended pellet was sprayed into a formvar-coated grid, allowed to dry, and observed with a Hiatachi electron microscope. Bacterial culture was not conducted on the first case. Culture of the second case was conducted on the lung, liver, kidney, spleen, and small intestine.

The laboratory findings on the first case revealed that, at the time of necropsy, the dog was in good postmortem condition. The perianal region and ventral tail were stained with small amounts of dark red to black tarry feces. There was a strong fetid odor emanating from the cadaver. The serosal surface of the small intestines was segmentally roughened and the jejunum and ileum contained multiple, small, red clots. The colon contained small amounts of dark red to black tarry feces. Multifocal petechia were scattered throughout the lungs. Gross diagnoses of moderate, diffuse, hemorrhagic enteritis and mild interstitial pneumonia were made and the changes were considered suggestive of CPV-2 infection with associated bacterial septicemia causing pneumonia. Within the small intestines, small numbers of randomly distributed crypts were mildly to moderately dilated and contained small amounts of necrotic cellular debris and occasional neutrophils. The lining epithelium of the villus tips was mildly to moderately attenuated, with occasional necrotic epithelial cells. The numbers of lymphocytes and plasma cells in the lamina propria were mildly increased. However, the lymphoid follicles were occasionally sparsely populated in the follicular center, with scattered necrotic lymphocytes. Small numbers of short rod-shaped bacteria were in the lumen and occasionally extended deep into the crypts. The periarteriolar lymphoid follicles in the spleen had mildly decreased cellularity, with scattered necrotic lymphocytes. Histologic diagnoses of mild, lymphocytic and plasmacytic enteritis, enteric and splenic lymphoid depletion, and necrosis were made. Although epithelial necrosis lining the proximal portions of the villi and the villus crypts was mild, pathologic lesions of the intestine varied in severity by segment. The lesions were considered consistent with viral infection, such as CPV-2. Electron microscopy on intestinal contents indicated the presence of coronavirus particles, but not parvovirus. Immunohistochemistry staining (red) identified CCV antigen within epithelial cells lining the villus tips, but not deep in the villus crypts (Fig. 1A). This was consistent with the epithelial tropism expected with CCV. No staining was seen with control antibodies (Fig. 1B). In addition, no CPV-2 antigen was identified in dilated crypts containing necrotic cellular debris (Fig. 1C).

The laboratory findings on the second case revealed that the puppy cadaver was in good postmortem condition. The perineal area had moderate amounts of adherent dried feces and the mucous membranes were dry, consistent with dehydration. An 8-cm section of distal jejunum and ileum was strangulated through the ileo-cecal orifice (ileo-cecal intussusception). The Peyer's patches were sunken and the small intestines and colon contained moderate amounts of yellow, liquid digesta and feces, respectively. The gross lesions were interpreted as enteritis with intussusception, consistent with viral diarrhea. The small intestine had diffuse blunting of the villi. Intestinal crypts within the duodenum and jejunum were widely spaced by edema, and the crypts were often lined by attenuated epithelia and dilated with eosinophilic cellular debris, pyknotic nuclei, and sloughed cells (crypt necrosis). Gut-associated lymphoid tissues were severely depleted and several crypts were prolapsed within the empty patches. An 8-cm section of ileum associated with the ileo-cecal intussusception lacked cellular details and was composed of pyknotic nuclei and eosinophilic cellular debris (ischemic necrosis). The jejunal–ileal and ileo–cecal junctions had severe crypt necrosis and villous blunting, and the submucosa, muscularis mucosa, and serosa were severely expanded by blood and edema (Fig. 2). Additionally, the thymus and lymph nodes were severely depleted of lympho-
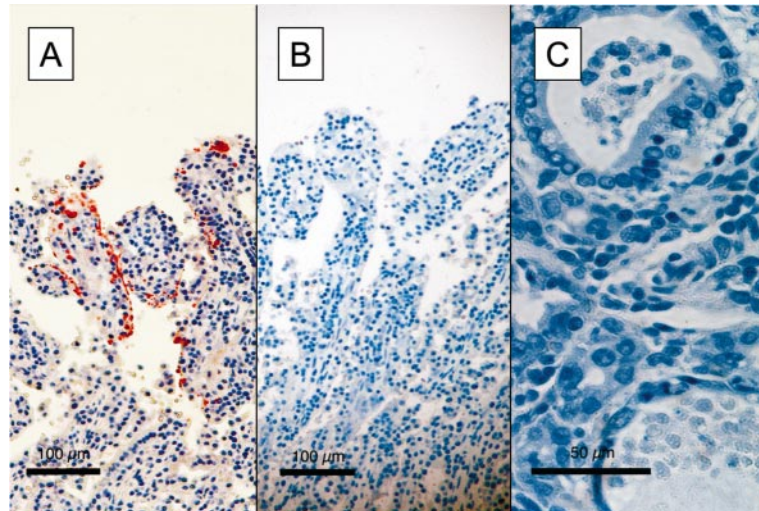
Figure 1. Photomicrographs of the small intestine from case 1. **A**, Immunohistochemistry staining with murine anticoronavirus antibody. **B**, Negative isotype control sections. **C**, Immunohistochemistry with murine anticanine parvovirus antibody. Hematoxylin and eosin. Bar size as noted.

cytes. The bone marrow was highly cellular, with a predominance of myeloid precursors. The histologic lesions were interpreted as severe necrotizing enteritis with diffuse lymphoid depletion, most consistent with a viral etiology and possibly secondary bacterial septicemia. Bacterial culture of the tissue pool and lungs was positive for a low to moderate growth of *Escherichia coli* and *Enterococcus* sp. Fecal culture for *Salmonella* sp. was negative, and Victoria blue stain for *Campylobacter* sp. was negative. Electron microscopy of the feces was positive for coronavirus and negative for parvovirus.

Immunologic staining of affected intestine for CCV from the paraffin-embedded blocks revealed immunoreactivity within the cytoplasm of small clusters of partially necrotic enteric epithelial cells. This staining was characterized as red intracytoplasmic staining of coronavirus-infected cells. Immunologic staining of the same intestinal sections for CPV-2 was negative. The low number of CCV-positive epithelial cells was attributed to the longevity of this puppy's disease and the fact that supportive therapy was administered for several days preceding death. The laboratory results indicated that severe necrotizing enteritis in this puppy was due to, or included, concurrent infection with CCV. Despite the similarity of these lesions to CPV-2, the virus was not identified by either EM or IHC, making the likelihood of infection with this virus low.

This report highlights 2 cases of fatal enteritis in which CCV was the only virus detected. The cases were initially
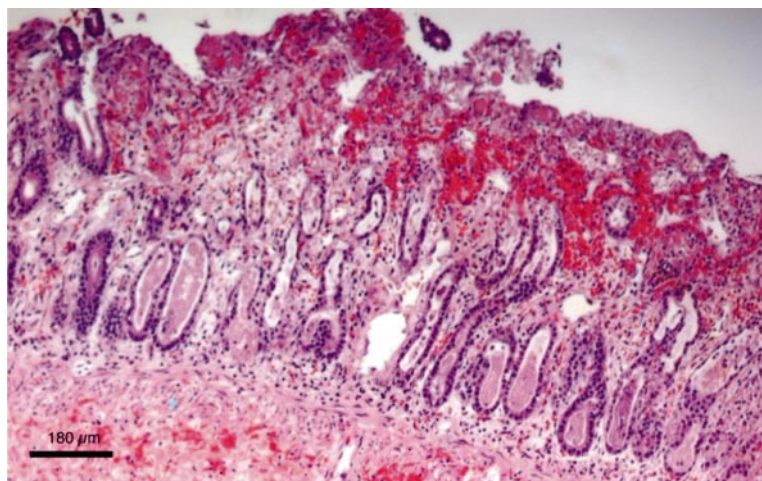
Figure 2. Photomicrographs of the section of ileum from case 2. Note severe villous blunting and villous crypts, which are distended with necrotic debris and lined by attenuated or necrotic epithelium. The lamina propria is widened by fibrin, proteinaceous material, and erythrocytes. These lesions were consistent with severe necrotizing enteritis, noted segmentally throughout the small intestines. Hematoxylin and eosin. Bar size as noted.

considered to be CPV-2 due to the acute onset and the severity of clinical symptoms.[5,7] Ancillary testing utilizing EM revealed the occurrence of coronavirus and absence of parvovirus in the gastrointestinal contents. This observation by itself provided support of a CCV-associated enteric disease.[3] However, the definitive test for viral enteritis in both cases was the CCV-positive IHC on gut sections.[14] This finding, together with negative CPV-2 IHC, provided direct evidence that CCV was not only present in high quantities in the enteric contents, but was actively replicating in the sections of the small intestine.[17] The results of this report raised several questions at the clinical and diagnostic levels regarding the severity of CCV as a primary enteric pathogen in dogs and the bias that can enter into a diagnosis based solely on clinical symptoms.[1,3,5,7] The readily available antemortem diagnostic assays for CPV-2 make it the first-step diagnosis when it comes to canine infectious enteritis.[3] Other than EM, there has not been a reliable antemortem assay for detection of CCV.[3,11,14] Recently, there have been studies conducted using more sensitive assays, such as polymerase chain reaction (PCR), on dogs with natural and experimental-induced enteritis, and the results support the importance of CCV in the canine enteritis complex.[2,9,10,16] Using PCR for detection of viral infections in dogs with diarrhea compared with those that were normal, it was shown that CCV was detected in 57.3% of feces from dogs with diarrhea, compared with 40% in normal dogs.[9] Detection of CPV-2 was 25% in dogs with diarrhea compared with 10% in normal dogs.[9] These results indicated that CCV is being carried by a much larger percentage of the normal population than has been previously recognized and that the virus contributed to the overall percentage of dogs with diarrhea.[1,3,9,22] Recent studies on the molecular epidemiology of CCV from Italy[13] and Australia[10] indicated that CCV is changing and that it appears to be increasing in virulence. On the basis of the cases presented, CCV was the only virus detected, with no evidence of concurrent CPV-2. It is conceivable that newer strains of CCV are emerging, possibly via recombination with related coronaviruses, and that CCV should be monitored by a reliable antemortem assay, such a CCV antigen enzyme-linked immunosorbent assay.[4,6,13,15] Also, clinicians and diagnosticians should be vigilant of possible CCV, especially in cases of canine enteritis in young puppies where CPV-2 has been ruled out using CPV-2-specific diagnostic assays, such as IHC, and where there is a cold-weather occurrence to the disease pattern more conducive to CCV than CPV-2.[19,21,26]

Sources and manufacturers

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b. Viro Stat, catalog #7321, Portland, ME.
c. Signet, catalog #2246, Dedham, MA.
d. Dako Cytomation, catalog #K3464, Carpinteria, CA.

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Eastern equine encephalitis in dogs

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Abstract. Eastern equine encephalitis virus (EEEV) is an *Alphavirus* that is endemic in the Southeastern United States. From 1993 to January 2005, the Veterinary Diagnostic and Investigational Laboratory in Tifton, Georgia, performed postmortem examinations on over 101 domestic canines exhibiting clinical neurological disturbances. In 12 of these dogs, brains were histologically suggestive of infection with EEEV. All dogs were less than 6 months of age, with no breed predilection. Clinical signs included pyrexia, depression, nystagmus, and lateral recumbency. Microscopically, brains from all 12 puppies contained infiltrates of lymphocytes, plasma cells, and histiocytes, with occasional neutrophils and random foci of astrocytosis and gliosis. There were mild to moderate perivascular infiltrates of neutrophils along with scattered lymphocytes, plasma cells, and macrophages in the meninges. Viruses isolated from brain homogenates of all 12 puppies were confirmed by indirect fluorescent antibody testing to be EEEV. Additionally, RNA extracted from the brains and viral cultures of 2 dogs were determined by a specific reverse-transcriptase polymerase chain reaction (RT-PCR) to contain EEEV. The single available serum sample exhibited a 1:8 serum neutralization titer to EEEV.

Key words: Alphavirus; canine; eastern equine encephalitis virus; PCR; perivascular cuffs; serology; virus isolation.

The etiologic agent of eastern equine encephalitis (EEE) is eastern equine encephalitis virus (EEEV), which is a member of the genus *Alphavirus* in the family Togaviridae and has been identified as a source of epizootics in domestic and wild animals since the 19th century.[16] The first disease outbreaks suspected to be EEE were reported in 1847, both in the eastern United States and in Texas and Michigan. Mosquitoes are often the vectors for EEEV, and these epizootics were usually concentrated around low-lying swampy areas.[7,8,16] In addition to transmission by mosquitoes, direct contact with contaminated blood, feces, and vomitus, chicken mites, semen, or assassin bugs can be a source of infection.[5,10,13] Clinical signs vary depending on species. Horses display pyrexia along with ataxia, head pressing, stupor, and depression. Mortality rates in horses may approach 90–100%.[8] Clinical signs in pigs, cattle, and goats are similar, with the addition of paddling and convulsions.[8,18] In humans,

there is an abrupt onset of high fever, lethargy, vomiting, convulsions, malaise, and myalgia.[8,19] The mortality rate is 50–75% in infected humans, and survivors may have neurological complications.[19] There have been anecdotal reports that suggest dogs are susceptible to EEEV, but there is little published information. During the last 12 years, the Tifton Veterinary Diagnostic and Investigational Laboratory in Tifton, Georgia (VDIL), has received over 101 cases of domestic dogs from the south Georgia area with neurological signs. Herein, we describe 12 of these cases that were diagnosed with EEEV infection.

All dogs were young (10 days to 6 months). Clinically, puppies were pyrexic (>40°C), anorexic, and exhibited diarrhea. These clinical signs rapidly (within 24–36 hours) progressed to include recumbency, nystagmus, depression, and seizures. All dogs died or were euthanized. There was no breed predilection, but affected puppies included bulldogs, Dalmatians, Walker hounds, Labrador Retrievers, an Australian cattle dog (Blue Heeler), and Boxers. All puppies were maintained outdoors. All but 1 case occurred in late spring or summer.

Submissions consisted of either whole cadavers (10) for necropsy or both fresh and fixed tissue specimens (2). Fresh tissues (brain and other organs if available) were cultured for viruses. Some tissues were collected in 10% neutral buffered formalin, processed, and embedded in paraffin. Four-

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