Genotypic Characterization of Canine Coronaviruses

Associated with Fatal Canine Neonatal Enteritis in the United States

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ABSTRACT

Emerging canine coronavirus (CCoV) variants that are associated with systemic infections have been reported in the European Union; however, CCoV-associated disease in the United States is incompletely characterized. The purpose of this study was to correlate the clinicopathological findings and viral antigen distribution with the genotypic characteristics of CCoV in 11 puppies from nine premises in five States that were submitted for diagnostic investigation at Cornell University between 2008 and 2013. CCoV antigen was found in epithelial cells of small intestinal villi in all puppies and the colon in two of the 10 puppies where colon specimens were available. No evidence of systemic CCoV infection was found. Comparative sequence analyses of viral RNA extracted from intestinal tissues revealed CCoV-II genotype in nine out of 11 puppies. Of the nine CCoV-IIIs, five were subtyped as group IIa, one as IIb, while three CCoVs could not be subtyped. One of the CCoV-IIa was isolated in cell culture. Infection with CCoV alone was found in five puppies, of which two also had small intestinal intussusception. Concurrent infections either with parvovirus (n=1), attaching-effacing *Escherichia coli* (n=4) or protozoan parasites (n=3) were found in the other six puppies. CCoV is an important differential diagnosis in outbreaks of severe enterocolitis amongst puppies between 4 days and 21 weeks of age that are housed in high population density. These findings will assist with rapid laboratory diagnosis of enteritis in puppies and highlight the need for continued surveillance for CCoV variants and intestinal viral diseases of global significance.
INTRODUCTION

Canine coronavirus (CCoV) was first recognized as a pathogen of dogs in 1971 (1) and together with transmissible gastroenteritis virus (TGEV) of swine and feline coronavirus (FCoV) is a member of the family Coronaviridae, subfamily Coronavirinae, genus Alphacoronavirus, species Alphacoronavirus-1 (2). Infection with CCoV is common in young dogs, particularly those housed in large groups such as kennels, shelters and breeding facilities (3-7). Traditionally, CCoV has been reported to infect the small intestinal villous absorptive epithelial cells resulting in mild and self-limiting diarrheal disease (8, 9). Young dogs, particularly those co-infected with other enteropathogens including parvovirus, can develop severe and often fatal disease (8, 10-12). The emergence of CCoV variants that are associated with severe clinical disease, mortality and systemic infections of dogs has been reported from several countries in the European Union (EU) (13-18). Although fatal CCoV-associated disease without other pathogens was reported in two puppies in the United States in 2005, the CCoVs were not characterized (19).

CCoVs circulate as two distinct genotypes: CCoV-I and CCoV-II, and both viruses can be detected in feces and tissues obtained from infected dogs by RT-PCR (7, 20). These genotypes can be distinguished on the basis of antigenic and genetic differences in the gene encoding the surface spike protein (21, 22). The viral spike protein binds to host cell receptor and triggers fusion of the viral and cellular membranes, making it an important determinant of cellular tropism and pathogenicity (23). Genotype I CCoV cannot be propagated in cell culture, and thus, is understudied compared to genotype II CCoV that is easily adapted to cell culture conditions. A similar situation exists with the closely related FCoV type I viruses and is suspected to be due to differential receptor requirements between genotypes (24). CCoV-II viruses use aminopeptidase N (APN) as receptor (25), while the receptor for CCoV-I viruses has not been identified. CCoV-
II viruses are classified into at least two subtypes, namely CCoV-IIa and CCoV-IIb, based on the sequence of the first 300 amino acids of the spike protein, a region known as the N-terminal domain (NTD). The NTD is an important determinant of intestinal tropism in the closely related TGEV (26, 27). Although the CCoV-IIa and -IIb classification is not part of the official CCoV taxonomy, these subtypes are widely referenced in the literature. Moreover, CCoV-IIa viruses also exist as two biotypes that differ in pathogenicity and tissue tropism and have an entirely CCoV-like NTD. Productive infection and replication of the classical CCoV-IIa biotype is restricted to intestinal epithelial cells. By contrast, an emergent pantropic CCoV-IIa biotype that can spread systemically is associated with profound leukopenia (28, 29), and has been detected from the tonsils, thymus, heart, lungs, liver, pancreas, mesenteric lymph node, spleen, kidneys, urinary bladder, muscles and brain of affected dogs by RT-PCR (13-18). Isolation of virus from extra-intestinal tissues has also been reported in some instances (13, 14, 16), but also failed in multiple other instances (18). The CCoV-IIb spike gene has a TGEV-like NTD (15, 30), and like TGEV, it causes enteritis in neonatal animals. Although it is generally restricted to the small intestine, CCoV-IIb RNA has been detected in extra-intestinal tissues of dogs co-infected with canine parvovirus (15-17) or with unknown co-morbidity (18). Finally, a third CCoV-II variant with a CCoV-I NTD has been reported in both the United States and Sweden (25, 31).

The purpose of the present study was to characterize the genotype of CCoV associated with outbreaks of fatal disease in young dogs submitted to the Animal Health Diagnostic Center (AHDC) at Cornell University between 2008 and 2013. Following localization of CCoV antigen in tissue sections by using immunostaining, the type and subtype of each virus was determined by sequencing of the NTD from purified viral RNA amplified by RT-PCR assay. Since changes in the proteolytic cleavage of the spike protein can modulate viral pathogenesis in FCoV (32), we
also characterized the sequence of the spike protein cleavage motifs. Lastly, we used phylogenetic analysis to compare the sequences of the spike NTD obtained in the present study with those previously reported in the EU. The results of our study will assist with rapid laboratory diagnosis of CCoV-associated enteritis in dogs and enhance surveillance for emerging intestinal viral variants of global significance.

MATERIALS AND METHODS

Diagnostic Investigation. The sample population consisted of dogs submitted to the AHDC at Cornell University between 2008 and 2013 with lesions of viral enteritis that were positive for the presence of CCoV antigen by immunohistochemical (IHC) staining. With the exception of puppies 4a and 4b in which selected tissues were collected by the referring veterinarian during a field necropsy, all cases were processed for complete necropsy including collection of multiple segments of gastrointestinal tract. In addition to gross and histopathological examinations of a standard set of tissues, bacteriological culture of intestinal specimens including *Salmonella* and *Campylobacter* species and fluorescent antibody (FA) tests on fresh frozen tissue sections for CCoV, group A rotavirus and canine parvovirus were performed on all cases. At the request of the referring veterinarian or according to the pathologist-in-charge, selected fresh tissues obtained from puppies 2, 4a, 4b and 8 were also processed for virus isolation. Dogs with respiratory signs or lesions were examined for the presence of canine distemper virus, canine parainfluenza and canine adenovirus by FA staining of frozen tissue sections.

Histopathology. Sections of brain, thymus, heart, trachea, lungs, liver, gall bladder, tongue, stomach, pancreas, small and large intestines, mesenteric lymph node, spleen, kidneys, adrenal glands, urinary bladder, skeletal muscles and bone marrow were fixed in 10% neutral buffered
formalin, embedded in paraffin, sectioned at 4-μm thickness, and stained with hematoxylin and
eosin. Selected sections of intestinal tract also were stained with tissue Gram stain and a modified
Steiner silver stain to further characterize bacteria when present. For IHC staining, sections of
tissues were deparaffinized and processed for antigen retrieval. After blocking endogenous
peroxidase activity with 3% hydrogen peroxide and treatment with normal goat or normal rabbit
serum for 5 minutes (Invitrogen, Carlsbad, CA), the slides were reacted with the coronavirus-
specific mouse monoclonal antibody FIPV3-70 (Custom Monoclonals International, Sacramento,
CA, USA) followed by biotinylated goat anti-mouse, streptavidin-peroxidase conjugate
(Invitrogen), chromogen, 3,3-diaminobenzidine-tetra hydrochloride and hematoxylin
counterstain. Duplicate intestinal sections from each puppy were stained with the group A
rotavirus-specific mouse monoclonal antibody 9-10 (33) and canine parvovirus-specific rabbit
polyclonal antibody CPV vp1/vp2 (Colin Parrish, Baker Institute, Cornell University). For
FIPV3-70 and 9-10 antibodies, heat antigen retrieval consisted of microwave in citrate buffer at
pH 6.0 for 20 minutes, whereas for CPV vp1/vp2 antibody, antigen retrieval was accomplished
by digestion with pronase for 30 minutes.

Virus Isolation. Ten percent tissue pools of lung, liver, spleen and intestine from puppy 2,
intestine from puppy 4a, lung and intestine from puppy 4b, and lung, liver, spleen, kidney and
brain from puppy 8 were prepared in Eagle minimal essential medium (MEM-E) containing 0.5%
bovine serum albumin and 10 μg/mL ciprofloxacin. After tissue disruption and low speed
centrifugation, 1 mL of the filtered supernatants from puppy 2, 4a and 4b were inoculated onto
monolayers of canine fibroblast-like A-72 cells (ATCC CRL-1542) and immortalized canine
kidney cells (AHDC, Cornell University), while supernatant from puppy 8 was inoculated onto
immortalized canine kidney cells and human colorectal adenocarcinoma HRT-18 (ATCC CCL-
244) cells grown in 25-cm² flasks as previously described (34). Supernatants from puppy 4a and 4b also were inoculated onto canine kidney MDCK cells (ATCC CCL-34) and HRT-18 cells, respectively. The extract was allowed to remain on the monolayer for 1–2 h and then rinsed off with phosphate buffered saline (PBS). Cells were cultured at 37°C in MEM-E containing 10% gamma-irradiated fetal bovine serum. At 5-7 day intervals, monolayers were disrupted with trypsin and new monolayers established at a 1:3 split ratio. Cultures were monitored on a daily basis for the presence of cytopathic effect. CCoV isolation was confirmed by FA staining with the mouse monoclonal antibody FIPV3-70.

RT-PCR and Genotyping. RNA was extracted from formalin-fixed and paraffin-embedded (FFPE) tissues with RecoverAll™ Total Nucleic Acid Isolation Kit according to the manufacturer’s instructions (Ambion, Foster City, CA, USA). The resulting RNA was reverse transcribed into cDNA using SuperScript™ III First-Strand Synthesis System for RT-PCR (Life Technologies, Carlsbad, CA, USA). The RT reaction was primed with random hexamers. The presence of coronavirus cDNA within the sample was confirmed as previously described by PCR directed at a conserved region of the 3’ UTR (35). Samples that tested positive for the presence of coronavirus RNA based on the 3’ UTR were further characterized as genotype I or II using oligonucleotide primers directed against S1/S2 and S2’ cleavage motifs (Fig. 1 and Table 1). Type II viruses were further characterized into subtypes on the basis of the NTD (Fig. 1 and Table 1). Previous studies on CCoV differentiate type I and type II CCoVs based on the sequence of the membrane (M) protein; however, it is unclear how changes in the M protein correlate with changes in the S protein. Therefore, we designed oligonucleotide primers against regions of the S protein that differ substantially between genotypes (Fig. 1 and Table 1). PCR was performed using Platinum Taq DNA Polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA).
according to the manufacturers’ instructions with an annealing temperature of 55°C. PCR products were analyzed by electrophoresis on a 0.8% agarose gel. Products of the expected size were purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA).

**Comparative Sequence Analysis.** The products of the RT-PCR assays were sequenced by using Sanger di-deoxy sequencing method (Biotechnology Resource Facility, Cornell University). The CCoV RNA extracted from clinical samples was classified as CCoV-I or CCoV-II based on RT-PCR and sequencing of the spike S1/S2 and S2’ cleavage sites. Sequencing was also used to distinguish CCoV-IIa and CCoV-IIb variants by using a combination of new and previously published spike-specific primers targeting the NTD (20). Oligonucleotides specific for CCoV-I NTD were also included in order to detect CCoV-I/CCoV-II recombinants (Fig. 1 and Table 1).

We sequenced and aligned the CCoV-II S2’ cleavage site, which is adjacent to the conserved coronavirus fusion peptide (36), to look for deviations from the CCoV-II consensus cleavage motif: K-R-K-Y-R-S, where K is the amino acid lysine, R is the amino acid arginine, Y is the amino acid tyrosine, and S is the amino acid serine. This amino acid motif is likely to be cleaved by a variety of trypsin- and cathepsin-like proteases, with cleavage occurring between the R and S residues. Comparative analysis of PCR amplified CCoV gene-specific sequences was performed on the N-terminus of the S gene using Clustal X (Conway Institute, UCD Dublin, Ireland) and viewed in Genious v6.1.7 (Biomatters Ltd., Auckland, New Zealand). Neighbor-joining trees were constructed in Clustal X using 10,000 bootstrap trials and viewed in FigTree v1.4.0 (Institute of Evolutionary Biology, Edinburgh, UK). The partial nucleotide sequences of CCoV spike genes have been deposited in the European Nucleotide Archive under the accession numbers: XXXXX.

**RESULTS**
**Clinical Findings.** The signalments and clinical presentations of 11 dogs from 9 premises investigated in the present study are presented in Table 2. No sex or breed predilections were noted. Affected puppies ranged in age from 4 days to 21 weeks with a median age of 7 weeks, and multiple puppies per litter and multiple litters were affected on most premises. The puppies were mostly housed in large groups that experienced severe clinical signs of intestinal illness and mortality in Indiana (n=1), Kansas (n=4), New York (n=4), Pennsylvania (n=1), and a litter in transit between shelters located in North Carolina and Rhode Island (n=1).

**Laboratory Findings.** The results of IHC staining of formalin-fixed and paraffin-embedded (FFPE) intestinal tissue sections for the presence of CCoV and other pathological, microbiological and parasitological findings in dogs investigated in this study are presented in Table 3. All puppies had lesions consistent with viral enteritis characterized by various degrees of atrophy of small intestinal villi (villous:crypt ratio approximately 1:2) that were lined with attenuated, low cuboidal to squamous epithelial cells (Fig. 2A). Immunostaining confirmed the presence of CCoV antigen within the cytoplasm of small intestinal villous epithelial cells in all of the puppies (Table 3 and Fig. 2B and 2C). Infection with CCoV extended from the villus:crypt junction to the tip of villi diffusely along the small intestine in puppies 5, 7, 8, and 9, multifocally in groups of epithelial cells in puppies 1, 3, 4a, 4b, and 6b, and within scattered individual epithelial cells in puppies 2 and 6a. Of the 10 puppies in which colonic sections were available, only puppies 8 and 9 showed CCoV antigen within epithelial cells along the surface and crypts of the colon. Although lymphoid depletion of Peyer’s patches was present in 7 puppies, none of 10 puppies where lymphoid tissues were examined by IHC showed positive staining for the presence of CCoV antigen. Rare individual CCoV antigen positive cells, most likely antigen presenting dendritic cells, were scattered within the mesenteric lymph nodes in
puppies 6a, 6b, and 9. None of the puppies were positive for the presence of group A rotavirus by FA and IHC staining or Salmonella and Campylobacter species by bacteriological culture of intestinal specimens. However, concurrent intestinal pathogens were present in six puppies. Puppy 4a had severe acute multifocal crypt epithelial cell necrosis that was associated with canine parvovirus antigen as determined by FA and IHC staining. None of the other 10 puppies were positive for the presence of canine parvovirus antigen by FA and IHC staining. In addition to diffuse attenuation of villous epithelial cells, sections of small intestines from puppies 2 and 5 also showed multifocal epithelial cell necrosis and sloughing into the lumen that was respectively associated with large and moderate numbers of cytoplasmic coccidian parasites. Parasitological examination of fecal samples confirmed the presence of *Isospora* species and *Cystoisospora ohioensis* in puppies 2 and 5, respectively. The small intestine of puppy 2, a young Yorkshire Terrier also showed multifocal crypt ectasia, a finding associated with protein-losing enteropathy in this breed of dogs (37). The lumen of many colonic crypts in puppy 4b contained small numbers of pale eosinophilic, pear-shaped flagellated protozoan parasites consistent with mild trichomoniasis. Closely adherent Gram negative coccobacilli consistent with attaching-effacing *Escherichia coli* (AEEC) were present multifocally along the apical membrane of villous epithelial cells in sections of small intestines from four puppies; large numbers of bacteria were present in puppy 4a, while puppies 5, 6a and 6b had small numbers of adherent bacteria (38). Bacteriological culture of segments of jejunum taken from puppies 6a and 6b yielded *E. coli* isolates that were typed as O untypable:H49 and O8:H14, respectively (*E. coli* Reference Center, The Pennsylvania State University). The isolate from puppy 6b also was positive for the presence of *stxII*, encoding Shiga-like toxin type II, and isolates from puppies 6a and 6b were negative for the presence of *eae*, encoding intimin-gamma. Consistent with clinical signs of weakness and
vomiting, aspiration pneumonia was present in puppies 2, 5, 6a, and 7. Other lesions including bronchopneumonia in puppies 4b and 6b and hepatocellular necrosis in puppies 8 and 9 were considered incidental findings. With the exception of puppy 4a where lung tissue was not available, none of the lung sections taken from the remaining 10 puppies were positive for the presence of CCoV antigen by IHC staining.

**Virus Isolation.** CCoV was isolated from puppy 4b and canine parvovirus was isolated from puppy 4a. CCoV cytopathic effect (CPE) consisting of cell rounding, cell death and syncytia formation was observed 24 hours post-inoculation of canine A-72 cells (Fig. 3A and 3B). The other two cell lines did not yield CCoV. Infected A-72 cells were positive for the presence of CCoV antigen by FA staining (Fig. 3C). Sequencing of CCoV RNA extracted from infected cell culture lysates further confirmed infection of puppy 4b with CCoV-IIa that corresponded to the RT-PCR assay and sequencing results from corresponding FFPE intestinal specimen.

**RT-PCR, Genotyping and Comparative Sequence Analysis.** Coronavirus RNA was detected by RT-PCR in nine out of 11 puppies; sufficient CCoV RNA was not recovered from puppy 1 and 6b (Table 4). Comparative sequence analysis revealed CCoV-II in all 9 cases. Consequently, the S1/S2 cleavage site was not analyzed because it is only present in CCoV-I genotype (Fig. 1) (39). The CCoVs from puppies 3, 4b, 5, 7 and 8 were subtyped as CCoV-IIa, while the CCoV from puppy 9 was subtyped as CCoV-IIb, and those from puppies 2, 4a, and 6a could not be subtyped. The S2’ site was sequenced in eight out of the nine puppies from which CCoV RNA was successfully extracted. No variations in amino acid sequence at the cleavage site were detected (Table 4). Based on a neighbor-joining phylogenetic tree of available CCoV NTDs (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/), CCoV-IIa viruses from puppies 3, 4b, 5, 7 and 8 clustered with other CCoV-IIa viruses, including those......
associated with previous reports of pantropic CCoV in the EU, while the CCoV-IIb from puppy 9 clustered with CCoV-IIb from the EU (Fig. 4).

**DISCUSSION**

Although closely related to pantropic CCoV-IIa, the CCoV-IIa associated with neonatal mortality in our study were restricted to the intestinal tract, and therefore, consistent with classical enteric CCoV infection (19, 40). The use of immunostaining to confirm CCoV disease in our study might explain the lack of detection of pantropic CCoV. Previous work on pantropic CCoV has relied mainly on RT-PCR to detect systemic spread. RT-PCR is very sensitive and can detect viral genome in tissues without productive viral replication or infection being present. IHC is arguably a better method for detecting clinically relevant infection because it requires high levels of viral antigen, and therefore viral replication, to yield a positive result. In addition, IHC can localize antigen to biologically relevant cellular compartments such as the cytoplasm of infected villous epithelial cells (Fig. 2B and 2C). Because detection of viral antigen by immunostaining is highly time-dependent, early infection with concentrations of CCoV antigen below the detection limit of the assay cannot be ruled out completely as a reason for the lack of detection of CCoV in extra-intestinal tissues of our puppies. Confirmation of pantropic CCoV in previous reports relied primarily on RT-PCR assays; however, whether viral replication or infection is present in individual tissues cannot be conclusively confirmed by this method alone. Conversely, isolation of CCoV viruses from extra-intestinal tissues has been reported, but the clinical significance of this finding is unclear without co-localization of extra-intestinal lesions with viral antigen. Previous reports with FCoV have confirmed viral RNA by RT-PCR in tissues obtained from otherwise healthy cats (41). As with CCoV, immunostaining is generally negative in these cases. In the same studies, the positive RT-PCR results were attributed to viremia, a
finding that is common among cats experiencing asymptomatic enteric infection with FCoV (42).

The possibility that viremia may be associated with low-level virus replication within tissues of healthy animals cannot be ruled out completely (41).

The negative virus isolation result from pooled samples of lung and intestine from puppy 2 might be attributed to low level of CCoV as suggested from the small numbers of epithelial cells with viral antigen in immunostained sections of small intestine from this puppy. Conversely, negative CCoV isolation results from multiple non-intestinal tissues taken from puppies 2, 4a and 8 further suggest a lack of viral replication in extra-intestinal tissues from these puppies.

Viral tropism extended to the large intestines in puppies 8 and 9, respectively infected with CCoV-IIa and CCoV-IIb. To our knowledge, colonic infection has only been documented in one of five experimentally-infected 10-week-old puppies, 10 days post-inoculation with the C54 reference CCoV-II (40, 43). Given that concurrent pathogens were not found, extensive intestinal infection with CCoV alone most likely accounted for the demise of puppies 8 and 9. In support of this interpretation was the presence of hepatocellular necrosis in these puppies; a common finding in animals with extensive loss of intestinal barrier integrity which results in showering of the portal circulation by toxic products. Interestingly, these were the youngest puppies (4 days and 2 weeks), and host factors such as age may have contributed to CCoV infection of colonic epithelial cells.

Lymphopenia is a common clinical finding in reports of dogs with pantropic CCoV infection. Although hemograms were not available, lymphoid depletion of the thymus, mesenteric lymph nodes or intestinal Peyer’s patches was present in eight out of 10 puppies that had lymphoid tissues available. Similar lymphoid depletion was found in two puppies with fatal
CCoV-associated enteritis previously described in the United States (19). Consistent with the previous report, CCoV infection of lymphoid tissues was not found in our study. Infections with other coronaviruses including Middle Eastern Respiratory Syndrome (MERS) coronavirus, severe acute respiratory syndrome (SARS) coronavirus, equine coronavirus (ECoV), and FCoV are associated with lymphopenia (44-47). Where the cause of lymphopenia has been investigated, it is attributed to indirect mechanisms secondary to the viral infection such as cytokine-mediated apoptosis (48, 49).

All but one of the puppies in our study originated from high-density housing where outbreaks of enterocolitis were ongoing. Over half (6/11 or 54%) of our cases had concurrent intestinal infections with various combinations of pathogens. Co-infection with canine parvovirus, a known risk factor for CCoV associated mortality, was found in only puppy 4a. Severe intestinal damage can result in translocation of toxic products to extra-intestinal tissues, particularly the lungs and liver. Consistent with this observation, bronchopneumonia and hepatocellular necrosis were present in four puppies. Additionally, aspiration pneumonia, a common clinical complication seen in young debilitated puppies that are vomiting was also present in four puppies. The clinical significance of intestinal infection with AEEC in four puppies is unclear; however, the presence of small intestinal epithelial colonization by these organisms likely contributed to clinical signs of intestinal dysfunction leading to mortality in these cases. Clearly, host and environmental factors such overcrowding of puppies, co-infections with intestinal pathogens, pathogen load and degree of maternal immunity can determine the outcome of CCoV-associated enteritis. Although mutations in the S2’ cleavage site were not found, it remains possible that unidentified viral factors also could have contributed to the fatal outcomes. These factors may include variations in the NTD, a region that is known to be
important for enterotropism in TGEV. Subtyping of CCoV-II was based on the amino acid sequence of the NTD (Fig. 1). Because both CCoV-IIa and -IIb viruses were associated with fatal outcomes, it appears that viruses with a CCoV-like NTD (subtype CCoV-IIa) or a TGEV-like NTD (CCoV-IIb) have the potential to cause fatal enteritis.

Intussusception was observed in the small intestine of two of the 11 puppies with fatal CCoV-associated enteritis (Table 3). Interestingly, puppy 3 was from a breeding facility where a littermate with small intestinal intussusception recovered following surgical resection and anastomosis. This breeder recalled having over a dozen other puppies with small intestinal intussusception over the last two years following the introduction of several breeders acquired from Sweden where CCoV outbreaks were documented around the same period (31). Interestingly, a similar association between CCoV infection and small intestinal intussusception was reported in one of the two puppies previously described in the United States (19). The pathogenesis of intestinal intussusception associated with enteric viral infection is not well understood; however, in human infants, intestinal intussusception has been associated with adenovirus and enterovirus infections as well as vaccination with a discontinued live-attenuated rhesus-human reassortant rotavirus tetravalent vaccine (50-52). The observation that CCoV-associated enteritis can sometime present with small intestinal intussusception suggests a similar pathogenesis.

Our study provides detailed pathological findings in 11 puppies with fatal CCoV-associated enteritis that originated from nine premises in five States in the United States between 2008 and 2013. Key regions of the viral spike gene were sequenced to determine if viral factors played a role in these CCoV-associated mortalities. Owing to the retrospective nature of the present study with available FFPE tissue samples collected at necropsy with variance in post-
mortem intervals, sample quality and degree of RNA crosslinking by formalin fixation combined with the relatively low ratio of viral to cellular RNA within tissues and the natural variability of CCoV S gene limited our ability to use next generation sequencing methods to capture a more complete assessment of the viral populations involved in these cases. Another limitation of the present study was our inability to subtype the CCoV-II viruses from three puppies which was likely attributable to divergent NTD that could not be amplified by our PCR primers. Lastly, approximately 50% prevalence of CCoV-I and CCoV-II co-infections has been reported previously; however, we were unable to detect any CCoV-I infections in our study. Our CCoV-I primers were based on multiple alignments of previously published CCoV-I virus sequences in the region of the S1/S2 and S2’ cleavage sites. It is possible that the puppies in our study were infected with divergent CCoV-I viruses that were not amplified by our S-specific primers.

This study revealed the presence of CCoV-IIb variants in the United States and highlighted the potential of CCoV-IIa and IIb to cause morbidity and mortality in puppies. Extended tissue tropism of CCoV to the large intestine was found in two puppies; however, pantropic CCoV infections were not identified. Isolation of CCoV-IIa from puppy 4b confirmed the validity of genotyping results obtained from the corresponding FFPE tissue sections. CCoVs should be considered as a differential diagnosis and specifically sought in outbreaks of severe enteritis among puppies up to 21 weeks of age particularly when housed in high population density, but also in cases with small intestinal intussusception or enteritis associated with infections caused either by parvovirus, AEEC or protozoan parasites.
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Figure Legends

Fig. 1. CCoV-I and CCoV-II spike genes with the location of N-terminal domain (NTD), S1 receptor binding domain, S1/S2 cleavage site (S1/S2), S2’ cleavage site (S2’), S2 fusion domain and transmembrane domain (TM). Note that the S1/S2 furin cleavage site is only present in CCoV-I viruses.

Fig. 2. Photomicrographs of small intestine from puppy 8 with typical lesions of CCoV infection. A. The villous epithelial cells are diffusely disorganized, attenuated and low cuboidal (arrows). Note that the space between the epithelial cells and the lamina propria is an artefact of processing (hematoxylin and eosin stain; bar=100μm). B. Cross-section of small intestine showing CCoV antigen in villous epithelial cells (arrows; immunohistochemical stain; bar=500μm). C. Higher magnification showing CCoV antigen in the cytoplasm of villous epithelial cells (arrows; immunohistochemical stain; bar=50μm).

Fig. 3. Canine fibroblast-like A-72 cells, 24 hours post-infection with puppy 4b CCoV. Phase contrast microscopy of uninfected cell culture monolayer (A) and CCoV-infected cell culture monolayer with cytopathic effect characterized by rounding and death of individual cells (B) (10X original magnification). Immunofluorescence microscopy of CCoV-infected cell culture monolayer (mouse monoclonal antibody FIPV3-70 followed by Alexa Fluor 488 conjugated anti-IgG; 20X original magnification).
Fig. 4. Phylogenetic tree based on the spike protein N-terminal domain (NTD). Sequences are identified as CCoV-IIa or -IIb followed by the case number.
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<th>Specificity</th>
<th>Sense</th>
<th>Sequence (5' to 3')</th>
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<td>2017*</td>
<td>Type 1 NTD</td>
<td>+</td>
<td>GGCTCAACATACAACCTACAGTCTAG</td>
<td>163</td>
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<td></td>
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<td>TACATACTGCTCTCAATC</td>
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</tbody>
</table>

*Previously described oligonucleotide (20)


lymphocytes and acute monocytosis after pantropic canine coronavirus infection in dogs. Virus Research 152:73-78.

30. 


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<table>
<thead>
<tr>
<th>Case</th>
<th>Location</th>
<th>Breed</th>
<th>Age</th>
<th>Clinical Signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>08-149076</td>
<td>NY</td>
<td>Golden Retriever</td>
<td>3 weeks</td>
<td>Breeder with 24 adults not affected; litter of 5 puppies with weakness, dehydration, vomiting and diarrhea; 2 died, 1 recovered; other litters; 3 puppies affected: 1 died, 2 recovered</td>
</tr>
<tr>
<td>09-89334</td>
<td>PA</td>
<td>Yorkshire Terrier</td>
<td>21 weeks</td>
<td>Central nervous system signs-hypoglycemia; a littermate with diarrhea died 4 days earlier</td>
</tr>
<tr>
<td>09-97736</td>
<td>ID</td>
<td>Spaniel</td>
<td>5 weeks</td>
<td>Breeder with 15-20 adults not affected; puppy with 4 days history of vomiting and diarrhea died during surgery for jejunoileal intussusception; 1 littermate with intussusception and 2 others ill</td>
</tr>
<tr>
<td>09-107207</td>
<td>KS</td>
<td>Maltese</td>
<td>8 weeks</td>
<td>Distributor with 800 puppies with history of respiratory signs, vomiting and diarrhea</td>
</tr>
<tr>
<td>09-110089</td>
<td></td>
<td>Basset Hound</td>
<td>8 weeks</td>
<td></td>
</tr>
<tr>
<td>09-123567</td>
<td>NY</td>
<td>Mixed</td>
<td>7 weeks</td>
<td>Breeding/research facility; puppies with pale mucous membranes, depression, dehydration</td>
</tr>
<tr>
<td>10-51534A</td>
<td>KS</td>
<td>Bichon Frise</td>
<td>8 weeks</td>
<td>Breeder with 200 adults not affected; 100 puppies with 20% mortality when 6 to 8-week-old, 1 to multiple puppies per litter died within 3-4 days of showing anorexia, vomiting and diarrhea</td>
</tr>
<tr>
<td>10-51534B</td>
<td>Mixed</td>
<td></td>
<td>8 weeks</td>
<td></td>
</tr>
<tr>
<td>12-120628</td>
<td>RI</td>
<td>Mixed</td>
<td>5 weeks</td>
<td>8 rescued weaned puppies in transit from NC; 4 died with weakness, lethargy, dehydration, hypothermia</td>
</tr>
<tr>
<td>12-159396</td>
<td>NY</td>
<td>Shepherd Mixed</td>
<td>2 weeks</td>
<td>Rescue bitch from KY; 5 puppies died from a litter of 8</td>
</tr>
<tr>
<td>13-47387</td>
<td>NY</td>
<td>German Shepherd</td>
<td>4 days</td>
<td>Breeding/boarding facility with 8 adults showing mild vomiting and diarrhea; 2 puppies died when 3- and 4-day-old from litter of 8 with abdominal pain and bloody stools</td>
</tr>
<tr>
<td>Case</td>
<td>CCoV IHC</td>
<td>Other findings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>----------</td>
<td>-------------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SI LI</td>
<td>Other tissues</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. 09-148076</td>
<td>Pos. Neg.</td>
<td>Neg.: lung, kidney, bone marrow Peysers’ patch lymphoid depletion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. 09-89334</td>
<td>Pos. Neg.</td>
<td>Neg.: lung, urinary bladder Protein-losing enteropathy, <em>Isospora</em> species, Peysers’ patch lymphoid depletion, aspiration pneumonia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. 09-97736</td>
<td>Pos. Neg.</td>
<td>Neg.: lung, liver Jeunoseal intussusception, Peysers’ patch lymphoid depletion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4a. 09-107207</td>
<td>Pos. NA</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4b. 09-110089</td>
<td>Pos. Neg.</td>
<td>Neg.: lung, MLN Trichomoniasis, Peysers’ patch lymphoid depletion, bronchointerstitial pneumonia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6a. 10-51534A</td>
<td>Pos. Neg.</td>
<td>Pos.: MLN (rare) AEEC, aspiration pneumonia, bone marrow depletion, thymic and MLN lymphoid depletion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6b. 10-51534B</td>
<td>Pos. Neg.</td>
<td>Pos.: MLN (rare) AEEC, bronchopneumonia, bone marrow depletion, thymic, MLN and Peysers’ patch lymphoid depletion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. 12-159396</td>
<td>Pos. Pos.</td>
<td>Neg.: thymus, lung, liver Mild, multifocal, acute hepatocellular necrosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pos. Neg.</td>
<td>Neg.: lung, stomach</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* SI, small intestine; LI, large intestine; Pos., positive; Neg., negative; NA, not available; MLN, mesenteric lymph node; AEEC, attaching-effacing *Escherichia coli.*
TABLE 4. Results of canine coronaviruses (CCoV) genotyping and subtyping in this study with S2’ and S1/S2 cleavage site sequences and comparison with reference CCoV-I and –II.

<table>
<thead>
<tr>
<th>CCoV (case)</th>
<th>Genotype</th>
<th>Subtype</th>
<th>S2’ Cleavage Site Sequence</th>
<th>S1/S2 Cleavage Site Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>89334-09 (2)</td>
<td>II</td>
<td>ND</td>
<td>KRKYRS</td>
<td>ARTR - - - - - G</td>
</tr>
<tr>
<td>97736-09 (3)</td>
<td>II</td>
<td>a</td>
<td>KRKYRS</td>
<td>ERTR - - - - - G</td>
</tr>
<tr>
<td>107207 (4a)</td>
<td>II</td>
<td>ND</td>
<td>KRKYRS</td>
<td>DRTR - - - - - G</td>
</tr>
<tr>
<td>110089-09 (4b)</td>
<td>II</td>
<td>a</td>
<td>KRKYRS</td>
<td>ARTR - - - - - G</td>
</tr>
<tr>
<td>123567-09 (5)</td>
<td>II</td>
<td>a</td>
<td>KRKYRS</td>
<td>ERTR - - - - - G</td>
</tr>
<tr>
<td>51534-10A (6a)</td>
<td>II</td>
<td>ND</td>
<td>KRKYRS</td>
<td>ARTR - - - - - G</td>
</tr>
<tr>
<td>120628-12 (7)</td>
<td>II</td>
<td>a</td>
<td>KRKYRS</td>
<td>ND</td>
</tr>
<tr>
<td>159396-12 (8)</td>
<td>II</td>
<td>a</td>
<td>KRKYRS</td>
<td>ERTR - - - - - G</td>
</tr>
<tr>
<td>47387-13 (9)</td>
<td>II</td>
<td>b</td>
<td>KRKYRS</td>
<td>ARTR - - - - - G</td>
</tr>
<tr>
<td><strong>Reference CCoV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB/05</td>
<td>II</td>
<td>a</td>
<td>KRKYRS</td>
<td>ARTR - - - - - G</td>
</tr>
<tr>
<td>1-71</td>
<td>II</td>
<td>a</td>
<td>KRKYRS</td>
<td>ERTR - - - - - G</td>
</tr>
<tr>
<td>341/05</td>
<td>II</td>
<td>b</td>
<td>KRKYRS</td>
<td>ARTR - - - - - G</td>
</tr>
<tr>
<td>Elmo/02</td>
<td>I</td>
<td>NA</td>
<td>QPGGRS</td>
<td>VRRARRAVQG</td>
</tr>
</tbody>
</table>

The reference CCoV-IIa “pantropic” CB/05 (AAZ91437.1), CCoV-IIa “enteric” 1-71 (AAV65515.1), CCoV-IIb 341/05 (ACJ63231.1) and CCoV-I Elmo/02 (AAP72149) are included to highlight the conserved nature of the S2’ cleavage site within genotype II viruses. Basic residues suspected to be important for cleavage activation of the spike protein are in bold. ND, not determined. NA, not applicable.