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Clinical, Pathological and Immunohistochemical Findings in Diarrheic Dogs and Evaluation of Canine Parvoviral and Coronaviral Enteritis

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Abstract: The aim of this study is to examine, clinicopathological findings of naturally occurring diarrhea in dogs and correlate clinical sings with pathological findings of canine parvovirus and coronavirus infection. Hematology, biochemistry, pathology and immunohistochemistry were applied. Vomiting and diarrhea were prominent findings in all affected dogs. The critical values for good prognosis in diarrheic dogs were $>2\times10^3$ mm^{-3} total leukocyte count, below 25 mmol L^{-1} anion gap and >7.36 blood pH at the admission. Diagnosis was made by immunohistochemical examination in died dog's guts tissue sections. Strong immunopositive reaction were seen in enterocytes, lymphocytes and macrophages in Payer's patches in both parvovirus and coronavirus cases. Lesions were more severe in dual infection cases. The most important point of the supportive treatment was fluid and electrolyte therapy against to dehydration in all cases and strong relationship was observed in treatment starting time to recovering dehydration and mortality rate.

Key words: Canine coronavirus, canine parvovirus, pathology, immunohistochemistry, treatment

INTRODUCTION

Canine viral enteritis is a disease of dogs with an acute onset of vomiting and diarrhea, especially in puppies and where several animals are affected simultaneously. Four viruses have been identified as the essential causes of severe enteritis in dogs: Canine Parvovirus (CPV), Canine Coronavirus (CCV), Canine Rotavirus and Canine Distemper Virus (Jones *et al*., 1997; Buonavoglia *et al*., 2006). CPV is a contagious viral disease of dogs and is one of the most important causes of death in puppies (Decaro *et al*., 2005). Puppies, aged between 6 and 20 weeks old, are the most susceptible to parvoviral enteritis. Diagnosis of viral enteritis is generally based on clinical signs, gross-histopathological lesions, immunohistochemistry, virological or serological examinations and the other laboratory techniques (Carman and Povey, 1985; Buonavoglia *et al*., 2000; Desario *et al*., 2005). Parvovirus replication is mainly seen in highly mitotically active tissue, such as lymphoid tissue, bone marrow, or gastrointestinal tract epithelium of dogs and cats (Martella *et al*., 2004, 2005).

CCV are mainly associated with respiratory, enteric, hepatic and central nervous system diseases. Nevertheless, organs such as kidney, heart and eye can also be affected. CCV was first isolated in 1971 from gastroenteric dogs. Subsequently, additional cases have been reported that were usually mild and self-limiting, unless complicated by CPV (Appel, 1987; Jones *et al*., 1997). Infections are usually self-limiting but may be fatal in young animals (Pratelli *et al*., 2004; Evermann *et al*., 2005). The main target of CCV is the small intestinal epithelium, where a lytic infection results in desquamation and shortening of duodenal and jejunal villi. Pathologic changes have been characterized by dilated intestinal loops filled with watery faces, congested or edematous mucosa and edematous mesenteric lymph nodes in experimentally infected dogs. The jejunum is the preferential site of infection (Appel, 1987; Pratelli *et al*., 2004).

The aims of this study were to examine clinicopathological findings of dogs with diarrhea, evaluate clinical, pathological and immunohistochemical findings of naturally occurring canine parvovirus and coronavirus infection and relation of these finding in single and dual infection of these viruses.

MATERIALS AND METHODS

Study material was consisting of 60 enteric diarrhetic dogs with various breed, sex and age. Ages of dogs from 5 months to 5 years and 31 of 60 dogs were male, 29 were

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female and 25 Turkish Anatolian sheep dogs, 12 Dalmatian, 9 Collie, 9 German shepherd dogs and 5 stray dogs 20 of them died and pathological examination was performed at the Department of Pathology. At necropsy, intestinal tissue samples were fixed in 10% buffered formalin, routinely processed and embedded in paraffin. Samples sectioned at 5 μm and stained with Haematoxylin and Eosin (HE). All samples were immunohistochemically examined for to detection of parvo and coronaviruses.

Blood samples collected from 20 dogs at admission and only 10 of them were sampled before death (remaining dogs did not brought before died). Hematological and biochemical examination of 15 blood samples of diarrheic and recovered dogs were made at admission and re-sampled 3 days after starting treatment.

For immunohistochemical identification of CPV and CCV antigens, Peroxidase-Anti-Peroxidase (PAP) technique was performed on formalin-fixed paraffin embedded intestine tissue, using poly L-lysine-coated slides. Tissue sections were deparaffinized and hydrated by immersion in xylene, graded alcohols and distilled water. Endogenous peroxidase activity was blocked by incubating section in 3% hydrogen peroxide solution for 30 min. Sections were incubated protein blocking to block any specific reaction. Sections were gently drained and incubated with monoclonal anti-CPV and CCV antibodies (Parvovirus antibody, CPV1-2A1, Abcam, ab7669, MA, USA and Coronavirus antibody, Novo Castra, NCL-304, Newcastle- UK), at a dilution 1:500 in PBS. Sections were consecutively incubated with commercial, peroxidase, conjugated, antibody polymer (Nichirei, Japan), stained with DAB and counterstained with Mayer's haematoxylin and examined under the microscope. A section of normal dog's intestine was used as a negative control and an intestinal positive control for checking technical errors was also performed, using PBS, instead of specific primary antibody. The immunoreactivity was evaluated by observing the positive brown-black color of DAB (3,3-diaminobenzidine) chromogen, which contrasted with the light blue background of Mayer's haematoxylin.

Blood samples were taken from jugular vein of the diarrheic dogs for hematological and biochemical examinations. MS9 blood counting equipment and IDEXX Vet-Test equipment and reagents were used for hematological and biochemical analysis. Roche OPTI CCA blood gas analyzer was also used for blood analyses. Surviving animals were treated with lactate ringer solution, antibiotics and B complex vitamins. Most of the diseased animals recovered within 5 days treatment only 20 dogs were died. One-way-Anova analysis of variance test was used to observe any differences between groups relating to blood values. In the determination of differences between died and recovered dog's blood levels at admission and before death or after 3 days starting treatment, Duncan multiple comparison method was used. Calculations were made using the SPSS 10.0 program pack.

RESULTS AND DISCUSSION

Clinically, diarrhea, vomiting and dehydration lasting longer than 2 days were the most prominent findings at admission. Critical findings for prognosis in diarrheic dogs were the total leukocyte count, anionic gap and blood pH. Severe leucopenia observed in died dogs and total leukocyte count was below the 2×10^3 mm^{-3} in all fatal cases at admission and this value was found as critical level for prognosis. Total leucocyte counts were decreased to $0.77\pm0.19\times10^3$ mm^{-3} before death. Metabolic acidosis was also severe in died dogs, anion gap was significantly higher in died dogs than recovered dogs at admission, anion gap was below the 25 mmol L^{-1} in recovered dogs. Total protein levels were 3.5-5.1 g dL^{-1} in died dogs and between 5.0-6.5 g dL^{-1} in recovered dogs. Blood pH was 7.35 in died dogs which was taken from before death but pH were between 7.37-7.39 in recovered dogs before treatment. pH 7.35 was found as a critical value for to evaluation of prognosis in diarrheic dogs. Biochemical, hematological and blood gas analysis results were shown in Table 1.

Leucopenia, hemorrhagic enteritis and decreased body temperature were marked in CPV cases. Blood parameters returned their normal ranges in recovered dogs within 3 days after starting the treatment. These results were statistically significant ($p<0.001$).

High mortality rate was observed in dogs with hemorrhagic enteritis and most of them were positive for CPV immunohistochemically. At necropsy, hemorrhagic contents were seen all of the fatal CPV cases and hemorrhages were present throughout the gut but it was more prominent at the ileocecal areas (Fig. 1 and 2). In this study, 12 out of 20 cases were positive for single CPV, 3 cases were only CCV positive fatal enteritis in puppies. Five of 20 cases were dual infection with CPV and CCV.

Hemorrhagic enteritis lesions were found in all CPV positive cases at the histopathological examination of intestine sections. The predominant features of the intestinal lesions were severe necrosis of epithelial cells of intestine, distension of the crypts lumen with hemorrhagic exudates and degenerative changes of crypt epithelium. Totally necrosis most of crypt epithelium and totally depletion of Payer's patches were commonly seen, furthermore in some cases no lymphocytes were seen in Payer's patches. While degeneration, necrosis and

Table 1: Biochemical, hematological and blood gas analysis results

Parameter	Died dogs		Recovered dogs		
	At admission	Before death	At admission	3 days after starting treatment	Reference values (Bilal, 2004)
n	20	10	15	15	
PVC (%)	61.05±0.94[a]	62.40±1.09[b]	59.26±0.72[b]	50.60±1.48[b]	37-55
pH	7.35±0.16[a]	7.35±0.16[a]	7.39±0.13[b]	7.37±0.26[b]	7.40±0.05
pCO_2 (mmol L^{-1})	31.55±0.30[a]	30.30±0.26[a]	30.26±0.18[a]	30.13±0.165[b]	30
HCO_3^- (mmol L^{-1})	16.70±0.31[a]	16.00±0.33[a]	19.86±0.40[b]	21.00±0.47[b]	20.8
Base exces (mmol L^{-1})	-4.15±0.20[a]	-4.60±0.26[ab]	-2.13±0.45[bc]	-2.8±0.45[c]	(1)-(-3)
Na^+ (mmol L^{-1})	137.90±0.39[a]	136.60±0.58[a]	150.40±0.81[b]	144.13±0.916[b]	140-155
K^+ (mmol L^{-1})	3.13±0.03[a]	2.94±0.03[a]	4.63±0.09[b]	4.68±0.10[b]	3.5-5.1
Cl^- (mmol L^{-1})	95.40±0.92[a]	92.70±0.51[ab]	91.26±0.61[bc]	98.80±1.51[c]	96-113
Anion gap (mmol L^{-1})	35.25±0.77[a]	40.80±1.19[b]	23.73±1.11[c]	18.13±1.12[d]	5-30
Total leukocyte (WBC)×10^3	1.51±0.27[a]	0.77±0.19[a]	6.01±0.22[b]	11.07±0.45[c]	5-17.1

PCV: Packed Cell Volume; Na^+: Sodium ion; K^+: Potassium ion; Cl^-: Chloride ion; pCO_2: Partial pressure of carbon dioxide; anion gap = (Na^+ + K^+)-(HCO_3^- + Cl^-), Data are expressed as mean±SD, a, b, c, d within a row, means with different superscript letters were significantly different ($p<0.001$)

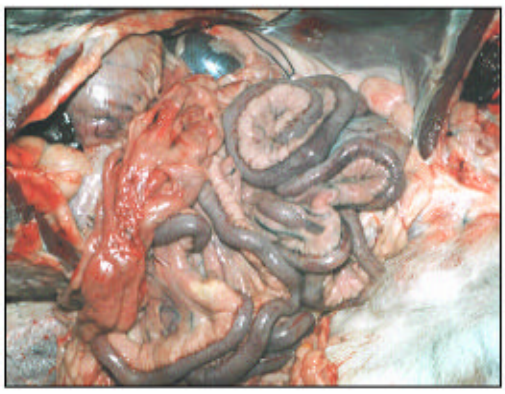

Fig. 1: Typical hemorrhagic enteritis in a CPV positive dog, serosal hemorrhages and hyperemic mesenterial vessels

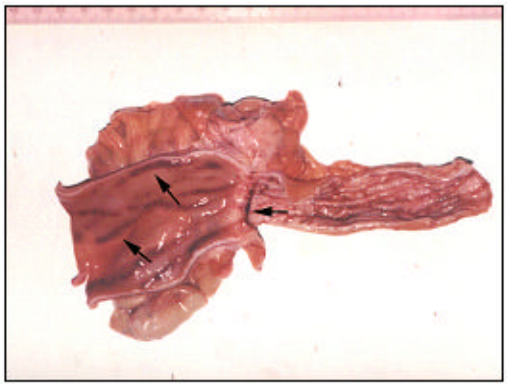

Fig. 2: Hemorrhage at the iliocecal area (arrows) in a CPV positive dog

Payer's patches depletion were prominent and severe in parvovirus cases, crypt dilatation, mild to severe Payer's patches depletion and regenerative changes in crypt epithelium were prominent in CCV positive cases (Fig. 3).

Similar gross findings were observed in single CCV positive cases, but lesions were milder in CCV than CPV. Over the evaluation of the histopathological lesions of the 3 coronavirus immunopositive cases, the lesions frequently seen the intestinal mucosa were atrophy of villi and necrosis of epithelial cells in the intestine, dilatation of crypt, degenerative-regenerative changes of crypt epithelium with infiltration of the lamina propria predominantly by mononuclear cells and some neutrophil granulocytes, necrosis some of the crypt and mild to severe depletion of Payer's patches (Fig. 4).

Inflammatory reaction was locally distributed throughout the gut wall. Edema, atrophy of lymphoid tissue and in one case mild hemorrhage was observed in the submucosa. Edema was also detected at the muscular layer of the intestine. Strong correlation was observed leucopenia and lymphocyte depletion at the lymphoid follicles. Severe depletion in lymphoid tissues was observed in dogs with leucopenia. While intranuclear inclusion bodies were scantly observed in CPV cases but not observed in CCV cases.

In dual infection cases with CCV and CPV, gross and histopathological findings were more severely observed than single virus infection cases. Hematological and biochemical values of the dually infected dogs were significantly different than normal levels.

The immunohistochemical examinations of the intestine showed positive reactions in the epithelial cells of the crypts, enterocytes, lymphocytes and macrophages in Payer's patches. Positive reactions were noticed in the apical part of the cytoplasm of epithelial cells and cytoplasm of inflammatory cells (Fig. 5-7).

These reactions were detected both of corona and parvovirus cases. While localization of positive reaction was prominent in enterocytes in CPV cases On the other hand, it was prominent in crypt epithelial cells in CCV cases. Strong positive reaction was observed in macrophages and lymphocytes in Payer's patches in CCV cases.

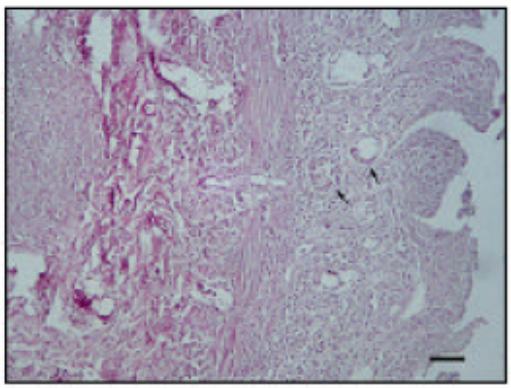

Fig. 3: Crypt dilatation, degeneration and regeneration (arrows) in a CCV positive case, gut, HE, Bar, 200 µm

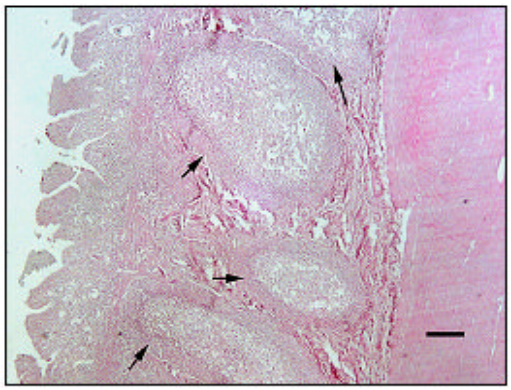

Fig. 4: Severe Payer's patches depletion (arrows) in CPV positive dog, center of the lymphoid foci almost empty, gut, HE, Bar, 200 µm

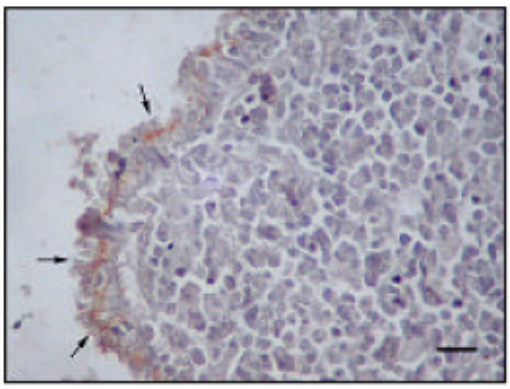

Fig. 5: Immunohistochemically, CVP positive reaction in the apical part of the cytoplasm of the enterocytes (arrows), gut, Streptoavidine-biotin, Bar, 100 µm

Parvovirus and coronavirus may have acted synergistically to cause disease. Parvoviruses cause more prominent and severe enteritis than corona viruses (Jones *et al*., 1997; Buonavoglia *et al*., 2006). Similar findings were also observed in this study. This study showed that most common cause of the fatal enteritis in dogs is parvovirus and dual infection with parvo and coronavirus. They can cause high mortality. It is well known that parvovirus infection causes severe lymphoid depletion; present finding indicated that coronavirus infection also cause lymphoid depletion as well. Parvoviral enteritis was observed more common than coronaviral enteritis and CPV diagnosed most of the cases. Seventeen out of 20 cases were CPV positive and 5 of them together with CCV in present study. Even dual infections, CPV lesions were more prominent. This study also indicated that histopathological examinations not enough for diagnosis, immunohistochemical or appropriate diagnostic methods must be support to diagnosis. Similar clinicopathological findings were seen in both 2 infection, but hemorrhagic enteritis more suggestive for CPV.

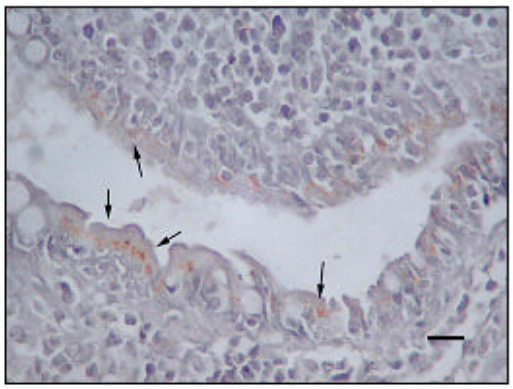

Fig. 6: CVP positive reaction in the enterocytes (arrows), Streptoavidine-biotin, Bar, 100 µm

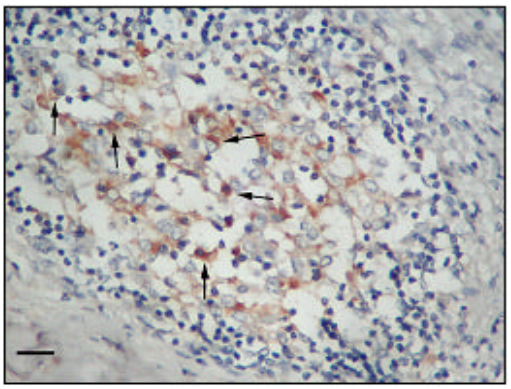

Fig. 7: CCP positive reaction in the macrophages at the Payer's patches, Streptoavidine-biotin, Bar, 200 µm

Parvovirus infections of dogs are characterized by widespread lesions in the digestive tract, lymphoid and hematopoietic tissue, which are frequently fatal. Those lesions have long been considered to be a direct consequence of virus attack of the susceptible cells resulting in necrosis (Martella *et al*., 2004, 2005; Decaro *et al*., 2005; Desario *et al*., 2005). The infections can lead to 2 distinct clinicopathologic presentations: An intestinal form, which is the main form in dogs older than 6 weeks and a cardiac form, which occurs in younger dogs. Intestinal form, which may occur in dogs of all ages but is most severe in young pups, is characterized vomiting, diarrhea and dehydration (Jones *et al*., 1997; Pratelli *et al*., 2004). In this study, the dog's ages were changing 5 days to 5 months old. Only one case was 5 years old and this finding showed that CPV infection affect not only puppies but also old dogs. There is no previous report about breed and sex disposition for CPV, but we observed that Turkish Anatolian Sheep Dogs were also susceptible to the viral enteritis especially CPV. The possible cause of this susceptibility can be attributed to the generally irregular vaccination programs in Anatolian Sheep Dogs.

Diarrhea is usually severe and often hemorrhagic, with sloughing of intestinal mucosa and replacement by cuboidal epithelium in CPV infections (Carman and Povey, 1985; Jones *et al*., 1997). Parvovirus and coronavirus may have acted synergistically to cause disease along with the cryptosporidia, which is most often a secondary invaded. Coronavirus infections have been occurred rarely and generally not fatal in dogs (Evermann *et al*., 2005). In this study, typical hemorrhagic enteritis was seen in fatal CPV cases and it was the cause of the death. Only five cases were together with 2 viruses and it was fatal. Although, ratio of to dual infection was low, mortality rate may be attributed to synergistically effect in present study.

Histopathologically, there is necrotic and hemorrhagic enteritis of the small intestine reminiscent of CPV, with dilated crypts and, often regeneration of epithelium. Intranuclear inclusion bodies are found in intestinal epithelial cells of dogs with this infection. There is lymphopenia and neutropenia resulting from necrosis of precursor cells (Jones *et al*., 1997; Pratelli *et al*., 2004). In this study, atrophy of villi, necrosis of epithelial cells, distension of the crypts lumen which contained erythrocytes, dilatation of crypts, depletion of Payer's patches and severe hemorrhagic enteritis were commonly observed in CPV cases. Tissue tropism of parvovirus is thought to be confined to highly mitotically active tissues, such as intestinal epithelium, bone marrow and lymphoid tissue (Buonavoglia *et al*., 2000; Pratelli *et al*., 2004; Decaro *et al*., 2005; Elia *et al*., 2005). In this study, intranuclear inclusion bodies were scantly observed. Immunohistochemically positive reactions were also seen in mitotically active intestinal epithelial cells and lymphoid cells in Payer's patches in present study. Alterations of the intestinal villi were typical of CCV infections, however, basophilic intranuclear inclusion bodies in crypt epithelial cells, as well as necrosis and depletion of lymphoid tissue, are characteristic findings in CPV but not CCV infections (Jones *et al*., 1997; Buonavoglia *et al*., 2006). Diagnosis mainly depends on their lesions and isolation of the virus. The myocardial form is unique, but the intestinal form may be confused with other causes of enteritis, such as coronavirus infections the latter is not usually milder clinically and is not associated with leucopoenia or significant necrosis of intestinal epithelium (Jones *et al*., 1997). Similar clinical and pathological finding were observed in our study, but immunopositive reaction in Payer's patches in CCV cases were observed and mild to severe lymphoid tissue depletion were seen in these dogs. These findings suggested that gross and histopathological diagnosis of parvovirus can cause miss diagnosis.

Coronaviruses infections are usually self-limiting but may be fatal in young animals (Pratelli *et al*., 2004; Evermann *et al*., 2005; Baunavoglia *et al*., 2006). The virus can be demonstrated by suitable immunfluoresan techniques in affected cells in the small and large intestine and in affected reactive lymph nodes of the mesentery. Virus can also be demonstrated specifically in intestinal epithelial cells shed into the intestinal lumen. Diagnosis depends on confirmed identification of the etiologic agent (Jones *et al*., 1997). In this study were used immunoperoxidase techniques and were obtained positive result in enterocytes, crypt epithelia and lymphoid tissue. Because of the similarity of clinical and pathological findings of CCV and CPV, definitive confirmation of the etiology by demonstrating the antigen within the lesions should be by appropriate methods like as immuno-histochemistry.

Strong immunopositive reaction in lymphocytes and macrophages in Payer's patches in both parvovirus and coronavirus cases indicated that histopathological evaluation of these 2 diseases can cause miss diagnosis. Because lesions in severe coronavirus cases were very similar that observed in parvovirus cases. Although, this finding very suggestive for CPV, this study showed that this finding may be seen in CCV cases. Immuno-histochemical observation should be done for evaluation.

Rapid dehydration usually contributes to the metabolic acidosis in the pathophysiology of secretory diarrhoea (Argenzio, 1992). The main prognostic factors were total leukocyte count, pH and anion gap in our study. Dogs with under $2\times10^3/mm^3$ total leukocyte levels

were commonly died although supportive treatment. In comparison with the died dogs and recovered dogs blood gas analysis results revealed a significant decrease in HCO_3^- and blood pH in died dogs. Significant increases in the mean concentrations for partial pressure of Carbon dioxide (pCO_2) were also found. Died dogs had a significant higher anion gap at admission than the recovered dogs and a lower plasma concentration of Na^+, K^+ and higher Cl^-. These findings may be explained the severity of diarrhea than vomiting. This study showed that the main point of the treatment fluid and electrolyte therapy in diarrheic dogs.

CONCLUSION

Although, CCV and CPV can be evaluated by clinical and histopathological examinations, for definitive diagnosis immunhistopathology or similar specific methods are necessary. The critical values for prognosis in diarrheic dogs were total leukocyte count (below the 2×10^3 mm^{-3} indicate bad prognosis), anion gap (below the 25 mmol L^{-1} indicate good prognosis) and blood pH (critical level 7.35). For recovery, early treatment is important in enteritis cases and severe infection can cause mortality. Regular vaccination programmes should be launched for dogs. Positive CPV cases generally causes to death especially non vaccinated dogs. In this study, Turkish Anatolian Sheep Dogs were also found very susceptible to the viral enteritis especially canine parvovirus.

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