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RESEARCH ARTICLE

Molecular Detection and Hematological Changes Associated with the Canine Diarrheic Viruses in Pakistan

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ABSTRACT

Enteric viruses like canine coronavirus (CCoV), canine parvovirus (CPV), and canine rotavirus (CRV) cause high morbidity and mortality in dogs, especially in young canines and hence pose great economic losses to dog breeders worldwide. This creates immense psychological distress and trauma for dog owners. CCoV is mistakenly diagnosed as CPV in many cases by clinicians though CCoV causes less severe disease than CPV. Sometimes both CCoV and CPV co-exist in a patient causing severe clinical disease and even death in dogs. In such cases, clinicians are uncertain about the prognosis. CRV is detected in several countries but no research has been carried out on it in Pakistan yet. The objective of this research was to observe if CCoV, CPV and CRV co-exist in diarrheic dogs. A total of 384 stool samples of dogs, that were reported at different clinics in Lahore, with the complaint of diarrhea, were collected and rendered for rapid detection of the viruses using immuno-chromatographic test kits followed by PCR-confirmation of the positive samples. The individual prevalence of CCoV, CPV and CRV turned out to be 21.6, 26.3 and 0% respectively. The prevalence of CCoV and CPV co-infection was 3.4%. Male German shepherd dogs under the age of 1 year are highly prone to get viral diarrhea in July and August.

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INTRODUCTION

People who face loneliness, mental trauma, and emotional breakdown have made pet dogs a necessary part of their lives because dogs give them amusement, support them emotionally and help fight friendlessness (Deng *et al.,* 2018). Livestock and other domesticated animals like dogs are richly inhabited by the conducive environment of Pakistan. Among these animals, dogs stand out to make a total population of three million in Pakistan and they have individualistic features like loyalty, retrieving, hunting, sniffing and guarding (Towakal *et al.,* 2010). In 1971, CCoV was identified as a deadly pathogen of dogs (Binn *et al.,* 1974). CCoV is an RNA virus with a single strand possessing a genomic length of 30kbp. The envelope imparts the virus with immense protection against the challenges of the environment and it survives acid and lipid solvents, this unique attribute renders the virus escape the inactivation in the upper GI tract.

Younger dogs are highly prone to get CCoV infection (Stavisky *et al.,* 2012). The epithelial cells of the intestinal villi are infected by CCoV leading to mild to severe diarrhea (Saif *et al.,* 1990). Among the common causes of neonatal morbidities and mortalities in young animals, diarrhea is the leading cause (Kim *et al.,* 2021). CPV, a DNA virus and the smallest virus with a diameter size of 25nm, belongs to *parvoviridae* family (Singh *et al.,* 2021). In both CCoV and CPV-infected canine patient, it is seen that the disease is very fatal in pups. Diarrhea with blood, vomiting, dullness, sluggishness, fever, anorexia, dehydration and loss of body condition are the symptoms observed in patients infected with CCoV and CPV (Dema *et al.,* 2022). Often CCoV is misdiagnosed as CPV infection and vice versa leading to wrong diagnosis and failure at the end of a veterinary clinician to educate the dog owner and to give a prognosis of the disease because CPV causes more serious clinical illness than CCoV and sometimes there is a mixed infection of CCoV and CPV that always leads

to the death of patient causing anxiety in veterinarian and the dog owner (Sulehria *et al.,* 2023).

Methods to diagnose CCoV are immune-chromatography (ICT) based test, PCR, and cell-line culture method (Yoon *et al.,* 2018). CPV may be diagnosed using ICT-based tests and PCR. CRV is a *Reovirus* that contains a double strand of RNA and has no envelope around its genomic material. It is prevalent in almost every species and has a huge range of hosts. In humans, the virus is very notorious and causes fatal gastroenteritis in children. It is transmitted via the oro-fecal route. (The 5-Minute Veterinary Consult). CRV has been identified as a zoonotic diarrheic pathogen in dogs. *Canine Rotavirus C* is more identical to *Bovine Rotavirus C* (Marton *et al.,* 2015). *Rotavirus A* causes diarrhea in humans, dogs, and pigs (Yan *et al.,* 2019). *Rotavirus* causes mild to severe enteritis in dogs and the infected dog is febrile, anorexic, suffers from diarrhea and vomiting, is sluggish and dehydrated and it confuses with *canineparvo* virus infection (Ortega *et al.,* 2017). There is a significant association between canine diarrhea and the type of food consumed by the dog, the age of the dog and the breed of the dogs (Khan *et al.,* 2022).

MATERIALS AND METHODS

Collection of Samples: A total of 384 samples (n=384) of the feces of the dogs (*Canis lupus familaris*) with diarrhea were collected in sterile containers from different veterinary clinics in Lahore and were transported at 4ºC to Animal Health Research Lab, Department of Veterinary Medicine, UVAS, Lahore (Fig.1).

Rapid Detection Test for CCoV: The fecal samples were rendered for rapid detection of the virus using ICT-based test kits (Fig. 2) from Quicking Biotech, China, Cat No.W81066 CCoV & CPV (Sulehria *et al*., 2020).

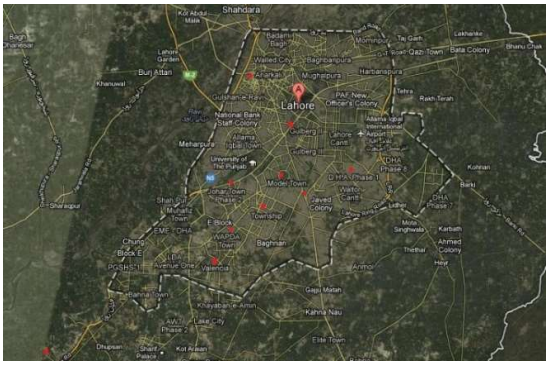

Fig. 1. GIS map of district Lahore showing sampling sites in red dots.

Fig. 2: CPV positive sample right column 1st kit (from left), CCoV positive sample left column 3rd kit (from left), and negative samples in kits 4,5 & 6 (from left).

Molecular Diagnosis of CCoV: This phase involved cDNA preparation, RNA extraction from fecal samples, PCR, and gel electrophoresis.

RNA Extraction for CCoV: RNA extraction was carried out using The Total RNA Fast Extraction Stool Kit, Cat# RP8001, Bioteke Corporation, China (Khan *et al.,* 2020) following the protocols by the manufacturer.

Molecular Detection of CCoV: To detect the virus, the 2X PCR Taq Master Mix kit (BioShop, Canada) was utilized to amplify an M-gene fragment of 321bp length using species-specific primers. A conventional PCR was run in a 25µL reaction mixture. The recipe for PCR was; 15µL master mix, 1.0µL forward primer (FP) and reverse primer (RP) each, 6µL nuclease-free water and 2.0µL template DNA (Agnihotri *et al.,* 2017). A confirmed detection was done by Gel-electrophoresis. For this purpose, agarose gel (1.5%) was made in TAE buffer. For each sample, 8µLs of PCR product was used with the marker (molecular weight 100bp, DNA Plus Ladder Bioshop, Canada) and was set to run for 40mins at 100V. For illumination, ethidium bromide dye was used. The bands on the gel were cut and rendered for gene sequencing (Macrogen-USA). The conditions of PCR are elaborated in Table 1.

Rapid Detection Test for CRV: The fecal samples were rendered for rapid detection of the virus using ICT-based test kits (Fig. 2) from Quicking Biotech China, Cat No. W81044 for detection of CRV (Sulehria *et al*., 2020).

Molecular Diagnosis of CRV: This phase involved cDNA preparation, RNA extraction from fecal samples, PCR, and gel electrophoresis.

RNA Extraction for CRV: RNA extraction was carried out using the total RNA Fast Extraction Stool Kit, Cat# RP8001, Bioteke Corporation, China (Sulehria *et al.,* 2023) following the protocols by the manufacturer.

Molecular Detection of CRV: To confirm the presence of CRV, VP6 Gene, a portion of *CRV* was amplified following the protocols (Ortega *et al.,* 2017). The 2X PCR Taq Master Mix kit (BioShop, Canada) was used which amplified a 379bp fragment of the VP6 gene. A conventional PCR was run in 25µL of volume. The recipe for PCR was; 8µL master mix, 1.5µL forward primer (FP), reverse primer (RP) each, 11µL nuclease-free water, and 3.0µL template DNA. A confirmed detection was done by gel electrophoresis. For this purpose, agarose gel (1.5%) was made in TAE buffer. For each sample, 8µLs of PCR product was used with the marker (molecular weight 100bp, DNA Plus Ladder Bioshop, Canada) and was set to run for 40 mins at 100V. For illumination, ethidium bromide dye was used. The bands on the gel were cut and rendered for gene sequencing (Macrogen-USA).The conditions of PCR are elaborated in Table 1.

Rapid Detection Test for CPV: The fecal samples were rendered for rapid detection of the virus using ICT-based test kits (Fig. 2) from Quicking Biotech, China, Cat No. W81066 for the detection of *CPV* and *CCoV* (Sulehria *et al*., 2020).

Table 1: PCR cycling conditions for amplification of VP2 (CPV-2) and M gene (CCoV).

Stage	CCoV	CPV	CRV
Initial Denaturation	94°C for 5mins (40 cycles)	94°C for 5mins (40 cycles)	94°C for 5mins (1 cycle)
Denaturation	94°C for 30secs	94°C for 30secs	91°C for 1 min
Annealing	50°C for 30secs (40 cycles)	54°C for 1 min (40 cycles)	61°C for 1 min (40 cycles)
Extension	72°C for 1 min	72°C for 1 min	72°C for 1 min
Elongation	72°C for 1 min	72°C for 1 min	72°C for 1 min

Molecular Diagnosis of CPV: This phase involved DNA extraction from fecal samples, PCR and gel electrophoresis.

DNA Extraction for CPV: DNA extraction was carried out using Stool Genomic DNA Rapid Extraction Kit (Spin-column) Model # 50, Bioteke Corporation, China following the protocols by the manufacturer.

Molecular Detection of CPV: For the confirmation of CPV, a portion of VP2 gene was targeted, and the primers used (Agnihotri *et al.,* 2018) amplified and targeted a 767bp gene fragment. The total volume of the reaction mixture was 12.5µL comprising 3.0µL template DNA, 6.25µL master mix, 0.5µL forward and reverse primers each and 2.25µL of nuclease-free water. A confirmed detection was done by gel electrophoresis. For this purpose, agarose gel (1.5%) was made in TAE buffer. For each sample, 8µLs of PCR product was used with the marker (molecular weight 100bp, DNA Plus Ladder Bioshop, Canada) and was set to run for 40mins at 100V. For illumination, ethidium bromide dye was used. The bands on the gel were cut and rendered for gene sequencing (Macrogen-USA). The conditions of PCR are elaborated in Table 1.

Blood Sampling: Blood collection (1mL) was done from each positive patient from cephalic vein and transferred into EDTA coated vacutainer to perform a complete blood count (CBC) to study hematological dynamics in CCoV, CPV and CRV positive and co-infected patients.

Statistical Analysis: Descriptive statistics for instance, percentages and proportions were applied to summarize the data regarding prevalence. The chi-square test was utilized to compare the prevalence of CCoV, CPV, and CRV using IBM SPSS Statistics V21.0 (IBM, USA).

RESULTS

Prevalence and descriptive epidemiology of Canine Diarrheic Viruses: Out of 384 dogs, 84 were positive for CCoV, 101 were positive for CPV, none (0) was positive for *CRV,* 13 patients had co-infection of CCoV and CPV. Hence, the computed overall prevalence for each of the viruses turned out to be 21.9, 26.3, 0, 3.4% for CCoV*,* CPV, CRV and CCo-CPV co-infection respectively (Table 3). Out of 84 CCoV-positive patients, 37 were between 0 to 4 months of age, 11 were 5 to 8 months old, 5 were 9 to 12 months old and 31 were above 1 year of age with their respective prevalence; 9.6, 2.9, 1.3 and 8.1% (Table 4). Among 101 CPV-positive patients, 38 were between 0 to 4 months of age, 44 were 5 to 8 months old, 17 were 9 to 12 months old and 2 were above 1 year of age with their respective prevalence; 9.9, 11.5, 4.4 and 0.5%. No prevalence was observed for *CRV*. Among the 13 CCoV-CPV co-infected patients, 4 dogs were between 0 to 4 months of age, 9 were 5 to 8 months old and none was observed above the age of 9 months with their computed prevalence; 1.04, 2.3, 0% respectively. Table 4 shows that out of 84 CCoV positive patients, 40 were males and 44 were females with their respective prevalence 10.4% and 11.5%. Out of 101 morbid dogs, 54 were males (prevalence 14.06%) and 47 were females (prevalence 12.23%). Likewise, 2.08% prevalence (8 positive out of 13) was observed for the patients with a co-infection of CCoV and CPV whereas 1.3% (5 positive out of 13) prevalence was seen in female patients with CCoV-CPV co-infection. Table 4 shows the highest prevalence (4.9%) of CCoV in July and the least prevalence was observed in December (0%). The highest prevalence of CPV was observed in August (7.3%) and the least prevalence in January i.e. 0.3%. The highest prevalence of the CCoV-CPV co-infection was observed in August (1.3%) and the least in June *i.e*. 0.3%. The highest prevalence of CCoV was seen in German Shepherd dogs *i.e*. and the least was observed for Pit-bull, Crossbreds and Mongrels *i.e*. 0.5% each. The prevalence of CPV was the highest in German Shepherd dogs *i.e*. 10.7%, least was observed in Poodles and Mongrels *i.e*. 0.3% each. The data revealed that the prevalence of CCoV-CPV co-infection in diarrheic dogs was the highest in Crossbreds *i.e*. 1.3%. The p-value of the results of Pearson's Chi-square statistical test showed that age, sex, month of the year and breed of dogs were statistically non-significant, the prevalence of the canine diarrheic viruses. However, the comparison of the values of X^2 and α showed that all the parameters were critically significant (Table 6).

Molecular detection and phylogenetic analysis of CCoV and CPV: Amplification of the M-gene for CCoV and of VP2 gene for CPV showed bands at 321bp and 767bp respectively. The accession numbers allotted by NCBI Gen Bank for M gene of CCoV are MT 259779 and MT 259780. The phylogenetic tree of CCoV, shown in Fig. 3, reveals that the isolates of the current study belong to the Alpha *Coronavirus* and show similarity with the isolates from the UK, Tunisia, China, Columbia, Brazil, and South Korea. Likewise, the phylogenetic tree of *CPV* from the current study is shown in Fig. 4, which shows evolutionary relationships of taxa. The *CPV* detected in this study is CPV-2c and the history of evolution was inferred from the Neighbor-Joining method. The optimal tree (length =0.01462851) is visible. The Tamura-Nei method was used to calculate the evolutionary distances. The analysis of the evolution was performed in MEGA7.

Clinical and hematological examination: Upon the clinical examination it was observed that among the CCoV-positive patients, 100% were anorectic, 63% were febrile, 100% were diarrheic, 69% had vomiting, 62%

Table 2: Primers used for the amplification of VP2 (CPV) and M gene (CCoV)

Target Gene	Primer	Length (bp)	Sequence 5' to 3'
M gene	pCCoV/M/FII	321	GTTATACAGAAGGACTAAGTCT
	pCCoV/M/RII		GTTGAGTAATCACCAGCTTTAG
VP2 gene	pCPV/VP2/1-19 (F)	767	ATGAGTGATGGAGCAGTTC
	pCPV/VP2/767-748(R)		CTTAGTAAGTGTACTGGCAC
VP6 gene		379	GACGGVGCRACTACATGGT
			GTCCAATTCATNCCTGGTGG

Table 3: Overall prevalence of canine diarrheic viruses.

-	CCoV	CPV	CRV	CCoV-CPV
Prevalence	21.6%	26.3%	0%	3.4%

Table 4: Prevalence of canine diarrheic viruses with respect to age, sex, month and breed

Parameter	Group	Prevalence % CCoV (+ve)	Prevalence % CPV (+ve)	Prevalence % CRV (+ve)	Prevalence % CCoV-CPV(+ve)
Age	0 to 4 months	9.6% (37)	9.9% (38)	0	1.04% (4)
	5 to 8 months	2.9% (11)	11.5% (44)	0	2.3% (9)
	9 to 12 months	1.3% (5)	4.4% (17)	0	0% (0)
	Above 12 months	8.1% (31)	0.5% (2)	0	0% (0)
Sex	Male	10.4% (40)	14.06% (54)	0	2.08% (8)
	Female	11.5% (44)	12.23% (47)	0	1.3% (5)
Month	January	0.3% (1)	0.3% (1)	0	0% (0)
	February	0.8% (3)	1.3% (5)	0	0% (0)
	March	1% (4)	0.5% (2)	0	0% (0)
	April	1.8% (7)	0.8% (3)	0	0.5% (2)
	May	2.3% (9)	4.2% (16)	0	0% (0)
	June	4.4% (17)	2.9% (11)	0	0.3% (1)
	July	4.9% (19)	4.9% (19)	0	0.8% (3)
	August	4.2% (16)	7.3% (28)	0	1.3% (5)
	September	1% (4)	1.8% (7)	0	0.5% (2)
	October	0.5% (2)	1.3% (5)	0	0% (0)
	November	0.5% (2)	0.5% (2)	0	0% (0)
	December	0% (0)	0.5% (2)	0	0% (0)
Breed	GSD	7.3% (28)	10.7% (41)	0	1.04% (4)
	Labrador	3.6% (14)	7.6% (29)	0	0.3% (1)
	Rottweiler	2.9% (11)	1.9% (7)	0	0.3% (1)
	Pug	2.3% (9)	1.6% (6)	0	0% (0)
	Shih Tzu	2.3% (9)	1.6% (6)	0	0% (0)
	Bully	1% (4)	0 (0)	0	0% (0)
	Poodle	0.8% (3)	0.3% (1)	0	0.3% (1)
	Pitbull	0. (2)	2% (8)	0	0% (0)
	Crossbred	0.5% (2)	0.5% (2)	0	1.3% (5)
	Mongrel	0.5% (2)	0.3% (1)	0	0.3% (1)
		Total CCoV +ve84			
		Total CPV +ve 101			
		Total CRV +ve 0			
		Total CCoV-CPV +ve13			

felt abdominal pain and mortality rate was 30.9%. The results of the hematological parameters of these patients showed that 100% CCoV positive patients were anemic, 8.3% had leukopenia, 0% had panleukopenia and leukocytosis. Among the 101 CPV patients, 100% were anorectic, 86.1% were febrile, 100% were diarrheic, 100% had vomiting, 100% felt abdominal pain and the mortality rate was 63.4%, 100% were anemic, 45.5% had leukopenia, 100% had panleukopenia and none had leukocytosis (Table 5).

DISCUSSION

The current study was a novel approach to computing the prevalence of major diarrheic viral diseases in canines and the existence of these diarrheic viruses in canines as co-pathogens. *Canine coronavirus*, *Canine parvovirus* and *Canine rotavirus* were focused in the study. The prevalence of CCoV was 21.6%. This finding coincides with findings of Sulehria *et al.* (2020) who reported CCoV in Pakistan with a prevalence of 23.8%. Duijvestijn*et al.* (2016) computed 31.7% prevalence of CCoV in Nederlands and in China it was 26% according to Lu *et al.* (2016). In Brazil, the prevalence was 0% as per Curie *et al.* (2016), In India, Agnihotri *et al.* (2017) and Agnihotri *et al.* (2018) reported 8% prevalence. The difference in the prevalence of CCoV in different parts of the world may be because the RNA of CCoV *i*s incapable of staying stable in feces and the virus is shed intermittently in feces. The prevalence of CPV was 26.3% which is very close to the report of Singh *et al.* (2021) who reported CPV with a prevalence of 28% and Tagorti *et al.* (2018) who reported CPV with a prevalence of 32.14% in Tunisia. The prevalence of CRV was 0%. In India, the same results were observed by Dema *et al.* (2022).The prevalence of CCoV and CPV co-infection was 3.4%. The phylogenetic analysis showed that the virus isolates of both CCoV and CPV showed close resemblance with the virus isolates of different parts of the world. The current study showed that the prevalence of CCoV was the highest *i.e.* 9.6% in the dogs aged between 0 to 4 months of age. The same was observed by Sulehria *et al.* (2020) and Deka e*t al.* (2013). CPV was the highest in dogs aged between 5 to 8 months.

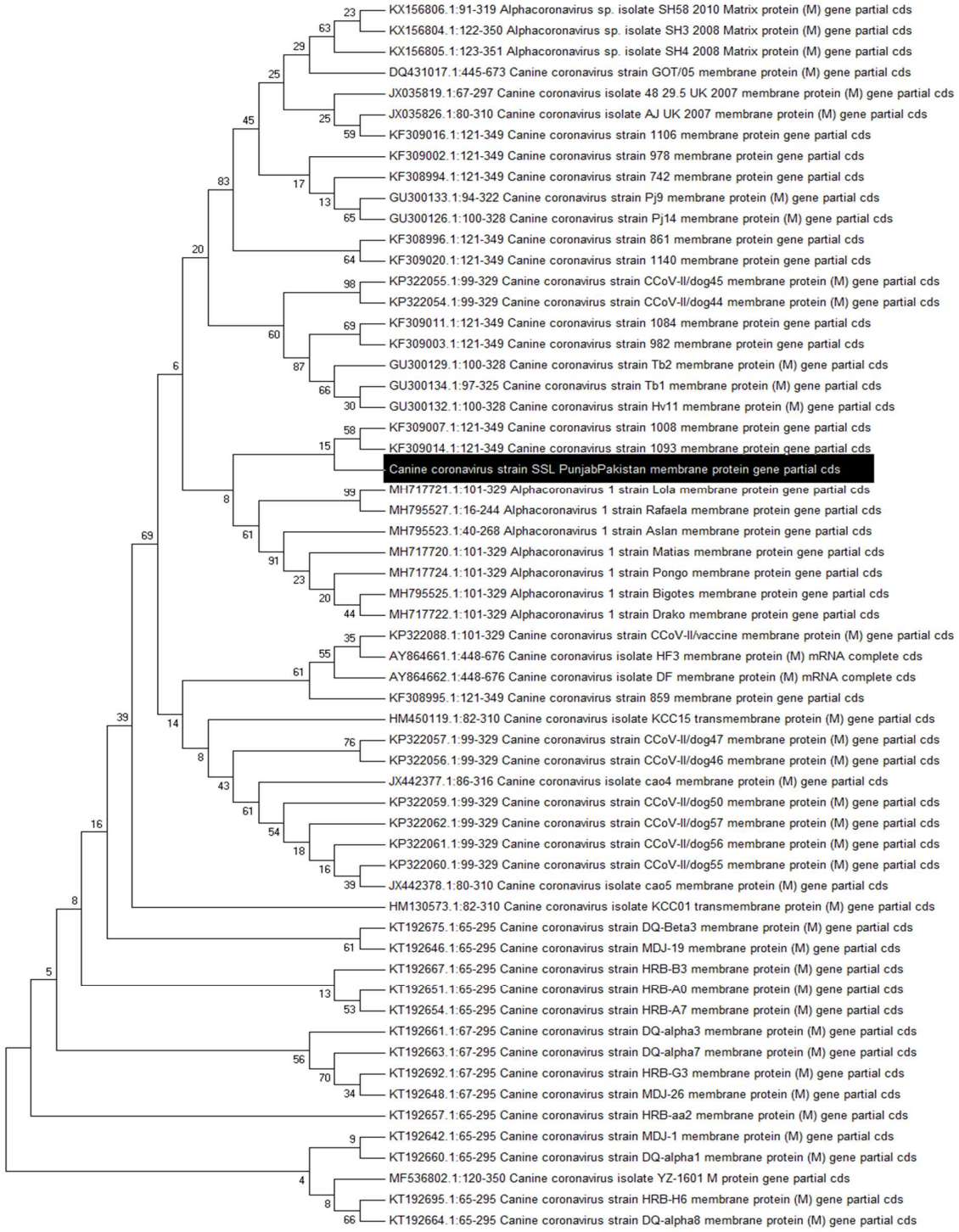

Fig 3: Phylogenetic tree of Canine Coronavirus.

Table 5: Hematological and clinical findings in the morbid dogs.

Clinical Sign	CCoV (%) (n=84)	CPV (%) (n=101)	Co-infection (%) (n=13)
Anemia	100% (84)	100% (101)	100% (13)
Leukopenia	8.3% (7)	45.5%(46)	100% (13)
Panleukopenia	0% (0)	100% (101)	100% (13)
Leukocytosis	0% (0)	0% (0)	0% (0)
Fever (>103°F)	63% (53)	86.1% (87)	100% (13)
Anorexia	100% (84)	100% (101)	100% (13)
Diarrhea	100% (84)	100% (101)	100% (13)
Vomiting	69% (58)	100% (101)	100% (13)
Pain in abdomen	62% (52)	100% (101)	100% (13)
Mortality	30.9% (26)	63.4% (64)	100% (13)

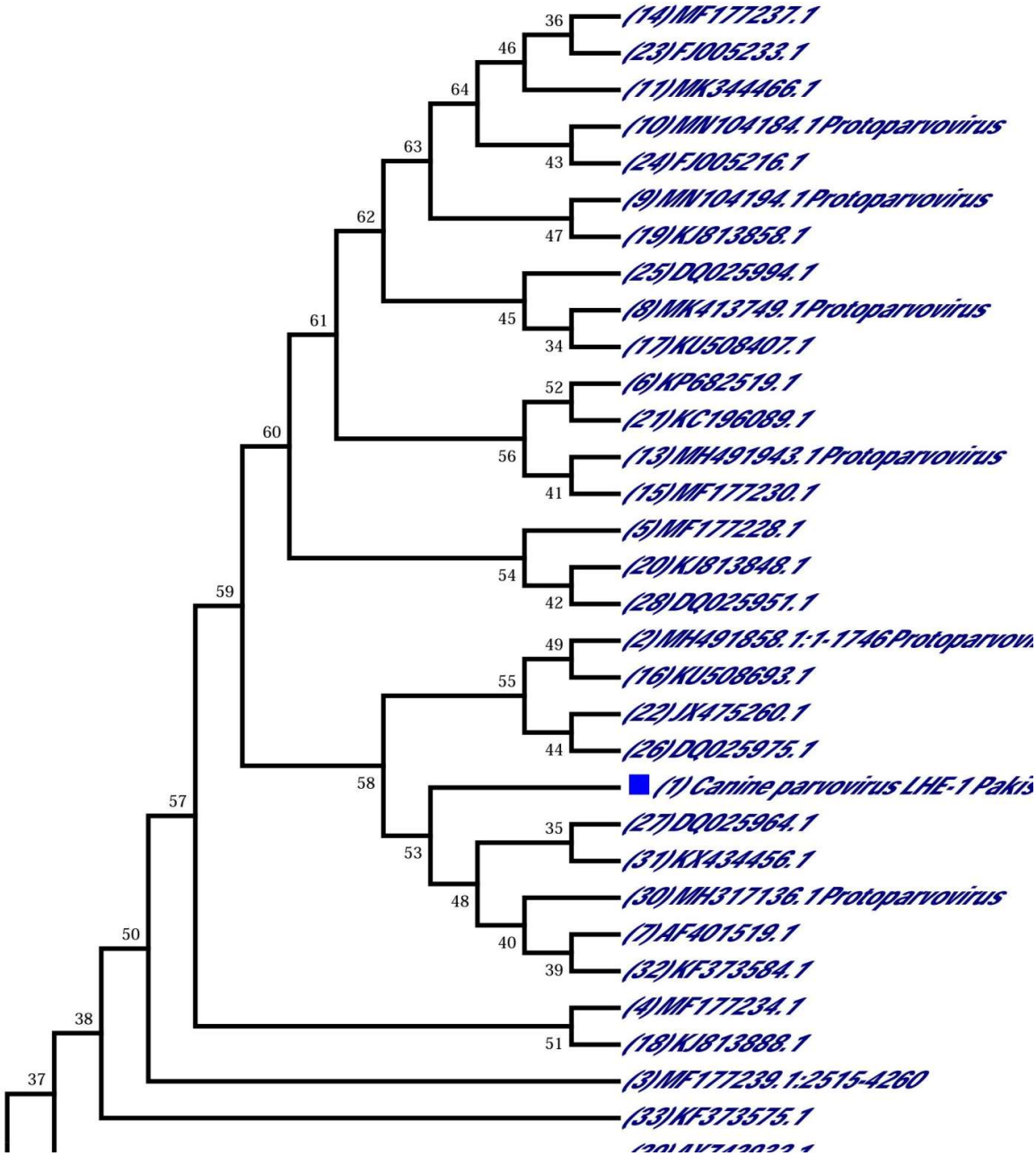

Fig. 4: Phylogenetic tree of Canine Parvovirus.

Table 6: Result of chi-square analysis of the parameters compared with p and α values.

Parameter	X^2 CCoV	α value	P value	X^2 CPV	α value	P value	X^2 CCoV-CPV	α value	P value	Degree of freedom
Age	33.9	16.27	1.0	44.5	16.27	1.00	16.8	16.27	1.00	3
Sex	0.2	10.83	0.3	0.5	10.83	0.51	0.7	10.83	0.59	1
Month	71.1	31.26	1.0	94.2	31.26	1.00	26.7	31.26	0.99	11
Breed	70.8	27.88	1.0	167.6	27.8	1.00	21.6	27.88	0.99	9

This coincides with the findings of Agnihotri *et al.* (2018).The highest prevalence of CPV and CCoV co-infection was observed in the dogs who were 5 to 8 months of age. The study showed that CCoV was more prevalent in female dogs. This finding was in harmony with the findings of Stavisky *et al.* (2012) and Sulehria *et al.* (2020). CPV was observed to be more prevalent in male dogs. This finding shows no discrepancy with the reports of Tagorti *et al.* (2018), Singh *et al.* (2021), Khare *et al.* (2019) and Agnihotri *et al.* (2018). The highest prevalence of CCoV was observed in July, the same trend was observed by Deka *et al.* (2013).

The CPV prevalence was observed to be the highest in August which endorses the findings of Dema *et al.* (2022). The prevalence of co-infection of CCoV and CPV was in August. The CCoV and CPV co-infection was most prevalent in German shepherd dogs. The same trend was reported by Dema *et al.* (2022). The CCoV-positive

patients were anemic, leukopenic, febrile, anorectic and had diarrhea, vomiting, and pain in the abdomen. These findings are in line with those of Sulehria *et al*. (2023), Agnihotri *et al.* (2017), Dongre *et al.* (2015) and Sharma *et al.* (2008). The CPV positive patients were anemic, febrile, diarrheic, anorectic, leukopenic, panleukopenic and had pain in abdomen. These findings coincide with findings of Ortega *et al.* (2017). The patients with CCoV-CPV co-infection showed anorexia, fever, pain in the abdomen, diarrhea, vomiting, anemia, panleukopenia and 100% mortality rate. This is endorsed by the findings of Ortega *et al.* (2017).

Conclusions: *Canine coronavirus* and *canine parvovirus* are circulating in the canine population of Pakistan and may exist as co-pathogens in a single canine patient causing a very severe clinical illness leading to death. *Canine rotavirus* could not be detected in the canine population of Pakistan. Male German shepherd dogs under the age of 1 year are highly prone to get viral diarrhea in July and August.

Declaration of competing interest: All the authors have declared no conflicts of interest.

Authors contribution: Conception, US, SS; methodological approach, US, SS, MI; critical examination, US, SS, MI, and HM; writing initial draft preparation, US, SS; writing, assessment, and proofreading, US, SS, and MI.

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Ethics approval: The study design was approved by the Ethics Committee of the University of Veterinary and Animal Sciences Lahore, Pakistan under diary No. 502/dated: 02-03-2018.

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