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Biotechnological and virological analysis of canine parvovirus infections by C-reactive protein levels, serological, hematological and molecular techniques

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ABSTRACT

The study aims to approach Canine Parvovirus (CPV) diagnosis using multi-method biotechnological techniques including molecular, serological, and hematological analyses. CPVs are causing severe global viral diseases with high dog mortality. Samples were taken from 52 unvaccinated dogs exhibiting symptoms between 2020 and 2021. These included stool, blood, serum, and patient data. CPV genomic DNA was extracted from fresh stools, with DNA concentration and purity measured using Nano drop-spectrophotometry. CPV genomic DNA was detected via RT-PCR in 29 samples (55.8%), CPV IgM-Ab and IgG-Ab were detected in the sera through ELISA in 27 samples (51.9%), and Canine parvovirus antigens were identified in the stool samples by immunochromatography in 20 samples (38.5%). Utilizing canine-specific quantitative ELISA kits, the average level of serum C-reactive protein (CRP) was determined to be 4.66 g/L (with a range of 3.27 to 6.05 g/L). Hematological analysis revealed lymphopenia in 89.6%, leucopenia in 44.8%, anemia in 68.9%, and low hematocrit in 82.8%. All the dogs examined were under 1 year of age, among which 21 (72.4%) were up to 3 months old, and 8 (27.6%) were up to 6 months old, testing positive for CPV. The highest CPV positivity, at 93.1% (n=27), was observed among dogs with outdoor access. The results indicated that hematological parameters and CRP alone were not specific for CPV diagnosis, but provided valuable data for prognosis and differential diagnosis. No significant differences were observed in RT-PCR and ELISA results. However, a noticeable reduction in positivity rates was evident in lateral immunochromatographic viral antigen detection in stool.

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Introduction

Parvoviruses are small, non-enveloped, single-stranded DNA viruses with a spherical capsid and belonging to *Parvoviridae* family (1). Parvoviruses exhibit remarkable environmental resilience, surviving for up to 5-7 months. These viruses can withstand high temperatures, extreme pH conditions, trypsin digestion, and various disinfectants, making them difficult to eradicate (2,3). However, certain agents like formalin, sodium hypochlorite, beta propiolactone, hydroxylamine, and UV rays can inactivate them. CPVs can also agglutinate red blood cells from various mammals and birds (4).

Dogs are among the most beloved companion pets worldwide, serving both physical and emotional roles for humans. However, Canine Parvovirus (CPV) infection remains a highly contagious and deadly acute viral disease, particularly affecting puppies aged 6 to 20 weeks. The disease is characterized by severe gastrointestinal symptoms, including acute, fibrinous, necrotic, and hemorrhagic enteritis, leading to foul-smelling diarrhea. Additionally, CPV infection can result in myocarditis, particularly in young dogs (2,4). Even vaccinated dogs and puppies born to immune-insufficient mothers are susceptible to CPV due to insufficient protective antibodies (5,6,7).

Two main etiological agents, Canine Parvovirus type-1 (CPV-1) and Canine Parvovirus type-2 (CPV-2), cause the infection (1,8,9). CPV-2 has further evolved into different antigenic forms, including CPV-2a, CPV-2b, and CPV-2c, all with similar pathogenesis (8-11). These types can affect a wide range of hosts and even cause feline pan leucopenia in cats (12). Despite the availability of vaccines, CPV-2 remains a significant health concern for dogs globally (13,14).

CPV targets rapidly dividing DNA-synthesizing cells, such as intestinal cells, bone marrow precursor cells, and myocardiocytes. It disrupts cell mitosis and multiplication, leading to cell death (2,15-17). The virus is primarily transmitted through fecal-oral and oronasal routes (18). Clinical symptoms include depression, vomiting, diarrhea (which can be mucoid or hemorrhagic), dehydration, anorexia, and fever (7). The disease can manifest as acute hemorrhagic enteritis or myocarditis, with mortality rates as high as 70% in puppies and less than 1% in adult dogs (18-22).

Leucopenia is a significant finding in CPV-2 infections, often dropping below 2000-3000 cells/ml during the disease. Although clinical and laboratory findings can aid diagnosis, they may not be sufficient on their own due to the presence of other pathogens causing similar symptoms. Therefore, a combination of diagnostic methods is necessary (2,4,22,23).

Various biotechnological diagnostic methods are employed for CPV infection diagnosis, including convention-

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al PCR, Real-Time PCR, electron microscopy, virus isolation, hemagglutination test, ELISA, latex agglutination test, virus neutralization test, and immunochromatographic tests. These methods have varying levels of sensitivity and specificity. There are limited studies on serum C-reactive protein (CRP) levels in the diagnosis of CPV infection and have not been combined with other diagnostic methods. This study seeks to diagnose CPV infection through a biotechnological strategy encompassing RT-PCR, Nano-drop spectrophotometry, ELISA, lateral immunochromatography, and hematological methods. Additionally, it evaluates serum CRP values to monitor variations in dogs impacted by the disease (16,22-25). This study harmonizes various biotechnological techniques to attain a thorough CPV infection diagnosis.

Materials and Methods

Experimental materials

During the study, a total of fifty-two unvaccinated dogs exhibiting symptoms such as weakness, lethargy, reduced appetite, high fever, vomiting, and diarrhea/bloody diarrhea were examined for suspected natural viral infections. Pertinent data including age, breed, gender, ownership status, and living environment were documented. Whole blood samples, comprising 5 ml of EDTA for hematology and 8 ml of serum for serology and CRP tests, were collected from the jugular and cephalic veins and preserved in a cold chain. For molecular and serological analysis, a sterile swab was used to collect a stool sample from the rectum of each dog.

Experimental methods

Hematological Evaluation

Blood analysis was conducted using the Mindray Veterinary Auto analyzer device and blood count kits comprehensively on the sampling day. The complete blood count, hematocrit and platelet count assessments were performed, including leukocyte (WBC), lymphocyte (LYM), erythrocyte (RBC), hemoglobin (HGB), hematocrit (HCT), and platelet (PLT) counts.

Blood smears were generated from peripheral blood samples for a semi-quantitative platelet count. Air-dried samples were treated with a 'wet preparation' technique by adding a drop of new methylene blue, followed by covering with a slip without fixation. A total of 10 immersion fields were examined. Platelet counts, stained in blue-purple, were determined for each immersion field and compared against a reference value table to establish a semi-quantitative platelet count.

The capillary centrifuge test was automated at a speed of 12,000 rpm using the Nüve-NF 048 device, and the results were assessed by comparing them with a reading scale provided with the device (a unit ruler ranging from 0 to 100 in a specific ratio). The capillary tube sample was filled with three-quarters of the blood drawn from the peripheral blood tubes, and its bloodless end was sealed. During evaluation, the entire blood volume was considered as 100, and the capillary tube was placed on the reading scale to obtain a reading.

Measurement of Canine Anti-Parvovirus IgM and IgG Antibody Levels using the ELISA Method

Canine Anti-Parvovirus IgM and IgG antibody (Canine Parvovirus Ab IgM and IgG) ELISA test kits were obtained from a commercial company (Shanghai Coon Koon Biotech Co., Ltd, IgM Cat No: CK-bio-20590, IgG Cat No: CK-bio-20589) were used for CPV Ab IgM and IgG serological tests using the ELISA method.

ELISA test: The kit uses the qualitative enzyme immunoassay method. The micro titer plate provided was precoated with antigen, the sample was added, and the horseradish peroxidase enzyme (HRP) was added respectively, incubated, and thoroughly washed. The Stop Solution changed color from blue to yellow, and the color intensity was measured at 450 nm using a spectrophotometer. The calibration standards were tested simultaneously with the samples and a CUT OFF value was obtained. Then, it was decided whether Parvovirus Ab IgM and IgG were present in the samples by comparing them with the CUT OFF value.

Measurement of Canine Serum C-Reactive Protein through ELISA Method

The canine serum C-reactive protein (CRP) level was assessed using a species-specific canine CRP ELISA kit obtained from a commercial company (Shanghai Coon Koon Biotech Co., Ltd, Cat No: CK-bio-18804).

The ELISA Kit employed the double-antibody sandwich technology principle to determine the level of canine CRP. All kit reagents were equilibrated to room temperature (20-25 °C) before use. Standard and samples were introduced into wells pre-coated with the target antibody. Subsequently, the HRP-Conjugate reagent was added to form an immune complex. Following incubation and washing, unbound enzymes were removed, and substrates A and B were added. The solution's color transformation, from initial blue to final yellow due to acid action, was observed. Color intensity was positively correlated with CRP concentration.

The test procedure was followed, thereby determining the correction of optical densities of the samples for blank adsorbent effects, and subsequently calculating the substance quantity corresponding to the optical densities of each sample on the curve. For retrospective control, substance quantities were entered as a function of the x-axis y, and the corresponding optical density was obtained. This process confirmed the accuracy of measurements.

Real-Time Polymerase Chain Reaction (RT-PCR) Tests

Preparation of Template DNA for RT-PCR; Stool samples were collected from dogs with diarrhea using sterile swabs and then homogenized in a phosphate-buffered saline solution (PBS) at a concentration of 10% (w/v).

To clarify the fecal suspensions, a brief high-speed centrifugation was performed using a microfuge. Subsequently, 200 ml aliquots of the supernatant were utilized for DNA extraction. The process involved inactivating PCR inhibitors and allowing for a 10-minute cooling period before further processing.

Primers and Probe Design for RT-PCR

In this study, a modification was made in the probe selection, and the methodology was followed by Decaro et al. (2007). The FAM-BHQ-1 probe was used to replace the FAM-TAMRA probe. TaqMan probe was labeled with the fluorescent dye 6-carboxyfluorescein (FAM) at its 5' end, and the quencher dye 6-carboxytetramethylrhodamine

Table 1. Primers used for RT-PCR.

CPV primer F	5'-AAACAGGAATTAACTATACTAATATATTTA-3'
CPV primer F	5'-AAATTTGACCATTTGGATAAACT-3'
CPV probe	5'-FAM-TGGTCCTTTAACTGCATTAAATAATGTACC-BHQ1-3'

Table 2. Components and their quantities used for RT-PCR reactions.

Component	Volume/Reaction
2x QuantiNova Probe PCR Master Mix	10.0 µl
Primer F	1.0 µl
Primer R	1.0 µl
Probe	0.25 µl
DNA	5.0 µl
Rox passive dye	1.0 µl
Nuclease-free water	1.75 µl
Total	20 µl

Table 3. Heat cycles of RT-PCR reactions.

Step	Period	Temperature
Enzyme Activation	5 min	95ºC
Denaturation	30 sec	95ºC
Annealing	45 sec	51 ºC
Extension	30 sec	60ºC
40 cycle	Real-time reading at the end	

(BHQ-1) at its 3' end.

For quantitative real-time PCR analysis of gene expression, Applied Biosystems TaqMan Gene Expression Assays were employed. These assays are furnished with a pair of unlabeled PCR primers and a dye tag (FAM) at the 5' end, along with a minor groove binder (MGB) containing a non-fluorescent quencher (NFQ) at the 3' end. The system encompasses a TaqMan probe as a crucial component.

Standard DNA Preparation

Extraction procedures were carried out together with the positive control (CPV-2). Viral DNA extraction was performed using the 'QIAamp DNA Stool Mini Kit' (QIAGEN, 51504, Germany) from the collected stool samples.

Positive Control of CPV DNA

As a positive control, Canine parvovirus DNA was used, which was obtained from the Konya Selçuk University Faculty of Veterinary Medicine Department of Virology.

Real-Time PCR

RT-PCR tests were performed using the Step One Plus (Applied Biosystems) RT-PCR device and the data were analyzed with the software of the device.

Copies of CPV standard dilutions and DNA templates were simultaneously subjected to real-time analysis. In order to determine the presence of CPV by RT-PCR in the samples used in the study, the following primers (Table 1) were used, as in the study by Decaro et al. (2007).

Real-Time PCR reactions; the components given in Table 2 were transferred to optical covered8 strip tubes for each sample.

Real Time PCR reactions heat cycles Decaro et al. (2007) protocol was modified and used as shown in Table 3.

Statistical Analysis

The study data were organized into tables as raw statistical data using the MS Excel program, and subsequently, they were subjected to analysis using the SPSS 17 statistical software. The resulting data are presented in Table 4 and Table 5.

Results

Epidemiological Data Findings

The study population consisted of naturally infected 52 dogs, obtained from Adana Metropolitan Municipality, Osmaniye Municipality Animal Shelters, and private veterinary clinics, displaying symptoms compatible with the canine parvovirus infection and diarrhea. The sampling was conducted by collecting EDTA blood, serum, and rectal stool samples between June 2020 and January 2021.

Table 4 displays the distribution of the 52 dogs based on their age, breed, gender, and clinical symptoms observed during their initial evaluation. All of these dogs ages were between 1 and 12 months old, comprising 27 males and 25 females. Among them, 31 dogs were owned, while 21 were strays. Notably, 29 (55.8%) were confirmed as CPV positive using the RT-PCR molecular testing method, and 27 (51.9%) were serologically diagnosed through ELISA tests.

Results of Hematology Tests

In Table 5, statistically, differences between races of WBC, RBC, HGB, HCT, PLT, and LYMPH values were found $0.05<p$ (n:3).

Upon examining the blood samples collected from the

Table 4. Descriptive statistics on the characteristics of dogs.

	Groups	Number	Percent (%)
Age range	1-3 months old	39	75
	4-6 months old	11	21,16
	≥ 7 months old	2	3,84
Breed	Kangal	11	21,15
	Mix	21	40,39
	Golden retriever	5	9,62
	German pointer	4	7,69
	German Sepherd	3	5,77
	Terrier	1	1,92
	Setter	1	1,92
	Cane corsa	2	3,85
	Beagle	1	1,92
	Alabay	1	1,92
	Maltese	1	1,92
	Tea cup	1	1,92
Gender	Male	27	51,92
	Female	25	48,07
CPV Positive	Yes	29	55,77
	No	23	44,23
Sick	Male	17	58,62
	Female	12	41,38
Healthy	Male	10	43,48
	Female	13	56,52
Stray	Yes	21	40,38
	No	31	59,62
Vomit	Yes	37	71,15
	No	15	28,85
Diarrhea	Yes	52	100
	No	-	0
Bloody diarrhea	Yes	39	75
	No	13	25
Free movement	Yes	49	94,23
	No	3	5,77
Dehydration	Yes	44	84,62
	No	8	15,38
Fever	Yes	38	73,08
	No	14	26,92
Weakness	Yes	44	84,62
	No	8	15,38
Anemia	Yes	35	67,31
	No	17	32,69
Mucosal pallor	Yes	43	82,69
	No	9	17,31

studied dogs, a comprehensive assessment was conducted using a veterinary auto analyzer, an automatic hematocrit centrifuge device, and blood smears stained with new methylene blue. The results revealed significant hematological findings among the afflicted canines. Specifically, the sick dogs exhibited diffuse lymphopenia at a rate of 89.6%, while leucopenia was observed in 44.8% of cases. Moreover, anemia was identified in 68.9% of the dogs, and a substantial portion—82.8%—displayed low hematocrit levels.

ELISA Test Results

Dogs displaying clinical symptoms consistent with viral infection during their initial examinations were subjected to serological evaluation using the ELISA method. Out of the total of 52 examined dogs, 27 were serologically identified as positive for CPV infection through ELISA testing.

Table 5. Statistical differences between races in hematology test results.

Dog Breeds Reference Values	WBC <6,7 10^9/L	RBC <5,6 10^{12}/L	HGB <12,9 g/L	HCT <37 %	PLT <200 10^9/L	LYMPH <4,4 10^9/L
Kangal.1	10,5±0,32^{i}	5,29±0,25^{h}	10,7±0,21^{n}	34,2±0,35^{ı}	355±2,14^{c}	1±0,05^{o}
Kangal.2	6,6±0,05^{m}	5,74±0,14^{f}	14,6±0,31^{d}	39,2±0,42^{c}	374±2,31^{a}	1,4±0,09^{lm}
Kangal.3	5,96±0,07^{n}	6,57±0,27^{c}	15,4±0,24^{c}	41,9±0,14^{b}	142±1,34^{l}	1,73±0,21^{i}
Kangal.4	6,6±0,08^{m}	5,74±0,18^{f}	14,6±0,17^{d}	39,2±0,34^{c}	174±1,28^{gh}	1,4±0,09^{lm}
Kangal.5	5,5±0,11^{o}	4,55±0,18^{o}	11,3±0,34^{l}	29,9±0,27^{m}	163±1,36^{ı}	0,00±0,00
Kangal.6	3,1±0,08^{r}	4,61±0,23^{n}	9,5±,28^{u}	29±0,37^{o}	148±1,21^{k}	0,8±0,02^{ö}
Kangal.7	5,1±0,04^{ö}	5,54±0,31^{g}	13,1±,28^{g}	38,2±0,19^{d}	104±1,09^{s}	1,6±0,09^{k}
Mix.1	10,5±0,15^{i}	4,68±0,14^{i}	12,8±0,04^{h}	24,5±0,25^{ü}	112±1,43^{r}	2,3±0,14^{g}
Mix.2	6,30±0,09^{m}	4,79±0,21^{k}	11,3±0,17^{l}	26±0,18^{u}	105±1,48^{s}	2,9±0,19^{e}
Mix.3	20,7±0,14^{e}	4,73±0,24^{l}	12,3±0,28^{i}	34±0,29^{i}	173±1,28^{gh}	5,1±0,31^{b}
Mix.4	20,42±0,31^{e}	14,78±0,38^{a}	10,1±0,11^{ö}	31,1±0,19^{l}	367±2,05^{b}	3,33±0,11^{d}
Mix.5	24,77±0,11^{b}	4,3±0,05^{ö}	9,8±0,10^{s}	27,8±0,37^{r}	196±1,09^{e}	2,06±0,08^{ı}
Mix.6	11,32±0,19^{ı}	5,06±0,21^{ı}	11,8±0,28^{k}	32,7±0,27^{k}	163±1,34^{ı}	1±0,05^{o}
Mix.7	21,08±0,25^{d}	4,21±0,14^{r}	8,7±0,36^{z}	26,1±0,24^{u}	179±1,28^{f}	1,43±0,21^{lm}
Mix.8	12,57±0,27^{g}	4,33±0,12^{ö}	9,9±0,28^{r}	28,1±0,29^{ö}	171±1,32^{h}	1,95±0,18^{ı}
Mix.9	11,08±0,17^{ı}	4,62±0,07^{n}	9,6±0,24^{t}	29,2±0,27^{n}	147±1,05^{k}	1,25±0,13^{n}
Mix.10	14,47±0,32^{f}	4,02±0,06^{s}	8,4±0,34^{x}	26,5±0,24^{ş}	137±0,96^{m}	1,4±0,09^{lm}
G.Sepherd.1	5,1±0,14^{ö}	5,57±0,28^{g}	14,6±0,38^{d}	36,7±0,28^{f}	177±1,64^{fg}	1,5±0,12^{kl}
G.Sepherd.2	23,6±0,19^{c}	6,69±0,13^{b}	17,6±0,35^{b}	48,2±0,34^{a}	180±1,46^{f}	4,5±0,42^{c}
G.Sepherd.3	5,2±0,14^{oö}	3,3±0,09^{v}	8,9±0,19^{v}	23,2±0,28^{v}	156±1,54^{i}	0,7±0,05^{o}
Pointer.1	11,1±0,21^{ı}	3,37±0,09^{u}	9,9±0,14^{r}	18,8±0,28^{z}	128±1,28^{n}	2,2±0,11^{g}
Pointer.2	2,9±0,05^{s}	6,46±0,32^{d}	18,4±0,32^{a}	47,8±0,35^{z}	272±1,63^{d}	1±0,24^{o}
Pointer.3	42,5±0,28^{a}	5,86±0,28^{e}	13,7±0,19^{e}	36,3±0,39^{g}	54±1,26^{ü}	9±0,15^{a}
G.Rertiever.1	0,98±0,17^{v}	6,47±0,39^{d}	13,4±0,42^{f}	37,6±0,39^{e}	122±1,28^{o}	0,79±0,24^{ö}
G.Retriever.2	5,32±0,08^{oö}	4,83±0,14^{i}	12,6±0,18^{ı}	35,9±0,28^{h}	117±1,24^{ö}	0,98±0,31^{o}
G.Retriever.3	9,9±0,28^{k}	4,67±0,14^{m}	8,8±0,09^{y}	27,1±0,34^{s}	124±1,64^{o}	3,3±0,08^{d}
Cane Corsa	2,5±0,14^{t}	3,86±0,05^{t}	11,1±0,09^{m}	20,5±0,38^{y}	176±1,34^{fg}	1,3±0,08^{mn}
Alabay	12±0,25^{h}	2,5±0,34^{z}	9,2±0,18^{ü}	10±0,18^{x}	94±1,42^{t}	1,2±0,05^{n}
Maltese	7,7±0,17^{l}	2,66±0,28^{y}	10,6±0,28^{o}	18,8±0,21^{z}	78±1,58^{u}	2,7±0,17^{f}

WBC (reference value = < 6,7 10^9/L) = m,n,o,ö,r,s,t,v < reference value < a,b,c,d,e,f,g,h,ı,i,k,l ($p<0,05$).
RBC (reference value = <5,6 10^{12}/L) = g,h,ı,i,k,l,m,n,o,ö,r,s,t,u,v,y,z < reference value <a,b,c,d,e,f ($p<0,05$).
HGB (reference value = < 12,9 g/L) =, h,ı,i,k,l,m,n,o,ö,r,s,t,u,ü,v,y,z < reference value < a,b,c,d,e,f,g ($p<0,05$).
HCT (reference value = < 37 %) = f,g,h,ı,i,k,l,m,n,o,ö,r,s,t,u,v,y,z < reference value < a,b,c,d,e ($p<0,05$).
PLT (reference value = <200 10^9/L) = e, f,g,h,ı,i,k,l,m,n,o,ö,r,s,t,u,v,y,z < reference value < a,b,c,d ($p<0,05$).
LYMPH (reference value = <4,4 10^9/L) = d,e, f,g,h,ı,i,k,l,m,n,o,ö,r,s,t,u,v,y,z < reference value < a,b,c ($p<0,05$).

Results of Canine Anti-Parvovirus IgM Antibody Level Measurements

To ascertain IgM test outcomes, the Optical Density (O.D.) of micro titer plates was read at 450 nm using a BioTek ELX 800 micro titer plate reader device. The analysis revealed that the average for positive wells was 2.256, meeting the validity condition set by the manufacturer's test protocol for values of ≥1.00. Notably, 27 out of the 29 samples were determined to exhibit positive results for the level of Canine Anti-Parvovirus IgM antibodies.

Calculation of Canine Serum C-Reactive Protein Values

Quantitative measurements were conducted using CurveExpert Professional (ver.2.6.5) by comparing the optical densities of samples with 2-fold dilutions of the standard solution provided with the ELISA kit. Curves were generated using Lowess Smoothing and Bleasdale methods, with optical densities from standard and blank readings on the y-axis and corresponding substance amounts (ng/dl, mg/dl) on the x-axis. To validate curve accuracy, entered standard data were tested against the generated curve, and curves yielding standard amounts without deviation and achieving a score of at least 970/1000 were employed for analysis. After correcting the optical density of samples as outlined in the test procedure, substance amounts corresponding to each sample's optical density on the curve were calculated. For retrospective verification, measurements were confirmed by inputting substance amounts on the x-axis and retrieving corresponding optical densities. Obtained results indicated that CRP values ranged from the highest of 6.05 g/L to the lowest of 3.27 g/L, with a mean value of 4.66 g/L.

Results of Real-Time PCR (RT-PCR) Test

RT-PCR were conducted on DNA extracted from the fecal samples of the 52 dogs included in the study. The

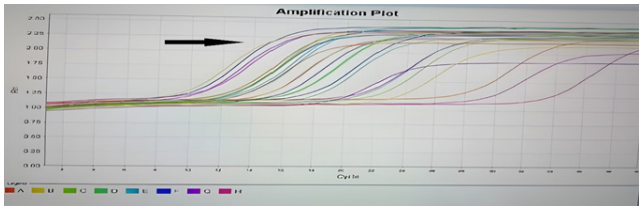

Figure 1. The amplification plot results of the RT-PCR test for CPV-positive samples (indicated by a black arrow).

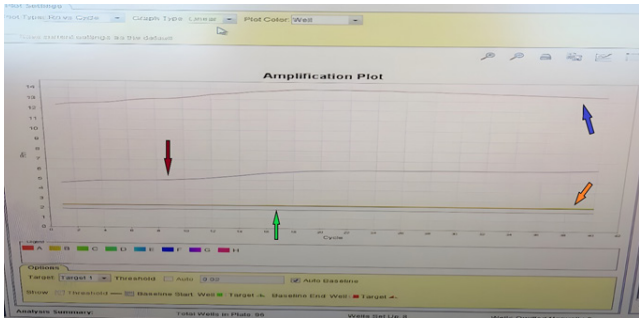

Figure 2. The amplification plot results of the RT-PCR test for CPV-positive samples are displayed with specific indicators: the positive control is marked by a red arrow, the negative control by a green arrow, the positive sample by a blue arrow, and the negative sample by an orange arrow.

approximate quantity of DNA nucleic acid in the samples was determined before conducting the RT-PCR test using a nano-drop spectrophotometer. Absorbance was measured at wavelengths 260 nm and 280 nm. The highest DNA concentration recorded was 39.61 ng/µl, the lowest was 2.13 ng/µl, and the average was 8.27 ng/µl. CPV DNA was identified in the feces of 29 dogs through RT-PCR, indicating a positive result rate of 55.8% (Figures 1 and 2).

Discussion

RT-PCR is a sensitive and specific test method to detect the presence of CPV DNA in the dog's feces. A positive RT-PCR result confirms the viral presence, aiding in early diagnosis even before clinical signs appear. Nano-drop spectrophotometry measures the concentration and purity of the DNA in samples. ELISA tests can detect CPV-specific antibodies in the dog's serum. A rising level of antibodies indicates an active immune response against the virus. However, serological tests might not be useful in the early stages of infection, as it takes time for the immune system to produce detectable levels of antibodies. Hematological changes associated with CPV infection include leucopenia and neutropenia. Blood tests can reveal these abnormalities, which are often indicative of a viral infection. CRP is an acute-phase protein that increases in response to inflammation and infection. Elevated CRP levels can indicate the presence of an active infection. Monitoring CRP levels can help assess the severity of the infection and the response to treatment. The study aims to enhance CPV infection diagnosis using multi-method biotechnological techniques including molecular, spectrophotometric, serological and hematological analyses.

The current study indicated a CPV-positive rate of 55.8% using RT-PCR, which was observed to be consistent with the previously published data. Studies conducted in different regions and canine populations have yielded varying results regarding CPV prevalence. Elbaz et al. (26) conducted a study in Egypt using immunochromatography and PCR. Hirasawa et al. (27) in Japan and Awad et al. (28) in Egypt reported CPV-2 positivity rates of 54.1% and 55-56%, respectively, through PCR tests, with similarities to the results of this study. In recent years, various researchers reported different outcomes in a limited number of studies conducted in Türkiye. For instance, Şahna et al. (2008) reported a CPV-2 detection rate of 76.5% in stool samples from stray dogs with diarrhea in Kayseri by PCR. Similarly, Dik (2017) indicated a positivity rate of 66% of CPV-2ab through PCR in dogs with diarrhea. Both of these findings exceeded the results of this study. The differences in the results of the studies are likely attributed to variations in the examined population and differences in sample sizes.

In contrast, according to the outcomes of this study, a 38.4% positivity rate for CPV-2 was identified in Ankara, Türkiye (31) in distinct examinations of canine fecal samples. This figure was lower than the results of this study. Similarly, Yilmaz et al. (32) and Yesilbag et al. (33) reported CPV-2 positivity rates of 35% and 35.5%, respectively, in the Bursa region. The variations between these studies could potentially be attributed to differences in dogs' living conditions, the absence of maternal antibodies and colostrum protection, and other individual factors. Consequently, it underscores the importance of studies conducted to demonstrate the ongoing presence of the virus at a significant rate among dogs in our country.

Blood sample analyses in the examined dogs in the study identified various hematological abnormalities. These included diffuse lymphopenia (89.6%), leucopenia (44.8%), anemia (68.9%), and low hematocrit (82.8%) levels. Several previous studies have also investigated the hematological effects of CPV infection in dogs. For instance, Castro et al. (34) conducted a study in Brazil on dogs under 1 year old with CPV infection, reporting anemia, lymphopenia, and leucopenia percentages of 61.5%, 77%, and 46.1%, respectively. Terzungwe (23) in Ukraine reported anemia (20.7%), leucopenia (31%), and lymphopenia (27.6%) in a study involving 29 dogs of various breeds and ages with CPV infection. Alves et al. (35) in Brazil studied 103 dogs under 1 year old with CPV infection and noted severe leucopenia in 75% of affected dogs, with 40.4% of the leucopenic patients succumbing to the infection. Arslan et al. (36) also reported that dogs with CPV infection exhibited a hematocrit (HCT) of 36.97%, lower than the control group. The destruction of red blood cells (RBC), lymphocytes (LYMP), hematocrit (HCT), and white blood cells (WBC) due to CPV infection results in severe diseases that can lead to mortality. The virus targets rapidly proliferating tissues such as intestinal epithelium and bone marrow, leading to conditions like lymphopenia, panleucopenia, anemia, and immunosuppression. These adverse effects arise from the virus's impact on myeloproliferative cells in the lymphopoietic system and bone marrow, ultimately contributing to the severity of the infection, particularly in young puppies.

In this study, we assessed the levels of Canine Anti-Parvovirus IgM and IgG antibodies. The average positive well value for CPV IgM was determined as 2.256, while for CPV IgG, it was 2.618. Ultimately, 27 out of 29 samples presented positive results for both CPV IgM and IgG antibody levels. The presence of positive IgM

antibody levels indicates an active infection, and the presence of IgG antibody levels signifies the immune system's response to CPV infection in dogs. In a study evaluating immunity and seropositivity of IgG antibodies against CPV infection with 48 vaccinated and unvaccinated dogs in Abeokuta, Nigeria, Babaloa et al. (37) reported notable findings. The study revealed a high anti-CPV antibody titer of 79.2% (38 dogs) and a lower titer of 20.8% (10 dogs), confirming the presence of IgG antibodies to CPV among the studied dogs. Waner et al. (38) documented that all dogs exhibited IgM and IgG antibodies of CPV-2 in naturally infected dogs using serum IgM and IgG Rapid Dot ELISA, and also noted that the titers of IgM antibodies in naturally infected dogs were quantitatively higher. Correction: The conducted studies demonstrate a significant relationship between the detection of CPV IgM and IgG antibody levels and the diagnosis of CPV infection.

In previous studies, Elbaz et al. (26) conducted in Egypt and Tekelioglu et al. (39) in Türkiye utilized lateral immunochromatography Ag (IC-Ag) as a rapid antigen screening test. Elbaz et al. obtained positive CPV-2 outcomes in 35 (70%) of the 50 samples, while Tekelioglu et al. found positive results in 47 (44.3%) of the 106 samples. Intriguingly, the same samples were found to contain CPV-2 DNA in 42 samples (84%) when analyzed using chain reaction (PCR) tests. Hirasawa et al. (27) scrutinized the prevalence of CPV-2 in Japanese dogs, assessing the genomic variations between virus strains, and reported a virus positivity rate of 54.1% based on PCR tests performed on 74 stool samples. Moreover, Awad et al. (28) determined the prevalence of CPV-2b infection to be between 55-56% among the study population in their research on the prevalence of CPV infection in Egypt. Consequently, in this study, a 55.8% positive rate was established through RT-PCR tests. Although the figures in Elbaz et al.'s (26) findings appear lower, a resemblance can be observed between the results of Hirasawa et al. (1996) and Awad et al. (2019), indicating commonalities despite geographical differences. A significant common point of studies conducted in different years and geographical regions is that they reveal the global prevalence of CPV infection.

The study recorded the highest CRP value as 6.05 g/L, the lowest as 3.27 g/L, and an average value of 4.66 g/L. Similarly, to our results, McClure et al. (40), in a study aiming to predict outcomes in CPV-infected puppies using serum CRP concentration, revealed an average CRP level of 9.73 g/L. Hindenberg et al. (41), on the other hand, reported a CRP concentration ranging from 4 to 281 mg/l in their research. Elevated CRP concentrations are typically indicative of inflammation, often associated with various conditions such as bacterial infections and certain types of cancers (40,41), and its connection to viral diseases in dogs is a newly researched topic. The findings of this study underscore the significance of hematological and serological parameters, including CRP, as prognostic factors in CPV-infected dogs. Dogs with severe anemia, lymphopenia, leucopenia, or a combination of these conditions are significantly more prone to a higher risk of mortality. Previous researches by different investigators suggests that CRP concentrations in dogs can increase in response to local or systemic inflammation induced by factors such as infections, sepsis, and trauma (42,43). In a prospective study involving 116 dogs (43), those with Systemic Inflammatory Response Syndrome or sepsis displayed notably higher CRP concentrations (165 ± 82 mg/l) than those with localized inflammation (108 ± 70 mg/l), demonstrating observable differences. Sasaki et al. (44) explored the differentiation between bacterial and viral infections through inflammatory markers in an early-stage study. The variations in CRP concentrations between infections led to moderate to high CRP increases. Importantly, CRP levels are influenced by the timing of sample collection during the course of the disease. In this study, samples were collected from dogs during the first 5 days of infection. During this period, CRP values peaked at 6.05 g/L and subsequently decreased to 3.27 g/L. The mean value was determined as 4.66 g/L, aligning with the observations made by Sasaki et al. (2002). These findings emphasize the correlation between CRP levels and disease progression.

In conclusion, this study aimed to comprehensively assess the CPV infection status in dogs through a multimethod biotechnological diagnostics approach. The rapid and severe progression of CPV infection underscores the critical importance of early and accurate diagnosis for timely intervention and treatment. The study introduces novel data that can serve as references for future investigations in this field. There were few studies and limited data focusing on the relationship between changes in serum CRP levels and CPV disease. In this study, the FAM-BHQ-1 probe was used to replace the FAM-TAMRA probe. This modification involves the replacement of the TaqMan probe labeled with the fluorescent indicator dye FAM at the 5' end and the quencher dye BHQ-1 at the 3' end, and it is considered an alternative probe option for CPV detection.

Interest conflict
None of the authors has a conflict of interest.

Consent for publications
The authors read and proved the final manuscript for publication.

Availability of data and material
All data generated during this study are included in this published article.

Authors' Contribution
All authors had equal roles in study design, work, statistical analysis and manuscript writing.

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