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Molecular detection and phylogenetic analysis of Canine Parvovirus (*CPV*) in diarrhetic k9 dogs in Thi-qar Province-Iraq

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ABSTRACT

The present study was designed to the PCR diagnosis and DNA sequencing of canine parvovirus type 2 in diarrheal canine dogs. The results of 35 clinically suspected CPV-2 infected puppies showed diarrhea, vomiting (100%), dehydration (77%), and 25% of animals were dead. A genetic fragment of 400bp length that partially covered a coding portion of the *CPV* gene, within viral samples was amplified in this study. A direct sequencing strategy was performed for the observed PCR amplicons in the amplified genetic locus. Subsequently, a phylogenetic tree was constructed in the observed variants to assess the accurate phylogenetic distances alongside other relative viral sequences. The present results recorded that the presence of about 99% homology between studied samples with Canine parvovirus. Nine genetic variations of *CPV* gene were identified in Canine parvovirus; several genetic variation detected in all studied samples, while some variation were detected only in (S4,S5,S7) samples, like: A54G, A215G and T69C.

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Introduction

Canine parvovirus (CPV) has been a significant concern since the late 1970s, causing severe of infection and fatal viral disease in dogs. Despite the use of vaccines, the virus remains a leading cause of gastroenteritis and high mortality, particularly in Non-vaccinated young dog puppies (Liu et al., 2017). All ages of dogs are susceptible to parvovirus, with young dogs showing clinical signs within 3–8 days, including fever, hemorrhagic gastroenteritis, vomiting, and bloody or watery diarrhea. CPV is a virus of the Parvoviridae family, known for its single-stranded DNA genome and two pathotypes in dogs: type 2 canine parvovirus (CPV2) and CPV-2a (Kim et al., 2017).

These viruses are considered highly resistant in the environment, contributing to their spread. This is mainly due to their resistance to disinfection of the environment and to their ease of transmission from environment to environment. Parvovirus affects young and growing dogs, where the number of infected individuals in a population attacked by the virus can reach 100%, with a high mortality rate. They are mainly young animals because they will acquire immunity to the virus, either through vaccination or by having formed an immune system capable of protecting the virus. Infected dogs can be seen with diarrhea, vomiting, anorexia, dehydration, and the gradual development of a severe septic shock state. (Maganga et al., 2023; Schirò et al., 2022; Milićević et al., 2023; Decaro et al., 2020).

The pathogenicity of CPV may be determined by different factors such as breed, immunological status, and viral load. The white blood cell count decreases in canine body fluids, elevating the susceptibility of these CPV patients to secondary infections. The direct

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detection and identification of CPV-2 through genetic material are of great importance for surveillance and control (Sarabandi & Pourtaghi, 2023; Maganga et al., 2023). The study by Al-Bayati et al., (2010) in Baghdad of Iraq recorded that the virus was detected in pups brought to private Vet Clinics in Baghdad. Pups suffered from bloody diarrhea, vomiting, dehydration and increased body temperature; Canine parvovirus antigen detected by using rapid antigen test kit and Haemagglutination – inhibition tests (HI).

Polymerase chain reaction is one of the most precise and rapid method for detection of CPV (Schunck et al., 1995). Outbreaks of parvovirus have been reported from worldwide area (Sharma et al., 2012).

The main objective of this study to determine CPV as causative agent in diarrheal canine dogs by PCR technique and DNA sequencing.

Materials and Methods

Sampling

Thirty five cases of dogs between the ages of 3-19 months were selected with clinical history and showed sever symptoms such as bloody or/ and watery diarrhea, unpleasant odors, and loss of appetite, depression and coarse coat of the study; during in the period from October 2023 to June 2024 conducted in Nasiriyah, Thi-Qar province, from veterinary hospital and private clinic, all dogs suffered from the above symptoms, swab stool samples transports in ice container to the lab, all data of collected samples of infected dogs were listed in Table 1.

Extraction of viral DNA

Used QIAamp Fast DNA Mini Kit according to the manufacturer's instructions for viral DNA extraction.

Diagnosis of *CPV* gene

The specific primer pairs of *CPV* gene encoded for capsid protein, as following: forward: 5'-CAAATAGAGCATTGGGCTTACC-3'and reverse:5'-CAATCTCCTTCTGGATATCTTC -3. The PCR program for *CPV* gene: first denaturation at 95°C for 3 min, followed 40 cycles at 95°C for 15sec then at 55°C for 20sec annealing, at 72°C for 40sec. extension and final extension for 4min at 72°C after the last cycle (Sharma et al., 2012). Electrophoresis of PCR product was carried out in 1.4% agarose gel and the presence of a 400bp band indicate a positive result of this gene.

DNA Sequencing

The PCR products of CPV2 gene were send to Macrogen Inc. Geumchen, Seoul, South Korea.

Table 1 Fecal samples of dogs collected from Thi-Qar province.

Sample No.	Breed	Age (Month)	Sex	Vaccination Status
1	Doberman	5	M	NV
2	Doberman	7.1	M	NV
3	Doberman	5.3	M	NV
4	Rottweiler	7	F	NV
5	Mallinois	16	M	NV
6	Mallinois	19	F	NV
7	Mallinois	17	F	NV
8	Doberman	18	M	NV
9	Mallinois	6	M	NV
10	Mallinois	19	F	NV
11	Doberman	4	M	NV
12	Doberman	4	F	NV
13	Rottweiler	5	M	NV
14	Rottweiler	4	F	NV
15	Rottweiler	5	F	NV
16	Doberman	6	F	NV
17	Mallinois	6.5	F	NV
18	Doberman	5.7	M	NV
19	Rottweiler	9	M	NV
20	Mallinois	6.5	M	NV
21	Doberman	6	M	NV
22	Doberman	5	F	NV
23	Mallinois	8	M	NV
24	Rottweiler	10	F	NV
25	Rottweiler	16	F	NV
26	Mallinois	3	F	NV
27	Labrador	12	F	NV
28	Doberman	6	F	NV
29	Mallinois	5	M	NV
30	Doberman	9	F	NV
31	Mallinois	4	F	NV
32	Mallinois	5	M	NV
33	Mallinois	3	M	NV
34	Labrador	6	F	NV
35	Labrador	8	F	NV

Analysis of sequencing results

The sequencing results of PCR products aligned, edited and analyzed by using BioEdit sequence alignment editor program 7.2, and compere with reference database sequence.

Phylogenetic tree

The detected variants in studied virus were compared with their neighbor homologous reference sequences using the NCBI-BLASTn server in specific phylogenetic tree, using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-476.47). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of

pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log likelihood value. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.00% sites). This analysis involved 33 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 336 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

Statistical analysis

The data of the current study was statistically analyses using SPSS, at p. value < 0.05, Descriptive statistics for classis organization.

Results

The present results of 35 animals diagnosed as suspected CPV-2 infections were clinically investigated based on the clinical signs observed in animals from Thi-Qar province. The clinical signs of CPV disease in the animals showed diarrhea and vomiting (100%), dehydration (77%), and 25% mortality, as shown in Table 2.

The results of clinical examinations of diseased puppies recorded a significant ($p > 0.05$) increase in body temperature (40.8 ± 0.21 °C), respiratory rate (50.5 ± 1.43 breaths per minute), and heart rate (158.3 ± 2.31 beats per minute) of diseased animals compared to the healthy group, as shown in Table 3.

Table 2 The clinical signs of infected puppies

No	Clinical signs	Infected puppies	Percentage (%)
1	Diarrhea and vomiting	35	100%
2	Dehydration and dullness	27	77%
3	Dead animals	9	25%

Table 3 Vital signs of infected puppies with CPV and control

Parameter	Healthy no=10	CPV no=35
Body temperature C°	38.6± 0.07b	40.8± 0.21a
Heart rate/ mint	94.7±2.02b	158.3± 2.31a
Respiratory rate/ mint	24.7± 0.86b	50.5± 1.43a

Values are mean ± standard error of mean. a,b ($P<0.05$).

To detect the presence of the CPV-2 gene in viral DNA samples, only 15 out of 35 samples (43%) tested positive, showing amplicons approximately 400 bp in length, as illustrated in Fig. 1.

The NCBI BLASTn engine detected approximately 99% similarity with the intended target, which partially overlapped with the coding region of the *CPV* gene sequences. This was determined through a comparison of the observed DNA sequences of the examined samples with the reference DNA sequence (GenBank accession no. NC001539).

Alignment analysis of the 400 bp sequences identified 9 nucleotide changes compared to the corresponding Canine Parvovirus reference sequence. The positions of the observed variations are shown in Table 4. Based on the investigated *CPV* gene sequences, a phylogenetic tree was generated for the 15 studied viral samples. Along with other deposited DNA sequences, this phylogenetic tree was aligned with closely related sequences using the Tamura-Nei model (Tamura and Nei, 1993).

There were 33 aligned nucleic acid sequences in the entire tree that was constructed. The canine parvovirus, represented by the nucleic acid sequences included in this tree, was present. The examined CPV sequences were grouped into two branches based on the genetic sequences of the canine parvovirus, indicating a closely related range of diversity within these sequences Fig. 2.
A total of 33 nucleic acid sequences were aligned in the constructed tree. Canine parvovirus, represented by the integrated nucleic acid sequences in this tree, was present. The examined CPV sequences were grouped into two branches based on the genetic sequences of the canine parvovirus, demonstrating a close relationship between them.

From the inference of this phylogenetic tree Fig. 3 the sequences most closely related to the studied samples were found to be very closely related to Canine parvovirus and Canine parvovirus 2 isolates. The analyzed CPV sequences clustered into adjacent phylogenetic branches. The studied samples (S1, S2, S3, S6, S8, S9, S10, S11, S12, S13, S14, S15) were closely related to Canine parvovirus 2 (MT078776.1,

MT078773.1, KX219742) and were also highly related to Canine parvovirus KR869669.1.

In contrast, samples S4, S5, and S7 were related in the current phylogenetic tree to Canine parvovirus strains (KX425923.1, MW048560.1, KY937665.1, MN258987.1) and other viral strains.

Table 4 Variations in the 400bp of the *CPV* amplicons in comparison with the reference gene acc. No. NC001539).

Samples	Variant	Position in sequence
All samples	C5G	5
All samples	G19T	19
S1-S3,S6,S8-S15	T21C	21
S4,S5,S7	A54G	54
S4,S5,S7	T69C	69
All samples	T76A	76
All samples	A77T	77
All samples	T205G	205
S4,S5,S7	A215G	215

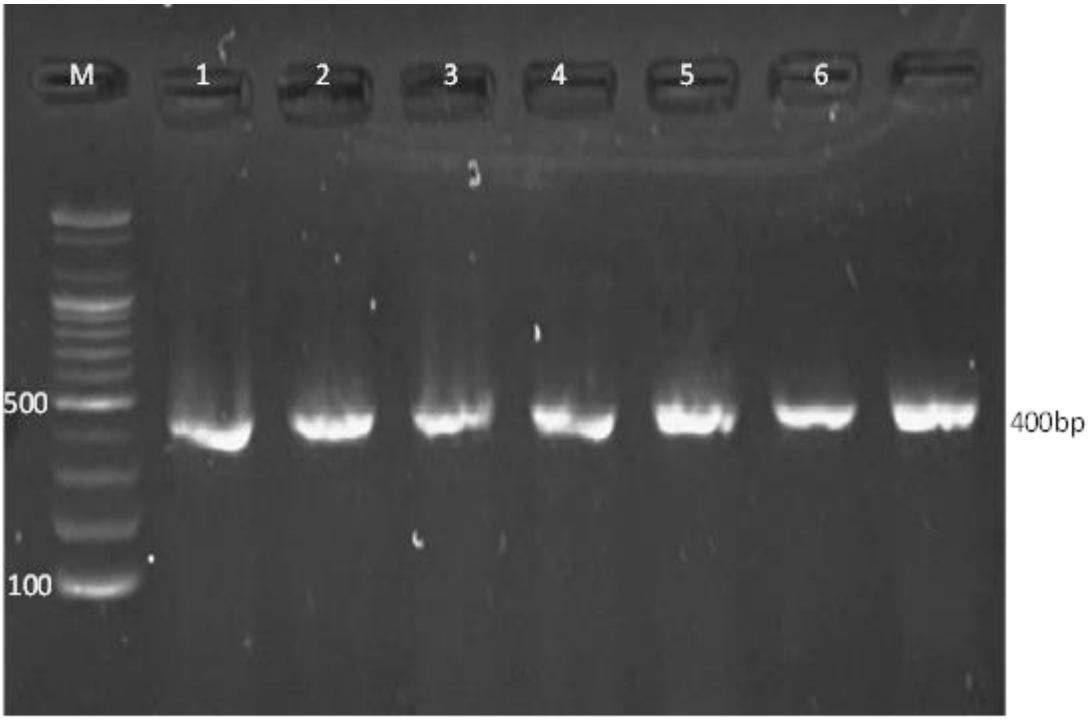

Fig 1. Agarose gel electrophoresis of CPV-2 amplifed,where M:DNA marker,Lanes, 1-7: these bands of size 400 bp represent the positive results of amplifiedica CPV-2 gene.

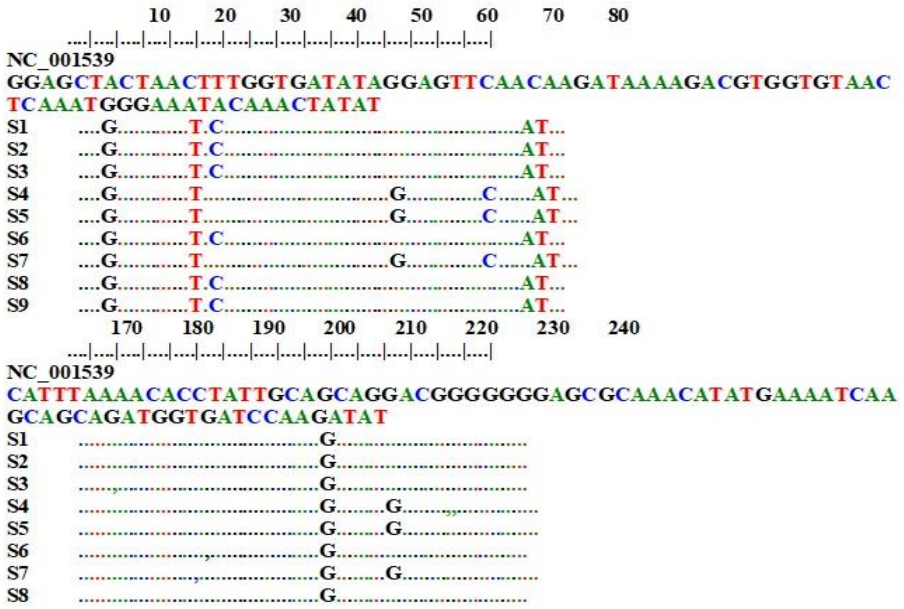

Fig 2. Sequences alignment of some viral samples with its corresponding reference sequences of the *CPV* within the Canine Parvovirus genomic DNA sequences. The symbol ref: refers to the NCBI reference sequences, "S: refers to sample numbers.

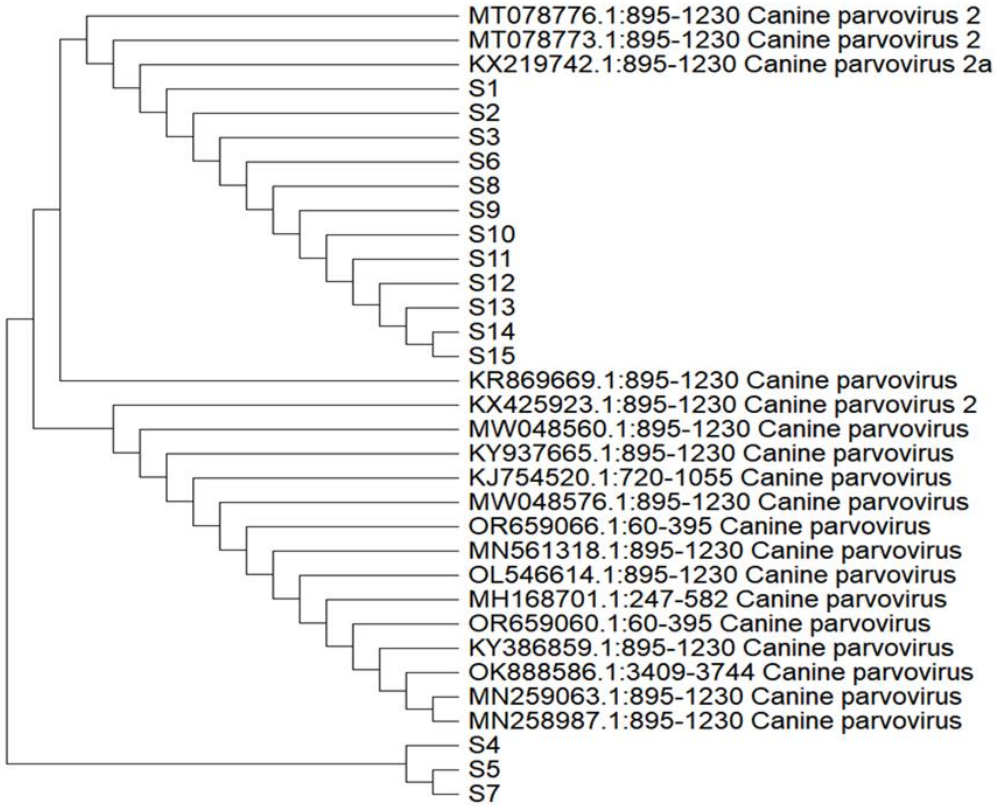

Fig 3. Phylogenetic tree of *CPV* gene in canine paravirus2.

Discussion

The present results of clinical signs of CPV diseases animal showed diarrhea, vomiting, dehydration and nearby 25% of animals were dead. Also, the clinical examinations of diseased puppies recorded that a significant increase in body temperature, respiratory rate and heart rate of diseased animals when compared to healthy group. During the first few months of their lives, puppies are susceptible to canine parvovirus, a dangerous infectious virus. Given the similarity of diarrhea and vomiting to other enteric disorders, clinical identification of CPV infection appears to be quite challenging (Parthiban et al., 2012).

In line with the theory that maternal antibodies are passed on through colostrum, shielding the young puppies from infectious diseases, the study's infected dogs ranged in age from 3 to 19 months. Additionally, the infection rate was higher in dogs younger than 6 months (Sharma et al., 2018). Additionally, Magouz et al. (2023) found that a large number of animals were unvaccinated, highlighting the necessity of stepping up vaccination efforts to lower the prevalence of CPV-2. Nevertheless, additional CPV-2 results indicated that there might be more contributory elements involved.

There were several variation detected in all studied samples, while some variation were detected only in (S4,S5,S7) samples, like: A54G, A215G and T69C.

The evolution of Canine Parvovirus (CPV) is critically linked to mutations in the VP2 gene, which serve as pivotal markers in differentiating CPV-2 variants. The article by Chen et al. (Chen et al., 2021) elucidates the significance of these mutations, highlighting that the VP2 protein is not only essential for host range determination but also plays a crucial role in the antigenic properties and receptor binding of the virus. This study underscores the importance of molecular and sequence analysis of the VP2 gene as the gold standard for identifying CPV-2 strains. (Magouz et al.2023). The diagnstic gene as 16SrRNA gene used to diagnosis Canine parvovirus and other microorganisme such as bacteria (Musa et al., 2019; Degaim et al., 2021).

The results of phylogenetic tree of *CPV* gene in Canine parvovirus, showed highly closely related groups of studied isolates with compared Canine parvovirus strains. The similarity of viral species in studied samples to other strains was measured by BLASTn analysis of the *CPV* gene, there were recorded in the National Center for Biotechnology Information (NCBI) are in concordance of 99% homology with Canine parvovirus that isolated from many countries like: (MT078776.1, MT078773.1, KX425923.1, KY937665).

The PCR results to detecte the *CPV* gene in Canine parvovirus from stool samples showed (43%) of samples harbored *CPV* gene.

The results of Mochizuki et al (1993) detected CPV-2 DNA from fecal samples of dogs with diarrhea by nested PCR. They found CPV-2 DNA in (22/59; 37.3%) fecal samples. Schunck et al (1995) detected CPV-2 DNA from fecal specimens derived from enteritic dogs by single round PCR. Also, using PCR technique for established the presence of CPV-2 DNA in 54 out of 65 (83.1%) samples tested. Strain of CPV present in India has been documented to be CPV 2a (Sagazio et al., 1998).

According to other research, neutralizing antibody tests revealed that dogs in Thailand were infected with CPV-2. In addition to being detected in Vietnam, CPV is gradually spreading throughout Italy, Portugal, Spain, and Germany (Decaro et al., 2006; Martella et al., 2004; Nakamura et al., 2004; Hassan and Degaim, 2024). As previously reported (Zaghawa and Abualkhier, 2019; Sayed-Ahmed et al., 2020), the high percentage of PCR-positive samples (85%) indicates that CPV2 plays a significant role in causing diarrhea in puppies in Egypt (Magouz et al., 2023; Kareem,etal.,2020). Parvovirus infection was the primary cause of vomiting and diarrhea in a study involving 3,864 sick dogs in Egypt (Rakha et al., 2015). Awad et al. (2019) reported the presence of CPV-2b; whereas Awad et al. (2018) reported isolates of parvovirus that Parvovirus isolates which were 100% related to Portugalian isolates. Also, the study performed in Iraq, whom found that all viral sequences were CPV-2b variants, which differed genetically by 0.8% to 3.6% from five commercially available vaccines, and alignment between eight nucleotides of field virus sequences showed 95% to 99.5% similarity (Baba Sheikh et al., 2017).

Conclusion

The PCR diagnosis and DNA sequencing of CPV2 gene recorded that CPV type-2 were the most prevalent strains circulating in Thi-qar province, Iraq.

Availability of data and material

Data are accessible upon request.

Conflict of Interest Statement

The author declare that there is no conflict of interest regarding the publication of this paper.

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