Molecular, pathological and hematobiochemical detection of canine parvovirus in dogs

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ABSTRACT:

Canine parvovirus (CPV) infection is considered a common cause of puppies mortality less than 6 months. Despite treatment with available therapies, many dogs still died from CPV related complications. This study aims to describe the clinical investigation, pathological findings, and molecular diagnosis of canine parvovirus infection in dogs.

A total of thirty five samples, including blood and fecal samples were collected from dogs of different ages and breeds (German shepherd, Husky, Loloo, and Rottweiler puppies) to be used in this study. The study considered the dogs' history, clinical examination for vital parameters, alteration of haemogram, and fecal examination for detection of canine parvovirus antigen.

10 rectal swabs collected from dogs showing clinical signs of canine parvovirus enteritis were initially tested for CPV-2 using CPV Ag test kits rapid test. Results showed 9/10 of swabs were positive for CPV-2. For further investigations, all swabs were carried out using molecular identification, through the extraction of Viral DNA and conventional PCR for the CPV-2, all samples gave positive results for CPV-2. Histopathological examination of the tongue, lung, kidney and intestine suggested the infection with CPV enteritis. leukopenia, neutropenia, lymphopenia, and thrombocytopenia with a significant increase in monocytic and eosinophilic count were recorded. A significant increase in serum total protein and serum globulin and a significant decrease in serum albumin were noticed. Serum ALT, AST, blood urea nitrogen (BUN), and serum creatinine were significantly increased. Samples that showed strong PCR products were sequenced and phylogenetic analyzed to detect the percentage of nucleotide sequence identity and divergence between CPV of this study and other reference strains. PCR   and the sequence analysis confirmed canine parvovirus-2a as the etiology of the disease. Good management is advised to avoid secondary or severe dehydration and marked gastrointestinal fluid hypovolemia with loss of protein and bacterial sepsis.

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INTRODUCTION

Canine parvovirus (also referred to as CPV, CPV2, or parvo) is a highly contagious viral disease mainly affecting dogs **(Allison et al. 2013)**.

Infection with CPV results in morbidity and mortality in young dogs around the globe. Although puppies between the ages of 6 weeks and 6 months and unvaccinated dogs are more vulnerable, CPV can infect dogs of any age. The most frequent form of the illness is acute enteritis, which typically manifests as anorexia, lethargy, fever, vomiting, and mucoid to hemorrhagic diarrhea finally may results in dehydration and hypovolemic shock **(Mylonakis et al. 2016; Sykes, 2014)**.

CPV spread from dog to dog by direct or indirect contact with their underlined feces. Vaccines can prevent this infection, but mortality can reach 91% in untreated cases *(***Stuetzer and Hartmann, 2014)**.

CPV is a non-enveloped single-stranded DNA virus that belongs to the family *Parvoviridae*, subfamily *Parvovirinae,* and genus *Protoparvo* virus according to the International Committee on Taxonomy of Viruses **(Battilani et al. 2019)**.

There are two types of canine parvovirus called CPV1 & CPV2. CPV2 causes the most serious disease and affects domesticated dogs. There are variants of CPV2 called CPV-2a and CPV-2b **(Woolford et al. 2017)**. In 2000, a third antigenic type, CPV-2c, was identified which also spread worldwide in the meantime **(Decaro et al. 2012)**. All of the variants of CPV-2 are genetically related as CPV-2c differs from CPV-2a and CPV-2b at only one point on the DNA strand **(Hong et al. 2007)**.

Although CPV-2 is a DNA virus, its genomic substitution rate is similar to RNA viruses, with a value of approximately 10^{-4} substitutions per site per year. Consequently, after its emergence in 1978 CPV-2 has been undergoing rapid evolution and, in just a few years, the original antigenic type 2 has been completely replaced by the new antigenic variants called CPV-2a, -2b and -2c, based on key amino acid substitutions in the VP2 protein **(Buonavoglia et al. 2001; Shackelton et al. 2005)**.

The typing of the CPV-2 variants is commonly based on the different amino acids observed in residue 426 of the VP2 protein (Asn in CPV-2a, Asp in CPV-2b, and Glu in CPV-2c), These amino acid changes have provided important biological properties and have enabled the CPV-2 variants to replicate and spread more effectively in susceptible hosts **(Ohshima et al. 2008)**.

In the following years, the original type CPV-2 was replaced by two antigenic variants: CPV-2a and CPV-2b **(Goddard and Leisewitz, 2010)**.

Rapid reliable diagnosis is essential to detect CPV infection. A polymerase chain reaction (PCR) shows the highest sensitivity compared to traditional methods however, PCR can only be performed in specialized laboratories which delays the diagnosis **(Decaro et al. 2013)**.

As infected dogs shed high quantities of CPV in their feces, several commercial tests are available for the detection of the CPV antigen in-house. Based on an enzyme-linked immunosorbent assay (ELISA) and detect CPV antigen in feces **(Decaro and Buonavoglia, 2012)**.

MATERIALS AND METHODS
Animals and Ethical Approval:

The examination was done on 35 dogs of different ages (from 5 to 12 weeks), breeds (German shepherd, Husky, Loloo, and Rottweiler), sex, and weight which were presented to different pet clinics and pet hospitals in Cairo. As instructed by the Animal Health Research Institute, Egypt, institutional and national criteria for the care and use of animals were followed.

Clinical Examination:

Complete clinical examination was performed on 35 dogs including general inspection of appearance, posture, gait, skin, conformation, behavior, and physical examination for

temperature, respiration, pulse, mucous membranes, and superficial lymph nodes **(Hill et al. 2011)**.

Sampling:

Fecal Samples

A total of 10 fecal swab samples were collected from suspected CPV infected dogs showing clinical signs .Samples were placed in a viral transport medium of phosphate buffered saline. Samples were given to Animal Health Research Institute for laboratory diagnosis while taking biosecurity precautions **(OIE, 2021)**.

Blood samples

Blood samples were collected from 10 apparently healthy dogs and 25 suspected CPV infected dogs. Two blood samples were collected from the cephalic vein of each dog; first sample was collected on EDTA as anticoagulant for complete blood count evaluation. The second sample was collected on a plain tube and centrifuged at 3000 rpm for 15 minutes for serum separation for biochemical assay analysis.

Canine Parvovirus CPV Ag test kits rapid test:

A commercial test was supplied by Wuhan J.H. Bio-Tech Co., Ltd. for the detection of CPV-2 antigens, following the manufacturer's instructions.

Hematological and Biochemical investigations:

Hematological profiles consisting of red blood cell (RBC) count, hemoglobin (Hb) concentration, hematocrit (PCV) valve, mean corpus-cular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total white blood cell (WBCs) count and WBCs differential count were recorded by using a Mindray BC-5000 Vet auto hematology ana-lyzer (Mindray, Shenzhen, China). liver and kidney function tests include (total protein, albumin, globulin, A/G ratio, ALT, AST, BUN and creatinine) were investigated by commercial analytical kit (Spinreact, S.A./S.A.U.Ctra. Santa Coloma, 7 E-17176 SantEsteve De Bas, Spain).

Statistical analysis:

The results were tabulated and statistically analyzed by using Statistical Package for Social Sciences (SPSS) and the difference between healthy and diseased dogs was analyzed using Student's t-test and presented as mean± standard error (SE) **(Snedecor and Cochran, 1989)**.

Pathological examination:

Four dogs died from twenty five due to the severity of the symptoms, bad prognosis, and late veterinary treatment. At necropsy, tissue specimens from the tongue, lung, kidney, and intestine from freshly dead infected dogs were fixed in neutral buffered formalin 10% for 48 hours, dehydrated in ascending grades of ethanol (70%-100%), cleared in xylene, embedded in paraffin wax. 5μm thickness of paraffin sections were obtained by using an automated microtome and then stained with routine Hematoxylin and Eosin (H & E) **(Suvarna et al. 2018)**.

Molecular Diagnosis:

DNA extraction:

DNA extraction from 10 samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 μl of the sample suspension was incubated with 10 μl of proteinase K and 200 μl of lysis buffer at 56°C for 10 min. After incubation, 200 μl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 μl of elution buffer provided in the kit.

Oligonucleotide Primer

Primers used were supplied from Metabion (Germany) According to **(Buonavoglia et al. 2001)**.
Forward primer: 5' CAGGTGATGAATTT-GCTACA -3'
Reverse primer 5'-CATTTGG TAAACTGG-TGGT -3'.

PCR amplification

Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmolconcentrations, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

Analysis of the PCR Products

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products were loaded in each gel slot. A generuler 100 bp ladder (Fermentas, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table 1. Target genes, amplicon sizes, and cycling conditions.

| Target gene | Amplified segment (bp) | Primary denaturation | Amplification (35 cycles) | | | Final extension |
			Secondary denaturation	Annealing	Extension	
Vp2	630	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.

Sequence materials

PCR products were purified using a QIAquick PCR Product extraction kit. (Qiagen, Valencia). Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used for the sequence reaction and then it was purified using Centrisep spin column. DNA sequences were obtained by Applied Biosystems3130 genetic analyzer (HITACHI, Japan), and a BLAST® analysis (Basic Local Alignment Search Tool) **(Altschul et al. 1990)** was initially performed to establish sequence identity to GenBank accessions. The phylogenetic tree was created by the MegAlign module of Laser gene DNAS tar version 12.1 **(Thompson et al. 1994)** and Phylogenetic analyses was done using maximum likelihood, neighbor-joining and maximum parsimony in MEGA6 **(Tamura et al. 2013).**

RESULTS
Clinical signs:

The signs of the affected dogs were more severe in younger ages than older ones. Out of the 35 dogs that were examined, 25 had symptoms like fever, dehydration, vomiting, diarrhea (ranging from yellowish to reddish), anorexia, and loss of body weight. Four dogs were died out of twenty-five. Two puppies showed neurological signs such as convulsion.

Canine Parvovirus CPV Ag test kits rapid test:

9 samples out of 10 were positive.

Hematological Finding:

Table 2. hematological and biochemical picture of canine parvovirus infected dogs.

Parameters	Healthy dogs N=10	CPV infected dogs N= 25
RBCs ×10^3/μl	7.3±0.22	5.2±0.21***
Hb gm/dl	15.4±0.39	11.44±0.43***
PCV %	47.2±0.48	36±2.26**
MCV	65±1.33	70.31±2.52
MCH	21.22±0.3	22.43±0.51
MCHC	32.7±0.59	31.98±0.91
TLC×10^3/μl	13±0.15	9.8±0.14***
Neutrophil×10^3/μl	8.17±0.08	5.62±0.08***
Lymphocyte×10^3/μl	3.59±0.06	2.2±0.05***
Monocyte×10^3/μl	0.94±0.09	1.47±0.07***
Eosinophil×10^3/μl	0.29±0.03	0.41±0.02***
Basophil×10^3/μl	0.02±0.02	0.08±0.02
Platelets×10^3/μl	332±5.83	179.4±18.56***
TB (g/dl)	5.36±0.07	5.96±0.07***
AL (g/dl)	3.68±0.09	2.94±0.02***
GL (g/dl)	1.68±0.07	3.02±0.1***
A/G ratio	2.18±0.14	0.97±0.04***
ALT (U/I)	73±0.95	158.4±3.7***
AST (U/I)	31.2±0.58	94.16±0.55***
BUN (mg/dl)	12.24±0.1	13.73±0.21***
Creat. (mg/dl)	1.2±0.19	4.49±0.12***

** Significant difference at P value (<0.01)
*** Significant difference at P value (<0.001)

As, demonstrated in **table (2)** hematological study of CPV infected dogs revealed a significant decrease in each RBC count, Hb%, and packed cell volume (PCV) values compared to healthy dogs giving a picture of normocytic normochromic anemia. Results also showed a significant decrease in total leukocytic count (TLC) (leukopenia), neutropenia, lymphopenia, and thrombocytopenia with a significant increase in monocytic and eosinophilic count in infected dogs than healthy ones.

The biochemical profile showed significant increase in serum total protein and serum globulin with significant decrease in serum albumin in CPV infected dogs compared to healthy dogs. Serum ALT, AST, blood urea nitrogen (BUN), and serum creatinine were significantly increased in infected dogs when compared to healthy ones.

Pathological findings:

At Necropsy, most cases evolved hyperemic gastrointestinal serous membranes, congestion of the heart, kidneys and pulmonary edema. Histopathologically, the tongue of infected dogs showed ulceration and the predermal inflammatory cells with round ones and neutrophils **(fig.1. A).**

The Lung of infected dogs showed focal area of consolidation and emphysema **(fig. 1. B)**.

The kidney of infected dogs showed glomerular tuft atrophy **(fig.1.C).** Sub-acute focal interstitial nephritis was also detected.

The intestine showed necrosis of the two-third of the upper intestinal villi **(fig.1.D)**, in addition to focal leucocytic aggregations and sub-serosal edema. Other sections demonstrated hemorrhagic enteritis, submucosal edema and depletion of the payer's patches.
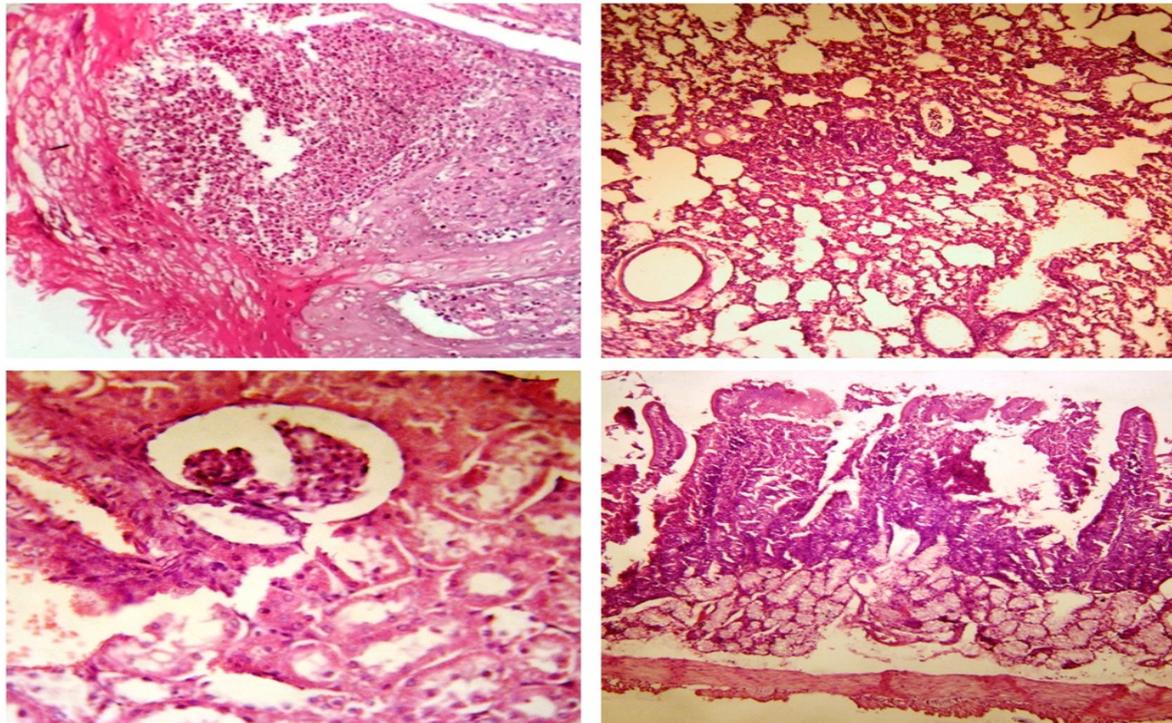
(Fig 1) A. Tongue of dogs infected with parvovirus showing ulceration, the predermal inflammatory cells are round ones and neutrophils H&E X 100
B. Lung of dogs infected with parvovirus showing focal area of consolidation and emphysema. H&E X 400.
C. Kidney of dogs infected with parvovirus showing glomerular tuft atrophy. H&E X 400.
D. Intestine of dogs infected with parvovirus showing necrosis of the two- third of the upper intestinal villi. H&E X 400.

Molecular characterization of CPV by PCR
10 samples were subjected to PCR using specific Primer targeted the **VP 2**for detection of the CPV strain. The primer succeeded to amplify the specific CPV products (630p) from the extracted DNA products **(Fig. 2).**
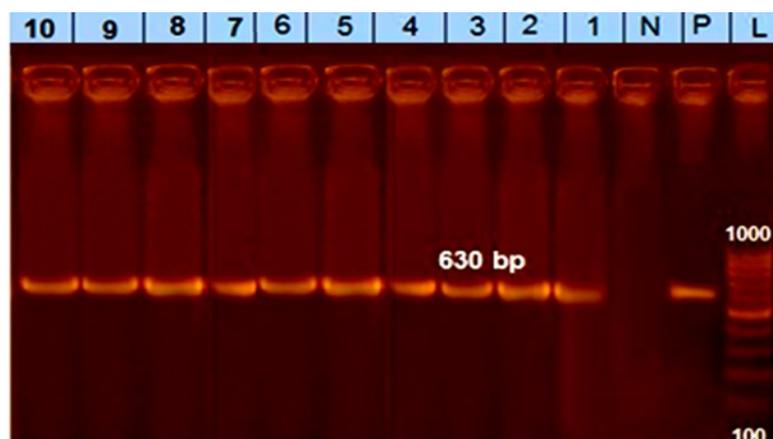
Fig. (2): Agarose gel electrophoresis of amplified PCR products of CPV. The specific primers set amplified a DNA fragment of 630 bp equivalent to the expected amplification product (amplicon) size from CPV. Lanes:
(L) 100 bp DNA ladder.
(P): positive PCR products from parvovirus reference strain.
(N): negative control (no primers). Lanes (1, 2, 3, 4, 5,6, 7, 8, 9, 10) +ve samples

Nucleotide sequencing of CPV

A samples representative of CPV was sequenced and submitted on Genbank with accession number:

Banklt OQ730213 CPV Egypt 1SS

Phylogenetic analysis of CPV

A phylogenetic tree was constructed based on the nucleotide sequence alignment of the VP2 of CPV showing that they are belonging to CPV- 2c **(Figs. 4&5).**
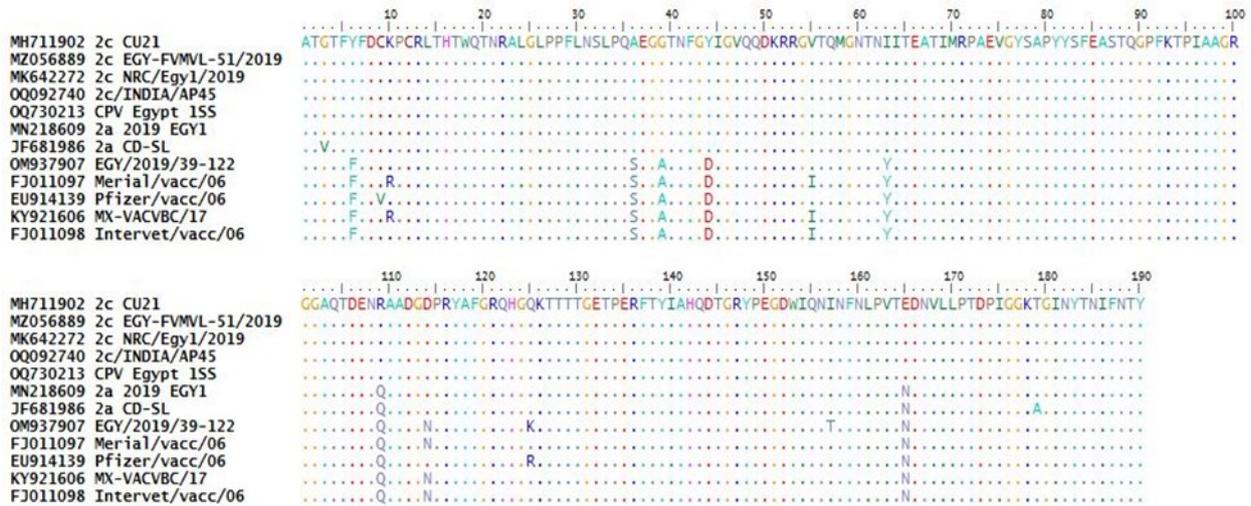
Fig 3. Amino acid alignment of CPV.

Fig. (4): CPV sequence distance, Percentage of nucleotide sequence identity and divergence between CPV of this study and other reference strains. The CPV of this study is in bold font labeled with black circles.
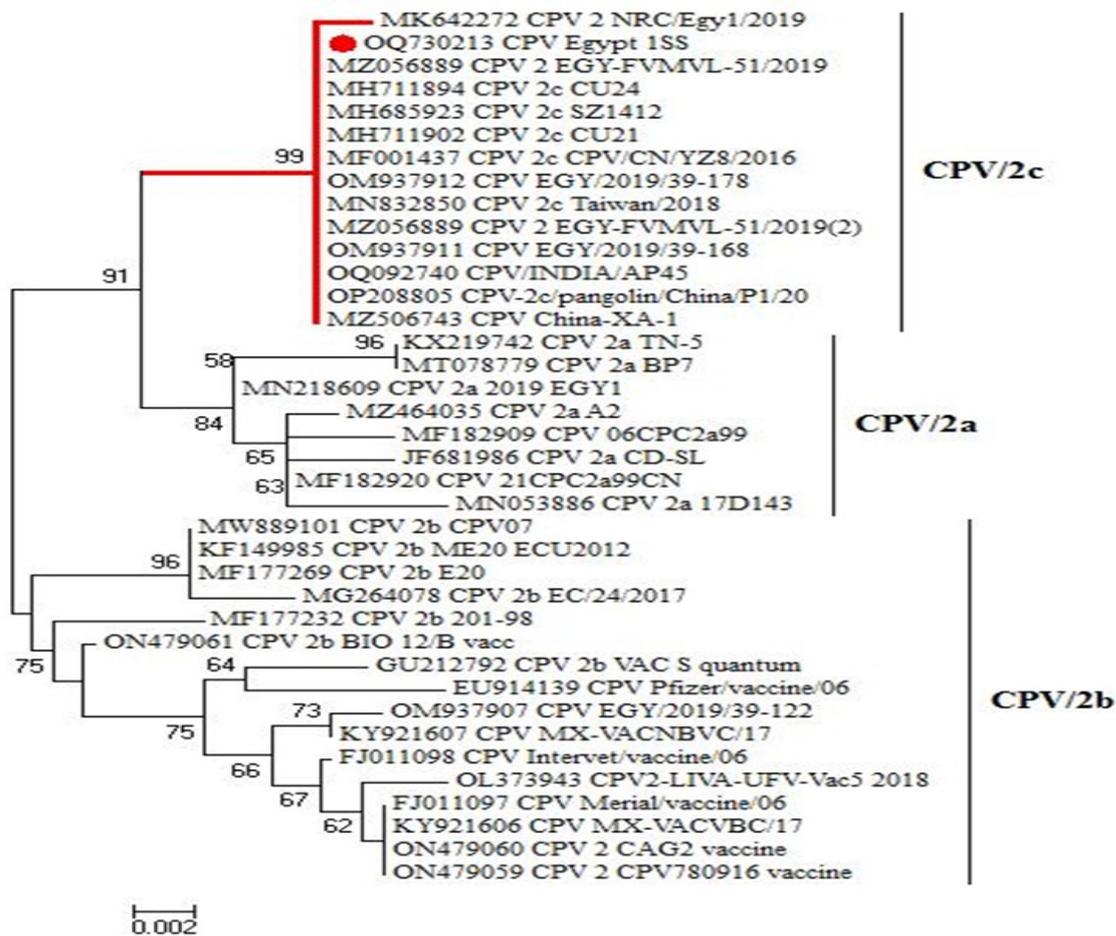
Fig 5. Phylogenetic tree of the VP2 sequence of CPV isolated from Egypt. The CPV of this study is in bold font labeled with red circles.

DISCUSSION

Canine parvovirus 2 (CPV-2) is one of the most common etiological agents that cause severe gastroenteritis in puppies. Early accurate diagnosis is very important to infected dogs for conservative treatment **(Castro et al. 2013).**

10 fecal swabs were tested by fecal CPV-2 Ag rapid kits. 9 /out of 10 samples were positive. One sample showed false-negative results with the rapid kit, possibly because the contact dog was in the first stage of the disease and not showing prominent clinical signs **(Decaro and Buonavoglia, 2012).** Similarly to our results, **Oliveira et al. (2018)** reported diarrhea, vomiting, hyperemia, and segmentation of the small intestine with focal pulmonary congestion with bacterial aggregation and fibrinous plaques.

As documented in table (2) our results revealed a reduction in RBCs count, Hb %, and PCV value in infected dogs which may be attributed to intestinal hemorrhage and bleeding **(El-Zahar et al. 2019),** these results are in harmony with **(El-Zahar et al. 2019; Terzungwe, 2018)** and in contrast with **Bhat et al. (2013)** who recorded unaltered total erythrocytic count and PCV value in canine enteritis infected dogs than healthy ones. **Oliveira et al. (2018)** sugested that massive CPV-2 virus replication in leukocytes frequently leads to cell lysis and causing acute lymphopenia .Moreover, our results showed leukopenia, neutropenia, and lymphopenia which may occur due to the cytotoxic effect of the virus on the hematopoietic cells and with **(Castro et al. 2013; El-Zahar et al. 2019; Weiss et al. 1999)** and disagreed with

Terzungwe (2018) who recorded lymphocytosis in male CPV infected dogs at age <6 months, on the other hand, **Kumar and Kumar (2017)** observed an increase in neutrophil count, they suggested that neutrophilia might occur due to the secondary bacterial infection associated with the CPV infection. In the present study, we reported thrombocytopenia in CPV infected dogs, this result was in harmony with **Castro et al. (2013)** who mentioned that CPV-infected dogs revealed significant thrombocytopenia compared to healthy dogs and with **El-Zahar et al. (2019)** who reported a non-significant decrease in platelets count in CPV infected dogs and in disagreement with **(Bastan et al. 2013; Nivy et al. 2011)** who reported thrombocytosis in CPV infected dogs. Thrombocytopenia might be because of decreased platelet production or as a result of a direct action of virus and/or immunologic component on platelets or endothelium as suggested by **(Shah et al., 2014).**

Furthermore, infected dogs manifested a significant increase in total protein and globulin levels with a significant decrease in albumin level, these changes are usually recorded in enteritis as a consequence of malabsorption, decreased dietary intake, and protein losses **(Craven et al. 2011)**. Our results were in agreement with **Nivy et al. (2011)** who recorded hypoalbuminemia in CPV infected puppies and **Bhat et al. (2013)** who reported significant decrease in albumin level and A/G ratio with an increase in globulin level in dogs with enteritis compared to control dogs, in contrary **Bastan et al. (2013)** recorded non-significant change in total protein level with significant increase in albumin level in CPV infected dogs.

Regarding kidney functional parameters we recorded significant increase in both BUN and serum Creatinine levels in CPV infected dogs than healthy ones which indicated acute kidney injury during infection with CPV. This increase was related to dehydration in infected dogs **(Macintire and Smith-Carr, 1997)**. Our result was in harmony with **Bhat et al. (2013)** who observed significant increase in BUN value as a result of pre-renal uremia which might reflect a serious problem in the kidneys during

the course of the CPV disease also, **(Bastan et al. 2013; El-Zahar et al. 2019)**. On the other hand van den **Berg et al. (2018)** demonstrated that BUN value was within the normal reference range and serum creatinine level was significantly lower in CPV infected dogs than healthy dogs.

Regarding ALT and AST values, the present study revealed significant increase in both parameters in CPV-infected dogs compared to healthy ones and this may be a consequence of the hepatic disorders (reactive hepatopathy) and the development of inflammatory bowel disease **(Berghoff and Steiner, 2011).** Our results in harmony with **Bhat et al. (2013)** who reported non-significant increase in ALT and AST in dogs with enteritis **El-Zahar et al. (2019)** recorded significant increase in both ALT and AST in CPV infected dogs compared to healthy ones.

Canine parvovirus rapidly spreads between dogs when exposed to contaminated feces and infected soil via oronasal exposure. After ingestion of the virus, the replication starts in the lymphoid organs such as the oropharynx, mesenteric lymph nodes, and thymus gland Cells. After that, the virus disseminated to the intestinal crypt within the blood causing viremia which is observed 1-5 days after infection **(Carmichael, 1981)**. Necrosis and destruction of the intestinal villi epithelium and fusion of the intestinal crypts were observed in combination with Leucopenia may be due to extensive replication of the virus in crypt cells and consequent attempts of intestinal epithelium regeneration and bone marrow aplasia which is favor to the secondary bacterial invasion **(Zachary, 2012)**. The author also observed dehydration, endotoxemia and hypovolemic shock developed in the severely affected cases causing death. Following exposure to viremia, the parvovirus localized in the gastrointestinal epithelium of the tongue, oral, esophageal mucosa, and the small intestine. Parvovirus is also demonstrated in lymphoid tissues of the thymus, lymph nodes, and bone marrow causing depletion of lymphocytes in lymph nodes and intestinal crypt necrosis. Parvovirus can be isolated also from other organs such as lungs, kidneys, spleen, myocardium and liver. **Lobetti (2003)** demonstrated that after viremia, the vi-

rus localized in the gastrointestinal epithelium of the tongue and other organs of lung hemorrhage demonstrated by **Robinson et al. (1980) while Turk et al. (1990)** recorded cases of pulmonary edema in dying dogs of complicated septicemia. Our results is different from **Matsui et al. (1993)** who mentioned necrosis of crypt epithelium in the small intestine within shored villi due to the lack of replacement by maturing crypt cells leading to collapsed lamina properia.

Our results are in line with **Al-Bayati (2016)** who demonstrated vacuolar degeneration of epithelial cells of the mucosa with moderate mononuclear cell infiltration in subepithelial layer of the tongue, shrinkage of the glomerular tuft with Bowman's capsule dilatation and cellular degeneration of the epithelial cell lining the renal tubule as well as edema of alveoli in addition to pathological changes of intestine represented in the regeneration of epithelial cells of lamina propria with mononuclear cell infiltration in mucosa and submucosa.

Ten samples were tested by PCR; the forward and reverse primers are located in the conserved region of the VP2 gene and reasonably avoid the mutation sites **(Zhuang et al. 2019a; Zhuang et al. 2019b)** . All ten samples were positive by PCR as highly sensitive and can detect low virus titer **(Faz et al. 2017).**

Nucleotide sequencing of CPV was conducted, and the scale bar shows the number of changes per nucleotide when compared to other reference strains in the Genbank database.
Samples representative of CPV was sequenced and submitted on Genbank with accession number: Banklt OQ730213 CPV Egypt 1SS and were compared with other isolates. The isolated sample shows 100% amino acid similarity with MH711902 CPV 2c CU21, MZ056889 CPV 2EGY-FVMVL-51/2019, OM937912 CPV EGY/2019/39-178,MN832850 CPV 2c Taiwan/2018, MZ056889 CPV 2EGY-FVMVL-51/51/2019, OM937911 CPV EGY/2019/39-168, OQ092740CPV/INDIA/AP45 and MZ506743 CPV China-XA-1.It shares 99.8% similarity with MK642272 CPV 2NR/Egy1/2019.IThe isolated sample shows 99.1%Similarity with

MN218609 CPV 2a 2019 EGY 1.It shares 98.8 % amino acid similarity with ON479061 CPV 2b_BIO_ 12/B_vaccand MZ464035 CPV 2a A2. Also shares similarity 98.6% with MF 182909 CPV 06CPC2a99 ,KX219742 CPV 2a TN-5 and JF681986 CPV 2a CD-SL. Similarity 98.4% with MF1777232 CPV 2b 201-98andMN053886 CPV 2a 17D143 .Similarity 97.9% with GU212792 CPV 2b VAC_S quantum , FJ011098 CPV intervet/vaccine/o6 and KY921607 CPV MX-VACNBV/17.It shares 98.1 % similarity with MG264078 CPV 2b EC/24/2017.

Similarity 97.7% with OM937907 CPV EGY/2019/39-122, FJ011097 CPV Merial/vaccine/06, KY921606 CPV MX-VACVBC/17 and ON479060 CPV 2CAG2_vaccine. Similarity 97.5% with EU914139 CPV Pfizer/vaccine/06

Conclusion:

We confirm the presence of CPV infection in our environment besides diagnostic tools' importance in identifying the disease nature and pattern for the rapid managemental response.

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