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CLINICAL, VIROLOGICAL AND MOLECULAR CHARACTERIZATION OF CANINE PARVO VIRUS IN DOGS

Rofaida M. Soliman*[1], Nabil M. Baker[1], Mohamed Y. Nasr[1] and Mohamed H. Khodeir[2]

[1]Animal Medicine Department, Faculty of Veterinary Medicine, Damanhour University.
[2]Veterinary Serum and Vaccine Research Institute, Abassia-Cairo, Egypt.

***Corresponding Author: Dr. Rofaida M. Soliman**

Animal Medicine Department, Faculty of Veterinary Medicine, Damanhour University.

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ABSTRACT

Canine parvovirus (CPV-2) remains the most important and potentially fatal viral disease seen in canine populations especially young pups causing acute hemorrhagic enteritis and myocarditis. Based on this, the present study aimed to investigate the different methods for diagnosis of CPV-2 infection, detection and genotyping of CPV-2 circulating in Egypt by polymerase chain reaction (PCR). A total of 50 fecal samples were collected from diarrheic dogs suspected to be infected with CPV-2 based on clinical findings. All the cases were screened for CPV-2 using immunochromatographic (IC) test, out of which 25 (50%) were positive while 25 (50%) were negative. 18 samples were chosen randomly (8 positive by rapid test kit and 10 negative) plus the local vaccine for PCR. It was found that all 18 samples (in addition to the local vaccine) were found clearly positive for parvoviruses by PCR assay using Hfor/Hrev primers. Two positive samples plus the vaccine were subjected to further genotyping. The three samples were positive CPV2b indicating that this CPV type 2b is the currently circulating genotype in Egypt. Virus isolation and identification were made to demonstrate the cytopathic effects of the virus. Phylogenetic tree revealed that CPV-2b VP2 partial sequences were phylogenetically associated with one of the most commonly used CPV vaccine strains (Fort-dodge vaccine). This study revealed that PCR is considered as the most reliable diagnostic technique having high degree of sensitivity and specificity in detecting CPV-2 from fecal samples, helping in identification of dogs shedding CPV-2 at low titers in their feces.

KEYWORDS: CPV-2, dogs, hemorrhagic enteritis, diagnosis, immunochromatographic (IC) test, PCR.

INTRODUCTION

Among the various gastrointestinal disturbances, enteritis is the most common clinical condition encountered in all breeds and age groups of canine population.[1] Amongst the viral etiologies responsible for gastroenteritis in dogs, canine parvovirus (CPV) is considered as the most pathogenic. Canine parvovirus 2, the causative agent of acute hemorrhagic enteritis and myocarditis in dogs, is a highly contagious and often fatal disease. CPV was first identified in 1978 as the etiological agent of an epizootic severe gastroenteritis of dogs characterized by depression, loss of appetite, vomiting, diarrhea (mucous or hemorrhagic) and leucopenia.[2] By the end of 1980, CPV-2 was completely replaced globally in dogs by a genetic and antigenic variant termed CPV-2a.2 Subsequently, the VP2 residue 426 changed from Asn to Asp and then from Asp to Glu in the so-called CPV-2b and CPV-2c antigenic variant strains, respectively.[3] The virus was named CPV-2 in order to differentiate it from a closely related parvovirus of canine known as CPV-1 or minute virus of canine (MVC**).**[4] Over the years, a number of diagnostic assays both serological and molecular have been developed for prompt, precise and sensitive diagnosis of the disease.[5] Diagnosis of CPV-2 infection is very important, especially in kennels and shelters in order to isolate infected dogs and prevent transmission to susceptible contact animals. Diagnosis on the basis of clinical signs is not definitive, since several other pathogenic organisms can cause diarrhea in dogs. Various laboratory methods have been developed to detect CPV-2 in the feces of infected dogs, for example electron microscopy (EM)[6], enzyme linked immunosorbent assay (ELISA), immunochromatographic tests (IC), viral isolation (VI), haemagglutination inhibition (HI) tests, conventional polymerase chain reaction (CPCR) and real-time polymerase chain reaction (RT-PCR).[7] Based on the importance of such disease in canine population, our study aimed to study the incidence of canine parvovirus infection in dogs presented to private and governmental pet clinics in Alexandria and El Behera governorates, isolation of the causative virus, using PCR as a diagnostic tool in a comparison with the rapid diagnosis and molecular typing of the CPV-2 to demonstrate the field and vaccinal strain currently found in Egypt.

MATERIALS AND METHODS

1. Animals

A total of 50 dogs of different breeds within age range from 2 months up to one year, distributed allover different localities of Alexandria and El Behera governorates. These dogs were subjected to clinical examination after asking the owners for the history complaining from diarrhea, lethargy and vomiting. The noticed signs were suspected to be parvovirus infection and all puppies were found suffering from different degrees of diarrhea, dehydration, emaciation and lethargy.

2. Samples

A total of 50 fecal samples/rectal swabs were collected between January and November 2017 from suspected cases for canine parvo virus infection admitted to private and governmental veterinary clinics from different districts of Alexandria and El Behera governorates as detailed in **table (1).** Fecal swabs were collected (separately from each animal) in labeled tubes containing normal saline(Phosphate buffer saline) with 10% of antibiotic stock solution and subjected to 2 cycles of freezing and thawing then for centrifugation at 2000rpm for 10 minutes. The supernatant fluid was separated and kept at -80ºC till used for virus isolation.

Table 1: Data of the CPV-2 suspected cases.

Breed	Number	Sex	Age group	Vaccination status	Date of vaccination
German Shepherd	14	Male (N=8) Female(N=6)	2-3 months (N=10) 3-4 months (N=3) 8 months (N=1)	Unvaccinated (N=11) Vaccinated (N=3)	At 1.5 months
Labrador	11	Male (N=7) Female(N=4)	2-3 months (N=5) 3-4 months (N=4) 6 months (N=2)	Unvaccinated (N=10) Vaccinated (N=1)	At 2 months
Rottweiler	10	Male(N=5) Female(N=5)	2-3 months (N=8) 3-4 months (N= 2)	Unvaccinated (N=8) Not known (N=2)	----------
Griffon	5	Male (N=3) Female(N=2)	2-3 months (N=2) 3-4 months (N=3)	Unvaccinated (N=3) Vaccinated (N=2)	At 1.5 months
Golden Retriever	3	Male (N=1) Female(N=2)	3-4 months (N=1) 5 months (N=1) 9 months (N=1)	Unvaccinated (N=1) Vaccinated (N=2)	At 2 months
Husky	3	Male (N=2) Female(N=1)	2-3 months(N=1) 3-4 months (N=1) 5 months (N=1)	Unvaccinated (N=2) Vaccinated (N=1)	At 2 months
Boxer	1	Male (N=1)	3-4 months (N=1)	Unvaccinated (N=1)	----------
Wolf dog	1	Male (N=1)	6 months (N=1)	Not known (N=1)	----------
Pitbull	2	Male (N=1) Female (N=1)	3-4 months (N=1) 7 months (N=1)	Unvaccinated (N=1) Vaccinated (N=1)	At 2 months
Total	50	Males=29 Females =21 Total =50	2-3 months= 26 3-4 months=16 5 months=2 6months=3 7 months=1 8 months =1 9 months =1	unvaccinated =37 vaccinated = 10 Unknown=3	----------

3. Virus Isolation on cell culture: For isolation of CPV-2, a confluent monolayer of Vero cell culture (African green monkey kidney cell line) using minimum essential medium (MEM) with new born calf serum. Three blind successive virus passages were carried out according.[8]

4. Virus titration: The micro-titer technique according to.[9]

5. Virus identification

5.1 Demonstration of the induced CPE in infected Vero cell culture: Normal and infected Vero cell cultures were stained with hematoxylin and eosin according to.[10]

5.2 Virus neutralization: According to.[11]

5.3 Direct fluorescent antibody technique (FAT): Direct FAT was carried out according to.[12]

5.4 Antisera

5.4.1-Canine Parvovirus antiserum:

Specific anti-canine parvovirus serum was kindly supplied by the Department of Pet Animal Vaccine Research, Serum and Vaccine Research Institute, Abbasia, Cairo.

5.4.2 Canine Parvovirus antiserum conjugated with fluorescein isothiocyanate
It was supplied kindly by the same department and used for identification of the obtained virus isolates using direct fluorescent antibody technique.

5.5 Rapid agglutination test (RAT): The test was carried out according to.[13] Staph protein-A was prepared according to.[14,15]

6. Detection of viral antigen
Antigen Rapid CPV Ag test kit was used for detection of canine parvovirus antigen in collected fecal samples (Antigen Rapid CPV Ag Test Kit, BioNote, Inc., Gyeonggi-do, Korea), following the manufacturer's instructions.

7. Local CPV vaccine
Live attenuated canine Parvo vaccine, was kindly supplied by Veterinary Serum and Vaccine Research Institute, Abassia, Cairo. Such vaccine was used for genotyping the vaccinal strain.

8. Extraction of DNA
It was carried out according to QIAamp DNA mini kit instructions.

8.1 Oligonucleotide primers used in PCR
Table 2: Oligonucleotide primers sequences (Metabion , Germany).

Virus	Gene	Primers	Primer/ probe sequence 5'-3'	Amplified Segment (bp)
Canine parvo	VP2	Screening primers	Hfor: CAGGTGATGAATTTGCTACA	630
			Hrev: CATTTGGATAAACTGGTGGT	
		Sequencing primers	Pbs: CTTTAACCTTCCTGTAACAG	427
			Pbas: CATAGTTAAATTGGTTATCTAC	

8.2 Cycling conditions of the primers during PCR: Temperature and time conditions of the different primers during PCR are shown in Tab. (3) according to[5] and Emerald Amp GT PCR master mix (Takara) kit.

Table 3: Cycling conditions of the different primers during PCR

Gene	Primary Denaturation	Secondary Denaturation	Annealing	Extension	Final Extension	No. of cycles
VP2	95˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	35

8.3 Sequencing of CPV-2b VP2 and phylogenetic analysis: A comparative analysis of sequences was performed using the CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign module of Lasergene DNAStar software Pairwise, which was designed by[16] and Phylogenetic analyses were done using maximum likelihood, neighbor joining and maximum parsimony in MEGA6.[17]

RESULTS
1. Clinical findings
The examined dogs had a significant elevated mean rectal temperature 40 ± 0.30; heart rate 170±3 /min and respiratory rate 40 ± 5 /min when compared to healthy dogs. All the 50 (100%) CPV suspected dogs had anorexia and diarrhea **(Fig.1)** with variations in the color of stools. Out of 50 dogs with parvoviral diarrhea 20 (40%) dogs had bloody diarrhea (Fig.2), 10 (20%) had chocolate brownish coloured foul smelling diarrhea, 9 (18%) had mucous coated with streaks of blood and 6 (12%) had blood tinged diarrhea and 5 (10%) had yellowish fetid smell diarrhea. The diarrhea was mostly watery in consistency and the frequency of diarrhea varied from 2 to 8 times in a day.

Thirty five dogs (70%) had vomitions, 30 (60%) dogs had moderate dehydration, 17 (34%) dogs had severe dehydration. The parvovirus infected dogs exhibited different combinations of above clinical signs.

Fig.1: A CPV-2 infected German shepherd puppy showing severe straining and diarrhea.

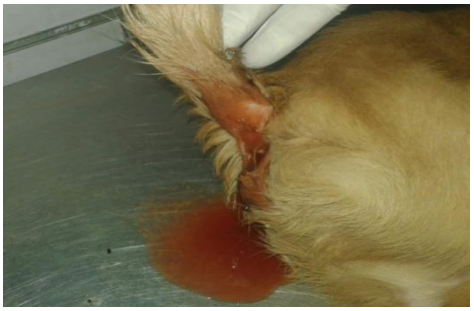


Fig.2: Severe bloody diarrhea in 3 old month Golden retriever puppy infected with CPV-2.

2. Prevalence of CPV infection in association with the season: Of the 50 samples collected during the period of study, a non-uniform distribution of the cases was found throughout the seasons. The highest number of cases was detected in the winter season where 30 (60%) dogs were suffering from the disease, while 6 (12%) in spring, 5 (10%) in summer and 9 (18%) in autumn **(Fig.3)**.

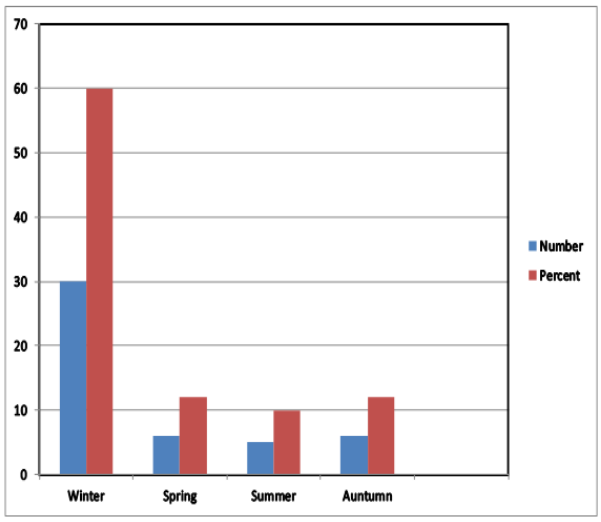


Fig. 3: Prevalence of CPV2 infection with respect to season.

3. Age and vaccination status of CPV suspected dogs Out of the 50 suspected dogs **,** the number of suspected cases was 26 at age of 2 months (26 dogs), however, it was 10, 6, 2, 3, 1, 1 and 1 at 3, 4, 5, 6, 7, 8 and 9 months respectively **(Fig. 4)**.

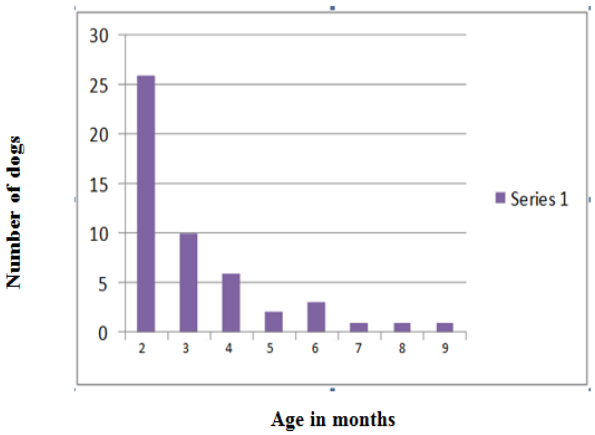


Fig.4: Age of the CPV-2 suspected dogs.

Of the 50 samples that were screened for CPV2 infection by immunochromatographic assay, 37 (74%) came from dogs with no vaccination history, 10 (20%) came from dogs that had been vaccinated for at least one time, the remaining 3 (6%) came from dogs with unknown vaccination history. Of the vaccinated dogs, 5 (10%) had been vaccinated once only, 4 (8%) had taken the two doses of vaccine and one dog had taken 3 vaccination doses (**(Fig.5)**.

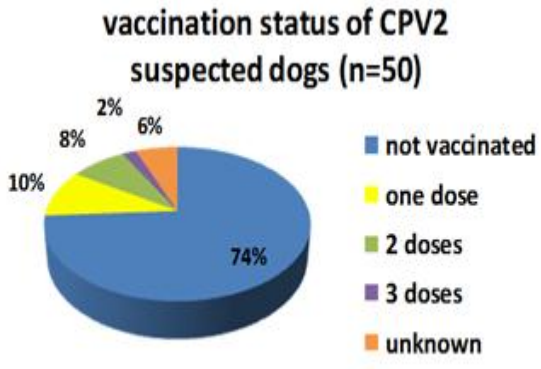


Fig. 5: Vaccination status of CPV2 suspected dogs as history taken from owners.

4. Detection of Canine Parvovirus

4.1. Rapid detection of CPV antigen by immunochromatographic(IC) test

After examination of the dogs administered to the clinics with a complain of different clinical signs suspected to be CPV infection, a rapid CPV test kit was performed. Rectal or fecal swap was taken as a sample for performing the test following the manufacturer's instructions. All the 50 cases were screened for CPV using Rapid test kits, out of which 25 (50%) were positive **(Fig.6)** while 25 (50%) were negative **(Fig.7).**

Fig. 6: A positive result of a rapid CPV test kit. (Two bands are visible).

Fig. 7: A negative result of a rapid CPV test kit. (Only one band is visible).

4.2 Virus isolation and demonstration of CPV cytopathic effect

Through three successive passages of fecal samples that showed positive results in rapid CPV detection test; 2 samples showed progressed cytopathic effect (CPE) with increased virus titer. At first the CPE was started by the 5 day post infection of Vero cell and ended within the 7 to 8 days later then began to appear by the 3rd day post cell infection and ended by the 7th day. We compared between normal vero cells (**Fig.8)** and infected vero cells (**Fig.9)**. Such CPE was characterized by cell rounding and aggregation followed by cell lysis and detachment from the culture surface **(Fig.9)**. The virus titer started by 2log10 TCID50/ml to reach 5log10 TCID50/ml by the third passage as tabulated in **Table (4)**.

Table (4): Passages of the obtained CPV in Vero cell culture.

Passage number	Onset of CPE (DPI*)	End of CPE (DPI)	Virus titer (log10 TCID50/ml)
1	5	8	2
2	4	8	3
3	3	7	5

*DPI= days post infection

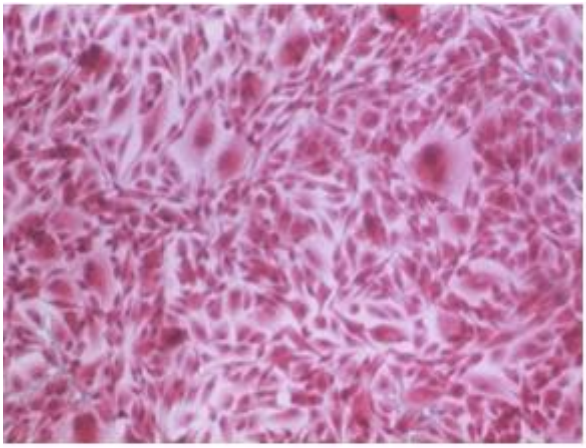


Fig. 8: Normal Vero cell culture (H&E, 100Xs).

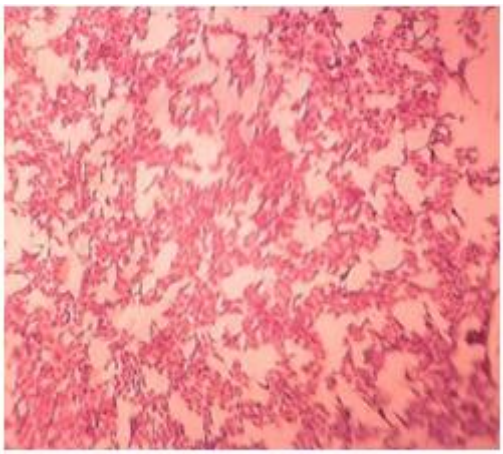


Fig. 9: Infected Vero cell culture with CPV type 2b (H&E, 100Xs)

4.3 Virus identification

4.3.1 Rapid slide agglutination (RAT) and virus neutralization (VNT) tests

It was found that both of RAT and traditional VNT were able to detect the antigens of CPV-2. Faster results obtained by RAT within 2-5 minutes while VNT needs 3-5 days. RAT carried out on Vero cell culture positive samples at the third passage. It was noticed that the strength of agglutination was strong with high titers of virus antigen and weak with lower titers as demonstrated in **(Fig. 10).**

Fig. 10: RAT showing agglutination with difference in strength regarding to titers of the virus on Vero cell culture.

4.3.2. Direct fluorescent antibody technique (FAT)

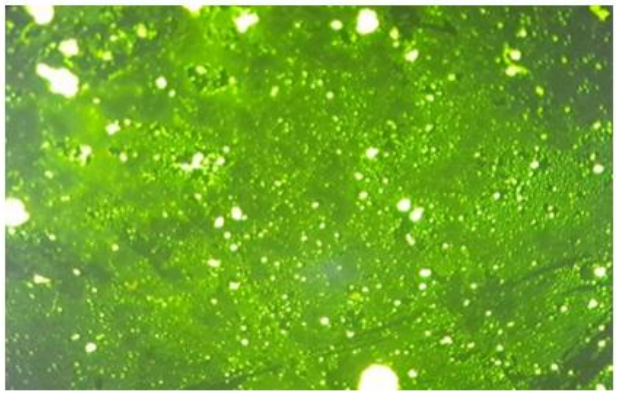


Fig. 11: Positive FAT on CPV infected vero cells showing intra cytoplasmic apple green reaction.

Application of direct FAT on infected Vero cell culture using specific anti-CPV serum conjugated with FITC revealed positive intra-cytoplasmic apple green reaction **(Fig.11)** indicating the presence of CPV.

4.4. Molecular detection and characterization of CPV2

4.4.1 Detection and genotyping of CPV2 by conventional PCR

Out of 50 samples collected and tested by immunochromatographic assay, 18 samples were chosen randomly (8 positive by Rapid test kit and 10 negative) plus the local vaccine. It was found that all 18 samples (in addition to the vaccine) were found clearly positive for parvoviruses by PCR assay using Hfor/Hrev primers, by yielding a specific product size of 630 bp **(Fig.12).**

After then two positive samples (Sample NO 17 and 18) plus the vaccine (Sample NO 19) were subjected to further genotyping using specific primers for CPV2a, CPV2b and CPV2c.The results showed that the three samples (2 virus isolates and the vaccine) were positive CPV2b as clear in **(Fig. 13)**.

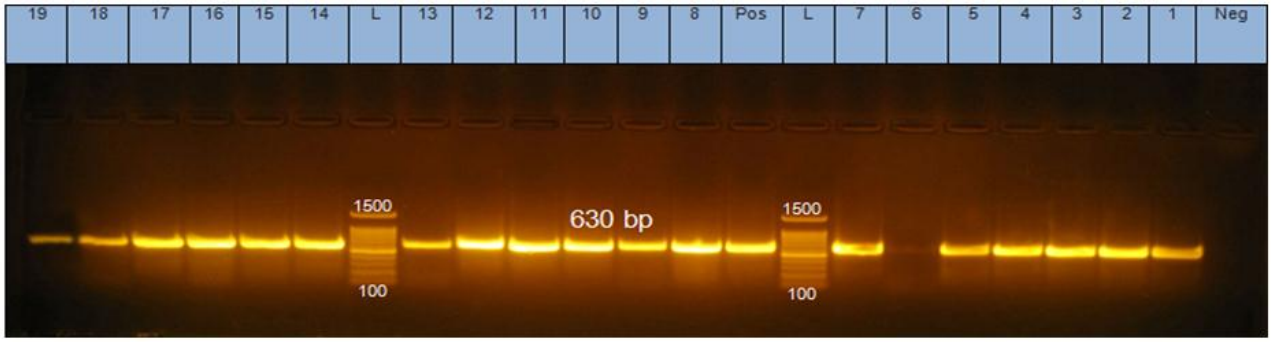


Fig. 12: Screening of clinical samples by PCR assay using CPV-2 Primers.

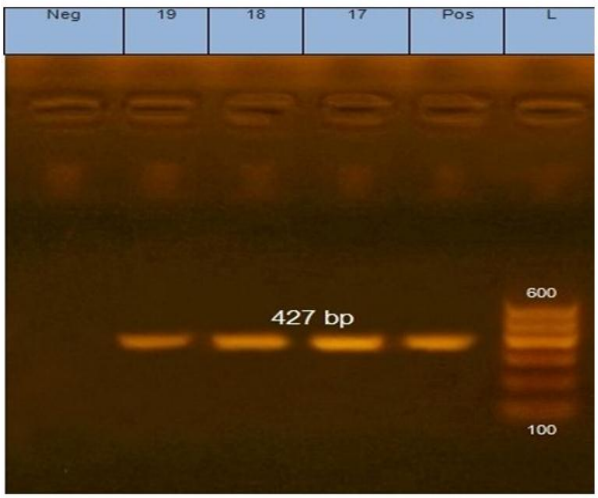


Fig. 13: Agarose gel showing gene (427 bp) using primer set CPV- 2b-F and CPV-2b-R. Ladder L= DNA marker 100 bp.

4.4.2 Sequencing of CPV2b and phylogenetic tree

The PCR product was sequenced using the suitable primer with the help of automated DNA sequencer for typing of CPV strain. The obtained sequence was compared with other CPV-2 isolates in Egypt and other countries **(Fig.14 and 15)**. Sequence comparison showed identities of 99.8% with CPV serotype 2b isolates from Thailand representing accession numbers on Gene bank of "KP715709.1","KP715701.1", "KP715700.1", "KP715699.1","KP715694.", whereas a similarities were 99.6% with CPV-2a isolate from China"MF467236.1" and CPV-2a isolate from India "KX219738.1" **Table (5).** The highest genetic variability was found among the U.S.A isolates (99.1%).

In Egypt, our isolate was found in same cluster with the CPV-2b isolate from Ismailia governorate "KX358526".

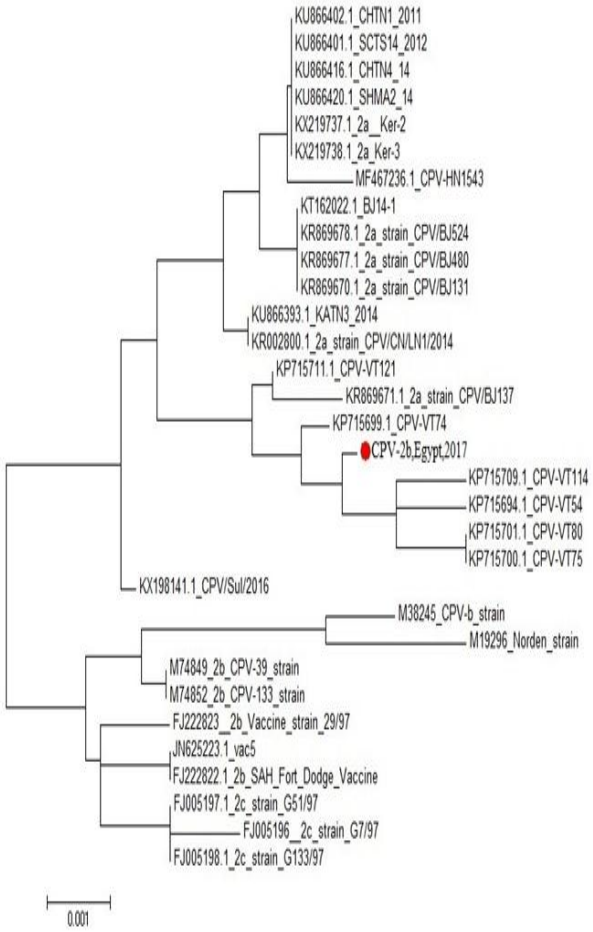


Fig. 14: Maximum likelihood tree illustrating phylogenetic relationship among parvovirus isolates. Canine parvovirus (CPV-2b) sequenced in this study is shown with solid circle.

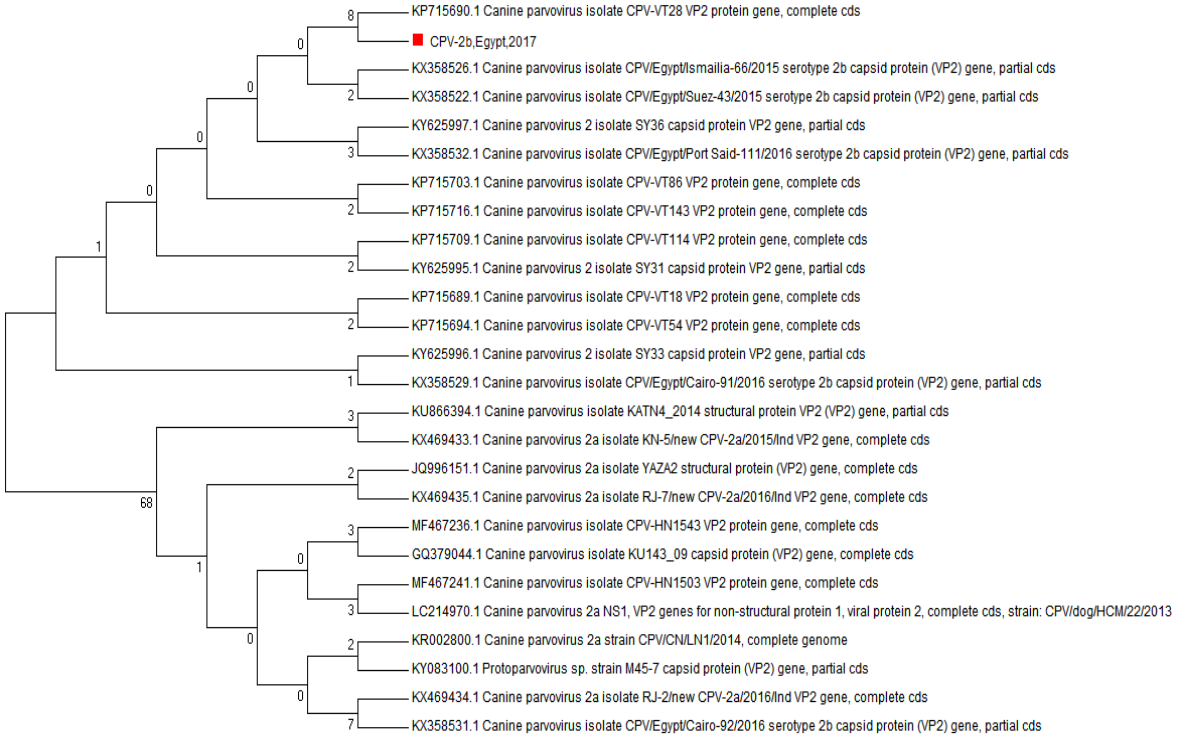
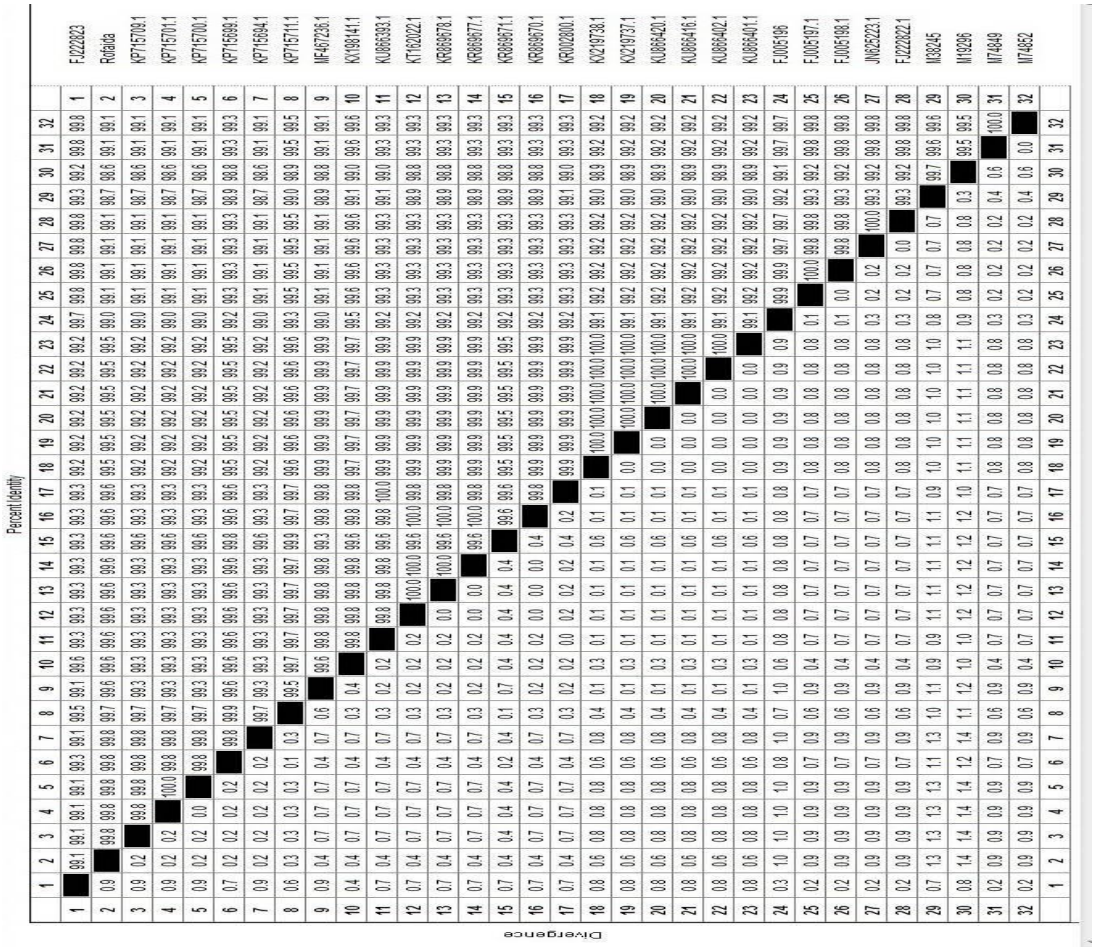


Fig. 15: Phylogenetic tree among parvovirus isolates. Canine parvovirus (CPV-2b) sequenced in this study is shown with solid square.

Tab. 5: Similarity and divergence of our CPV-2b isolate and reference strains. CPV-2b in our study represented by NO.2.



DISCUSSION

In the present study CPV-2 infected dogs exhibited clinical signs such as diarrhea, significant elevated mean rectal temperature, heart rate, respiratory rate, vomitions, dehydration, pale buccal mucus membrane. Similarly[18] recorded elevated body temperature and heart rate, paleness of mucous membrane, diarrhea, anorexia, vomition and dehydration. Observed variations in the diarrhea were in accordance with the reports of earlier worker.[19]

Bloody diarrhea was one of the most clearly observed signs in 40% of dogs, this is in accordance with[20] who reported hematemesis in dogs with CPV infection. Such diarrhea may be due to destruction and collapse of the germinal epithelium of the intestinal crypts and the resulting villous atrophy.[21] Dehydration was also one of the characteristic clinical sign in the present study which may be as a consequence to large quantity of fluid loss from vomiting and diarrhea.[22] Vomiting was one of the major clinical sign in infected dogs which developed early within 24 to 48 post infection as reported by.[23,24]

Our study showed that there was a non-uniform distribution of the cases was found throughout the seasons in Egypt where the highest number of cases was detected in the winter season where 30 (60%) dogs were suffering from the disease, while 6 (12%) in spring, 5 (10%) in summer and 9 (18%) in autumn **(Fig.5).** On the other hand,[25] reported that Dogs in warmer months have been reported as having a higher risk and be more susceptible for CPV infection. The probable reasons for this could be increased availability of susceptible population and favorable environmental condition for the growth, multiplication and dispersion of the pathogen during winter season and stress factor on the puppies as the temperature, wind speed and rainfall tend to have an impact in the spread of infection and outbreak of the disease in addition to the various predisposing factors.

Our study revealed that the majority of the samples originated from dogs under the age of 6 month old with a percent of 94% **(Fig.4).** This finding correlates with that of the other studies carried out around the world which have shown that dogs in that age category are at the highest risk of CPV-2 infection.[26]

The results of this study suggested that unvaccinated dogs were more likely to acquire CPV-2 infection as 37 (74%) came from dogs with no vaccination history, while only 10 (20%) of the dogs were vaccinated (with difference in number of vaccine shots). These results are in agreement with.[23,27] who reported that the absence of a protective immunity (no vaccination scheme or vaccine

failure due to interference with maternal antibodies), is a significant risk factor to the CPV infection.

All the 50 were screened for CPV-2 infection by Rapid antigen test kit, Out of which 25 (50%) were positive while 25 (50%) were negative. While taking random 10 negative samples by the immunochromatography and detecting them by conventional PCR, they were positive. Not surprisingly, these results confirmed the previous studies of[7] who stated that an immunochromatographic (IC) test was compared to molecular techniques, showing that the relative sensitivity of the test did not exceed 50% with respect to the nucleic acid-based methods, whereas the specificity was 100%. The poor sensitivity of the IC test was associated with the low amounts of virus shed in the feces during the late stages of infection and/or the early presence of high CPV antibody titers in the gut lumen that may sequestrate most viral particles. A more recent study compared the performances of three different commercially available, antibody-based tests for rapid detection of CPV antigens with PCR and immunoelectron microscopy, confirming the high specificity and low sensitivity of the antigen-detection kits.[28]

The CPEs were not so prominent in the beginning, a mild CPE in the form of increased granularity, distorted cell morphology and detached cells were seen in one of the infected cells at third passage level **(Fig.9).** This was in agreement with.[29]

The rapid slide agglutination Test is being used successfully in the laboratory to detect many viruses like FMD, CPV harvested from infected animal cells and tissues as mentioned in the previous study.[13] Our results revealed that the strength of agglutination was strong with high titers of virus antigen and weak with lower titers. The RAT because of its simplicity and rapidity of performance and its low cost, has a great potential for use for direct detection and identification of CPV as a screening strategy.

Specific intracellular fluorescence observed in infected cell culture of the two samples demonstrated the presence of CPV in infected cell lines **(Fig.11).** This was in agreement with the previous studies made by.[30]

Recently the PCR technique has been increasingly used as a tool for the diagnosis of canine Parvoviral infection.[31]

Our results showed that all 18 samples (in addition to the vaccine) were found clearly positive for parvoviruses by PCR assay**.** Of these 18 samples, 10 samples were tested negative by the rapid test and found to contain CPV-2 DNA by conventional PCR. This is not surprising, as[7] previously declared the higher sensitivity of PCR assay over other diagnostic techniques. Our results revealed that both samples and the local vaccine were clearly positive for CPV-2b **(Fig.13)**. Such results are the first of its kind to demonstrate the current circulating field and vaccinal strain in Egypt as few studies were done on this point. Previously, the prevalence of CPV-2b has been reported in several countries within the five continents and that was found to be the predominant antigenic variant in African countries[32], Ireland[33], the UK[34], the USA[35] and in four of nine Asian countries.[36] PCR based sequence analysis has revolutionized our knowledge of the spatial and temporal dynamics of CPV infection and also helps to gain new insights into pathogenesis and antigenic differences between CPV-2 types.[37]

Our CPV-2b isolate was found in same cluster with the CPV-2b isolate from Ismailia in 2015 governorate "KX358526", also there was 99.9% identity of our isolate with CPV-2b isolates from Thailand **(Fig.14)** and **Table (5)** that may give a new insight into epidemiological transmission of the virus in our country.

Phylogenetic investigations of our Egyptian CPV-2b VP2 partial sequences identified monophyletic relations among the close geographically associated CPV samples. In addition, CPV-2b VP2 partial sequences were phylogenetically associated with one of the most commonly used CPV vaccine strains (Fort-dodge vaccine) as clear in **(Fig.14).**

CONCLUSIONS

The present study provides a new and valuable insight into the epidemiological dynamics of parvovirus type 2 infection and declared that CPV-2b is the currently circulating field strain that also present in the local vaccine. The high sensitivity of the PCR assay may help in identification of dogs shedding CPV-2 at low titers in their feces, aiming adequate measures of prophylaxis to prevent CPV infection, especially in kennels and shelters, where this virus is often responsible for dramatic epizootics.

Conflict of Interest

The author(s) declared no potential conflicts of interest.

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