Journal of Current Veterinary Research

ISSN:4026-2636

Journal homepage: http://www.jcvr.journals.ekb.eg

Genetic and Molecular Typing of *Canine Parvovirus* Strains Circulating in Symptomatic Dogs in Egypt

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ABSTRACT

Canine parvovirus (CPV) is considered one of the serious and problematic diseases in young puppies. It causes hemorrhagic enteritis and myocarditis in affected dogs. The aim of the present study was to detect CPV-2 in feces of clinically diseased puppies by polymerase chain reaction (PCR) followed by VP2 gene partial sequencing and molecular characterization of circulating strains in Egypt as well as studying some factors associated with the disease incidence. Forty fecal samples were collected from clinically suspected dogs with CPV-2. The clinical diagnosis was confirmed in 35 suspected clinical cases (87.5%) by PCR using common and specific primers sets for detection of (CPV-2, CPV-2a, CPV-2b, CPV-2c) validating that presumptive clinical diagnosis is practically dependable. Nucleotide sequencing of parvovirus (CPV-2) isolates showed that all the three antigenic types (2a, 2b, and 2c) are currently circulating in Egypt.

Keywords: CPV2; Dogs; PCR; Diarrhea

INTRODUCTION

Canine Parvovirus is an acute enteric viral disease particularly fatal in young puppies characterized by hemorrhagic gastroenteritis and myocarditis forms with high morbidity and mortality (Bargujar et al., 2011). The etiological agent, canine parvovirus type 2 (CPV-2), is a small non-enveloped, single stranded DNA virus, genus Parvovirus, family Parvoviridae (Nandi & Kumar, 2010). The original CPV-2 strain was subdivided into two distinct antigenic variants; CPV-2a and CPV-2b. Subsequently, new variant strain called CPV-2c, was detected in Italy (Wang et al., 2016). These variant strains (CPV-2a, CPV-2b and CPV-2c) are circulating among dogs breeds globally (Apaa et al., 2016). The virus has higher replication in lymphoid, intestinal epithelial tissues and bone marrow in puppies during the first two weeks of life with high dissemination of infection through fecal-oral route (Zourkas et al., 2015). Following 3-7 days incubation period, fever, depression, anorexia vomiting, mucoid to hemorrhagic diarrhea of foul smelling and dehydration were predominant features noticed in affected dogs (Kalli et al., 2010). CPV- 2a and CPV- 2b were the circulating genotypes in Egypt (AL-Hosary, 2018; Soliman et al., 2018). The current study designed to detect CPV-2 variant strains circulating in Egypt using molecular based approach depends on PCR and gene sequencing, as well as studying some factors associated with the disease incidence.

MATERIALS AND METHODS

Clinical examination

Puppies were clinically examined as described previously (Decaro et al., 2005). Data regarding age, breed, sex and vaccination status of each puppy was collected and recorded accordingly.

The guidelines of the Committee of Animal Care and Welfare, Faculty of Veterinary Medicine, University of Sadat City were applied with adequate measures taken to reduce pain or discomfort.

Samples:

Forty fecal samples were collected from puppies with diarrhea presented to small animal veterinary clinics in Cairo and Menoufia governorates during a period of 6 months from October 2017 to March 2018. Fecal samples were mixed (10%, wt/vol) in 1 ml sterile phosphate-buffered saline (PBS, pH 7.2) then, centrifuged at 10,000 rpm for 10 min at 4ºC. The supernatants were used for DNA extraction.

DNA extraction:

Total DNA extraction was performed using G-spin™ Total DNA Extraction Kit (iNtRON biotechnology) following the manufacturer' recommendations.

PCR:

The PCR was developed using differential PCR primers for identification of CPV-2a, CPV-2b and CPV-2c (Table 1) that were designed using NCBI Primer3 software (Kaur et al., 2014) to identify different antigenic types of CPV-2 in a single PCR reaction.

Table (1) primers of CPV2

Antigenic Type	Primers	Sequence	Product Size
2	CPV-2GM F CPV-2GM R	5′-CTGCTACTCAGCCACCAACT-3′ 5′-AGGTGTTTCTCCTGTTGTGGT-3	**719**
2a	CPV-2aGM F CPV-2aGM R	5′-AGAGCATTGGGCTTACCACC-3′ 5′-ATCTTCCTGTATCTTGATGTGCT-3′	**379**
2b	CPV-2bGM F CPV-2bGM R	5′-TGTATTGCTACCAACAGATCCA-3′ 5′TGGTGCATTTACATGAAGTCTTGG-3′	**178**
2c	CPV-2cGM F CPV-2cGM R	5′-GTGGTTCTGGGGGTGTGG-3′ 5′-AGCTGCTGGAGTAAATGGCA-3′	**470**

The PCR was carried out as described by (Kaur et al., 2014) with some modifications. Two µl of DNA sample were mixed with 8.5 µl water, nuclease free (Promega); 12.5 µl Taq DNA Polymerase 10X Buffer and primers 10 pmol each. The thermocycling format: denaturation at 95ºC/2 min; 35 amplification cycles with denaturation at 95ºC/30 s, annealing 59ºC/30 s, extension at 72ºC/30 s; and a final incubation at 72ºC/10 min.

Genotypic and sequencing analysis of CPV2 variant strains:

The PCR products were purified and sequenced using an AB1 PRISM 3100 genetic analyzer (Applied Biosystems, USA, gene link DNA Sequencing service, New York, USA). The obtained sequences were aligned with other sequences retrieved from the GenBank using CLUSTALW algorithms available in the Molecular Evolutionary Genetic Analysis (MEGA version X) software (Kumar et al., 2018) and blasted using NCBI Nucleotide BLAST (Morgulis et al., 2008). The phylogenetic trees were built in MEGA version X using the Neighbor-Joining method (Studier & Keppler, 1988). The evolutionary distances were computed using the Jukes-Cantor method. One thousand bootstrap replicates were conducted to assess statistical support for the tree topology.

Statistical analysis

SPSS Statistical Package Social Sciences software program (2007) (Babbie et al., 2007) was used for descriptive analysis as well as for Chi-square analysis and odds ratios.

RESULTS

Clinical manifestations of CPV-2.

The most prominent recorded clinical signs included loss of appetite, depression, vomiting, fever and bloody diarrhea. There was a rise of body temperature (greater than or equal to 39.4ºC) which progressively turned to subnormal with progression of vomiting and diarrhea. The vomiting and bloody diarrhea were recorded in 85% (34/40) and 77.5%

(31/40) of examined clinical cases respectively.

Molecular identification of CPV-2 in fecal samples using PCR:

Out of 40 samples from puppies, displaying clinical signs of canine parvovirus infection 35 (87.5 %) were positive using the CPV-2 primer yielding a product size of 719 bp (Fig. 1).

Antigenic characterization of CPV-2 using differential PCR

For the purpose of antigenic characterization of CPV-2, differential PCR primers for identification of CPV-2a, CPV-2b and CPV-2c (Table 1) were designed using Primer3 software from the NCBI website by (Kaur et al., 2014) to be used in a single PCR reaction to detect various antigenic types of CPV-2.

All the 35 samples were positive using differential PCR primers individually yielding 379 bp with CPV-2a primers (Fig.2), 178 bp with CPV-2b primers (Fig.3) and 470 bp with CPV-2c primers (Fig.4) respectively. Analysis of these four pair of primers using the SnapGene® 4.2.6 software reveled that these primers are not specific and can be used for molecular identification of CPV-2 but cannot be used to differentiate its variants.

All the four pairs of primers were able to bind to any of CPV-2, CPV-2a, CPV-2b and CPV-2c genome yielding the corresponding PCR product at the expected size.

Sequencing and phylogenic analysis of CPV-2

The nucleotide blast and phylogenetic analysis of the generated sequences revealed that all CPV-2 variants CPV-2a (Fig. 5), CPV-2b (Fig. 6) and CPV-2c (Fig. 7) are circulating in Egypt.

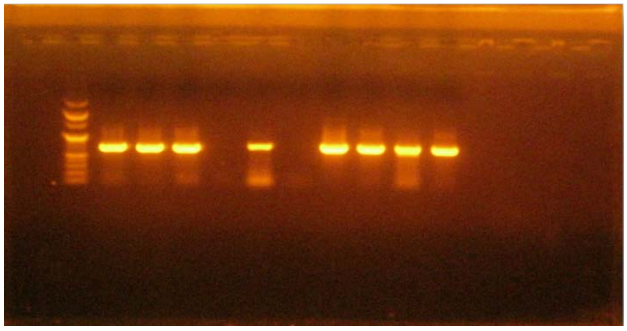

Fig.(1): PCR results of CPV-2 fecal samples using primers for CPV-2 (719 bp). Lanes 1, 2, 3, 5, 7, 8, 9 and10 are clinical positive samples; lanes 4 and 6 are clinical negative samples; M: Molecular weight marker.

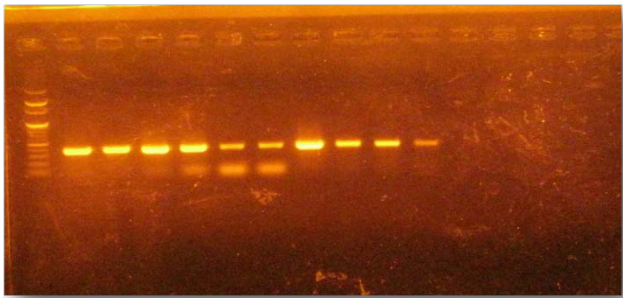

Fig.(2): PCR results of CPV-2 fecal samples using primers for CPV-2a (379 bp). Lanes 1 to 10 are clinical positive samples; M: Molecular weight marker.

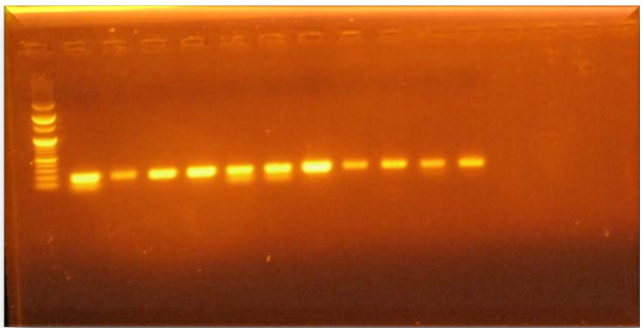

Fig. (3): PCR results of CPV-2 fecal samples using primers for CPV-2b (178 bp). Lanes 1 to 11 are clinical positive samples; M: Molecular weight marker.

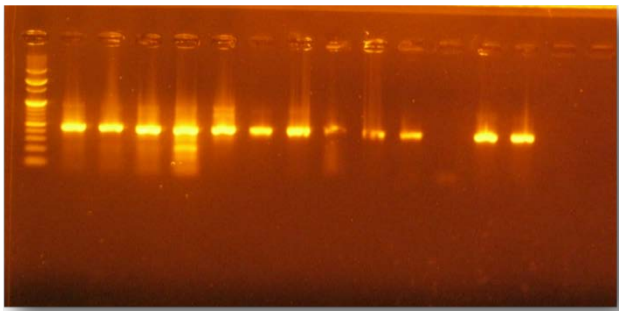

Fig.(4): PCR results of CPV-2 fecal samples using primers for CPV-2c (470 bp). Lanes 1, 2, 3, 5, 6, 7, 8, 9, 10, 12 and 13 are clinical positive samples; lanes 11 is clinical negative samples; M: Molecular weight marker.

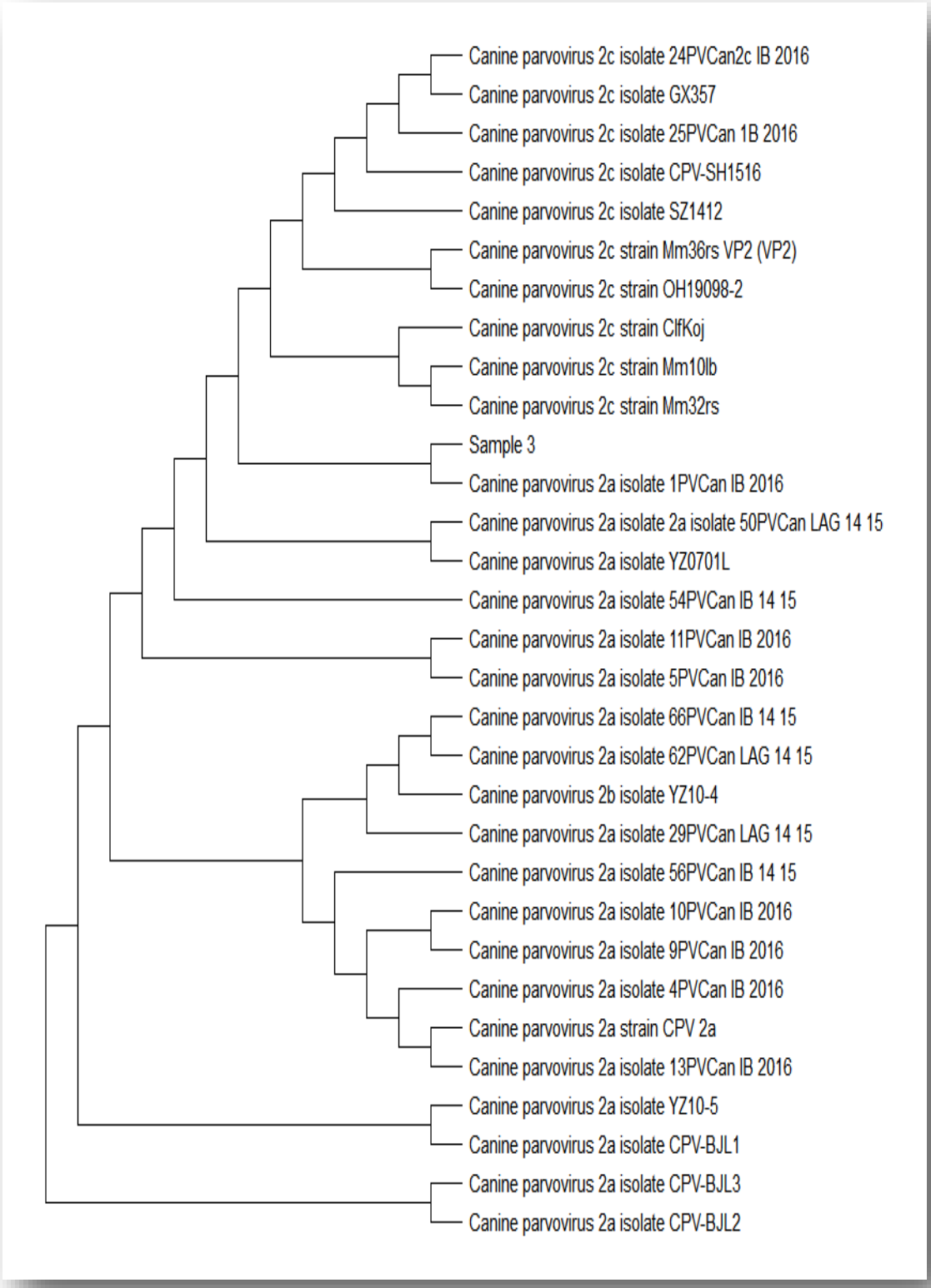

Fig.(5): Phylogenetic tree based on the VP2 gene displaying the genetic relationships between this study CPV-2a isolate and CPV-2 strains of various genotypes.

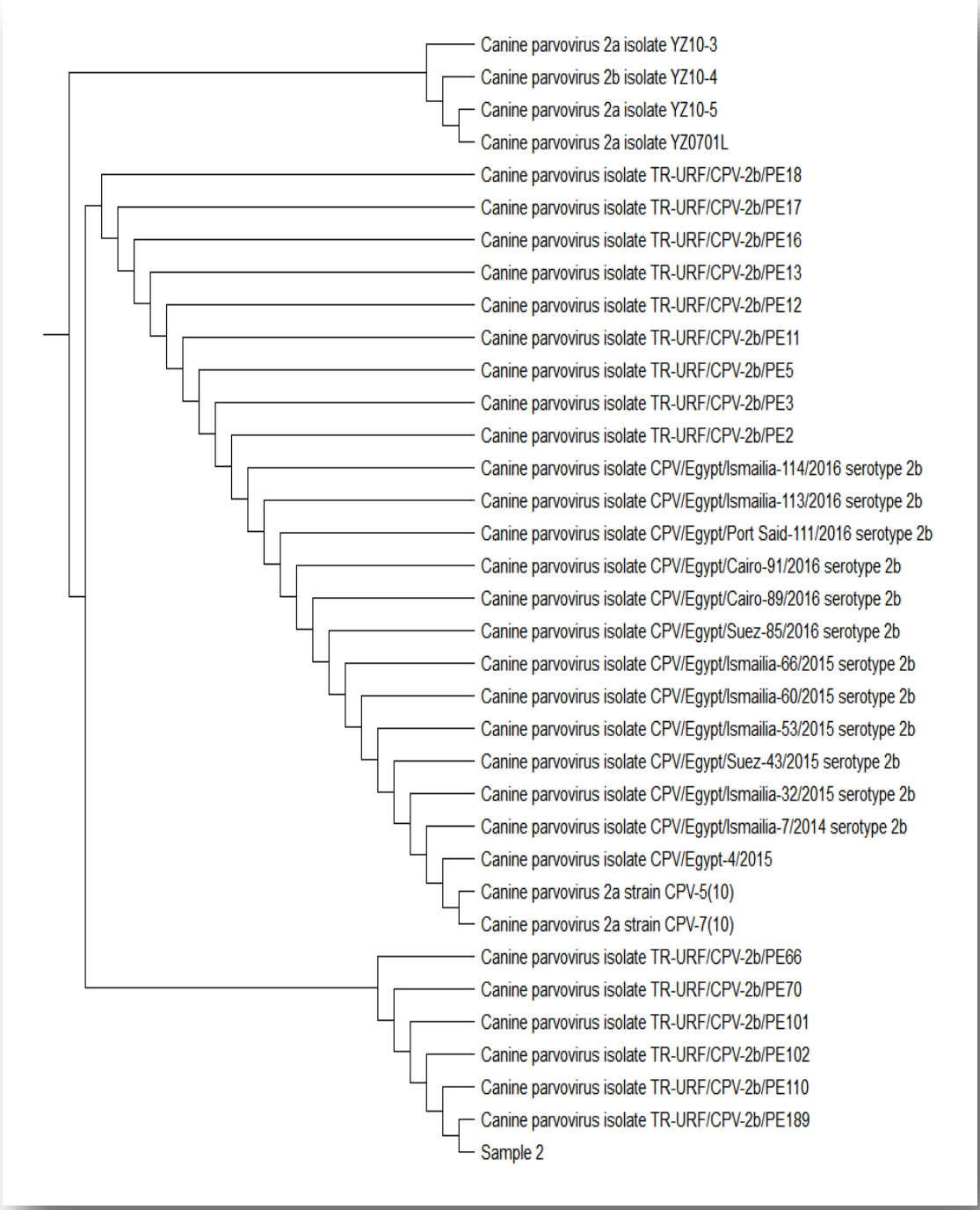

Fig.(6): Phylogenetic tree based on the VP2 gene displaying the genetic relationships between this study CPV-2b isolate and CPV-2 strains of various genotypes.

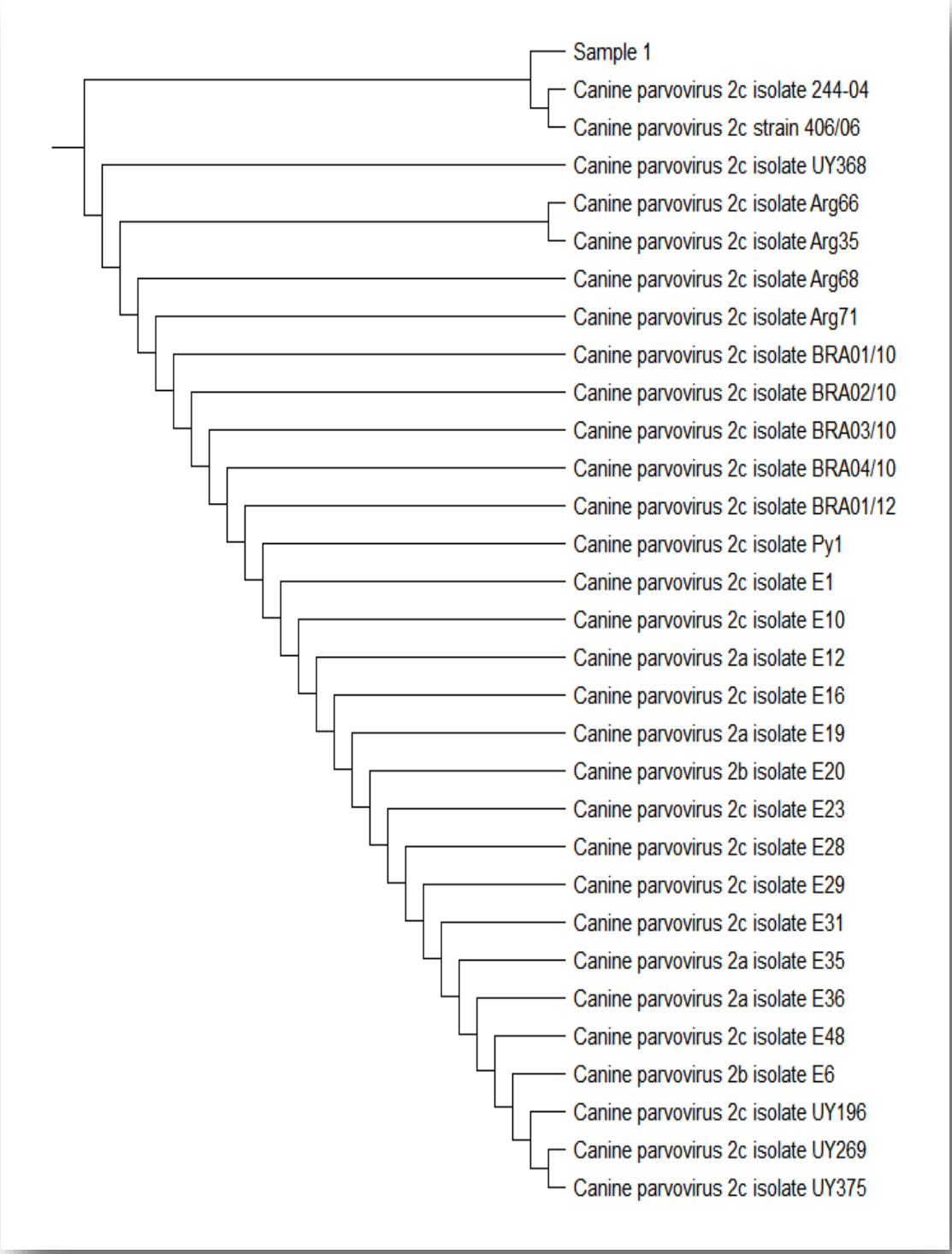

Fig.(7): Phylogenetic tree based on the VP2 gene displaying the genetic relationships between this study CPV-2c isolates and CPV-2 strains of various genotypes.

Epidemiological features of CPV-2

The prevalence of CPV-2 infection in dogs

The prevalence of CPV-2 varied with age, sex, breed, and vaccination state of examine dogs (Table 2). The prevalence decreased with age from 92.3% in age group 1-6 weeks to 80% in age group > 12 weeks. The prevalence in males was 91.8% (34/37) and in females 82.3% (14/17). The prevalence was 92.3% (21/23) in non-vaccinated dogs and 33.33% (1/3) in vaccinated dogs. Concerning breeds, the prevalence rates were 89.4%, 80%, 66.6%, 90% and 100% in German shepherd, Rottweiler, Pit bull, Native and Labrador respectively.

Univariate analysis of risk factors

The association between the prevalence rate of CPV-2 infection and each of the four risk factors was examined by univariate analysis.

The results of these four analyses are described in the following sections.

1. Age

The prevalence of CPV-2 infection decreased insignificantly with age from 92.3% (12/13) in age group 1-6 weeks to 80% (4/5) in dogs aged > 12 weeks. The risk of being infected with CPV-2 increased insignificantly with decreasing age (Table 3).

2. Sex

Though the prevalence of CPV-2 in males (91.30%; 21/23) was higher than in females (82.35%; 14/17), the difference was not statistically significant (X^2 = 0.71; P=0.3) (Table 4).

3. Vaccination

The prevalence of CPV-2 infection in vaccinated dogs was 33.3% (1/3), which was significantly lower than in non-vaccinated dogs (91.89%; 34/37) (X^2 = 8.7; P=0.03) (Table 5). Vaccinated dogs had about 4 times lower chance of contracting the disease than non-vaccinated dogs (OR = 4.2). One dog, which was positive for CPV-2 in PCR, was vaccinated for CPV-2.

4. Breed

The prevalence of CPV-2 infection varied with dog breed, but the differences were not statistically significant (X^2 = 2.05; p = 0.5) (Table 6).

Table 2: The prevalence of CPV-2 in dogs.

Risk factors	Category	No. of samples	No. of infected dog	Prevalence (%)
Age	1-6 weeks	13	12	92.3%
	7-12 weeks	22	19	86.3%
	> 12 weeks	5	4	80%
Sex	Male	23	21	91.3%
	Female	17	14	82.3%
Vaccination	No	37	34	91.8%
	Yes	3	1	33.3%
Dog breed	German shepherd	19	17	89.4%
	Rottweiler	5	4	80%
	Pit bull	3	2	66.6%
	Native	10	9	90%
	Labrador	3	3	100%

Table 3: Univariate analysis of association of age with the prevalence of CPV infection.

Age (weeks)	Infected	Uninfected	Total	Prevalence %	X^2 (*p* value) *	OR (CI 95%)
1-6	12	1	13	92.3%		
7-12	19	3	22	88%	0.53	0.62 (0.05-7.7)
> 12	4	1	5	80%		
Total	35	5	40			

* *p* value in comparison to age group 1-6 weeks (Pearson Chi-square; 0.131; P=0.781)

Table 4: Univariate association of sex with the prevalence of CPV infection

Sex	Infected	Uninfected	Total	Prevalence %	X^2(*p* value)	OR
Male	21	2	23	91.30	0.7	0.4(0.06-3.01)
Female	14	3	17	82.35		
Total	35	5	40			

Pearson Chi-square (0.71; P=0.3)

Table 5: Univariate association of vaccination with the prevalence of CPV infection

Vaccination	Infected	Uninfected	Total	Prevalence %	X2 (p value)	OR (95% CI)
No	34	3	37	91.89	0.03	4.2 (1.2-64.3)*
Yes	1	2	3	33.33		
Total	35	5	40			

Pearson Chi-square (8.7; P=0.03)

Table 6: Univariate analysis association of dog breed with prevalence of CPV infection

Dog breed	Infected	Uninfected	Total	Prevalence %	X^2 (*p* value)	OR (95% CI)
German shepherd	17	2	19	89.4%		
Rottweiler	4	1	5	80%		
Bit bull	2	1	3	66.6%	0.8	0.9 (0.4-1.8)
Native	9	1	10	90%		
Labrador	3	0	3	100%		
Total	35	5	40			

Pearson Chi-square (2.05, P=0.5)

DISCUSSION:

Canine parvovirus infection has emerged as one of the most important diseases of young puppies. Although adult dogs showed less severe symptoms of disease, they serve as source of infection. Also, due to its immunosuppressive nature it reduces animal's ability to resist various infectious diseases (Kaur et al., 2014). CPV-2 was first recognized in 1977 and since then it has been well established as an enteric pathogen of dogs throughout the world with high morbidity (100%) and frequent mortality up to 10% (Appel et al., 1979). CPV-2 has changed genetically and antigenically and was rapidly replaced by an antigenically variant strain named CPV-2a, as well as two other single mutant viruses with substitutions of VP2 residue 426 to Asp (called CPV-2b) or to Glu (called CPV-2c) (Miranda et al., 2015). Moreover, genetic variation among CPV-2 isolates were used to classify the viruses into four genotypes (2, 2a, 2b, and 2c) that differ in their amino acid sequence and VP2 gene phylogenetic relationships (Baba Sheikh et al., 2017).

In the present study, the identification and molecular characterization of CPV-2 in fecal samples obtained from clinically suspected dogs were carried out using conventional PCR, sequencing and phylogenetic analysis.

Forty clinically suspected CPV-2 infections, of approximately 6 to 18 weeks old different breeds puppies were clinically examined in small animal veterinary clinics in Cairo and Monofeya governorates between October 2017 and October 2018. The most prominent recorded clinical signs were fever, depression, loss of appetite, vomiting, and severe bloody diarrhea. Similar clinical signs including elevation of body temperature and heart rate, paleness of mucous membrane, diarrhea, anorexia, vomiting and dehydration in diseased dogs were recorded in this study that agreed with numerous reports (Kapil et al., 2007; Soliman et al., 2018)

The vomiting and bloody diarrhea were recorded in 85% and 77.5% of examined clinical cases respectively. Vomiting and bloody diarrhea were of the major clinical sign in CPV-2 infected dogs which developed early within 24 to 48 post infection (Soliman et al., 2018; Woods et al., 1988). Bloody diarrhea may be due to destruction and collapse of the germinal epithelium of the intestinal crypts and the resulting villous atrophy (Bastan et al., 2013). Dehydration was also one of the characteristic clinical complication in the present study, which may be as a consequence to large quantity of fluid loss from vomiting and diarrhea as recorded by (Greene, 2013).

Various approaches have been used for CPV-2 diagnosis that includes virus isolation, hemagglutination (HA), hemagglutination inhibition (HI), Electron Microscopy (EM), Immunofluorescence (IFT), ELISA and PCR. However, sensitivity of the traditional diagnostic approaches (HA, HI, EM, IFT, and ELISA) has been confirmed to be lower than molecular assays and therefore, PCR has been used for the identification of CPV-2 in fecal samples with high sensitivity and specificity (Schunck et al., 1995).

Out of 40 samples from dogs exhibiting clinical signs of CPV-2, 35 were positive by PCR using the CPV-2 primer indicating that presumptive clinical diagnosis is reasonably reliable considering PCR assay highly sensitive to detect CPV-2 more than other diagnostic techniques (Desario et al., 2005).

Differential PCR primers for identification of CPV-2a, CPV-2b and CPV-2c designed by (Kaur et al., 2014) were used for the purpose of further antigenic characterization of the 35 CPV-2 positive PCR samples. All the samples were positive by the all variants primers. In order to elucidate the dilemma, all the four pairs of primers were subjected to analysis using the SnapGene® 4.2.6 software. It was clear that all these primers are not specific, can be used for molecular identification of CPV-2 but unable to differentiate CPV-2a, CPV-2b and CPV-2c variants.

The molecular characterization of CPV-2 provides important information about the strains circulating in a region at a particular time and explained the relationship between CPV-2 strains from various parts of the world (Dogonyaro, 2010).

Nucleotide sequencing showed that all the three antigenic types (2a, 2b, and 2c) are currently circulating in Egypt. CPV- 2a and CPV- 2b were the circulating genotypes in Egypt and has been reported from Cairo, Ismailia, Port Said, Suez, Alexandria and El Behera governorates between 2014 and 2017 (Soliman et al., 2018). To our knowledge, this is the first confirmed report of CPV-2c in Egypt. Similarly, typing by nucleotide sequencing showed that all three antigenic types (2a, 2b, and 2c) are currently circulating in Tunisia (Touihri et al., 2009). A particular geographic location may harbor any of CPV-2 variants or a mixture of them with predominance of one type over the others (Ramadas, 2011). CPV-2a is the predominant variant in Asia and Europe, CPV-2b is the predominant antigenic variant in African countries while CPV-2c is thought to be the predominant variants in Latin America (Dogonyaro, 2010; Yi et al., 2016; Zhou et al., 2017).

The risk of being infected with CPV-2 increased insignificantly with decreasing age. These results agree with similar results obtained in other studies concluded that puppies aged ≤ 3 months have a higher rate of infection than older dogs confirming the already well-known fact that the CPV-2 infection is more severe in puppies (Banja et al., 2002).

No significant change in prevalence of CPV-2 infection between male and female dogs ($X2 = 0.71$; $P=0.3$), the prevalence of CPV-2 infection was higher in males when compared with females. The above observations are in accordance with other similar reports (Srinivas et al., 2013).

In this study, the prevalence of CPV-2 in vaccinated dogs was 33.3% (1/3), which is significantly lower ($X2 = (8.7$; $P=0.03$) than in non-vaccinated dogs (91.89%; 34/37). Vaccinated dogs were 4 times less likely to develop the disease (OR = 4.2) that, clearly shows the importance of vaccination for reducing CPV-2 infections.

One dog, which was positive for CPV-2 in PCR, was vaccinated before for CPV-2. This may be because of the mismatching of the CPV-2 strain causing infection in that dog and the vaccine strain. CPV-2 vaccine is effective against the strain of CPV-2 existing in the vaccine and not against the other antigenic strains of CPV-2 that may cause infection and even mortality in dogs (Martella et al., 2005). Recently, several reports recommended the development of new vaccines based on the currently circulating CPV-2 variants especially CPV2c. Nevertheless, other reports revealed that dated vaccines based on the CPV-2 strain are still effective (Touihri et al., 2009).

There was no significant association between dog breed and prevalence of CPV-2. Although prevalence was higher in Labrador, native,

German shepherd breeds than Rottweiler, and Bit bull breeds, the number of dogs of some breeds was too small to draw a reliable conclusion. Singh et al. (2013) reported that in India German shepherd breed followed by Labrador and Pomeranian breeds of dogs are most predisposed for CPV-2.

It can be concluded from the above study that CPV-2a, CPV-2b and CPV-2c variants are circulating in Egypt. CPV-2 presumptive clinical diagnosis is reasonably reliable. The differential PCR primers described by (Kaur et al., 2014) cannot be used for the purpose of antigenic characterization of CPV-2. Vaccination play an important role in reducing CPV-2 infections but matching between vaccinal and field strains should be considered.

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