

Detection and Molecular Characterization of Parvovirus Serotypes in Egypt

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ABSTRACT

Parvovirus infection is one of the most common enteric diseases affecting dogs in Egypt. A total number of 49 dogs younger than six month old, suffering from severe watery to bloody diarrhea, vomiting and lethargy were examined to confirm the infection with parvovirus through detection of the viral antigen serologically by FASTest® PARVO Card Test and genetically by using specific primers for both conventional and nested polymerase chain reaction (PCR). Serological assay confirmed the infection in 81.63% (40/49) of the examined animals, while the molecular techniques confirmed the infection in 81.63% (40/49) by the conventional PCR and in 97.96% (48/49) by the nested PCR. Sequencing revealed that CPV2a and CPV2b serotypes are circulating in the Egyptian field. The obtained sequences were submitted to the GenBank under the following accession MG764314, MG764315, MG930487 and MG930488. Serotype CPV2b showed some mutation in different sites.

Keywords:

Parvovirus
Serological fastest, PCR,
Egypt

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Introduction

Canine Parvovirus (CPV) infection was identified during the 1970s then becomes endemic worldwide (Decaro and Buonavoglia, 2012). It is a non-enveloped single strand DNA virus affecting dogs especially young puppies; some strains of this virus have zoonotic importance especially in children (Makhlouf *et al.*, 2017). It has two different strains, namely CPV-1 and CPV-2; both are characterized by high evolution rate. Canine parvovirus 2 has three variants (CPV-2a, CPV-2b and CPV-2c) (Shackelton *et al.*, 2005). The infection has two forms (i) the intestinal form, which is the most common; it is characterized clinically by vomiting, bloody diarrhea, anorexia and weight loss. (ii) Cardiac form is less common and usually affects the cardiac muscles of very young puppies and ended with death (Streck *et al.*, 2009).

This virus causes a highly contagious disease because during the enteric form the infected dogs usually shed the virus

in their stools and contaminate the environment. Susceptible dog takes the infection through direct or indirect contact. This infection characterized by high morbidity up to 100% and case fatality up to 10% (Appel, 1978; Appel *et al.*, 1979; Chollom *et al.*, 2013).

This study aimed to compare between serological and molecular detection of Parvovirus infection in household dogs with special reference to the molecular characterization and phylogenetic analysis of the circulating serotypes in Egypt.

Materials and methods

Animals, clinical examination and sampling

Forty nine dogs aged between one and six months suffering from bloody diarrhea and vomiting were sampled for serological and molecular confirmation of parvovirus infection. Clinical examination was carried out according to Cote (2011). Fecal sample was collected from each dog and divided to two aliquots, one was used for serological detection of the virus antigen and the second was stored at -20°C until used (Coles, 1986).

This work and the used procedures were performed ac-

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cording to the ethical standards of Assiut University and Veterinary authorities in Assiut province, Egypt.

Serological diagnosis

FASTest® PARVO Card Test Kit by Vetlab Supplies Ltd, UK was used for direct detection of parvovirus specific antigen according to manufacture instructions. FASTest® PARVO Card contains two monoclonal antibodies. The mobile monoclonal antibodies bound to colloidal gold particles located in the conjugate pad; the second is immobile and located on the test line. The positive result was demonstrated by detection of antigen-antibody-complex that forms pink-purple test line in the test zone.

Molecular diagnosis

DNA was extracted from the fecal samples using ZYMO Research Quick-DNATM Universal Kit (ZYMO Research, Co., USA, Cat. No D4068 & D4069) for solid tissue and special samples according to the manufacture instruction.

Conventional PCR was performed in total volume of 25µl using pCPV2ab specific primers pCPV2ab forward primer (5'-GAA GAG TGG TTG TAA ATA ATT-3') and pCPV2ab reverse primer (5'-CCT ATA TCA CCA AAG TTA GTA G-3') that amplifies of 681 bp fragment. Nested PCR carried out using pCPV2N forward primer (5'-TGAGCTGCATTTAGTTAGTTTTGA-3') and pCPV2N reverse primer (5'-TGTTTGCCATGTATGTGTTAGTCT-3') was used for amplification of a 442 bp fragment using conventional PCR product as template. PCR and nPCR products were electrophoresed in 1.5% agarose gel stained with ethidium bromide to visualize the specific bands under UV transilluminator (Pereira *et al.*, 2000).

Sequencing and phylogenetic analyses

Four positive samples were subjected to the same PCR reaction in 50 µl total volume then purified by using ZYMO Research DNA Clean & Concentrator™-5 kits (ZYMO Research, Co, USA) before sequencing in both directions using the same primers and Dideoxy chain termination method by BigDye® Terminator kit (Applied Biosystems, USA) according to the manufacturer's instructions. Sequence reactions were analyzed by PRISM 310 genetic analyzer (Applied Biosystems, USA) at the Molecular Biology Unit, Assiut University, Assiut, Egypt. The obtained sequences were assembled and subjected to BLAST search for similarity then each sequence was translated to its open reading frames (amino acids) using ExpASY translation tool (<https://www.expasy.org/>). The percent of identity and similarity between the obtained sequences and other sequences on the GenBank was calculated by using the DNASTAR's "MegAlign" sequence alignment software. Phylogenetic tree was constructed by using neighbor joining method on Mega 6.0 program with Kimura two parameters and bootstrap 1000, Canine Distemper partial sequence (AB025271) was used as an out-group (Kimura, 1980). Translated amino acids of CPV2b sequences were subjected to multiple alignments with previously detected Egyptian isolates on the GenBank database using Clustal omega, EMBL-EBI.

Statistical analysis

The obtained data were compared using Chi Square test (SPSS, Chicago, USA) at 5% threshold value.

Results

Dogs younger than six months old suffered from severe

watery to bloody diarrhea, vomiting and lethargy were infected with parvovirus. The infection was confirmed by the FASTest® PARVO Card Test and conventional PCR in 81.63% (40/49) of the examined dogs. While nPCR confirmed the infection in 97.96% (48/49) of the examined samples with significant difference ($P \leq 0.005$) (Figs. 1 and 2).

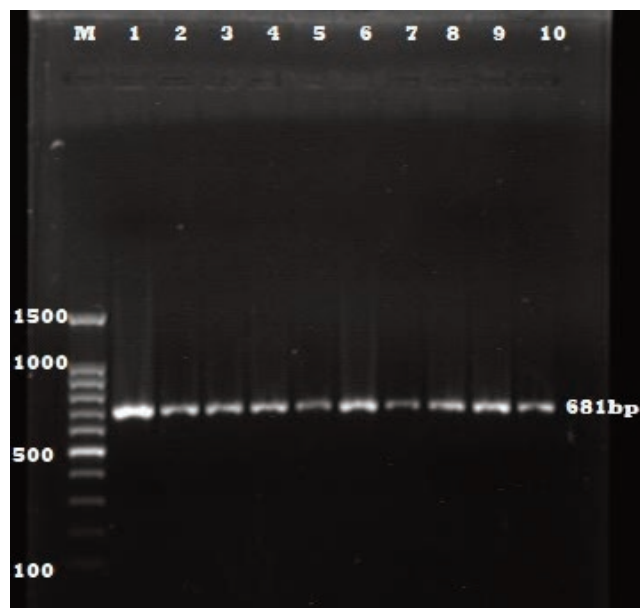


Fig. 1. Agarose Gel (M) DNA ladder 100 bp, lanes (1: 9) positive samples and specific bands at 681 bp. Lanes (10) Positive control samples.

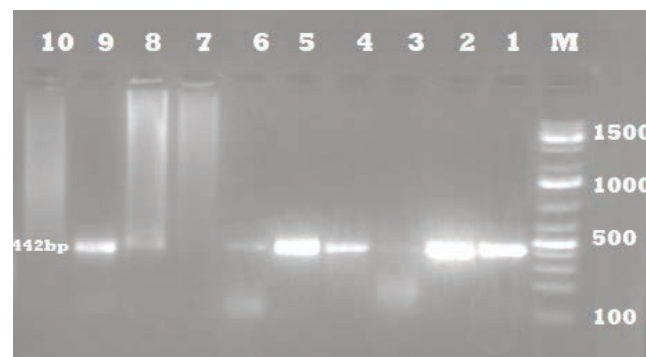


Fig. 2. Agarose Gel (M) DNA ladder 100 bp, lanes (1:6 & 8) positive and specific bands at 442 bp. Lanes (7) negative sample and Lanes (9, 10) Positive and negative control samples.

The four sequenced samples were assembled and submitted to the GenBank (Accession numbers: MG764314, MG764315, MG930487 and MG930488). In the phylogenetic analysis these sequences were clustered into two clades one for CPV2a and the second one for CPV2b. MG764314 and MG764315 sequences of CPV2a serotype are more related to each other and showed 100% of identity. On the other hand, MG930487 and MG930488 amplicons were more related to CPV2b serotype and recorded 99.5% of identity. In respect to the previous sequences recorded in Egypt, the identity was 98.7 up to 99.5% and 98.3 up to 100% with other sequences from different localities all over the world, respectively. Multiple alignments of CPV2b amino acids showed some mutation in different sites (Figs. 3, 4 and 5).

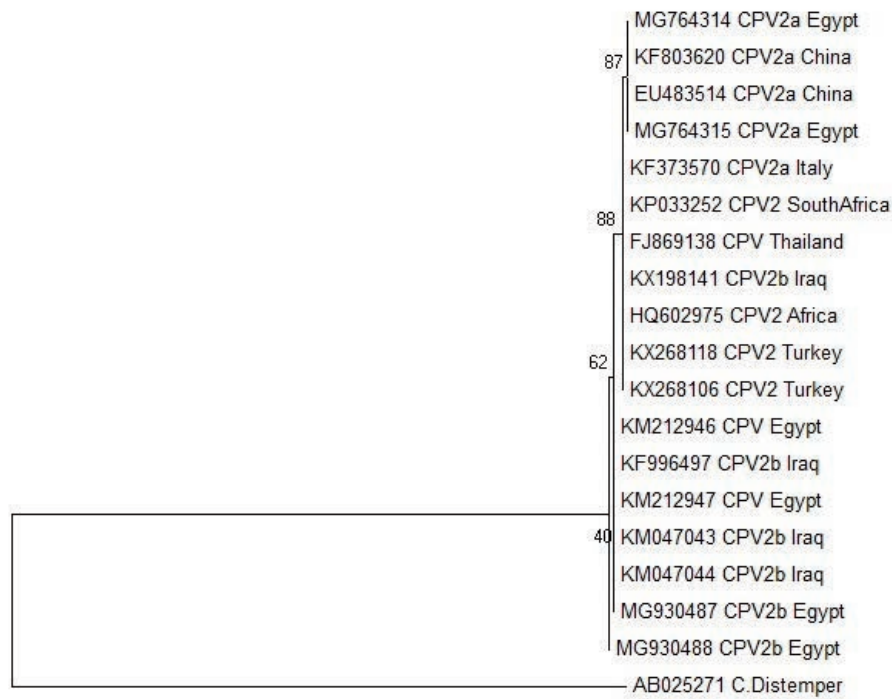


Fig. 3. Phylogenetic analyses for the Egyptian isolates against reference sequences of canine parvovirus.

Percent Identity																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
1	█	21.2	20.6	20.6	21.7	21.5	21.3	21.4	23.3	21.6	21.3	21.2	20.6	20.6	22.8	22.8	20.9	23.1	1	AB 025271_C.Distemper.seq	
2	111.9	█	99.5	99.8	99.7	99.8	98.7	98.7	98.6	98.9	98.7	99.5	99.7	99.4	99.4	100.0	100.0	98.5	98.3	2	EU 483514_China.seq
3	111.9	0.5	█	99.8	99.9	99.5	99.0	99.0	98.9	99.2	99.0	99.6	99.6	99.8	99.8	99.7	99.7	98.8	98.7	3	FJ 869138_Thailand.seq
4	111.8	0.2	0.2	█	99.9	99.7	99.0	99.0	98.9	99.2	99.0	99.8	99.8	99.7	99.7	99.7	99.7	98.8	98.7	4	HQ 602975_Africa.seq
5	111.6	0.3	0.1	0.1	█	99.5	99.0	99.0	98.9	99.2	99.0	99.6	99.4	99.8	99.8	99.7	99.7	98.8	98.7	5	KF 373570_Italy.seq
6	112.0	0.2	0.5	0.3	0.5	█	98.7	98.7	98.6	98.9	98.7	99.4	99.5	99.4	99.4	100.0	100.0	98.5	98.3	6	KF 803620_China.seq
7	112.9	1.3	1.0	1.0	1.0	1.3	█	99.5	99.5	99.2	99.2	99.0	99.0	99.0	99.0	98.7	98.7	98.6	99.5	7	KF 996497_Iraq.seq
8	112.4	1.3	1.0	1.0	1.0	1.3	0.0	█	99.8	98.7	98.7	99.0	99.0	99.0	99.0	98.7	98.7	99.0	99.5	8	KM 047043_Iraq.seq
9	112.9	1.3	1.0	1.0	1.0	1.3	0.0	0.0	█	98.7	98.7	98.9	98.9	98.9	98.9	98.6	98.6	98.9	99.5	9	KM 047044_Iraq.seq
10	112.7	1.2	0.8	0.8	0.8	1.2	0.0	0.0	0.0	█	100.0	99.2	99.2	99.2	99.2	98.9	98.9	97.9	99.5	10	KM 212946_Egypt.seq
11	112.5	1.3	1.0	1.0	1.0	1.3	0.0	0.0	0.0	0.0	█	99.0	99.0	99.0	99.0	98.7	98.7	97.7	99.5	11	KM 212947_Egypt.seq
12	114.1	0.3	0.2	0.1	0.2	0.5	1.0	1.0	1.0	0.8	1.0	█	99.6	99.5	99.5	99.7	99.7	98.8	98.7	12	KP 033252_S.Africa.seq
13	112.5	0.3	0.3	0.1	0.2	0.5	1.0	1.0	1.0	0.8	1.0	0.2	█	99.7	99.7	99.7	99.7	98.8	98.7	13	KX 198141_Iraq.seq
14	112.3	0.6	0.2	0.3	0.2	0.6	1.0	1.0	1.0	0.8	1.0	0.4	0.3	█	100.0	99.7	99.7	98.8	98.7	14	KX 268106_Turkey.seq
15	112.3	0.6	0.2	0.3	0.2	0.6	1.0	1.0	1.0	0.8	1.0	0.4	0.3	0.0	█	99.7	99.7	98.8	98.7	15	KX 268118_Turkey.seq
16	116.3	0.0	0.3	0.3	0.3	0.0	1.3	1.3	1.3	1.2	1.3	0.3	0.3	0.3	0.3	█	100.0	98.5	98.3	16	MG 764314_Egypt.seq
17	116.3	0.0	0.3	0.3	0.3	0.0	1.3	1.3	1.3	1.2	1.3	0.3	0.3	0.3	0.3	0.0	█	98.5	98.3	17	MG 764315_Egypt.seq
18	111.3	1.4	1.1	1.1	1.1	1.4	0.2	0.2	0.2	0.0	0.2	1.1	1.1	1.1	1.1	1.4	1.4	█	99.5	18	MG 930487_Egypt.seq
19	110.7	1.5	1.2	1.2	1.2	1.5	0.3	0.3	0.3	0.3	0.3	1.2	1.2	1.2	1.2	1.5	1.5	0.3	█	19	MG 930488_Egypt.seq

Fig. 4. Similarity and divergence between the Egyptian and reference sequences of canine parvovirus serotypes, the upper right values are the percentage of identity and the lower left values are the percentage divergences between sequences.

Discussion

Canine parvovirus infection is a common destructive enteric viral disease leading to high morbidity and mortality rates in unvaccinated puppies as well as in neglected untreated infected dogs (Decaro *et al.*, 2006). The virus is transmitted from the diseased to susceptible dogs through direct or indirect contact due to heavy shedding of the virus in feces and the excretion of infected dogs that leads to high opportunity of contamination in food staff, water and fomites (Zhao *et al.*, 2011). The virus shedding continue for some time after recovery and the animals considered as asymptomatic convalescent carriers (Clegg *et al.*, 2012). Canine parvovirus infection was

characterized by severe dehydration, leathery, vomiting and foul-smelling watery to bloody diarrhea because of erosive gastritis and enteritis, these findings come in agreement with Amthal, (2014) and Al-Hosary (2016), who reported similar clinical signs in dogs infected with parvovirus. FASTest® PARVO Card Test is a cheap, fast, accurate test for detection of the virus antigen in clinically infected dogs. The test detected the real infection in 81.63% of the examined dogs. So, it is a recommended approach for confirmation of parvovirus infection in dogs. Early and accurate diagnosis helps the diseased animals to recovery before any complication like severe anemia and dehydration, which are fetal in young animals according to Al-Hosary (2016).

CLUSTAL O(1.2.4) multiple sequence alignment

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VIRT26284_CPV_VP2gene_Egypt      -----MSELHLVSEFEQEIFNVLKTVSE
VIRT48193_CPV_VP2gene_Egypt      -----MSELHLVSEFEQEIFNVLKTVSE
MG930487_CPV2b_Egypt             MALDDPQAQIVTPWISLVANAMGVWIFNPGDWQLIVNTMSELHLVSEFEQEIFNVLKTVSE
MG930488_CPV2b_Egypt             -----MSELHLVRFEQEIFNVLKTVSE
                                  *****

VIRT26284_CPV_VP2gene_Egypt      SATQPPTKVYNNDLTASLMVALDSNNTMPFTPAAMRSETLGFYPWKPTIPTPWRYFQWD
VIRT48193_CPV_VP2gene_Egypt      SATQPPTKVYNNDLTASLMVALDSNNTMPFTPAAMRSETLGFYPWKPTIPTPWRYFQWD
MG930487_CPV2b_Egypt             SATQPPTKVYNNDLTASLMVALDSNNTMPFTPAAMRSETLGFYPWKPTIPTPWRYFQWD
MG930488_CPV2b_Egypt             SATQPPTKVYNNDLTASLMVALDSNNTMPFTPAAMRSETLGFYPWKPTIPTPWRYFQWD
                                  *****

VIRT26284_CPV_VP2gene_Egypt      RTLIPSHTGTSGTPNIIYHGTDPPDVQFYTIENSVPVHLLRTGDDEFATGTFYFDCKPCRL
VIRT48193_CPV_VP2gene_Egypt      RTLIPSHTGTSGTPNIIYHGTDPPDVQFYTIENSVPVHLLRTGDDEFATGTFYFDCKPCRL
MG930487_CPV2b_Egypt             RTLIPSHTGTSGTPNIIYHGTDPPDVQFYTIENSVPVHLLRTGDDEFATGTFYFDCKPCRL
MG930488_CPV2b_Egypt             RTLIPSHTGTSGTPNIIYHGTDPPDVQFYTIENSVPVHLLRTGDDEFATGTFYFDCKPCRL
                                  *****

VIRT26284_CPV_VP2gene_Egypt      THTWQTNRALGLPPFLNSLPQAEGGTNFG-
VIRT48193_CPV_VP2gene_Egypt      THTWQTNRALGLPPFLNSLPQAEGGTNFGY
MG930487_CPV2b_Egypt             THTWQTNRALGLPPFLNSLPQAEGGT----
MG930488_CPV2b_Egypt             THTWQTNRALGLPPFLNSLPQAED-----
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Fig. 5. Multiple alignments of CPV2b amino acids showed some mutation in different sites.

Molecular techniques were more sensitive and specific diagnostic tool, especially nPCR. It confirmed the infection in 97.96% of the examined animals, while serology and conventional PCR confirmed the infection in only 81.63% of the examined cases. This finding may be attributed to the ability of nPCR to detect the lowest amount of viral particles according to previous studies by Hirasawa *et al.* (1994); Mochizuki *et al.* (1996) and Kumar *et al.* (2010), who confirmed that nPCR is the most sensitive test to detect the viral particles.

According to the author's knowledge, the current study is the first report that provides data about the molecular characterization of the circulated parvovirus serotypes in Egypt, the obtained results confirmed that there are two different variants of CPV2 serotypes circulating in the Egyptian field that include CPV2a and CPV2b. The Egyptian strains were clustered into two clades. The first clade includes CPV2a isolates, which are closely related to Chinese isolates, while the second clade includes the isolates of CPV2b, which were closely related to previously isolated serotypes from Cairo, Egypt (Amthal, 2014) and serotypes isolated from Slemani Province, Iraq (Sheikh *et al.*, 2017). CPV2b is the main circulated serotype in Egypt and was recorded in some previous reports. Detection and molecular characterization of CPV2a and its close relationship with Chinese serotypes for the first time during this study may be attributed to lack of studies that concerned on molecular characterization of the Egyptian circulating serotypes in general and especially in Egypt. Also, this new finding suggested that this virus serotype (CPV2a) may be introduced from China to Egypt through contaminated dogs, contaminated premises like dog toys, plates for feeding and/or some feed stuff, which serve as source for indirect infection because the virus has the ability to be pathogenic under suitable environmental conditions during up to five months (Jacobs *et al.*, 1980). Analysis and alignment of the translated amino acids confirmed the occurrence of mutations in the Egyptian circulated isolates of CPV2b obtained during this study in comparison to the previously recorded sequences of the same serotype in Egypt. These findings confirmed that CPV2b circulated in different regions in Egypt has unstable DNA genome and in agreement with previous study by Miranda and Thompson, (2016), who reported some mutation in CPV2 variants. Viral protein 2 (VP2) is one of the major structural protein encodes the viral capsid protein and responsible for the antigenicity and involved in

the host immune response. So, any mutations will result in more pathogenicity of the virus (Lin *et al.*, 2014). Previous studies reported that CPV is characterized by a high genetic substitution rate like that observed in RNA viruses and this character is responsible for continuous antigenic evolution and rapid displacement by new antigenic variants (Decaro *et al.*, 2007a,b). This finding should be considered especially when establish vaccination protocol against this infection because these mutations perforate animal's immunity and may lead to outbreaks.

Conclusion

Canine parvovirus (CPV) is one of the most common diseases affecting dogs in Egypt; especially, young unvaccinated dogs. Fast sero-diagnostic kit is a recommended tool for rapid routine diagnosis while sophisticated technique like molecular assay is recommended for wide scale and epidemiological studies specially to detect the infection in carrier animals. There are two serotypes of CPV circulating in Egypt include CPV2a and CPV2b. CPV2b characterized with continuous antigenic evolution, which affect the efficacy of vaccination in Egypt.

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