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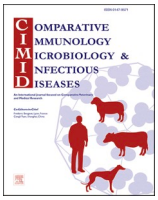

Molecular characterization of full-length VP2 gene of canine parvovirus type 2 strains circulating in Egypt 2019–2021

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ABSTRACT

Canine parvovirus type 2 (CPV-2) is a major cause of fatal gastroenteritis and myocarditis in puppies of domestic and wild carnivores. CPV-2 has accumulated changes over time lead to the emergence of three antigenic variants CPV-2a, CPV-2b, and CPV-2c. VP2 is the major capsid protein that determines virus antigenicity, and host range. Although the three CPV-2 variants were previously identified in Egypt, most reports covered a restricted geographic region and/or time period, and only analyzed partial fragments of VP2 gene. Therefore, this study was designed to test 100 rectal swabs collected from 7 Egyptian governorates between 2019 and 2021 for CPV-2 using PCR. A total of 65 positive samples were identified, mostly in pure dog breeds of young age. The three variants co-circulated in 2019, while CPV-2b was not detected in 2020 and 2021. The frequency of CPV-2b and CPV-2c was higher in 2019 and 2021, respectively. Analysis of CPV-2 full-length VP2 gene sequence from 19/65 positive samples has identified four common amino acid substitutions F267Y, S297A, A300G, Y324I, which are characteristic for the new CPV-2 variants currently circulating worldwide. Unique substitutions including A5G, G36R, V38E, Q370R, and G392V were recognized in certain samples, and appears to have distinct effect on receptor binding, nuclear translocation, and inter-species transmission. Phylogenetic analysis showed separation of CPV-2 strains into two clades. All strains of this study were classified in clade I with Asian strains. In conclusion, this study provides updated comprehensive molecular analysis of CPV-2 variants in Egypt.

1. Introduction

Canine parvovirus type 2 (CPV-2) is a highly contagious pathogen that causes severe hemorrhagic gastroenteritis and, less frequently, myocarditis in domestic dogs and wild carnivores worldwide [24]. The virus infection is mostly fatal, especially in young unvaccinated puppies and in those lacking sufficient passive immunity from dams [14,34]. CPV-2 is classified as a member of Protoparvovirus carnivoran 1 species within the genus *Protoparvovirus* of the family *Parvoviridae*, along with feline panleukopenia virus, mink enteritis virus, and raccoon parvovirus [37,42,5] CPV-2 has first described in 1978 as a novel virus that may originate from feline panleukopenia virus [2]. The virus has embraced many changes over time that resulted in the emergence of three antigenic variants, CPV-2a in 1980, CPV-2b in 1984, and CPV-2c in 2000 [4, 33] These variants had distinct biological properties from the original virus including the ability to infect cats and feline cell culture. The different CPV-2 variants are currently co-circulating globally and completely replaced the original type [38]. Considerable predominance of CPV-2a was recognized in Asia and Australia [17,45,46], CPV-2b in North America and Africa [10,29], and CPV-2c in South America and Europe [6,23].

CPV-2 is a small, spherical, and non-enveloped virus with an average diameter of 25 nm. The icosahedral virus capsid encloses a linearized negative single-stranded DNA genome of approximately 5.2 kb in length and two palindromic sequences on both ends. The genome includes two open reading frames (ORFs); one encodes the structural proteins (VP1, VP2, and VP3) that build up the virus capsid, and the second expresses the non-structural proteins NS1 and NS2, which play an important role in virus replication [1,31,8]. The high mutation rate of CPV-2 enables the virus to evolve rapidly like RNA viruses. The major capsid protein, VP2, is the key determinant of virus antigenicity, receptor binding affinity, and host range [19,21]. Divergence of CPV-2 into CPV-2a was attributed to six amino acid changes in residues 87, 101, 300, 305, 375, and 555 of VP2 protein. Two additional amino acid changes in VP2

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protein (N426D and I555V) were responsible for emergence of CPV-2b, while a single amino acid change (D426E) developed CPV-2c variant [33]. New variants of CPV-2a and CPV-2b were originated in the 1990 s by replacing serine with Alanine in the position 297 [28,47].

In Egypt, a limited number of studies has been conducted during the last few years [11,12,27]. All studies have utilized samples collected from 1 to 3 Egyptian governorates (mostly Cairo and Giza) during the period extending from March 2012 till September 2019, except few samples collected from 2020 in two recent studies [32,44]. The three variants of CPV were recognized in Egypt with variable predominance during the different time periods. Sequence analysis of the entire VP2 gene/protein for identification of the mutations in the circulating Egyptian strains is remarkably lacking. Therefore, the current study was designed to identify CPV-2 in samples collected from wider geographical region (seven Egyptian governorates) and broader time range (three successive years; 2019–2021). The complete VP2 gene of positive samples (n=19) was analyzed for better understanding of the genetic diversity of CPV-2 in Egypt.

2. Materials and methods

2.1. Clinical samples and ethical statement

A total of 100 rectal swabs were collected from dogs with acute gastroenteritis located in seven Egyptian governorates (Cairo, Giza, Gharbia, Sharqia, Qalubia, Behera, and Fayoum) during the period from September 2019 to June 2021. The diseased dogs represent both genders, variable age ranges (86% younger than 6 months), and a wide spectrum of pure and mixed breeds (Table 1). Samples were collected and transported using protocols approved by the Institutional Animal Care and Use Committee, Cairo University (CU-II-F-14–21). Swabs were prepared by soaking in 1 ml of PBS (Lonza, Basel, Switzerland), followed by vigorous squeezing, and centrifugation of suspension at 3000 xg for 15 min. The clarified supernatant was collected for immediate testing or stored at −20℃ till use.

2.2. Canine parvovirus type 2 detection

CPV-2 DNA was isolated from the prepared rectal swabs by using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Virus detection was performed by PCR amplification of a 504-base fragment of VP2 gene using CPV-2-F and CPV-2-R primers (Table 2). The assay was conducted by mixing 12.5 µl 2x TOP simple™ DyeMIX-n Taq master mix (Enzynomics, Soul, South Korea), 400 nM of both primers, and 5 µl DNA extract. The volume was completed to 25 µl by PCR grade water (Qiagen). The cycling conditions included initial denaturation at 95°C for 10 min, 35 cycles of denaturation (95°C for 30 sec), annealing (54°C for 30 sec), and extension (72°C for 1 min), and final extension at 72°C for 10 min. PCR amplicons were separated in 1.2% agarose gel stained with ethidium bromide and visualized by Gel Doc XR gel documentation system (BioRad, Milan, Italy).

Table 2
Oligonucleotide primers used in the study.

Name	Sequence (5' – 3')	Position*	Product size
Detection primers			
CPV2-F	GCG CAA ACA GAT GAA AAT CA	3754 – 3773	503
CPV2-R	CCT TTC CAC CAA AAA TCT GAG	4236 – 4256	
Sequencing primers			
Par-F	ACCAGATCATCCATCAACATCAAG	2532 – 2555	2007
Par-R	GCT GTA ATT TAA TCA ATC TTA AAA TAA TAT G	4508 – 4538	

* Nucleotide positions relative to CPV-2 complete genome sequence (Accession number JX660690).

2.3. DNA sequencing

The complete sequence of VP2 gene (1755 bases) of 19 CPV-2 positive samples was amplified for genetic analysis. Samples were selected to cover all the study variables including time, place, age, gender, breed, and vaccination. The amplification reaction was conducted using Phusion High-Fidelity PCR Kit (Thermo Fisher Scientific, Waltham, MA) and the primers Par-F and Par-R (Table 2) in GeneAmp 9700 thermal cycler (Thermo Fisher Scientific) according to the kit's instructions. Successful amplification was verified by visualization of the specific bands in agarose gel, while amplicons were purified using the QIAquick PCR Purification Kit (Qiagen). The purified PCR products were sequenced on both strands using Par-F and Par-R primers at Macrogen Inc (Seoul, South Korea). Sequence contigs were edited using BioEdit software, version 7.0.9.1 [16], and were assembled using Editseq program of Lasergene software, version 3.18 (DNAStar, Madison, WI). All sequences were deposited in GenBank (Accession numbers: MZ056881 to MZ056892, and OM100696 to OM100702).

2.4. Sequences and phylogenetic analysis

A sequence dataset was generated by downloading the entire VP2 gene sequences of 86 Egyptian and international CPV-2 strains from GenBank. These strains were chosen to represent the different CPV-2 variants on temporal and geographic bases. All sequences generated in this study and those included in the database were aligned together using Clustal W algorithm of MegaAlign program, Lasergene software to

Table 1
Distribution and characteristics of dogs used in sample collection.

Governorate	Cairo	Giza	Gharbia	Sharkia	Qalubia	Behara	Fayoum	Total
Year								
2019	13	30	7	0	1	6	1	58
2020	1	1	11	0	0	0	2	15
2021	2	4	20	1	0	0	0	27
Gender								
Male	6	16	12	1	1	3	3	42
Female	10	19	26	0	0	3	0	58
Age range								
0–6 month	14	32	30	1	1	5	3	86
6–12 month	1	3	4	0	0	1	0	9
Over 1 year	1	0	4	0	0	0	0	5
Vaccination								
Yes	2	12	21	0	1	5	1	42
No	14	23	17	1	0	1	2	58
Total	16	35	38	1	1	6	3	100

identify sequence variations, mutation sites, and deduced amino acid changes. The phylogenetic tree was constructed according to Tamura-Nei model [36], using the maximum likelihood method of MEGA 10.1.1, with uniform rates and Nearest-Neighbor-Interchange (NNI) tree interface. The bootstrap value was adjusted to 1000 replicates ensuring the accuracy of tree topology.

3. Results

3.1. Epidemiology of CPV-2

Among the collected samples (n=100), a total number of 65 samples tested positive for CPV-2 by PCR. Positive samples were retrieved from different pure breeds of dogs, including Labrador, Pitbull, Huskey, Griffon, German Shepherd, Rottweiler, Boxer, Dalmatian, Golden Retriever, Armant, Cocker, Akita, and Lolo. Two positive samples were identified in stray dogs of mixed native breed.

PCR positive samples were identified in five (out of seven) governorates with a higher incidence rate in Gharbia (35; 92.1%), followed by Cairo (12; 75%), Giza (16; 45.7%), and Behara (1; 16.7%). The only sample collected from Sharkia governorate was CPV-2 positive, while those collected from Qalubia and Fayoum were all negative. The distribution of positive samples during the study period was inconsistent with higher incidence rate in 2020 (14; 93.3%) and 2021 (23; 85.2%) as compared to 2019 (28; 48.3%). As expected, all but three positive samples were recovered from puppies younger than 6 months of age. CPV-2 prevalence in female dogs was higher (41; 70.7%) than in males (24; 57.1%). Similar results were shown in non-vaccinated (42; 72.4%) and vaccinated dogs (23; 54.8%) (Supplementary figure).

3.2. Distribution of CPV-2 variants

Typing of CPV-2 variants was based on the amino acid residue located in position 426 of VP2 gene. Sequence analysis of the full-length VP2 gene of 19 selected positive samples typed 5 CPV-2a, 5 CPV-2b, and 9 CPV-2c strains (Table 2). CPV-2a and CPV-2c variants were detected all over the study period with higher frequency of CPV-2a in 2019 and 2020, and CPV-2c in 2019 and 2021, whereas all CPV-2b variants were detected only in 2019. In general, CPV-2b and CPV-2c were the most detectable in 2019, while CPV-2a and CPV-2c were common in 2020 and 2021, respectively. Analyzing the distribution of CPV-2 variants has elucidated that there is no obvious correlation between the CPV-2 variant and the animal breed or gender. CPV-2a and CPV-2b were only linked to the young ages (0–6 months), while CPV-2c strains were recovered from all age groups. A noticeable higher rate of CPV-2c was identified in vaccinated dogs, compared to CPV-2a and CPV-2b.

3.3. Sequence analysis

The complete sequence of VP2 gene of the same 19 selected positive samples was obtained by Sanger sequencing and sequences were aligned with the sequence dataset downloaded from the GenBank. Four key residues were recognized in all samples (namely, 267Y, 297 A, 300 G, and 324I). Alanine was specifically identified at position 440 (440 A) in all CPV-2a and CPV-2b positive samples, while arginine at position 370 (370 R) was characteristic for all CPV-2c samples (Table 3).

A distinctive feature for many strains sequenced in the current study is the replacement of valine to glutamic acid at position 38 (V38E). This substitution was recognized in all CPV-2b strains, three CPV-2a strains (FVMVL-CPV-21/2019, FVMVL-CPV-59/2020, and FVMVL-CPV-87/2021), and two CPV-2c strains (FVMVL-CPV-51/2019 and FVMVL-CPV-74/2021). All CPV-2c strains recovered in 2020 and 2021 has demonstrated insertion of Glycine in instead of Alanine at position 5 (A5G), while three of them (FVMVL-CPV-88/2020, FVMVL-CPV-64/2021, and FVMVL-CPV-74/2021) have shown insertion of valine instead of glycine at position 392 (G392V). In addition, glycine was replaced by Arginine at position 36 (G36R) of a single CPV-2b (FVMVL-CPV-46/2019) and a single CPV-2c (FVMVL-CPV-29/2019) strains. Two amino acid substitutions [T44A and N323H] were unique to a single CPV-2c strain (FVMVL-CPV-74/2021).

3.4. Phylogenetic analysis

To identify the molecular relationship between the Egyptian CPV-2 strains identified in this study and the different strains collected from around the globe, a phylogram was generated using the entire VP2 gene sequence (Fig. 1). Two major clades were identified on the phylogenetic tree, and termed clades I and II. Clade I included all the Egyptian strains identified in the current study alongside with some Asian strains, and is subdivided into two genogroups: G I and G II. The former contained CPV-2c strains and the latter included CPV-2a and CPV-2b strains. The original CPV-2 including vaccine strains are all members of clade II, beside representatives of the three CPV-2 variants from Europe, Asia, and the Americas.

Table 3
Record of amino acid sequence variation in the full-length VP2 gene of CPV-2 strains identified in the current study.

STRAIN IDENTIFIC ATION	SUBTYPE	ACCESSION NUMBER	AMINO ACID RESIDUE												
			5	36	38	44	267	297	300	323	324	370	392	426	440
REFERENCE-790312	Prototype	M38245	**A**	**G**	**V**	**T**	**F**	**S**	**A**	**N**	**Y**	**Q**	**G**	**N**	**T**
FVMVL-CPV-21/2019	CPV2a	MZ056883	-	-	E	-	Y	A	G	-	I	Q	-	N	A
FVMVL-CPV-58/2019	CPV2a	MZ056891	-	-	-	-	Y	A	G	-	I	Q	-	N	A
FVMVL-CPV-59/2020	CPV2a	MZ056892	-	-	E	-	Y	A	G	-	I	Q	-	N	A
FVMVL-CPV-87/2021	CPV2a	OM100700	-	-	E	-	Y	A	G	-	I	Q	-	N	A
FVMVL-CPV-97/2020	CPV2a	OM100702	-	-	-	-	Y	A	G	-	I	Q	-	N	A
FVMVL-CPV-17/2019	CPV2b	MZ056881	-	-	E	-	Y	A	G	-	I	Q	-	D	A
FVMVL-CPV-18/2019	CPV2b	MZ056882	-	-	E	-	Y	A	G	-	I	Q	-	D	A
FVMVL-CPV-40/2019	CPV2b	MZ056887	-	-	E	-	Y	A	G	-	I	Q	-	D	A
FVMVL-CPV-46/2019	CPV2b	MZ056888	-	R	E	-	Y	A	G	-	I	Q	-	D	A
FVMVL-CPV-55/2019	CPV2b	MZ056890	-	-	E	-	Y	A	G	-	I	Q	-	D	A
FVMVL-CPV-26/2019	CPV2c	MZ056884	-	-	-	-	Y	A	G	-	I	R	-	E	T
FVMVL-CPV-29/2019	CPV2c	MZ056885	-	R	-	-	Y	A	G	-	I	R	-	E	T
FVMVL-CPV-36/2019	CPV2c	MZ056886	-	-	-	-	Y	A	G	-	I	R	-	E	T
FVMVL-CPV-51/2019	CPV2c	MZ056889	-	-	E	-	Y	A	G	-	I	R	-	E	T
FVMVL-CPV-64/2021	CPV2c	OM100696	G	-	-	-	Y	A	G	-	I	R	V	E	T
FVMVL-CPV-69/2021	CPV2c	OM100697	G	-	-	A	Y	A	G	H	I	R	-	E	T
FVMVL-CPV-74/2021	CPV2c	OM100698	G	-	E	-	Y	A	G	-	I	R	V	E	T
FVMVL-CPV-78/2021	CPV2c	OM100699	G	-	-	-	Y	A	G	-	I	R	-	E	T
FVMVL-CPV-88/2020	CPV2c	OM100701	G	-	-	-	Y	A	G	-	I	R	V	E	T

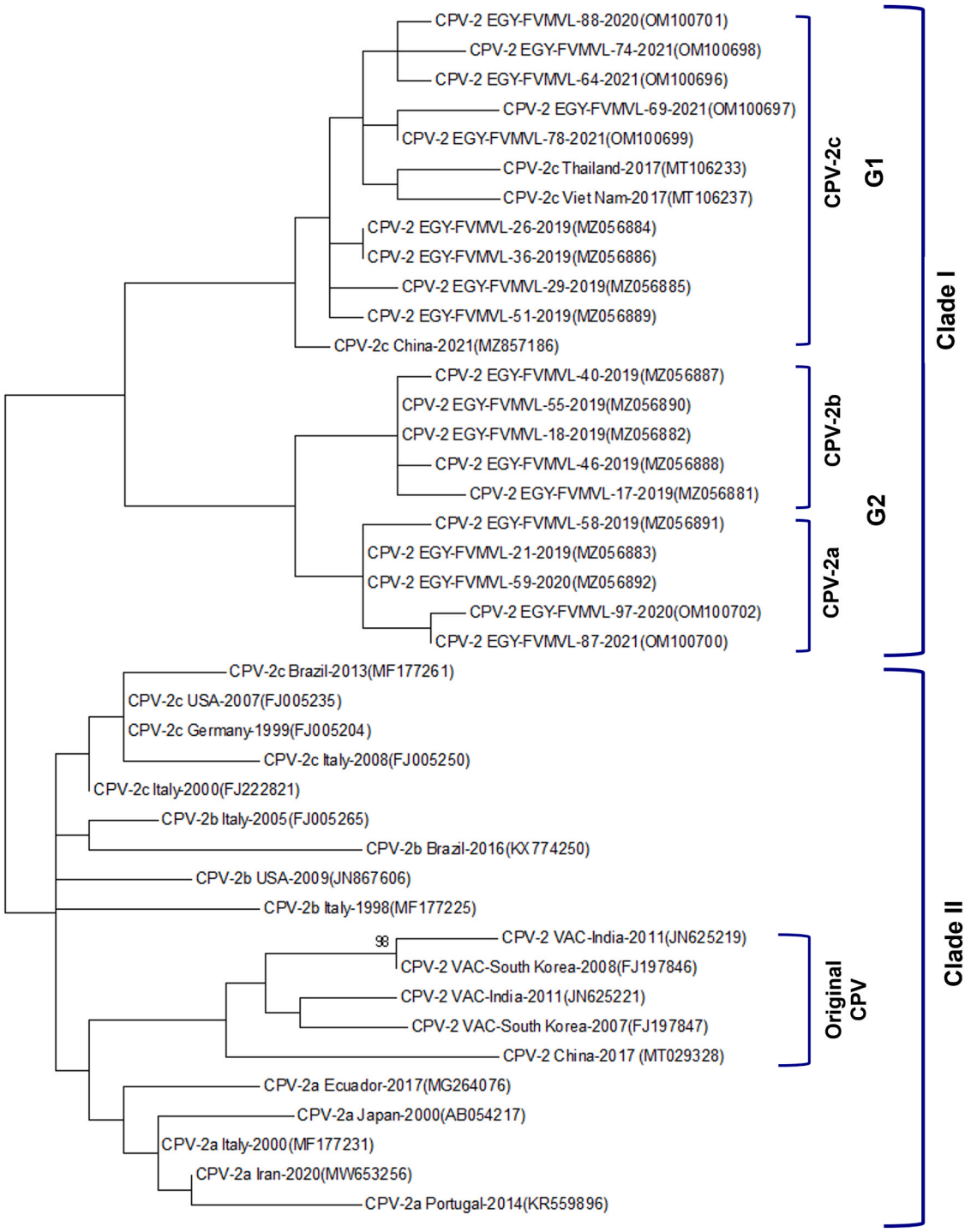

Fig. 1. Phylogenetic analysis of canine parvovirus type 2 strains. The full-length VP-2 gene sequence of Egyptian and international strains were aligned together using Clustal W algorithm, available within MegAlign program, Lasergene software. Tree construction was performed using maximum likelihood method of MEGA 10.1.1 according to Tamura-Nei model, with uniform rates and Nearest-Neighbor-Interchange (NNI) tree interface. Accuracy of the tree topology was ensured by applying bootstrapping of 1000 replicates, and values are denoted at the branch nodes. CPV-2 type, origin, date of identification, and accession number are all indicated. Clades and genogroups are indicated at the right side of the phylogram.

4. Discussion

CPV-2 is a principal cause of acute gastroenteritis in dogs with significant mortalities in puppies younger than three months of age [6,24]. Although many reports have been issued on the circulation of CPV-2 in Egypt during the last decade [11,3,44], there is a considerable lack of a comprehensive study that consider wider geographic region and time period. Molecular characterization of the major capsid protein VP2 was only restricted to partial fragments of the gene with the exception of a single report that analyzed the entire sequence of VP2 in 7 canine samples collected before September 2019 [27]. Therefore, this study was designed to analyze CPV-2 positive samples collected from 7 Egyptian governorates over 3 consecutive years starting from September 2019. The full-length VP2 gene of 19 representative samples was sequenced and analyzed for better understanding of CPV-2 diversity among the Egyptian population of dogs.

All three CPV-2 variants were identified in the current study in different provinces. The three variants co-circulated in the domesticated dog population in 2019, while CPV-2b was not detected in 2020 and 2021. It is notable that the vast majority of CPV-2 variants detected in the last year of the study (2021) were CPV-2c. This may imply that CPV-2c may replace CPV-2b in recent years. However, it`s important to conduct more comprehensive large-scale studies over an extended period of time to justify such conclusion. Consistent with other studies, most of the positive samples were detected in dogs less than six months of age (84.6%), which indicate preference of the virus to juvenile dogs [18]. However, CPV-2c was detected in dogs aging more than six months; one of them was adult four-year-old dog. Although such observation was not recorded before in Egypt, others identified CPV-2c in dogs aging 20 months and 12 years in Italy [7].

It is hypothesized that CPV-2 is evolved by a mechanism that new subtypes replace the older ones [6]. In this study, CPV-2b was not detected in 2020 and 2021, while the prevalence of CPV-2c significantly increased in 2021. This may be regarded to the vaccination stress favoring CPV-2c to prevail. The finding that 70% of CPV-2c positive samples came from vaccinated animals (compared to 40% and 20% for CPV-2a and CPV-2b, respectively) may support this assumption (Supplementary figure). Additionally, molecular analysis of the full-length VP2 gene has shown that all CPV-2c strains display three specific key amino acid residues; Q370R, Y324I, and T440A, which may be responsible for altered antigenicity and/or virulence causing vaccination failure [9,49]. This may explain the higher prevalence of CPV-2c in recent years.

Sequence analysis of the full-length VP2 gene has shown four key residues; F267Y, S297A, A300G, Y324I, in all tested samples. Although located close to the top of loop 3 in the shoulder of the three-fold axis spike, residue 297 does not appear to have major antigenic advantage leading to generation of vaccine escape mutants [40]. In contrast, residue 300, which is also located on the top of the three-fold axis spike, has a direct role in host jumping by interaction with TFR (CPV-2 receptor) [30,35]. On the other hand, A300G substitution lies in a critical antigenic region but it does not interfere with neutralizing antibodies like other mutations; A300V and A300D [21]. Unlike Y324I substitution, which have an effect on host adaptation globally [20,25], F267Y is not exposed on the capsid surface, and its exact role in spread of the new variants requires more investigation surface [26,43].

Unique amino acid substitutions were identified in some sequences recovered in this study like G36R, V38E, and G392V. As residues 36 and 38 located at the N-terminus of VP2 protein, they play an important role in determining the flexibility of the five-fold cylinder and nuclear translocation [22,41,43]. Change of the hydrophobic amino acids (glycine and valine) to hydrophilic residues (arginine and glutamic acid) in positions 36 and 38, respectively may affect the conserved poly-glycine chain nature close to the N-terminus and consequently interferes with virion translocation. On the other hand, G392V substitution might have an effect on receptor binding since residue 392 is located near the canyon of the capsid [41].

Interstingly, two amino acid substitutions; A5G and Q370R, were recorded only in subtype CPV-2c. Although both substitutions are common in Asia [13,48], Q370R was detected in CPV-2c from Panda, suggesting a role in viral adaptation [15]. A5G exists within the N-terminus on the surface epitope that readily interacts with the neutralizing antibodies. Lopez de Turiso et al., [22]. Another unique feature of a single CPV-2c strain is N323H. This residue is responsible for controlling transferrin attachment of canines, and therefore such substitution was thought to be the reason of introduction CPV-2 from feline host to canines [39].

Phylogenetic analysis using full-length VP2 gene sequence, has enabled classification of the strains analyzed in this study as members of clade I along with Asian strains (Fig. 1). Taking into consideration that the strains recovered from Europe and the Americas were all grouped in clade II, this may indicate that the strains circulating in Egypt may originate or co-evolute with strains of Asian origin. This assumption is supported by the presence of Asian specific amino acid residues: 267Y and 324I in all sequences analyzed in this study. Divergence of CPV-2c into a specific genogroup within clade I establishes the genetic distance with CPV-2a and CPV-2b, and with the vaccine strains that locate in clade II. As the newest emergent subtype, further investigations should follow to dissect the molecular evolution of CPV-2c and clearly justify the protection coverage of the vaccination programs to date.

In conclusion, the current study has identified the three CPV-2 subtypes/variants among diseased dogs in different Egyptian governorates from September 2019 to June 2021. Although CPV-2b was the predominant in 2019, it disappeared in the following years and replaced by CPV-2c that dominate in 2021. The full-length VP2 gene sequence was retrieved from samples selected on temporal, spatial, and demographic basis, and utilized in mutational and phylogenetic analysis. Significant amino acid substitutions were recorded in the analyzed sequences and phylogenetic analysis has suggested an Asian origin of the Egyptian strains.

Declaration of Competing Interest

The authors have no relevant financial or non-financial interests to disclose.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cimid.2024.102190.

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