Research Article

Feline Pan Leukopenia Molecular Detection and Viral Phylogeny in Egypt

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Abstract | Feline panleukopenia virus (FPV) causes severe disease in cats, as well as in a variety of wild carnivores including many endangered animals. Regular appearance of the virus increases the importance of evaluating the percentage of identity in relation to vaccine commercially used, detecting sequence changes related to virus virulence or tropism. In this study Fifty-seven fecal samples were collected from suspected cases of cats. 19 fecal samples were positive using Rapid FLV Ag test kits kit (VDRG FLV/FIV Ab rapid kit). Fifty-seven blood samples of the same animals were subjected for hematological examination to investigate the severity of leukopenia. Thirteen out of nineteen samples were positive with a polymerase chain reaction assay (PCR) targeting VP2 gene. Three positive fecal samples in animal showing sever and moderate leukopenia were successfully sequenced Partially. Sequence analysis results showed no variation in their sequence across all isolates when compared to the published FPV genome, which could imply that FPV appears to be in genomic stasis as compared to other Parvoviruses. Genomic stasis appears among the three Egyptian samples in this study under the GenBank accession number OP594200, OP574201, and OP574202, in addition to the high similarity of the isolates the vaccine strains of EU498680 and EU498681.No amino acid substitution was detected.

Keywords | Feline Pan-leucopenia, PCR, Sequencing, vaccination, screening, VP2and Egypt

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INTRODUCTION

Feline panleukopenia Virus (FPL) causes immune cell deficiencies (lymphoid depletion and pancytopenia), leading to life-threatening signs due to immunosuppression which is the major cause of death, in addition severe enteritis, dehydration, and sepsis were also common signs (Barrs, 2019). This virus can persist for months or even years due to its extreme resistance to physical environment and chemical agents (Uttenthal et al., 1999). The virus spreads via contact indirectly with contaminated people and improperly disinfected items. Shelter employees and equipment can act as carriers, putting unprotected cats at risk (Tizard, 1995). FPV and canine parvovirus can be shed by immunocompetent, healthy cat which is infected with silent sign thought to be a significant source of environmental contamination (Clegg et al., 2012; Bergmann et al., 2019). Unvaccinated, incorrectly vaccinated cats are at risk. Additionally, after receiving the live attenuated vaccine, cats may shed the FP viral antigen

in their faces (Tizard, 1995; Abd-Eldaim et al., 2009).

Animal parvovirus FPV belongs to the Protoparvovirus genus (feline panleukopenia virus) (Leisewitz, 2017). Protoparovirus is one of eight genera found in vertebrates belonging to the Parvovirinae subfamily of the Parvoviridae family (FPV). Carnivore protoparvovirus 1 was classified as a genus that included Feline Panleukopenia Virus (FPV), Canine Parvovirus (CPV), Mink Enteritis Virus (MEV), and Raccoon Parvovirus (Mietzsch et al., 2019).

It has a typical length of about 5 kb and is an icosahedral, single-stranded DNA virus that is not enveloped. Four ORFs can be found in the genomic sequence of this virus (ORFs). Nonstructural proteins 1 (NS1) and 2 (NS2) are encoded by ORF1 and ORF2, respectively, and structural proteins VP1 and VP4 are encoded by ORF3 and ORF4, respectively, which encode the major capsid protein of the viruses. These nonstructural proteins control DNA replication, assembly, and gene expression (Leisewitz, 2017).

Parvovirus VP2 encodes the major capsid protein, which accounts for roughly 90% of the total capsid protein. It has host range control. VP2 has been used to create subunit vaccines because it contains several crucial B cell epitopes that cause protective (neutralizing) antibodies during infection (de Turiso et al., 1991). The ORF encoding VP2 has received the most attention regarding genetic and epidemiological characteristics.

Canine parvovirus type 2 (CPV), feline panleukopenia virus (FPV), and mink enteritis virus (MEV) their antigenic relationships were studied. Results confirm that identifying the viral type can be difficult and often necessitates the use of more than one diagnostic method (Cotmore et al., 2014).

Viral diagnosis using immune chromatography test followed by VP2 targeting primers-based PCR specific for virus detection were routinely used. The complete blood count (CBC) is regarded as a valuable tool for determining disease severity and predicting prognosis. Sequence analysis is a definite method of diagnosis, as infection with FPV is usually associated with infection by canine parvovirus (CPV). When comparing the DNA sequences, they found that FPLV and CPV-2 are very similar; the main differences are due to six amino acid substitutions (at positions 80, 93,103, 323, 564, and 568). Protoparvovirus in carnivores consists of FPV, CPV, and related variants found in mink and raccoons, among others (Cotmore et al., 2014).

However, the origin of CPV is still a mystery. The origin of CPV is unclear; hypotheses range from feline panleukopenia virus (FPLV) to a carnivore parvovirus (Truyen, 1999; Truyen et al., 1996). CPV and FPV are available all over the world. The CPV was first found in Egyptian military police dogs in 1982 and manifested clinically and pathologically. FPV has been identified; the circulating genotypes in Egypt were PV-2a and CPV-2b (El-Neshwy et al., 2019; Zaher et al., 2020; Elbaz et al., 2021).

FPV appears to have a low genomic mutation rate compared to other Parvoviruses, whereas CPV-2 evolves rapidly. Inter-FPV subspecies recombination is strongly supported. It has been reported that FPLV and CPV recombination occurs. Canine and feline parvoviruses can be transmitted to one another, and this may be due to cross-infections (Decaro et al., 2008; Ohshima and Mochizuki, 2009; Mietzsch et al., 2019). Our study primary objective was to characterize multiple FPL strains by comparing and contrasting their VP2 gene sequences to those of isolates worldwide.

MATERIALS AND METHODS

Sampling

A clinically infected cat with a detailed clinical history of diarrhea, vomiting, and an absence of FPV vaccination during winter season had 114 samples collected, 57 of which were fecal. Fifty-seven blood samples were collected on EDITA and used for hematological examination. In 2019, 2020, and 2021, samples were collected from cats brought to veterinary clinics in Egypt.

Kit for Rapid Ag Testing Rapid VDRG FLV Ag/FIV Ab Kit supplied by MEDIAN Diagnostics was applied on fecal samples as recommended on the cat. No. PF-FEI-11.

Hematological studies were performed on 57 blood samples using (Exigo vet®) hematology analyzer, including RBC count, PCV, Hb concentration, red cell indices (MCV and MCHC), TLC, and platelet count**.** Differential leukocyte count (DLC) was performed manually by the method described by (Decaro et al., 2005).

Fecal sample preparation and concentration

Stool samples (1 ml each) were homogenised in 10 ml of phosphate-buffered saline (PBS) pH 7.2, centrifuged at 8000 rpm for 15 minutes. The sediment was discarded. Each sample was passed through a cellulose ester filter pore size 200nm.Penicillin and streptomycin were applied to the samples overnight at 4 degrees Celsius. Ultracentrifugation with a Beckman L7-65 (rotor70.1Ti) with 45,000 rpm/2 hours at 4 °C. The sediment was suspended in PBS at a 1:1 ratio.

Negative contrast electron microscopy

A negative contrast electron microscope was done according to (Amo et al., 1999) with some modification. One drop of homogenate was placed on a cooper grid then Grids were negatively stained with phosphotungstic acid, pH 7, 2% for 1 minute. Finally, the analysis was performed using a JEM 1200 EX II electron microscope.

Cs Cl equilibrium centrifugation

Stools were suspended in a 7.3-pH CsCl in PBS solution and centrifuged (with a refractive index of 1.370) for 24 hours before being centrifuged at 110,000 xg. A 0.5 ml fraction was collected. Each fraction's refractive index and absorbance at 260nm were calculated. After that, the sample was examined under E.M (Williams, 1980).

Polymerase chain reaction (PCR) convention Extraction of DNA

The easy pure stool genomic DNA kit (Trans) Cat. No, EE301 was used to extract DNA from 30 faecal samples. Mix 200mg stool and 0.25 glass Beed, add 1ml of LB21, and incubate at 70 °C. Centrifugation 150000 for 2 minutes. After that, add 250l of pb21. Incubate on ice for 5 minutes before centrifuging at 15,000xg for 2 minutes and transferring the supernatant to a new tube. Add 650µl ethanol and BB21; mix by vertexing and add 650 of the solution to the spin column add 500µl cb21 then centrifugation 12000xg for 30 seconds. Add 500 zb 21, then centrifugate 12000 for 30 sec. Centrifugation at 15000xg for 2 min. Place the spin column into a new tube, add50µl of elution, incubate for 2 minutes, then centrifugate at 12000xg for 1 min

PCR amplification

We used Emerald Amp Max PCR Master Mix (Takara, Japan), 12.5 µl of VP2-specific primers, 1 µl of each primer at 20 pmol, 7.5 µl of water, and 3 µl of DNA template in a 25 µl reaction. AB biosystem2720 thermal cycler was utilized for the reaction as showmen in Table 1.

Partial sequencing of VP2 region

Sequencing of the DNA templates were performed by Sanger dideoxynucleotide sequencing after purification of PCR product using BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher, USA) according to the manufacturer's instructions. A 3.2 pmol of reverse primers were used. The sequencing product was purified by using Centri-Sep™ Spin Columns (Thermo Fisher, USA) and the electro-kinetic injection on capillary electrophoresis systems 3500 Genetic analyzer (Applied Biosystems, USA).

Sequence analysis

Obtained sequences were assembled multiple nucleotide and deduced amino acid sequence alignment was performed using clustal W (Chenna et al., 2003) algorithm in the BioEdit software-version 7.1 (Hall et al., 2011). The sequences were aligned in MEGA11 software (Tamura et al.,2021). The identity percentages of the obtained sequences were detected using the GenBank using the alignment tool of Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees were constructed for partial VP2 sequence. Phylogenetic trees were inferred using the maximum likelihood approach implemented within MEGA11 software, the topology was estimated by bootstrapping over 1000 replicates (Saitou and Nei, 1987; Tamura et al., 2004, 2021).

RESULTS AND DISCUSSION

Rapid Ag test kit VDRG FeLV Ag/FIV Ab rapid kit and haematological analysis

Nineteen were positive out of 57 suspected cases in 3 successive years. WBC counts in infected cats showed deviation from Normal WBC count (5.5 to 19.5×10^3/µl), which ranged as showmen in Table 2 from mild (4.5×10^3/ µl) to severe (1.5×10^3/ µl) leukopenia as shown in Figure 1.

Electron microscopy on faecal samples of FLV-infected cats

Ultracentrifugation followed by negative staining technique, as showmen in Figure 1 showing characteristic icosahedral capsids of family *Parvoviridae.*

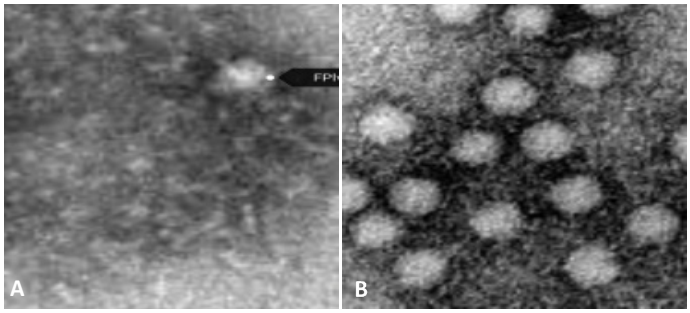


Figure 1: Shows parvovirus-like particles (P) in the feces of cats. (A) No surface details, but stain-penetrated particles with precise hexagonal shape. (B) the average size of the particles was 22.70.5 nm on which placed it within the parvovirus groups reported rang (Magnification 40,000 X).

Molecular characterization for FPV

Polymerase chain reaction

By using PCR, the VP2 gene's genomic DNA was successfully identified, and positive isolates amplified the target 583 pb fragment. Figure 2 and Table 2 shows that the nucleotide sequences of the incomplete VP2gene from the three selected isolates, with accession numbers OP594200, OP574201 and MW039141, have been deposited in GenBank.

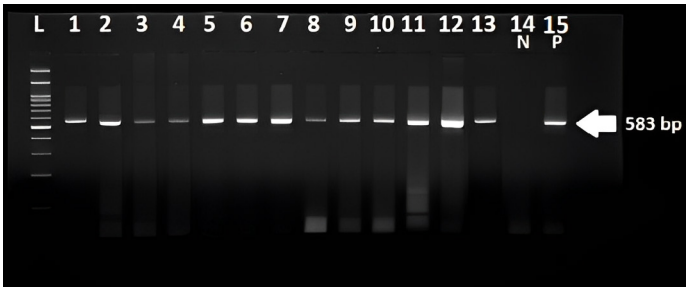


Figure 2: specific band with expected size 583 bp were observed. Lanes L ladder 100 pb, lane 1 to 13 were positive Samples Lane 14 negative control. Lane15 is a positive control.

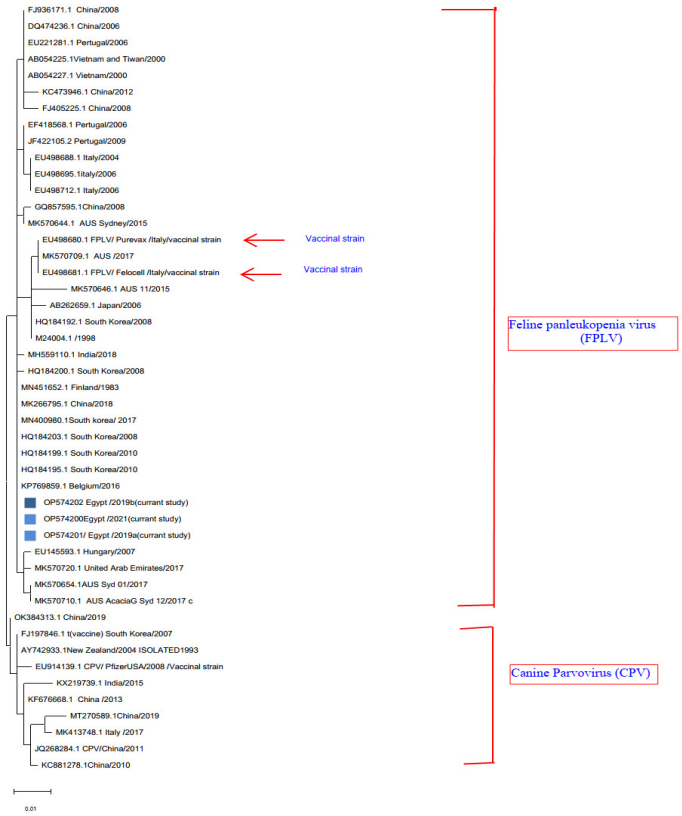


Figure 3: A phylogenetic tree of Feline panleukopenia based on partial VP2 gene conducted in MEGA11 (Tamura et al., 2021) using Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (–1930.82) is shown. The colour blue represents the FLP strain isolated in this study. Gen Bank IDs are located in each sequence, vaccine strains were indicated by arrow. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithma to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 47 amino acid sequences. There was a total of 526 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

DNA SEQUENCE ANALYSIS AND SEQUENCING

The partial-length VP2 gene were amplified and sequenced and deposited in gene bank under accession number OP574200, OP574201, and OP574202, yielding a 580-bp fragment, 534 nucleotide sequence, and 178 amino acid sequence (aa). Nucleotide (nt) and predicted amino acid (aa) sequences from the VP2 gene were used to create a multiple sequence alignment (Figures 3, 4); these isolates showed that they shared more than 98% percent identity of the three samples with isolates from Italy, Portugal, Finland, Vietnam, South Korea, India, and China and with the vaccinal strains (Table 4).

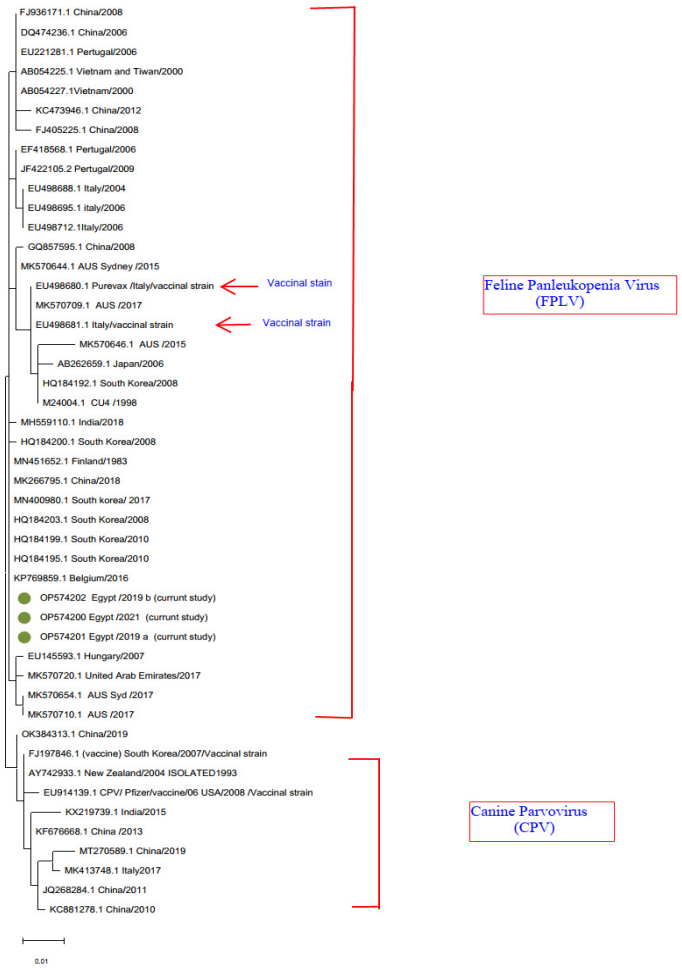


Figure 4: A phylogenetic tree was created using the partial sequence of VP2's deduced amino acid sequence. The vaccine strain is shown by arrow on the tree, while the FPLV strains used in this study are shown in green. The accession numbers with brief GenBank ID are located for each sequence.

AMINO ACID

Each of the three isolates (accession number OP574200, OP574201, and OP574202) has a VP2 protein with identical amino acid residues. Our studied sample from 2019, 2020, and 2021 has 100% nucleotide similarity to isolates from South Korea, China, and Finland Tables 3, 4. Each specimen's VP2 region was examined at key

Table 1: Primers, target genes, amplicon sizes, and cycling conditions used in this study.

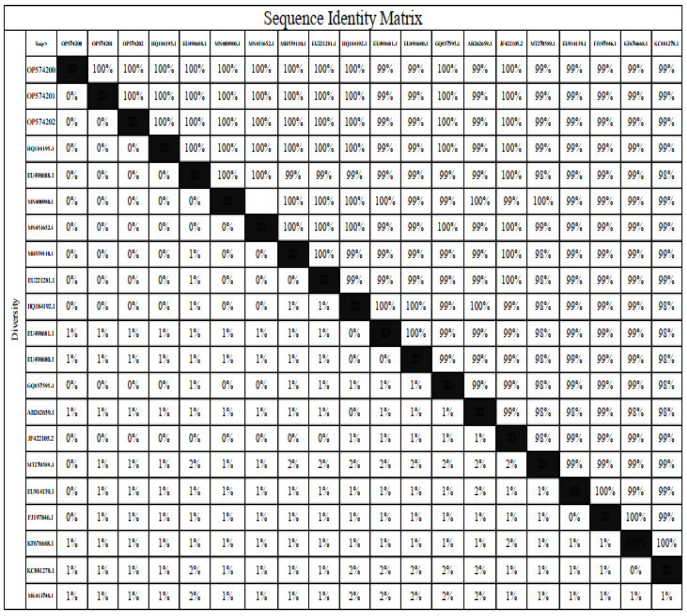
Primers sequences	Position	Amplified segment (bp)	Amplification (35 cycles)			Reference
			Secondary denaturation	Annealing	Extension	
555-F CAGGAAGATATCCAGAAGGA	4003-4022	583	94^{0}c /30 sec	47^{0}c /45 sec	72^{0}c /1 min	(Kapil et al., 2007)
555-R GGTGCTAGTTGATATGTAATAAACA	4561-4585					

The products of the PCR reactions were separated by electrophoresis in 1.5% agarose containing 10 g/ml ethidium bromide and viewed under UV light. Electrophoresis of the PCR reaction products in 1.5% agarose with 10 g/ml ethidium bromide allowed for their visualization under UV light.

Table 2: Summarized result of antigen detection, hematology on blood samples and PCR, and sequence on fecal samples under this study.

Year	No.	Blood sample		No.	Fecal sample	
		Rapid test	Hematology leukopenia		PCR	sequencing
2019	20	9	Average Severe (1.5×10^3/L)	20	9	2
2020	22	3	Mild (4.5×10^3/L)	22	1	0
2021	15	7	Moderate (3×10^3/L)	15	3	1

Table 3: Identity percent of the recently isolated strains gene bank accession numbers OP574200, OP574201, and OP574202 and isolates illustrated in table 4 and vaccine strain used in Egypt.



amino acid positions reflecting feline panleukopenia and canine parvovirus subtype. FPV specimens are ordered and classified as shown in (the phylogram). Canine parvoviruses 2a, 2b, and 2c are derived from newly emerging viruses with variability at the 426 a position

FPV is a fatal highly contagious viral disease which cause leukopenia and severe diarrhea. FPV infections are most common infection in *Felidae* family in addition it affects wild life. It is also detected in Egyptian mongoose (Duarte et al., 2013). Although all cats are susceptible to FPV, kittens are especially vulnerable. Infants who have neither been immunized nor exposed to maternal antibodies have a mortality rate of at least 5%. In spite of the virus importance but to date, there have been scant reports on FPVs in Egyptian cats, even though diagnosis depend mainly on clinical symptoms (vomiting and bloody diarrhea) and laboratory examination using PCR (Abdel-Baky, et al., 2022).

In the current study virus was detected in faecal sample of cats confirm its prevalence among cats population (Abd-Eldaim et al., 2009; Abdel-Baky et al., 2022). Different methods for viral diagnosis and identification from clinically infected cases used to avoid overlapping FPV and other related viruses, especially CPV2a.CPV2b, CPV2C, MEV. Since cats are reservoirs for maintaining FPV and CPV2 infection, On the other hand shedding CPV2a or 2b, rather than FPLV were previously detected in cats (Clegg et al., 2012). The rapid test has a relative sensitivity of less than 50% and a specificity of 100%, a positive result from confirms infection of cat with parvovirus. Moreover, the sensitivity of faecal antigen tests for the detection of parvoviruses is influenced by viral load in faeces (Neuerer, et al., 2008). The rapid test was used as a preliminary assay but the most reliable and precise methods for identifying viruses involved viral DNA isolation, amplification, and sequencing (Abd-Eldaim et al., 2009; Abdel-Baky et al., 2022).

Hematological examination confirms that the severity of leukopenia correlates with clinical illness, risk of death, and virus shedding level. Two isolates were identified from cases showing severe leukopenia in 2019 it may be due to higher viral load and 1 isolate was identified from moderate leukopenia in 2021 whereas mild cases showing negative results in sequence analysis Table 3, which is agreed with previous studies Infection with FPV causes bone marrow suppression, results in leukopenia (Breuer et al., 1998).

Table 4: Key amino acid position in VP2 genes of FPV, available vaccine strains in Egypt and CPV strains.

		Mutation sites in the VP2 protein of FPLV and CPV																
	Isolate ID	**408**	**411**	**418**	**419**	**420**	**421**	**426**	**440**	**469**	**526**	**540**	**554**	**562**	**564**	**568**	**573**	**576**
1	OP574200 (Current study)	R	E	I	N	F	N	N	T	I	D	R	N	V	N	A	Y	S
2	OP574201 (Current study)	R	E	I	N	F	N	N	T	I	D	R	N	V	N	A	Y	S
3	OP574202 (Current study)	R	E	I	N	F	N	N	T	I	D	R	N	V	N	A	Y	S
4	HQ184195.1	R	E	I	N	F	N	N	T	I	D	R	N	V	N	A	Y	S
5	EU498688	R	E	I	N	F	N	N	T	I	D	R	N	V	N	A	Y	S
6	MN400980	R	E	I	N	F	N	N	T	I	D	R	N	V	N	A	Y	S
7	MN451652	R	E	I	N	F	N	N	T	I	D	R	N	V	N	A	Y	S
8	MH559110	R	E	I	N	F	N	N	T	I	D	R	N	V	N	A	Y	S
9	EU22128.1	R	E	I	N	F	N	N	T	I	D	R	N	V	N	A	Y	S
10	HQ184192	R	E	I	N	F	N	N	T	I	D	R	N	V	N	A	Y	S
11	EU498681	R	E	I	N	F	N	N	T	I	D	R	N	L	N	A	Y	S
12	EU498680	R	E	I	N	F	N	N	T	I	D	R	N	L	N	A	Y	S
13	GQ857595.1	R	A	I	N	F	N	N	T	I	D	R	N	L	N	A	Y	S
14	AB262659.1	R	E	I	N	F	N	N	T	I	D	R	D	V	N	A	Y	S
15	JF422105	R	E	I	N	F	N	N	T	I	D	R	N	V	N	A	Y	S
16	MT270589.1	R	E	I	N	F	N	E	T	I	D	R	N	V	S	G	Y	S
17	EU914139.1	R	E	I	N	F	N	N	T	I	D	R	N	V	S	G	Y	S
18	FJ197846.1	R	E	I	N	F	N	N	T	I	D	R	N	V	S	G	Y	S
19	KF676668.1	R	E	I	N	F	N	N	A	I	D	R	N	V	S	G	Y	S
20	KC881278.1	G	E	I	N	F	N	D	A	I	D	R	N	V	S	G	Y	S

Samples screened Successfully by PCR targeting VP2 gene yielding 583 bp amplicon**.** The sensitivity and specificity of viral nucleic acid detection using PCR targeting the VP2 sequence agreed in many previous studies which improve the investigation of virus circulated in the field globally (Decaro et al., 2008; Kapil et al., 2007) whereas, in 2018, PCR and sequence analysis were done targeting VP1 as a confirmatory tool for virus diagnosis in Egypt isolate deposited in Gen Bank with accession number KY466003 (Awad et al., 2018).

The phylogenetic analysis for construction of the Maximum Likelihood tree of theVP2 sequence indicate FPV isolates clustered together to form a single consolidated clade genetically distinct from the CPV-2 isolates. However, their genome homology reach 98%. Moreover, the identity 100% of the Egyptian isolates and up to 99 % with world widely distributed FPVs isolates from Australia, China and Korea retrieved from Gene bank confirm the genetic stasis and the slow rate of random genetic drift of FPV in comparison to CPV show genomic substitution rates similar to those of RNA (Hoelzer et al., 2008). Low bootstrap values for each node were previously determined due to low antigenic variation in FPV virus (Chung et al., 2020).

This study highlights on the presence of missense mutation in position 564 and 568 appears in FPL isolates differentiating it from CPV2 Asn564Ser and Ala568Gly detected in all three Egyptian isolates OP574200, OP574201, and OP574202. These 2 amino acids are the key for FPLV replication in the feline species (Truyen et al., 1995). Regularly analysis of VP2 amino acid in CPV and FLV have provided important biological properties. It has been proved that FPLV evolved primarily due to random genetic drift. Interspecies transmissions of FPV are common due to amino acid changes (Duarte et al., 2009; Yang et al., 2010; Foley et al., 2013; Barrs, 2019; Inthong et al., 2019; Yi et al., 2022). In addition, Antigenicity also affected by several amino acid at positions 93 and 323 residues in the VP2 protein and cause variations in viral surface architecture (Parrish, 1991).

FPV strains (OP574200, OP574201, and OP574202) were related to the FPV strains under accession number (HQ184200) but were different in amino acid 469 and also there were mutation Val562Leu in VP2 protein of FPV vaccinal strain used (EU498680 andEU498681, Consistent with the prior research (Li et al., 2018).

Similarity between FPLV field strains and vaccine strain should be evaluated. Isolates under accession numbers OP594200, OP574201 and OP54202 sequences are showing 99.9 percent identical to those of an FPV vaccine strain (Genbank accession no. EU498680), validating the vaccine's effectiveness.

CONCLUSIONS AND RECOMMENDATIONS

FPV circulates widely among cats in Egypt. Inadequate vaccination coverage was identified in the FPL which considered as a major contributor to the re-emergence of this disease. Continuous and regular virus monitoring is needed, further research is needed on complete VP2 in order to construct a more reliable tree.

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NOVELTY STATEMENT

This study proved the circulation of feline panleukopenia among Felidae species in Egypt and its regular contribution in cases suffering from diarrhoea in 2019 to 2021. This study emphasis the genetic stasis of the virus in comparing to CPV belong to the same family. The PCR reveal higher efficacy in viral detection, and we recommend it as a rapid routine diagnostic test. Viral VP2 revealed a higher similarity among FPLV isolated from Egypt and globally.

AUTHOR'S CONTRIBUTION

Omnia Mohamed Khattab: Analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Sara M. Elnomrosy: Performed laboratory work, analyzed the data and wrote the manuscript.

Morcos Yani: Prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Hala Kamel Abdelmegeed: Prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Mahmoud Akram: Samples collection and hematological analysis.

Naglaa M. Hagag: Analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Conflict of interests

The authors declare there are no competing interests.

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