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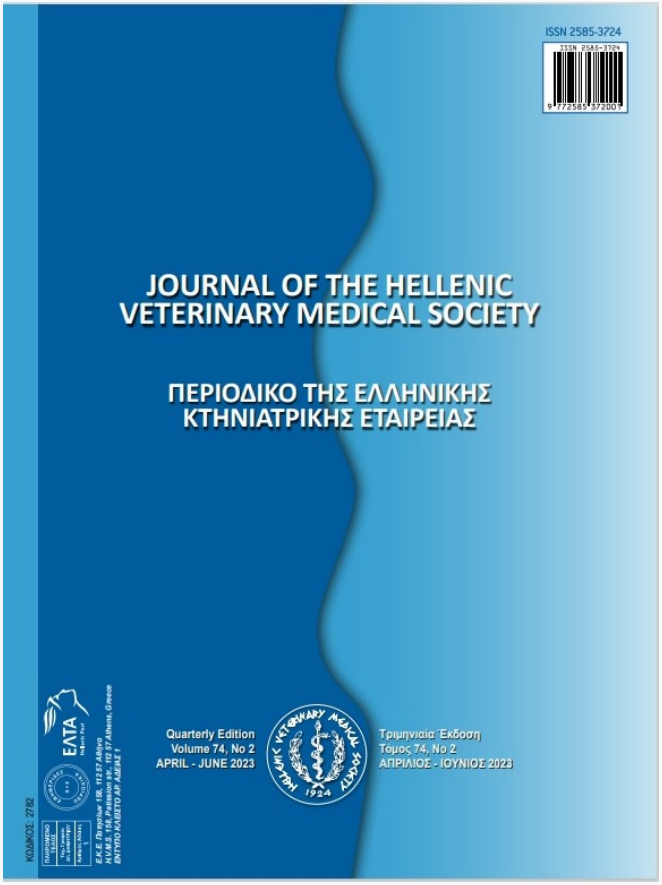


# First report of detection of Canine Parvovirus type 2 in naturally infected domestic cats in Egypt by duplex PCR for simultaneous detection of Canine Parvovirus type 2 and Feline Panleukopenia virus

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Research article
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# First report of detection of Canine Parvovirus type 2 in naturally infected domestic cats in Egypt by duplex PCR for simultaneous detection of Canine Parvovirus type 2 and Feline Panleukopenia virus

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**ABSTRACT:** Feline Panleukopenia virus (FPLV) and Canine Parvovirus (CPV) type 2 cause fatal gastroenteritis in cats and dogs. In this study we developed a duplex polymerase chain reaction (dPCR) assay for the concurrent detection of FPLV and CPV-2 in a single PCR tube. Two primers were used based on nucleic acid conserved regions of the two viruses which specifically amplify 237 bp of the VP2 gene of FPLV and 583 bp of the VP2 gene of CPV-2.Sensitivity and Specificity of the dPCR were evaluated. A total of 30 rectal/fecal swabs were collected from domestic cats suspected for parvovirus infecton in Kafrelsheikh province, Egypt and were tested for FPLV and CPV-2 viruses using the dPCR assay. The results revealed that this dPCR assay could detect a minimum of $1 \times 10^5$ copies of genomic DNA of the two viruses. The dPCR assay was highly specific as there was no amplification of nucleic acid of other feline and canine pathogens. The positive ratio was 83.3% (25/30) for FPLV and 16.6% (5/30) for CPV respectively. Further analyses of CPV samples by Restriction Fragment Length Polymorphism (RFLP) revealed that they are classified as CPV 2a/2b variants. This study reports the first detection of CPV 2a/2b from symptomatic cats in Egypt using dPCR assay that can detect FPLV and CPV in a single tube reaction.

***Keywords***: Egypt-Canine parvovirus - dPCR - Feline panleukopenia-VP2 gene

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## INTRODUCTION

Feline Panleukopenia virus (FPLV) and Canine Parvovirus (CPV) type 2 are the most significant fatal enteropathogens affecting domestic and wild cats and dogs (Sun *et al*. 2019). The disease is characterized by fever, diarrhea, dehydration, and vomiting especially in neonatal kittens and pups from 1-2 months and up to 6 months of age (Kim *et al*. 2017; Zobba *et al*. 2021).The fecal-oral route is the main pathway for infection with the parvoviruses causing acute hemorrhagic gastroenteritis (Liu *et al*. 2017); Ahmed *et al.* 2018; Balboni *et al.* 2018; Niu *et al.* 2018). In unprotected susceptible hosts, the infection spreads rapidly especially in the lymphoid tissues and intestinal crypt cells leading to death within a week after the onset of clinical signs (Capozza *et al*. 2021).

CPV and FPLV are members of family Parvoviridae, and genus Protoparvovirus (Nakamura *et al*. 2001). Parvoviruses have a linear single-stranded DNA genome approximately 5 kb in length with two main open reading frames (ORFs), one encodes the nonstructural proteins and the other encodes the capsid proteins. The VP2 is the main capsid protein that determines the host range determinant and elicits the production of neutralizing antibodies against parvovirus infection (Chung *et al*. 2020). FPLV was first isolated in 1965, while the CPV type 2 was first detected in dogs in the late 1970s, and then it was widely circulated over the world and became endemic in canids populations. The virus's actual origin is unknown, however it is thought to have evolved in a wildlife intermediary (Battilani *et al*. 2011).

In 1979, a CPV-2 variant designated as CPV-2a was identified in many countries. After five years the virus went through another antigenic change, yielding a new variant which was designated as CPV-2b. In the year 2000, a third type, CPV-2c, was discovered in Italy and quickly spread around the world, with the exception of Australia (Capozza *et al*. 2021). Based on virus genomic research, several investigations found that the original CPV-2 was totally replaced by CPV2a and CPV-2b variants (Brindhalakshmi *et al*. 2016). Although the main host of the CPV-2 is the dog, CPV variants had shown the ability to infect other carnivores, including cats, leading to a disease similar to feline panleukopenia (Capozza *et al.* 2021).

Although natural CPV infections in cats have been observed, FPLV remains the most common parvovirus that causes sickness in cats. The host range of the virus has also evolved with the advent of new CPV variants, infecting both cats and dogs, while the original CPV-2 cannot reproduce in cats in spite of the remarkable similarity in DNA sequences between the two viruses (Ahmed *et al.* 2018).CPV strains can reproduce in both canine and feline cell cultures, while FPLV strains couldn't replicate in canine cells (Niu *et al.* 2018).

Since the first report of feline infection with CPV-2a in the late 1980s, the two variants CPV-2a/2b have been demonstrated in cats worldwide (Neuerer *et al.* 2007; Muz *et al.* 2012; Barrs 2019). Recent studies have also reported that CPV-2c can infect cats leading to a more serious disease (Mukhopadhyay *et al*. 2016). Unlike the original CPV-2 strain, the CPV 2a, 2b, 2c antigenic variants can naturally infect cats, foxes, cheetahs, Siberian tigers and lions causing disease identical to feline panleukopenia ( Ohshima et al. 2009; Battilani *et al.* 2011 ; Balboni *et al.* 2018 ) .In a previous study, Truyen *et al*.(1996) reported that most of the FPLV cases detected in the United States and were CPV-2a or CPV-2b, suggesting that CPV-2 variants have begun to adapt to the feline tissue (Steinel *et al.* 2000; Decaro *et al.* 2010).

Recent investigations about cats with hemorrhagic enteritis have reported the prevalence of FPLV over CPV-2 in domestic and wild cats (Battilani *et al.* 2011). Conversely, it was reported that over 70% of parvovirus detected in domestic and wild cats in Taiwan and other nearby countries were CPV strains (Sun *et al.* 2019).

Mixed infections with more than one strain have been documented in domestic cats together with tigers, mountain lions, and leopard cats because they can be infected with both FPLV and CPV strains (Ikeda *et al.* 2000).These coinfections increase the frequency of genetic recombination of CPV and FPLV and indicate the epidemiological role of cats as an origin of novel emerging variants of parvovirus which can infect cats, dogs, and other wild animals (Niu *et al*. 2018).

Early and rapid detection of the parvovirus with accurate diagnostic tests is decisive for disease control. Clinical diagnosis is not accurate as many viruses can induce indistinguishable clinical signs in dogs and cats including canine distemper, coronavirus, and rotavirus (Neuerer *et al.* 2007). Moreover, cases of FPLV and CPV co- infections are consistently making the detection of specific pathogens difficult. A technique that can simply detect CPV-2 and FPLV

in one step would be of great value. Some regular techniques, including HA and HI assays, ELISA and rapid immunochromatography tests, have insufficient accuracy (Yi *et al.* 2018).Thus, it's crucial to develop a diagnostic technique that can detect and distinguish CPV from FPLV in the same sample. In the present study, we developed and applied a duplex PCR assay for simultaneous detection and differentiation of CPV and FPLV in feline clinical samples.

## MATERIALS AND METHODS

### Ethical Statement

No ethical approval was needed as there is no precise law for rectal swabs collection. All samples were collected under the permission of dog's owners**.**

### Viruses

Canine parvovirus type 2 nucleic acid was extracted from lyophilized Vanguard Plus CPV vaccine (Zoetis). DNA of Canine Adenovirus type 2 virus was extracted from Vanguard DAPi vaccine (Zoetis). The Canine distemper virus (CDV) isolate was provided by the Veterinary serum and vaccine research institute Cairo, Egypt .Nucleic acid extracted from *Dipylidium caninum* tapeworm was provided by the Department of Veterinary Parasitology of Kafrelsheikh University. DNA of Feline panleukopenia virus was extracted from lyophilized Felocell 4 vaccine (Zoetis). Feline Rhinotracheitis virus local isolate was provided by the Department of Veterinary Virology of Kafrelsheikh University, Egypt .Viral nucleic acids were purified using Thermo Scientific Gene Jet Viral DNA/RNA Purification Kit (Catalog No. K0821) as per manufacturer's protocol. Genomic DNA concentrations of viruses were estimated by a NanoDrop (Quawell UV-Vis spectrophotometer Q5000). cDNA of CDV was obtained from 5 µl of genomic RNA as described previously( Kasem et al. 2014). DNA and cDNA samples were preserved at − 80 °C until used.

### Primers used in duplex PCR

Two sets of primers that simultaneously amplify two different DNA fragments of the VP2 gene of FPLV (236bp) and of CPV (583bp) were used in this assay. Primers, FPLV (Forward CATACATGGCAAACAAATAGAGCA, Reverse TGTTTTAAATGGCCCTTGTGTAGA) (Zhang *et al*. 2019) and CPV (Forward CAGGAAGATATCCAGAAGGA, Reverse CAGGAAGATATCCAGAAGGA) (Buonavoglia *et al.* 2001) have been previously described but never used in a combination.

### Duplex PCR reaction mixtures and conditions

The optimal reaction conditions were tested by different gradient annealing temperatures, primer concentration, and cycle numbers. Premixed gDNA of CPV and FPLV were used as templates in the dPCR. The dPCR was conducted in 50 µl volumes, consisting of 25 µl of 2X TOPsimple DyeMix-nTaq (enzynomics) (Korea) Catalogue No.(P510T) ,1 µl of FPLV forward and reverse primers, 1 µl of CPV forward and reverse primers, 3 µl of (100 ng)FPLV gDNA , 3 µl of (100 ng)CPV gDNA and 15 µl of PCR grade water. The PCR thermal profile involved primary denaturation at 94 °C for 5 min, 30 cycles of 94 °C /45 sec, 55 °C /45 sec 70 °C/1min then 72 °C /5 min (Zhang *et al*. 2019) (Buonavoglia *et al.* 2001) .A negative reaction with no template DNA was also involved. Amplified PCR products were visualized through 1 % agarose gel electrophoresis using 8 µl of 100 bp DNA Ladder (Thermo Scientific).

### Sensitivity of the duplex PCR

The sensitivity of the duplex PCR assay was estimated by 10-fold serial dilutions of 100ng FPLV ($1.021 \times 10^{10}$-$1.021 \times 10^{0}$) copies and 100ng CPV gDNA ($1.032 \times 10^{10}$-$1.032 \times 10^{0}$) copies.

### Specificity of the duplex PCR

The specificity of the duplex PCR assay was estimated using DNA/RNA purified from Feline and canine pathogens (Feline Rhinotracheitis, Canine Distemper, Canine Adenovirus type 2 and Dipylidium caninum tapeworm) .A negative control without template was also included.

### Evaluation of dPCR on collected clinical samples:

A total of 30 rectal or fecal swabs were collected from pet animal veterinary clinics located in Kafrelsheikh province, Egypt from July to September 2021. The fecal/rectal swabs were collected from domestic cats showing clinical signs including fever, anorexia, bloody diarrhea, and vomiting with no previous vaccination history. Swabs were dipped in labeled tubes with 2 ml sterile phosphate-buffer saline (PBS) supplemented with 10% antibiotic solution then centrifuged at 12,000 rpm for 10 min at 4 °C .The supernatant fluid was stored at −80ºC till subjected to DNA extraction and dPCR.

### Restriction Fragment Length Polymorphism (RFLP) analysis

Purified amplicons of CPV positive samples were

digested with Mbo II restriction enzyme (Fast Digest, Thermofisher Scientific, USA) .10 µl of purified DNA products were mixed with 2 µl of 10X Fast Digest green Buffer and 1 µl of Fast Digest enzyme. 17 µl of nuclease free water were added to the mixture to prepare a total volume of 30 µl .The components were mixed gently and spined down. The tubes were incubated at 37°C in a heat block for 5 min. Then, amplicons were loaded on 2 % agarose gel electrophoresis with ethidium bromide stain (Polat *et al.* 2019).

## RESULTS

### Optimization of dPCR reaction conditions

The FPLV primers amplified a 236 bp fragment and the CPV primers amplified a 583 bp fragment when visualized in 1% agarose gel stained with ethidium bromide (Fig. 1). To estimate the ideal temperature for amplification, a number of temperatures from 49 °C to 56 °C were tested. The results revealed that the specific FPLV and CPV genes could be amplified at all of the tested annealing temperatures except 49 °C which amplified only 236 bp band using FPLV and CPV mix primers. The strongest dPCR bands were noticed at 52.7 and 55.2 °C and with no detected primer dimers or nonspecific bands as shown in Fig. 2.

### Sensitivity of dPCR technique

To evaluate the sensitivity of the duplex PCR assay, ten-fold serial dilutions of the purified FPLV and CPV genomic DNA were used. The concentrations of the viral gDNA were measured by a NanoDrop (Quawell UV-Vis spectrophotometer Q5000).The results revealed that the dPCR has the minimum simultaneous detection limit of $1 \times 10^5$ copies for both FPLV and CPV gDNA (Fig. 3A).

### Specificity of dPCR technique

For detection of the specificity of the dPCR assay, a group of nucleic acids purified from feline and canine pathogens were tested. The duplex PCR assay has proven to be specific for the detection of FPLV and CPV genes only with no cross-reaction with the tested pathogens (Fig. 4). No specific amplicons were detected in the lanes representing Canine distemper virus, Feline Rhinotracheitis virus, Canine Adenovirus type 2 and *Dipylidium caninum* tapeworm and negative control, while the lanes representing CPV and FPLV showed specific amplified bands (Fig 3B).

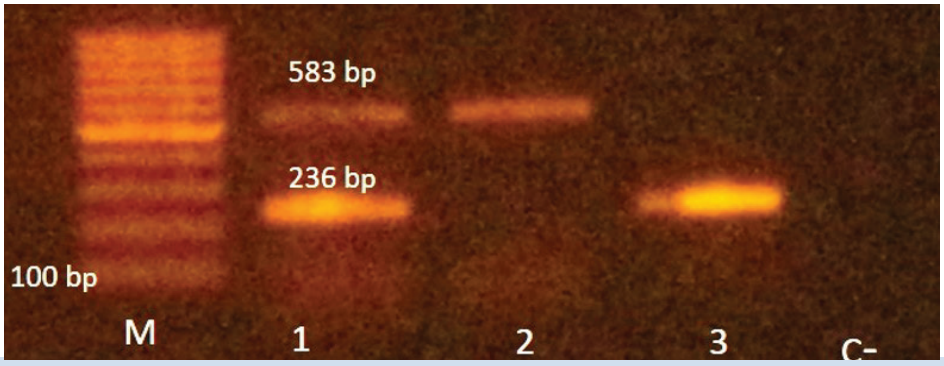


**Fig. 1:** Single and Duplex PCR for simultaneous detection of FPLV and CPV-2. Lane M: 100bp DNA size Marker. Lane 1: FPLV 236 bp and CPV 583 bp; Lanes 2: CPV 583bp; Lane 3: FPLV 236 bp; Lane C- : Negative control sample.

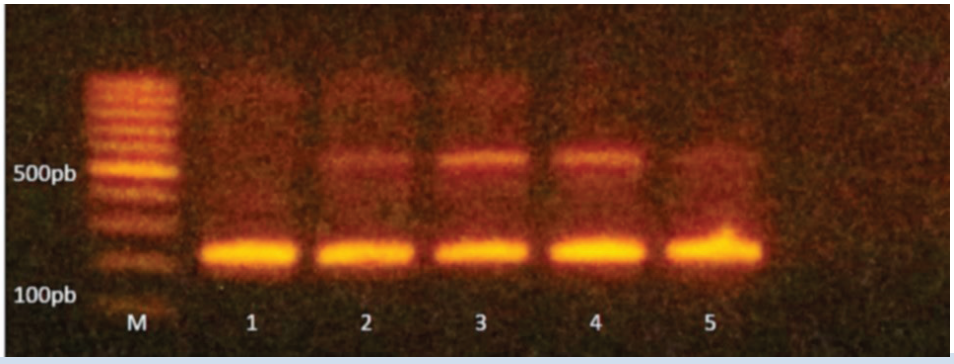


**Fig. 2:** Optimization of annealing temperature for dPCR using mixed primers. Lane 1-5 is 49 °C, 50.8 °C 52.7 °C, 55.2 °C, and 56 °C respectively; Lane M: 100 bp DNA Marker sample

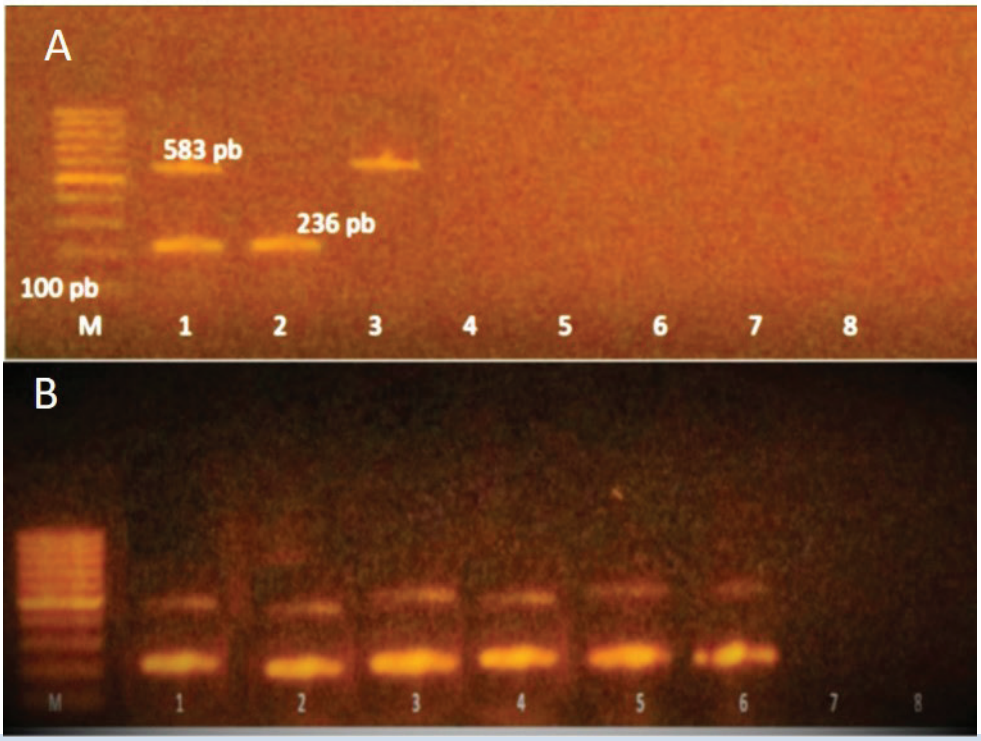


**Fig. 3:** (A) Duplex PCR sensitivity. Amplification using 10-fold serial dilutions of FPLV and CPV gDNA as template. Lane M: 100 bp DNA Marker; Lane 1-8: 1 $\times 10^{10}$-1 $\times$ $10^{0}$ copies as template, (B) Duplex PCR specificity Lane M: 100 bp DNA Ladder; Lane 1: FPLV 236bp and CPV 583 bp; Lane 2: FPLV; Lane 3: CPV; Lane 4: Canine Distemper virus; Lane 5: Feline Rhinotracheitis virus; Lane 6: Canine Adenovirus-2; Lane 7: Dipylidium caninum tapeworm; Lane 8: Negative control sample

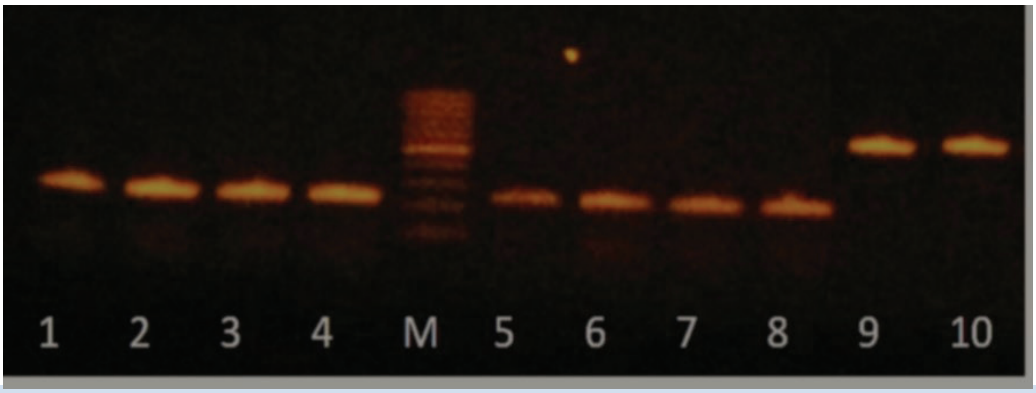


**Fig. 4:** Duplex PCR assay applied on clinical samples collected from diarrheic cats. Lane M: 100bp DNA marker; Lanes 1-8: FPLV positive samples; Lanes 9-10: CPV positive samples.

**Application of the dPCR technique on field samples**

The collected samples were tested by the dPCR assay. The results showed that 83.3% (25/30) samples were found positive for FPLV and 16.6% (5/30) samples were found to be CPV by the dPCR assay. Coinfection with both viruses was not detected in this study (Fig 4).

**Restriction Fragment Length Polymorphism (RFLP) analysis**

RFLP was applied successfully on all the CPV samples. The RFLP analysis showed undigested purified DNA fragments of all tested samples indicating that they are of the CPV 2a/2b strains (data not shown).

## DISCUSSION

FPLV and CPV-2 are the main etiological agents of fatal gastroenteritis in domestic cats and dogs in addition to many wild species (Niu *et al*. 2018).Many studies have reported CPV as a cause of gastroenteritis in cats which is indistinguishable from that induced by FPLV (Capozza *et al.* 2021) First reports of natural CPV infection in cats were reported in a five-month-old and in a one- year- old cat with classic signs of panleukopenia (Nakamura *et al*. 2001). Subsequently, CPV variants were isolated from cats in Europe, Asia and the United States (Capozza *et al.* 2021). Mochizuki et al. (1996) isolated a CPV-2a variant from a cat with clinical signs similar to feline panleukopenia. CPV-2 was reported in about 10% of cat samples in Germany, but studies from some Asian countries reported that around 80% of the infected cats were diagnosed with CPV-2 (Nakamura *et al*. 2001; Stuetzer *et al.* 2014).

In Egypt, there are only two studies that have reported FPLV infection in Egypt as they detected FPLV in 75/165 and 40/40 of the examined fecal samples from diarrheic cats by PCR (Awad *et al.* 2018a; Awad *et al*. 2018b). To date, there is no report of CPV infection in domestic cats in Egypt whereas reports of CPV in cats are common in other Asian and European countries (Stuetzer *et al.* 2014; Niu *et al.* 2018). Therefore, the present study is the first report of detection of CPV-2 infection in naturally infected domestic cats in Egypt.

Clinical diagnosis of parvoviruses is always indecisive and slow, as other viral pathogens can cause similar symptoms in cats including coronavirus and rotavirus (Sun et al. 2019). Different diagnostic techniques were used for CPV and FPLV diagnosis, such as PCR, DNA sequencing, virus isolation, ELISA, HA, HI and immunochromatography (Yi et al. 2017). Decaro *et al.* 2008 developed a real-time PCR assay, based on minor groove binder (MGB) probe technology, for rapid differentiation between FPLVs and CPVs. Also, (Streck *et al.* 2013) updated a TaqMan real-time PCR for canine and feline parvoviruses detection. In addition, a high resolution melting analysis (HRM) assay was applied and could be used for genotyping CPV and FPV in clinical samples instead of DNA sequencing as it can detect single or co-infections in one reaction (Sun *et al*. 2019). Moreover, Liu et al. 2020 developed a denaturation bubble-mediated strand exchange amplification assay for simultaneous detection of CPV-2 and FPLV in canine and feline fecal samples. Yet, the conventional tests cannot detect the viruses with high accuracy causing delay in starting specific treatment. Hence, the present study developed a sensitive, accurate and rapid technique for detection of CPV and FPLV in a single PCR test. Moreover, the duplex PCR technique has the advantage of detection of coinfections with FPLV and CPV-2 variants in one assay which is cost effective (fewer reagents are needed) and its time saving, fewer pipetting errors which decrease the possibility of samples contamination.In this dPCR assay, the primer combination yielded amplicons clearly distinguished from each other, the annealing temperatures were similar and with no dimers or nonspecific bands. The PCR product of CPV VP2 gene used in this assay encodes a nucleotide sequence which allows differentiation of FPLV from CPV-2 (Abdeldaim *et al.* 2009). No cross-reaction of CPV and FPLV primers with RNA/DNA extracted from other canine and feline pathogens was detected indicating the specificity of the dPCR assay. Results of sensitivity test of dPCR showed that the duplex PCR assay has a sufficient minimum detection limit of 1 × 105 for both FPLV and CPV gDNA. Thirty clinical samples were tested using dPCR assay showing 25/30 positive FPLV samples and 5/30 CPV positive samples. Coinfection with both viruses was not detected in this study while other studies reported co-infection cases of both CPV and FPLV (Sun *et al.* 2019). Further analyses of the field CPV PCR products by RFLP showed undigested DNA fragments of all five samples which proves that they belong to the CPV-2a/2b variants (Buonavoglia *et al*. 2001).The field samples were examined only by RFLP (without DNA sequencing) as it have accuracy of 99.5-100 % compared to sequencing (Hubáček *et al.* 2015 and Gunadi *et al*. 2016). The detection of CPV-2a and/or CPV-2b viruses in cat samples proves that both variants can cause clinical symptoms in feline suggesting that these CPV-2 variants may possibly replace FPLV in the future (Steinel *et al.* 2000)

Although CPV and FPLV viruses show very high DNA similarity, the CPV strains have gone through a number of antigenic changes leading them to earn the canine and to lose the feline host susceptibility and then readapt to the feline tissue (Tryuen *et al.* 1996). In a previous study (Mochizuki *et al*. 1996). CPV was able to replicate in cats after experimental subcutaneous infection but without any clinical signs while in another study, CPV-2 was not able to replicate in the feline tissue after intramuscular inoculation of the virus (Mochizuki *et al.* 1996). It has been reported

that CPV-2 strains can replicate in feline and canine cells in vitro but cannot infect cats in vivo, whereas the CPV antigenic variants CPV-2a/2b/2c can infect domestic cats in vivo (Mochizuki *et al*. 1996). Thus, veterinarians should pay closer attention to both CPV and FPLV infections, particularly interspecies transmission .Additional epidemiological and molecular inspections of the CPV-2 variants isolated from cats are strongly needed for controlling CPV and FPLV in domestic cats and dogs. Moreover, the novel presentation of CPV in the feline host population raises a debate about the efficiency of FPLV-based vaccinations in immunizing cats against CPV variant infections , (Decaro *et al*. 2010) as well as the need for increased CPV infection surveillance in domestic and wild cats ( Battilani *et al.* 2011).

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