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Evidences for Diagnosis of Feline Panleucopenia Viral Infection in Cats in Egypt: Molecular, Pathological and Histopathological Findings

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Abstract: Fifty cats of different of ages, sex, breeds naturally infected with Feline Panleucopenia virus (FPV), four healthy control cats. All 50 cats hospitalized for supportive & palliative treatment .Each cat was examined clinically to detect clinical manifestations of the disease showing symptoms suggestive for FP as well as ELISA, gross pathology, histopathology and PCR amplification analysis were conducted. 1-clinical signs detected in all diseased cats in the form of lethargy, fever, anorexia, thirst, diarrhea, vomiting and dehydration. 2-Rapid ELISA snip test for qualitative detection of viral antigen were positive when carried out on feces of infected cats and negative for the control one. The prognosis was bad and all diseased cats were died. 3- Postmortem examination showed dehydration, vomiting, diarrhea of offensive odour, Jejunum and ilium filled with gases, were odematous with petechial hemorrhage on both mucosa and serosal surfaces, mesenteric lymph nodes were hemorrhagic and odematous. Liver & spleen enlarged and severely congested. 4- Histopathological findings on intestine villi showed sever apical necrosis with atrophy segmental loss of crypts, lamina pro4pia were collapsed contained sloughed epithelium, debris and fibrin, severely infiltrated with inflammatory cell in submucosal area. Mesenteric lymph nodes, follicles showed focal necrosis, sever sinusoidal congestion and some of them contain fibrin. 5- PCR amplification analysis were conducted , revealed that the amplification products from tissue samples (Jejunum, ilium and mesenteric lymph nodes) were confirmed as FPV (VP1) gene by nucleotides sequence analysis presented in database.

Key words: Feline Panleucopenia · FPV · Cats · ELISA · Gross and Histopathology PCR · Sequencing

INTRODUCTION

Feline Panleucopenia (FP) is serious infectious disease for kittens and adult cats, FP caused by small minute viron belong to parvovirridae, the virus particles spread systemically post orinasal infection, its tropism affinity was high for rapidly dividing cell in lymphoid tissue and covering mucosal epithelium of small intestine resulting in sever enteritis. The disease manifested clinically by severe depression, vomiting, diarrhea, sharp decrease in circulating white blood cells and destruction of intestinal mucosa resulting in enteritis, dehydration, sharp drop in circulating WBCs end by death [1, 2].

Both live attenuated vaccine and killed vaccine were adapted for control. FP in cats [1] in Egypt, in spite of vaccination against FP, vaccination failure and lack of booster dose may lead to developing the disease. No published data regarding FPV infection in Egypt and no accurate diagnosis for FP disease [3].

In the current study, we have used pathological and histopathological studies for the first time in Egypt in addition to clinical signs and ELISA [1]. Viral RNA isolation used to achieve absolute confirmatory diagnosis.

Until now no available scheme system for diagnosis of FP so we planned this study to demonstrate gross pathology, histopathology and isolation of causative viron particle as main parameters for diagnosis and control of FP in cat in Egypt.

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MATERIALS AND METHODS

Chemicals:

- Trizol was bought from Invitrogen (Carlsbad, CA, USA). The reverse transcription and PCR kits were obtained from Fermentas (Glen Burnie, MD, USA). Direct ELISA kits were purchased from Sigma (St. Louis, MO, USA).10% Carson neutral buffered formalin (Sigma)
- TRIzol reagent was bought from Invitrogen (Germany). The reverse transcription and PCR kits were obtained from Fermentas (USA). SYBR Green Mix was purchased from Stratagene (USA).

ELISA: Direct ELISA (the antigen rapid FPV Ag test kit) for qualitative detection of FP viral antigen in feline feces were carried out on 50 fecal samples of cats suffered from clinical signs of FP viral infection and four healthy control cats (Bionote Inc. Korea) [1, 4].

Experimental Animals: Fifty diseased hospitalized cats, from different, breeds, ages and sex suffered from clinical manifestations caused by FP viral infection and four healthy control cats.

Sampling:

- Fecal samples were collected from 50 diseased hospitalized catsand 4healthy cat as a negative control checked by Rapid ELISA test (Rapid FPv Ag test kit for qualitative detection of viral antigen in feces of all examined cases) [1, 4].
- Four control cats exposed to soft death according to protocol issued by animal rights organizations:Step1- They received the first injection as sedation using tranquilizer Acepromazin 1% intramuscular followed by ketamine 10%intramuscular.

Step2- Pentobarboturate intravenous at rate 150mg/kg b.w rapid injection [5, 6].

- Pieces of jejunum, ilium and mesenteric lymph nodes of all 50 scarified cat carcasses had been sent for histopathological examination and viral isolation [1].

Pieces of jejunum, ilium and mesenteric lymph nodes healthy control cat (exposed to soft death then scarified, had been sent for viral isolation [7].

Methods:

Clinical Examination:

- Fifty cats were received at Germen Vet. Clinic at 6th October City, Giza, Egypt. Cats subjected to general and specific clinical examination according to Gaskell *et al.* [1] and were examined by measuring body temperature, examination of buccal and conjunctival mucous membranes, examination of superficial lymph nodes by palpation, abdominal palpation, appetite, body condition, skin, respiratory illness, digestive disturbances *e.g.*, vomiting and diarrhea were recorded for each case as a recommended routines check up at the clinic. History of the examined cases including bread, sex, age, past medical history and registered vaccination for each cats were conducted. All cases checked clinically for detection dehydration rate according to, we found that dehydration rate ranged from 8-12% [3, 8, 9].
- Gross pathology for recording the gross pathological picture of FP [1] in all disease cats.
- Histopathological Examination: jejunum, ilium and mesenteric lymph nodes were taken. Then fixed in 10% carson neutral buffered formalin. 3 to 5 µm section taken from blocks of paraffin wax, then stained with Heamotoxyline-eosm(HE) and microscopically examined [1, 10].

RNA Extraction: RNA was extracted from jejunum, ilium and mesenteric lymph nodes samples of infected cats. Total RNA was extracted from above mentioned samples using the QIAamp Blood Mini Kit (Qiagen, Hombrechtikon, Switzerland) and stored at -80^{o}C until further use.

Primer Design: Specific primer used in this study was designed according to complete genome of PFv genome using primer3 program as illustrated in Table (1).

First-Strand cDNA Synthesis and PCR Amplification: Extracted total RNA samples were reverse transcribed into cDNA. First-strand cDNA was synthesized in duplicate using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Rotkreuz, Switzerland) and oligo(dT) primer according to the manufacturer's instructions.

Purified RNA was reverse transcribed using the ImProm-II Reverse Transcription System using 250 ng oligo(dT) primer, 1–9 µL RNA combined with water to a

Table 1: Primers used in cDNA amplification

Primer name	Oligonucleotide Sequence (5'-3')	Estimated Product Size[a]
LEFT PRIMER	TGC CTC AAT CTG AAG GAG CT	881-1105 bp
RIGHT PRIMER	TTT CAT CTG TTT GCG CTC CC	

a-Based on available feline FPv genome sequences

total volume of 20 µL.The resultant cDNA was amplified by PCR using illustra™ puReTaq Ready-To-Go PCR Beads using 1 µL of each primer and 1 µL cDNA template, combined with water to a total volume of 25 µL. Amplifications were performed with the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 1 min and 10 min final extension at 72°C.

C-Nucleotide Sequence Analysis: Database searches with determined sequences were conducted by using the BLASTN programs in the GenBank on web site http://blast.ncbi.nlm.nih.gov/Blast.cgi. The sequences were aligned online by using BLASTN, version 2.2.21+ (on the web site http://blast.ncbi.nlm.nih.gov/Blast.) and the alignments were refined by visual inspection.

RESULTS

Clinical Examination: All the examined hospitalized cats suffered from clinical signs, which were suggestive for FP and cheeked by Rapid ELISA (the antigen rapid FPv Ag test kit) for qualitative detection of FP viral antigen in feline feces. These cats suffered from a cute and per acute sever forms of FP in addition to high degree of hydration rate 8-12%.Supportivetreatment, fluid therapy and uses of immune stimulant and antibiotics were introduced for all hospitalized diseased cats. All 50 cats died due to the severity of the disease.

ELISA Result for FPV: The results showed that 50 clinically infected cats with FPV were positive for Rapid ELISA test carried for qualitative detection of FP antigen in the feces, while 4 control cats were negative for rapid ELISA.

Gross Pathology Findings: Dehydration is a prominent feature, evidences of vomiting and diarrhea of bad smelling odour, subculenous blood vessel congested, enlarged spleen and ascites with presence of small yellowish amount of fluids in abdominal cavity, enlarged congested kidneys, mesenteric lymph nodes highly congested with small petechial hemorrhages on surface and enlarged, jejunum and ilium hypermic, congested, filled with amounts of gases, odometeus with petechial Hemorrhages on both luminal mucosa and outer serosa. Liver severally congested, enlarged. Gall bladder distended and filled with bile. Both lungs with bright red hypremicpatches. Red petechial patches on heart surface with highly congested coronary arteritis.

Histopathological Findings: Intestinal Villi on jejunum and ileum showed sever necrosis at apical part and atrophy.

Villi were short, clubbed and regionally fused and lined by attenuated epithelium. Segmental loss of crypt, crypt cells showed sever cytoplasmic degeneration with nuclear pyknosis, lamina propia were collapsed and their lumen contain sloughed epithelium, fibrin and necrotic debris sever infiltration with inflammatory cells in submucosal area, sever damage in tunica muscularis.

Mesenteric Lymph nodes showed focal necrosis in the follicles. Sever sinusoidal congestion (acute inflammatory form). Some follicles contain fibrin.

Molecular Determination for FPV: Template cDNA collected from cat samples (jejunum, ilium and mesenteric lymph nodes) with signs compatible with Feline Panleucopenia was amplified using the specific FPV primer. All samples were tested by conventional PCR assay using a primer that was previously designed according to complete genome or complete CDS of capsid protein gene of FPV genome. The cDNA from all examined FPV samples produced clear bands upon amplification with its primer with a 220bp product size. The primer was specific for FPV (PV2) gene fragment, the size of the amplicons corresponded to the expected size and no additional or non–specific bands were observed Fig. (1). In addition, healthy cats (control) exhibited negative FPV bands.

Analysis of the genomic region encompassed by this primer and its predicted amino acid sequence allowed discrimination of FPV from all its variants tissues. The amplification products from all positive samples were confirmed as FPV (VP1) gene by nucleotide sequences analysis. The sequence obtained for each isolate was aligned with the sequences available in the database using BLAST and the sequences with the highest coverage and highest degree of similarity were selected.

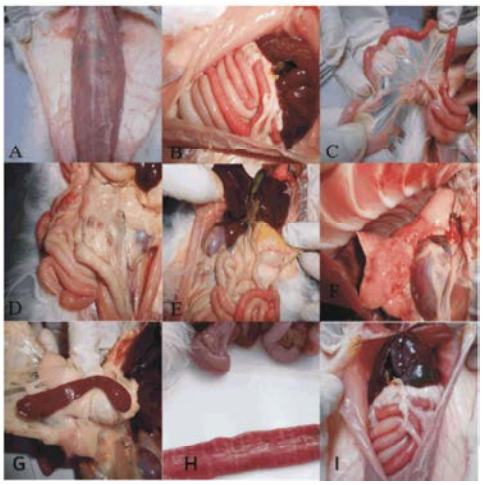

Fig. 1: Gross pathology findings:
A-Congestion of subcutaneous blood vessels& muscles.
B- Hyperemic and dilated jejunum and ileum & enlarged liver.
C-Hyperemic jejunum& odematous and enlarged mesenteric lymph nodes.
D- Enlarged mesenteric lymph nodes& minimal yellowish ascetic fluid.
E-Enlarged, congested liver & dilated enlarged gall bladder.
F- Red hyperemic batches on lung.
G- Congested enlarged spleen.
H- Red hemorrhagic patchs on mucosal surface of jejunum (rose red appearance) .
I-Severely congested and enlarged liver&jejunum and ileum hyperemic, filled with gasses.

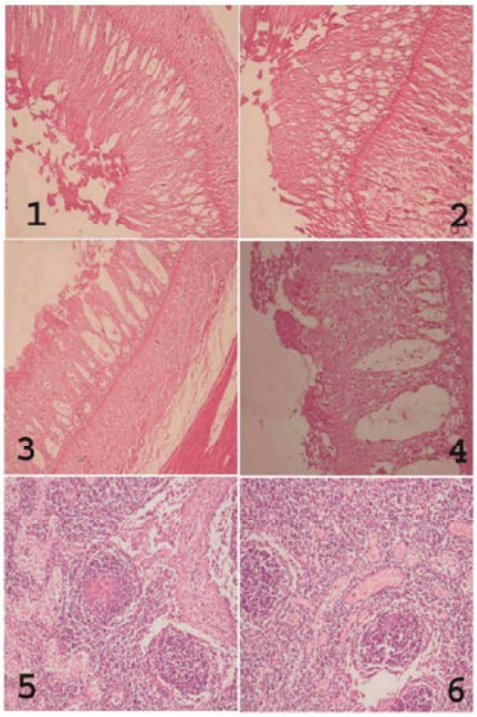

Fig. 2: Histopathological findings:
Plate: 1, 2 Ilium :
Plate: 3, 4 Jejunum:

Villi were short with sever necrosis at the apical portion, clubbed, regionally fused lined with attenuated epithelium, completely damage and atrophied.

Segmental loss of crypts, showed sever cytoplasmic degeneration with nuclear pyknosis sever infiltration with inflammatory cells in submucosal area, crypts lumen contained sloughed epithelium, fibrin and necrotic debris

Plate: 5, 6 Mesenteric lymph nodes: showed focal necrosis in the follicles. Sever sinusoidal congestion (acute inflammatory form). Some follicles contain fibrin

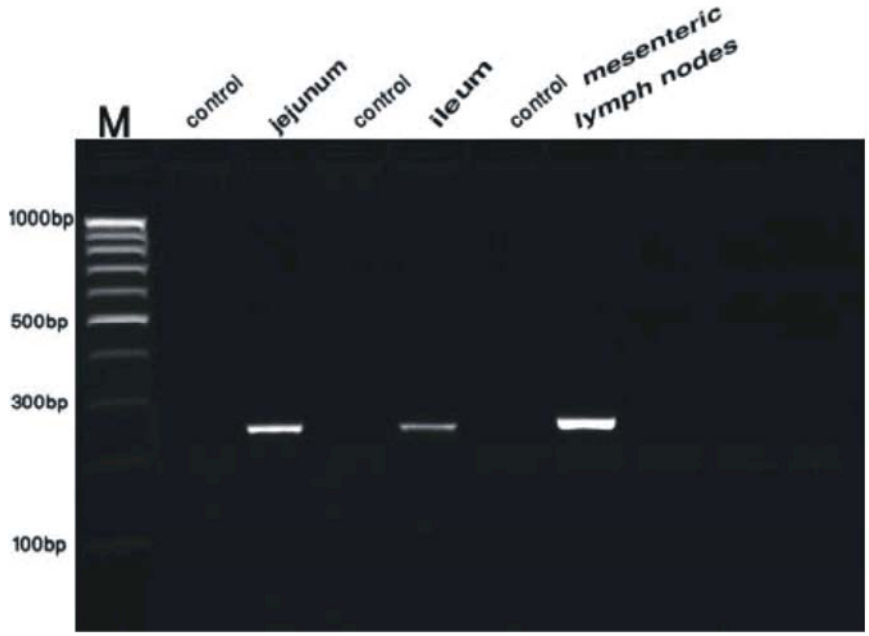

Fig. 3: Amplified cDNA of control and FPV infected cat samples. Only positive FPV DNA bands were showed at 220bp for the infected cats. M, DNA 100bp ladder markers.

The identification according to BLASTN programs version 2.5.1+ at the GenBank NCBI on web site http://blast.ncbi.nlm.nih.gov/Blast.cgi, using FASTA format the sequencing to align online, that identified the isolates as Feline Panleucopenia virus strain PT265/14 capsid protein VP1 (VP1) gene.

Nucleotide Sequence Accession Number: Nucleotide sequence was submitting using BankIthttps: // submit .ncbi.nlm .nih.Gov/subs/ genbank. The FPV gene fragment sequence was deposited in ncbi Nucleotide Database gene bank under accession no. MG894397.

DISCUSSION

Feline Panleucopenia is fatal infectious disease affect cats of all ages, FP clinically varies from per acute, acute and when cats founded died to subclinical form. FP is a disease of high morbidity 100% and high case fatality may reach to 100%in kittens less than 3 month age, 85% in adult cat, 90 in elderly cats, 100% if dehydration rate was 8% or more [1, 2, 3, 7].

FP virus has affinity tropism for rapidly dividing cells in lymphoid tissue and intestinal mucosa result in destruction of circulating white blood cells and damaging intestinal mucosa lead to enteritis, dehydration end by death [1].

Results of rapid ELISA for qualitative detection of FP viral antigen in the feces of all examined cats correlated with the results recorded [11, 12, 13].

Four control healthy cats exposed to soft death (Euthanasia of animals used for scientific purposes) [4, 5] and 50 cats included in this study were died after infection with FP virus, after death of all 54 cats , post mortem examination were done. Following findings were recorded: dehydration was a prominent sign, evidences of vomiting and diarrhea of bad smelling odour, subcutaneous blood vessels congested, enlarged spleen, enlarged congested kidneys, mesenteric lymph nodes highly congested, odematous and enlarged, ascites. Jejunum and ilium congested, filled with gases, odematous with petichael hemorrhages [1, 2].

Histopathological findings of specimens take from mesenteric lymph nodes, jejunum and ilium of died cats stained with haematxylin and eosin [1] showed the following findings: Intestine :intestinal villi atrophied with crypt necrosis , villi were shortened, with necrosis of apical portion, clubbed and regionally fused and lined by attenuated epithelium. Segmental loss of crypts , crypt cells showed sever cytoplasmic degeneration with nuclear pycknosis and lamina propia were collapsed and submucosal area severely infiltrated with inflammatory cells contain sloughed epithelium, fibrin , necrotic debris.

Mesenteric lymph nodes: showed focal necrosis of lymphoid follicles. Sever Sinusoidal congestion, some follicles contain fibrin.

All these microfindings were due to rapid destruction of highly divided cells in intestinal mucosa and lymphoid tissue as result of invasion of these cells by FP virus and its subsequent replication result in death of these dividing cells by apoptosis and necrosis [14, 15].

Cytopathic effect occurred in dividing cells in targeted tissues of infected cats as result of invasion of this cells by FP virus particles, invasion of these cells by FP viral DNA and its nucleocapsid lead to fragmentation of DNA of infected cell then death of these cells .Also release of virus particles after multiplication resulted in death of these infected cells with its replication ruminants as inclusion bodies intranuclearly [11, 12].

The results of the sequence analysis in the present study indicated that PCR products of the FPV cDNA exhibited very low variation in their nucleotide sequence of all isolates compared with the published FPV genome in the GenBank. These results are similar to previous other studies suggesting that FPV appears to be genomic stasis compared with other viruses such as Parvo viruses [16, 17].

The amplification products from all positive samples were confirmed as FPV (VP1) gene by nucleotide sequences analysis. The identification according to BLASTN programs version 2.5.1+ at the GenBank NCBI on web site http://blast.ncbi.nlm.nih.gov/Blast.cgi, using FASTA format the sequencing to align online, that identified the isolates as Feline Panleucopenia virus strain PT265/14 capsid protein VP1 (VP1) gene. The FPV gene fragment sequences isolated in this study was deposited in ncbi Nucleotide Database gene bank under accession no. MG894397.

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