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Journal of Genetic Engineering and Biotechnology

journal homepage: www.elsevier.com/locate/jgeb

Original Article

Feline panleukopenia viral infection in cats: Application of some molecular methods used for its diagnosis

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ARTICLE INFO

Article history:

Received 16 January 2018

Received in revised form 5 August 2018

Accepted 13 August 2018

Available online 27 August 2018

Keywords:

Feline panleukopenia

FPV

Cats

ELISA

qRT-PCR

DNA fragmentation

Apoptosis

ABSTRACT

Forty diseased cats and seven healthy control cats from different sex, ages and breeds had examined clinically to confirm presence or absence of clinical symptoms of *Feline panleukopenia* disease (FP). Several tools including ELISA, gene expression analysis (qRT-PCR), DNA fragmentation test and apoptosis assay were conducted to determine the *Feline panleukopenia* disease in cat tissues. Clinical symptoms in the form of depression, fever, anorexia, vomiting, diarrhea, dehydration, anaemia and leucopenia were recorded in the diseased cats while no clinical signs were observed in control healthy cats. ELISA results showed that all of diseased (n = 40) cats were positive while control cats (n = 7) were negative for FP viral antigen. After carrying out of ELISA assay, supportive treatment trials including fluid therapy, immunostimulant, antibiotics to overcome dehydration, restoring electrolytes imbalances, combating secondary bacterial infection were conducted but all diseased cats were died and control cats exposed to soft death. Gene expression analysis detected high levels of FP viral gene in several cat tissues in which ilium exhibited high viral expression levels compared with jejunum. Also, viral expression levels in jejunum were higher than in mesenteric lymph nodes. In addition, viral expression levels were not detected in tissues of control cats. The results of the DNA fragmentation assay observed that DNA extracted from different tissues of infected cats exhibited damaged DNA bands as compared with DNA of control cats. DNA fragmentation rates in infected tissues increased significantly ($P < 0.01$), the highest rates were showed in ilium and jejunum tissue than in mesenteric lymph nodes. Determination of apoptosis in cat tissues showed that rate of apoptosis/necrosis increased significantly ($P < 0.05$) in infected cats tissues in comparison to control cats. Moreover the highest apoptotic ratios of infected cats were observed in ilium and jejunum tissues compared with mesenteric lymph nodes.

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1. Introduction

Feline panleukopenia (FP) is serious infectious disease for kittens and adult cats. FP caused by small minute viron belong to parvoviridae, 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 [1–4]. 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 white blood cells (WBCs) end by death [1–4].

Palliative treatment was recommended for overcoming dehydration and restoring electrolytes balance, antibiotic to control secondary bacterial infections, immune stimulants for enhancing natural immunity in adult cats [4]. Both live attenuated vaccine and killed vaccine were adapted for control. FP in cats in Egypt, in spite of vaccination against FP, vaccination failure and lack of booster dose may lead to developing the disease. For our knowledge no published data regarding FPv infection and diagnosis of FP disease in Egypt are available.

Apoptosis, or programmed cell death, is a physiological process important for normal cellular turnover and is characterized by pronounced morphological changes and internucleosomal DNA degradation [5–8]. Studies have shown that it can be triggered

Peer review under responsibility of National Research Center, Egypt.

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by several viruses, and there is mounting evidence that induction of apoptosis contributes directly to the pathogenesis of a number of viruses, such as feline leukemia virus subgroup C, feline immunodeficiency virus (FIV), influenza A and B viruses, measles virus, and, most significantly, human immunodeficiency virus type 1 (HIV-1) [9–11]. In FP virus infected cats, the decrease of leukocyte counts are marked and lymphocytes disappear from the circulation, lymph nodes, bone marrow, and thymus [9–11]. It is probably that polymorphonuclear leukocyte stem cells are also destroyed [9–11].

The current study was planned to demonstrate the qualitative and quantitative symptoms of FP viral infection using most accurate confirmatory tools such as clinical signs, ELISA and viral gene expression analysis. In addition, DNA fragmentation and cell apoptosis in cat cells was determined to clarify the cytopathic effect of FP viral infection on different tissues (mesenteric lymph nodes, jejunum and ileum) of infected cats.

2. Materials and methods

2.1. Chemicals

TRIzol reagent was purchased 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).

2.2. Ethical approval

Ethical approval is necessary for completing this study; we informed and received the permission of the owners of cats included in this study for taking samples used in this work.

2.3. Examined animals

A – Forty diseased hospitalized cats, from different breeds, ages and sex were examined clinically to detect clinical manifestations caused by FP viral infection.

B – Seven healthy control cats of different breeds, ages and sex were examined clinically to prove that they were apparently healthy and free from FP viral infection.

2.4. ELISA

Cat samples were tested by Direct ELISA (the antigen rapid FPV Ag test kit) according to Esfandiari and Klingeborn [12]. Qualitative detection of FP viral antigen in feline feces were carried out on 40 fecal samples of cats suffered from clinical signs of FP viral infection and seven healthy control cats. For fecal swabs, the extraction buffer or conjugate was dispensed into the sample tube via the kit swab. Then, the sample swab was inserted into the tube containing the liquid and vortexed briefly. The extracted fecal sample–conjugate liquid was transferred to the kit device by the swab pipette from the test kit according to manufacturer's instructions.

2.5. Clinical examination

Forty cats were received and examined at a Veterinary Clinic, namely Germen Veterinary Clinic, at 6th October City, Giza governorate, Egypt. Cats were subjected to general and specific clinical examination according to Gaskell et al. [1]. Cats 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 and digestive disturbances. Also, vomiting

and diarrhea were recorded for each case as a recommended routine check up at the clinic. History of the examined cases including breed, sex, age, past medical history and registered vaccination for each cat was conducted [13].

All cases checked clinically for detection of dehydration rate according to Brown and Otto [13] in which the dehydration rate was ranged from 8 to 12%. All Forty cats were hospitalized for supportive treatment to help cats self-defense to overcome infections, plus administration of antibiotics to control second bacterial infection, combating dehydration and restoring electrolytes balance [13] but prognosis of these infected cats ended by death [1–3]. Additionally, clinical examination also had done for the seven healthy control cats.

2.6. Sampling

Fecal samples were collected from 40 diseased hospitalized cats and seven fecal samples from healthy control cats checked by Rapid ELISA test (Rapid FPV Ag test kit for qualitative detection of viral antigen in feces of all examined cases [1,12]. Carcasses of all 40 dead hospitalized cats were taken after death and exposed to post mortem examination. Seven control cats exposed to soft death according to protocol issued by animal rights organizations as follows: (a) They received the first injection as sedation using tranquilizer Acepromazin 1% intramuscular followed by ketamine 10% intramuscular. (b) They received Pentobarbiturate intravenous at rate of 150 mg/kg b.w as rapid injection [14–17]. Moreover, pieces of mesenteric lymph nodes jejunum and ileum, of all 47 scarified cat carcasses had taken and used for viral gene expression analysis, DNA fragmentation and cell apoptosis.

2.7. Gene expression analysis

2.7.1. Extraction of total RNA and cDNA synthesis

Different cat tissues (mesenteric lymph nodes; jejunum and ileum) were used to extract the total RNA using TRIzol[®] Reagent (Invitrogen, Germany) Kit. The isolation method was carried out according to the manufacturer's instructions of the above Kit. Approximately 50 mg of the cat tissues were mixed with some drops of liquid nitrogen and homogenized in 1 ml of TRIzol[®] Reagent in autoclaved mortar. Afterwards, total RNA was dissolved and preserved in diethylpyrocarbonate (DEPC)-treated water up to use.

To assess the RNA yield and purity of the total RNA, RNase-free DNase I (Invitrogen, Germany) was used to digest DNA contamination. A small drop of isolated RNA was examined photospectrometrically at 260 nm. The purity of total RNA was determined between 1.8 and 2.1 to be good purified when it examined by spectrophotometer at the 260/280 nm ratio. To avoid RNA damaging, aliquots of RNA were prepared after isolation for either reverse transcription reaction or otherwise for storing at –80 °C up to use.

To synthesize the complementary DNA (cDNA) isolated RNA from cat tissues was reverse transcribed into cDNA. The reaction volume was carried out in 20 µl. The reaction volume was prepared according to the instructions of the RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). The reverse transcription (RT) reaction was performed for 10 min at 25 °C. Afterwards, the tubes of the reaction were put in thermo-cycler machine for 60 min at 42 °C, and then the reaction was terminated for 5 min at 99 °C. The PCR products containing the cDNA were kept at –20 °C up to use for DNA amplification [18,19].

2.7.2. Quantitative real time-PCR (qRT-PCR)

A StepOne Real-Time PCR System (Applied Biosystem, USA) was used to assess the copy of the cDNA of cat tissues to detect the expression values of the tested genes. To perform the PCR reaction,

Table 1
Primers used in qRT-PCR 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 FLV genome sequences.

a volume of 25 µl of reaction mixtures was prepared containing 12.5 µl of SYBR® green (TaKaRa, Biotech. Co. Ltd.), 0.5 µl of 0.2 µM forward and reverse primers, 6.5 µl DNA-RNA free water, and 2.5 µl of the synthesized cDNA. The cDNA was propagated using reaction program consisted of 3 steps. In the first step the PCR tubes was incubated at 95 °C for 3 min. In the second step the reaction program consisted of 50 cycles. Each cycle of them consisted of 3 sub-steps: (a) 15 sec at 95 °C; (b) 30 sec at 60 °C; and (c) 30 sec at 72 °C. In the third step the reaction program consisted of 71 cycles. The first cycle of them started at 60 °C for 10 sec and then the followed cycles increased about 0.5 °C every 10 sec up to 95.0 °C. A melting curve of the reaction was performed for each qRT-PCR termination at 95.0 °C to assess the quality of the primers. To verify that the reaction of the qRT-PCR does not have any contamination PCR tubes containing non template control were used.

Specific primers used in this study for *Feline panleukopenia virus* gene (GenBank: KU248464.1, complete cds) were designed according to complete genome of PLV genome using Primer3 software (version 0.4.0, <http://primer3.ut.ee>) as illustrated in Table 1. The relative quantification of the target genes to the reference (GAPDH: F: 5'-GCG CCT GGT CAC CAG GGC TGC-3'; R: 5'-GAC TCC ACA ACA TAC TCA GCA CCA GCA TCA C-3') was determined by using the 2-ΔΔCT method.

2.8. Cellular DNA fragmentation using gel electrophoresis laddering assay

Apoptotic DNA fragmentation was qualitatively analyzed by detecting the laddering pattern of nuclear DNA as described according to Gibb et al. [20]. Briefly, cat tissues (mesenteric lymph nodes; jejunum and ileum) were homogenized, washed in PBS, and lysed in 0.5 ml of DNA extraction buffer (50 mM Tris-HCl, 10 mM EDTA, 0.5% Triton, and 100 µg/ml proteinase K, pH 8.0) for overnight at 37 °C. The lysate was then incubated with 100 µg/ml DNase-free RNase for 2 h at 37 °C, followed by three extractions of an equal volume of phenol/chloroform (1:1 v/v) and a subsequent re-extraction with chloroform by centrifuging at 15,000 rpm for 5 min at 4 °C. The extracted DNA was precipitated in two volumes of ice-cold 100% ethanol with 1/10 vol of 3 M sodium acetate, pH 5.2 at -20 °C for 1 h, followed by centrifuging at 15,000 rpm for 15 min at 4 °C. After washing with 70% ethanol, the DNA pellet was air-dried and dissolved in 10 mM Tris-HCl/1 mM EDTA, pH 8.0. The DNA was then electrophoresed on 1.5% agarose gel and stained with ethidium bromide in Tris/acetate/EDTA (TAE) buffer (pH 8.5, 2 mM EDTA, and 40 mM Tris-acetate). A 100-bp DNA ladder (Invitrogen, USA) was included as a molecular size marker and DNA fragments were visualized and photographed by exposing the gels to ultraviolet transillumination.

On the other hands, to determine the DNA fragmentation values the cat tissues were lysed in 0.5 ml of lysis buffer containing, 10 mM tris-HCl (pH 8), 1 mM EDTA, 0.2% triton X-100, centrifuged at 10 000 r.p.m. (Eppendorf) for 20 min at 4 °C. The pellets were resuspended in 0.5 ml of lysis buffer. To the pellets (P) and the supernatants (S), 0.5 ml of 25% trichloroacetic acid (TCA) was added and incubated at 4 °C for 24 h. The samples were centrifuged for 20 min at 10 000 r.p.m. (Eppendorf) at 4 °C and the pellets were

suspended in 80 ml of 5% TCA, followed by incubation at 83 °C for 20 min. Subsequently, to each sample 160 ml of DPA solution [150 mg DPA in 10 ml glacial acetic acid, 150 ml of sulfuric acid and 50 ml acetaldehyde (16 mg:ml)] was added and incubated at room temperature for 24 h. The proportion of fragmented DNA was calculated from absorbance reading at 600 nm using the formula:

$$\% \text{Fragmented DNA} = [\text{OD(S)}/\text{OD(S)} + \text{OD(P)}] \times 100$$

2.9. Determination of the apoptosis using acridine orange/Ethidium Bromide dye

Apoptotic changes in cat tissues (mesenteric lymph nodes; jejunum and ileum) were determined morphologically by fluorescent microscope after labeling with acridine orange/Ethidium Bromide (AO/EB) [4–6]. Briefly, cat tissues were washed in PBS, chopped finely and centrifuged at 7000 rpm for 5 min. The pellet obtained was suspended in trypsin-EDTA (0.25%, 53 mM) in PBS for 1 hr at 37 °C and smeared on clean glass slides. Finally, all cells smears were air-dried and fixed in a solution of methanol/acetic acid (3:1). The slides were stained with AO/EB mixture 25 µl of dye mixture (4 µg/ml AO and 4 µg/ml EB in PBS, pH 7.4). The cells were divided into four categories as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), and late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation) and necrotic cells (uniformly orange-stained cell nuclei). In each group, a total of 100 cells were observed under fluorescent microscope and the apoptotic/necrotic ratio was calculated as number of apoptotic and necrotic cells/total cell count ($\times 100$).

3. Results

3.1. Clinical examination and ELISA assay

This study exhibited that all hospitalized cats were suffered from clinical signs, which were suggestive for FP. Also, these cats were checked by Rapid ELISA for qualitative detection of FP viral antigen in feline feces. The symptoms of the studied cats included a cute and per acute sever forms of FP in addition to high degree of dehydration above 8%. In spite of supportive treatment, fluid therapy and uses of immune stimulant and antibiotics. All hospitalized cats (n = 40) died due to the severity of the disease (Fig. 1).



Fig. 1. Clinical pictures of *Feline panleukopenia* viral infection in Cats.

Moreover, the results showed that forty clinically infected cats with FPV were positive for Rapid ELISA test carried for qualitative detection of FP antigen in the feces, while, seven control healthy cats were negative.

3.2. Expression levels of FP viral gene in cat tissues

Fig. 2 represents the levels of FPV gene expression in different cat tissues (mesenteric lymph nodes, ileum tissues and jejunum).

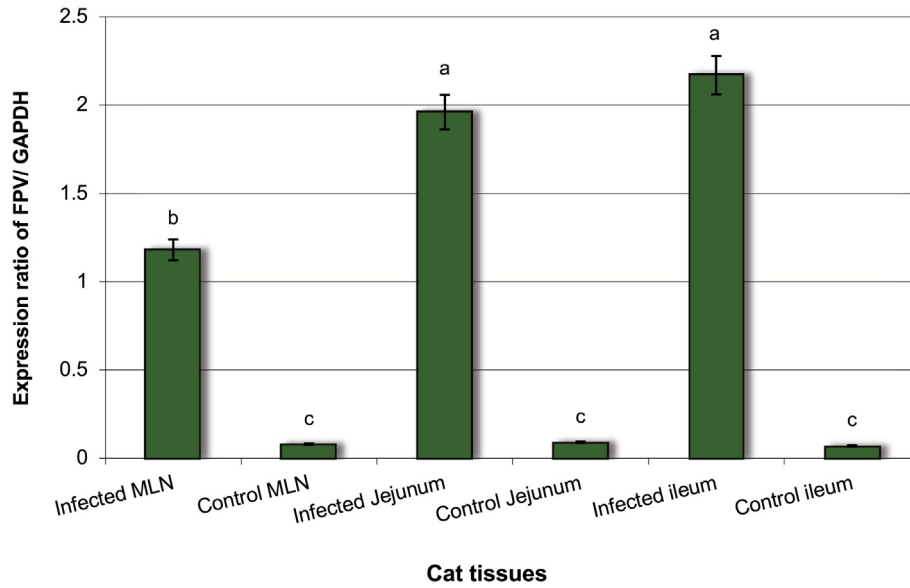


Fig. 2. Expression levels of FPV gene in different cats tissues. Data are presented as mean ± SEM. ^{a,b,c} followed by different superscripts are significantly different (P < 0.05).

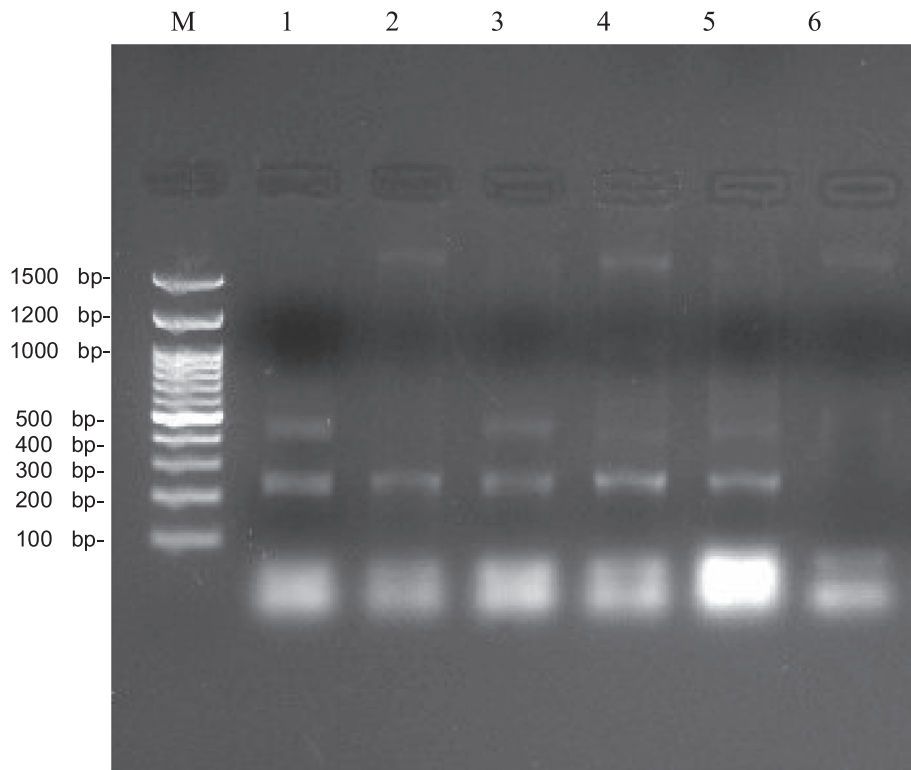


Fig. 3. DNA fragmentation detected with agarose gel of DNA extracted cat tissues by DNA gel electrophoresis laddering assay. Lanes 1 and 2 represent infected and normal mesenteric lymph nodes, respectively. Lanes 3 and 4 represent infected and normal jejunum, respectively. Lanes 5 and 6 represent infected and normal ileum, respectively. Lane M represents DNA ladder.

The relative quantification values of the FPV gene to the reference gene were determined by using the $2^{-\Delta\Delta CT}$ method as follows:

$$\Delta C_{T(\text{test})} = C_{T(\text{target, test})} - C_{T(\text{reference, test})},$$

$$\Delta C_{T(\text{calibrator})} = C_{T(\text{target, calibrator})} - C_{T(\text{reference, calibrator})},$$

$$\Delta\Delta CT = \Delta C_{T(\text{Test})} - \Delta C_{T(\text{calibrator})}$$

The results showed that the FPV gene was very low and not detectable in all control cat tissues. However, the expression levels of FPV gene in different infected cat tissues were very high compared with the control cats. Additionally, the expression levels of FPV gene increased with highly significant ($P < 0.01$) differences in ileum tissues compared with jejunum and mesenteric lymph nodes (Fig. 2).

3.3. Assessment of DNA fragmentation in cat tissues

The impact of infection with *Feline panleukopenia virus* on the genomic DNA of different cat tissues is summarized in Figs. 3 and 4.

The results showed that the DNA extracted from different tissues of infected cats showed damaged DNA bands as compared with the DNA of control cats (Fig. 3). Moreover, the DNA fragmentation rates in infected cat tissues increased significantly in comparison to control cats. Furthermore, the highest DNA fragmentation rates of infected cats were showed in ileum and jejunum tissues compared with mesenteric lymph nodes (Fig. 4).

3.4. Determination of the apoptosis in cat tissues using acridine orange/Ethidium Bromide dye

The effect of the FPV infections on the apoptosis in the cat tissues is summarized in Figs. 5 and 6.

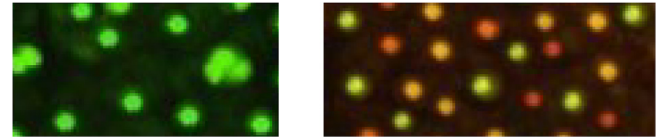


Fig. 5. Fluorescent microscope examinations demonstrating normal living cells (A) and apoptotic cells (yellow to orange cells, B) as detected by acridine orange/Ethidium Bromide staining.

The results revealed that most of infected cat tissues increased significantly the rate of the necrotic/apoptotic compared with control cats. Moreover, the highest apoptotic ratios of infected cats were observed in ileum and jejunum tissues compared with mesenteric lymph nodes (Fig. 6).

4. Discussion

Feline panleukopenia disease is a serious infectious disease affect cats of different ages resulting in high morbidity and mortality rates, it cause severe damage in mucosal lining of small intestine lead to sever enteritis subsequent diarrhea dehydration in addition to destroying circulating WBCs and lymphocytes in lymph nodes end by death of most infected cats [1,12,21]. Overall 40 diseased cats included in this study were diagnosed by detection of clinical signs and confirmed by Rapid ELISA test for qualitative detection of FP viral antigen in faces. In spite of all trials done to treat infected cats to restore dehydration, electrolyte imbalance resulted from sever enteritis induced by FP viral infection all diseased cats were died. These results are in agreement with those of Gaskell et al. [1], who reported that FPV is known to cause severe damage in mucosal lining epithelium of small intestine, circulating WBCs and lymphocyte of mesenteric lymph nodes which are coincided with death of infected cats. These results were confirmed by gene expression analysis which was used as qualitative accurate

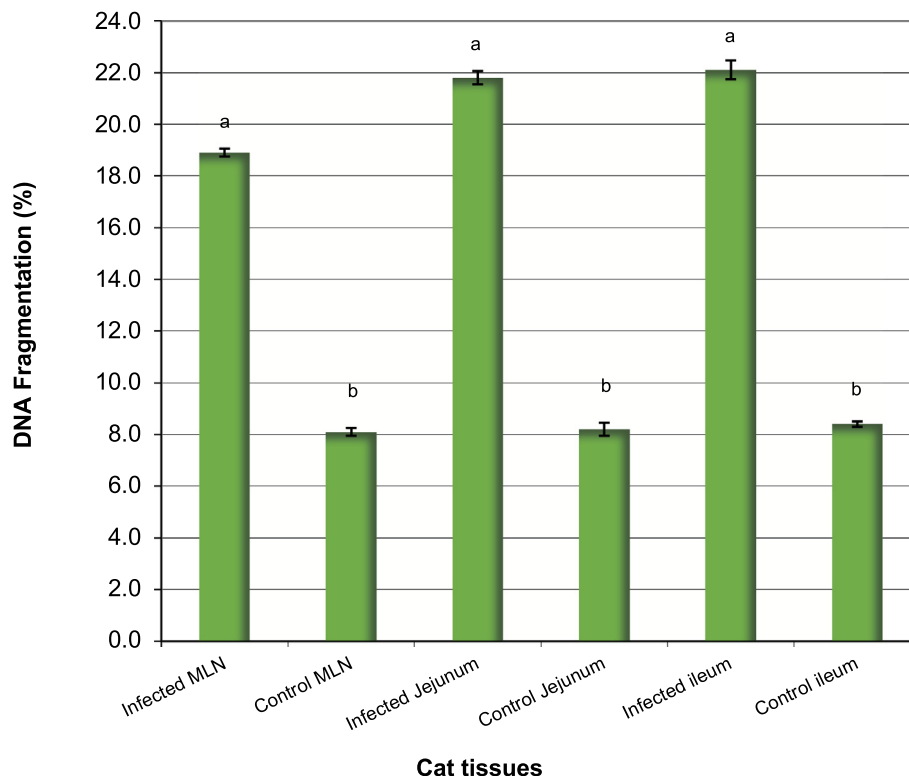


Fig. 4. DNA fragmentation in different cat tissues (mesenteric lymph nodes; jejunum and ileum). ^{a,b,c}Mean values within tissue with unlike superscript letters were significantly different ($P < 0.05$).

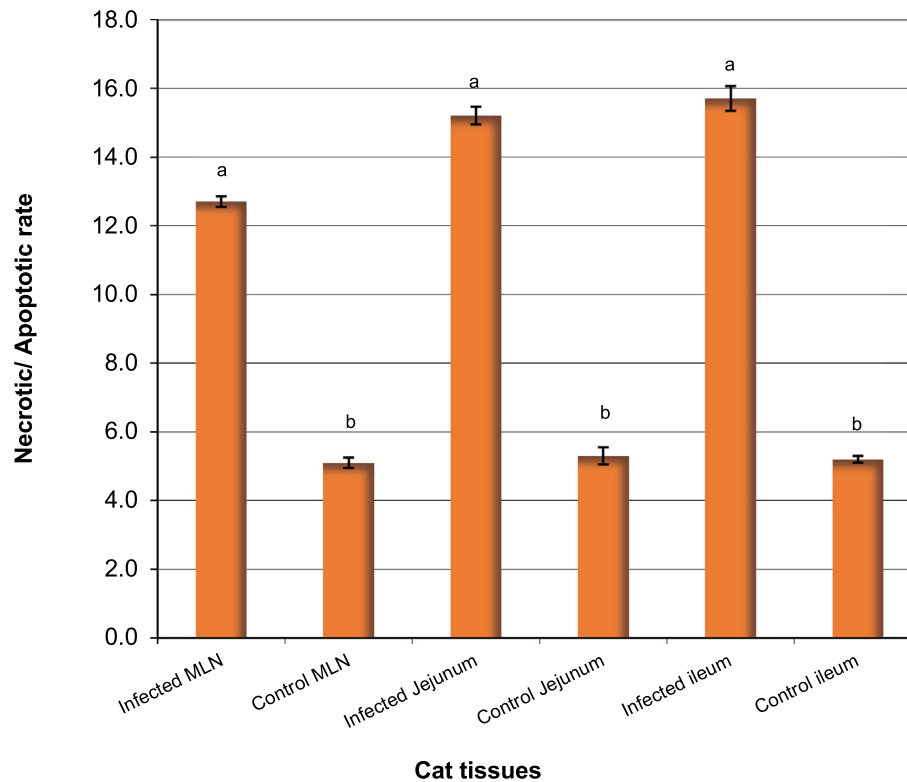


Fig. 6. Necrotic/Apoptotic rate in different infected and normal cat tissues determined by acridine orange/Ethidium Bromide staining. ^{a,b,c}Mean values within tissue with unlike superscript letters were significantly different ($P < 0.05$).

confirmatory test for detection of FP viral genes in targeted tissues of infected cats, while no FP viral gene was detected in targeted tissues of control healthy cats.

Gene expression analysis is a molecular technique not only used as a qualitative tool but also as a quantitative diagnostic test with high accuracy values for detection level of FPV gene in infected cat tissues. Gene expression analysis detected a high level of FPV gene in ileum than jejunum and mesenteric lymph nodes. The difference in increased levels of FPV gene expression was significantly higher between ileum and jejunum, jejunum and mesenteric lymph nodes. These findings may be due to the high affinity tropism of FP virus to the mucosal cell lining of ileum than in jejunum than lymphocytes of mesenteric lymph nodes [5–8].

There was a correlation between results of DNA fragmentation and cell apoptosis. The highest DNA fragmentation rates of infected cats were shown in ileum and jejunum tissues compared with mesenteric lymph nodes. The results revealed that most of infected cat tissues showed a significantly high rate of necrosis/apoptosis compared with control healthy cats. The highest apoptotic ratios of infected cats were observed in ileum and jejunum compared with mesenteric lymph nodes. Cytopathic effect induced during parvovirus infection is mediated by either apoptosis or necrosis and was caused by cell cycle arrest [22]. Cell cycle arrest and caspase activation create an optimum environment for parvovirus replication at the early stage of infection [23,24], the ultimate apoptosis is believed to be beneficial for virus egress at the late stage.

The mechanism of action that infected cats showed high levels of DNA fragmentation and apoptosis is explained by Chen and Qiu [8], who reported that large nonstructural protein (NS)1 and the small NS proteins (e.g., the 11 kDa of parvovirus B19), as well as replication of the viral genome, are responsible for causing infection-induced cell death. So cellular DNA damage response as a result of invading of single stranded parvovirus genome via the terminal repeats and left handed promoter region [8].

Furthermore, several studies indicated that cell death induced in tissue samples of cat infected with *panleukopenia* as well as enteritis apoptosis, widespread tissue damage caused by parvoviruses [5–7]. Also, apoptosis induced by virus infection is thought to be a means for the host to defend itself against viral invasion [20]. Additionally, Galluzzi et al. [25] reported that FP virus kills both erythroid and myeloid colony progenitors, resulting in anemia and leucocytopenia.

Conclusion: The results of the current study conclude that RT-PCR is a highly specific tool and appeared its sensitivity in detecting true infected animals. It can be used for FPV detection even in low log of viraemia which cannot be detected by ELISA but in comparison to ELISA is both costly and time consuming. Moreover, DNA fragmentation, cell apoptosis/necrosis rates can be used as non-specific diagnostic tools for FPV infection. Also, DNA fragmentation is an advanced tool for detecting intracellular damage occurred by FPV inside target tissue by measuring damage (defragmented cellular DNA of target tissue) and supplied us with explanation for cell death mediated either by apoptosis or necrosis. However, these tools could be very useful in studying explanation of the various tissue damages, virus pathoethal pathway measuring damage which irreparable involves vital organs as intestine, bone marrow, lymph nodes and resulting in the death of infected cats in most cases [26,27].

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