Pathological and Molecular Characterization of Feline Panleukopenia in Shirazian Cats

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Abstract
To throw light on the importance of cats as a potential source for genetic diversity of parvoviruses and, since feline panleukopenia virus has re-emerged as a major cause of mortality in felines, the present study has explored for the first time the genomic properties of feline panleukopenia virus (FPLV) detected in shirazian cats population in Egypt during 2012. Panleukopenia viral infection was primary diagnosed from the case history and the pathological findings. A feline panleukopenia virus (FPLV) was detected by PCR from frozen tissues of dead shirazian cats. Specific fragments were obtained by PCR using consensus primers of parvovirus and specific primers of FPLV. Sequence of the VP2 gene of the PCR product was determined and analyzed by comparison with reference FPLV and canine parvovirus (CPV) isolates. Phylogenetic analysis indicated that the isolated FPLV formed a monophyletic branch in FPLV cluster which differed from the other 11 FPV isolates from other countries (available in the GeneBank). The detected virus caused typical clinical symptoms and histopathological changes of FPLV in cats. The most significant histopathological finding was detection of intranuclear inclusion bodies in tissues from different body organs, mainly in highly proliferating tissues as liver. This is the first report on detection of FPV from shirazian cats in Egypt.

Key words: *parvoviruses, feline panleukopenia , shirazian cats .*

Introduction

Feline panleukopnia virus is one of parvoviridae which was identified at the beginning of the 20th century **(Verge and Christoforoni, 1928)**, maintaining a certain degree of genetic stability (**Battilani *et al*., 2006a)**.

FPL virus (FPLV) is a host-range variant of the feline parvovirus (FPV) subgroup consisting of canine parvovirus (CPV), mink enteritis virus (MEV), and racoon parvovirus **(Berns *et al*., 1995)**. These are at least 98% homologous in DNA sequence **(Martyn *et al*., 1990)**, and their antigenic properties are almost identical. The classification of FPVs, therefore, has traditionally been based on the hosts from which they were isolated. That is, FPV strains isolated from cats are classified as FPLV, and those from dogs are classified as CPV. However, it has been shown that some VP2 amino acids are responsible for the specificity of the host range as well as antigenic epitopes of CPVs **(Truyen *et al*., 1995)**. Since cats are susceptible to both CPV-2 and FPV viruses, superinfection and co -infection with multiple parvovirus strains may occur, potentially facilitating recombination and high genetic heterogeneity.

As molecular studies have not been carried out for several decades; therefore the antigenic properties of the currently circulating strains are not well known **(Decaro *et al*., 2008)**.

These viruses cause a variety of serious diseases, especially in young animals; they have a remarkable predilection for replication in rapidly dividing cells **(Ohshima and Mochizuki, 2009)**. Signs of disease include diarrhoea, lymphopenia and neutropenia, followed by thrombocytopenia and anaemia, immunosuppression (transient in adult cats), cerebellar ataxia (in kittens only) and abortion **(Truyen *et al*., 2009)**.

Feline panleukopenia pathogen may survive in the environment for several months and is

highly resistant to some disinfectants. Transmission occurs via the faecal-oral route. Indirect contact is the most common route of infection, and FPV may be carried by fomites (shoes, clothing). Intrauterine virus transmission and infection of neonates can occur. Cats of all ages may be affected by FPV, but kittens are most susceptible. Mortality rates are high - over 90% in kittens **(Truyen *et al*., 2009)**.
The aim of the present study is to record the pathological and molecular characters of FPL-virus in Egypt.

Material and Methods

1. Case History: Thirteen female sherazian cats from a pet shop, at 5-6 months- old were presented with complaints typical of FPL, including vomiting, anorexia, bloody diarrhea, and severe dehydration in some cases. Symptomatic treatments were applied. However, the clinical conditions did not improve significantly, and deaths of all cats within one week of symptoms appearance.
At necropsy: Careful postmortem examination was carried out and specimens from the lungs, liver, kidneys, spleen, cerebrum, cerebellum and different parts of the intestinal tract were collected from the dead cases. Tissue specimens were fixed on formalin for histopathological examination and the other half stored frozen [at – 20°C for genetic analysis].
2. Histopathological Examination: Formalin-fixed tissues were dehydrated in alcohol, cleared in xylene and embedded in paraffin wax. Sections of 4-5 um were stained routinely with haematoxyline and eosin (H&E). Phloxine & tartrazine was used for the detection of inclusion bodies **(Bancroft *et al*., 1996)**.
3. Molecular Investigation: DNA was extracted from 20µg frozen tissue specimens using genejet "Fermentas Cat. No. K0721" according to the manufacturer's instructions.
The entire VP2 gene was amplified with a set of primers designed for both feline and canine parvovirus (FMF forward 5'- GCTTTAGATGATACTCATGT -3' and FMR reverse 5'- GTAGCTTCAGTAATATAGTC -3') **(Mochizuki *et al*., 1996)**. Reactions of amplification were carried out Using Taq PCR Master Mix Kit (Qiagen, Cat. No. 201443). PCR reactions were done in 100 µL mixture reaction, according to the instruction of the manufacturer. The temperature cycling protocol consisted of hot start at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 30 sec., annealing at 55°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. A 5µL aliquot of PCR product was analyzed by electrophoresis using a 1% agarose gel and ethidium bromide staining. The targeted DNA fragments were purified using QIA-quick Gel Extraction Kit (Qiagen, Cat. No.28704). Purified PCR products were sequenced directly using a Prism Big Dye v3.1 kit (Applied Biosystems, Cat. No. 4336917) on an ABI 310 DNA automated sequencer **(Applied Biosystems) (Nguyen *et al*., 2004)**. All samples were analyzed in both forward and reverse directions. The Partial VP2 nucleotide sequences obtained were compared to sequences available from the GenBank and alignment of the sequences was carried out using the CLUSTAL W web interface.
Isolate consensus sequences were published previously and are available under GenBank accession number JQ801345

Results

1.Necropsy Findings: On postmortem examination the prominent pathological findings were hemorrhage at the mucous membrane of the colon and an anaemic appearance of abdominal organs.
2.Microscopic Findings: Histopathological changes were observed in the brain, intestinal tract, spleen, liver, lungs and kidneys.
Different stages of neural degeneration were recorded in the cerebrum, including shrunken and pyknotic (eosinophilic necrosis) neurons with neurophagia and microglial nodules formation. Mild to moderate hypertrophy of astrocytes was also a prominent feature (Fig. 1). Some neurons had intraneural vacuole formation, while others had extra neuron vacuole (Fig. 2).
Cerebellar changes showed hypocellularity of

molecular layer with marked depletion of granular cell layer. Meanwhile, the Purkinje cells appeared pyknotic, shrunken and were randomly placed (Fig. 3). Ependymal cells were markedly swollen.

The covering villi of small intestine were short, thickened and flattened. The large intestine had large hypertrophoid mucus glands distended with mucus (Fig. 4). Eosinophilic intranuclear inclusion bodies were seen in the lining epithelia (Fig. 5).

Examination of the spleen showed depletion of splenic white follicles with trabecular thickening (Fig. 6).

The liver revealed hepatocellular degeneration in form of hepatocytes vacuolation with distortion of hepatic cords (Fig. 7). Intranuclear inclusion bodies were also detected (Fig. 8).

The alveoli were filled with serous exudates (Fig. 9) associated with massive alveolar macrophage infiltration (Fig.10). Bronchial lining epithelia were hypertrophoid.

The kidneys showed marked enlargement of the proximal convoluted tubules with granular degeneration of the cytoplasm in addition to periglomerular edema (Fig. 11).

Intranuclear eosinophilic bodies were detected in the renal lining epithelia (Fig. 12).

3. Molecular Characterization: In this study we analyzed parvovirus strains detected in domestic cats, Consensus primers of parvovirus were used to distinguish parvovirus from other viruses. Molecular detection of the parvovirus was carried out by applying PCR, detection of FPLV specific region (a fragment of 700 ═ bp) in VP2 gene, and only 614 bp of the VP2 gene sequences were determined (Fig.13).

Sequence analysis: Isolate consensus sequences were published previously and are available under GenBank accession number **JQ801345.** Mutations detected in the course of this study were compared to all publicly available FPV sequences covering the respective genome regions (alignments were identical to those published previously).

Replacement recorded in two position at nt. 304 T→C and common replacement at nt. 570 G → A with FPL –virus had an accession number AB000061 Japan , which were further, analyzed using Megalign (DNASTAR, Window version 3.12e). These sequences were aligned separately to the consensus sequence. Gene rearrangements were confirmed only if the exact boundaries of the deletion or duplication event could be inferred from the sequence information (Fig.14).

A phylogenetic tree was constructed from the partial-length VP2 nucleotide sequence obtained here and additional sequences retrieved from the GenBank database. We constructed a phylogenetic tree by using the entire nucleotide sequences determined from the **JQ801345** EGY **FPV1** isolate and representative nine reference sequences retrieved from GenBank. The **JQ801345** EGY **FPV1** isolate was illustrated in (Fig.15). This tree was established that the new isolate are relatively clustered to FPL-virus.

Discussion

In the present study, the pathological investigation showed clinical appearance of severe emaciation and dehydration. These findings were in close contact with findings of **Kubba *et al.* (2006**) that could be attributed to diarrhea **(Truyen *et al*., 2009)**.

The histopathological findings demonstrated the predilection site of FPLV to be particularly TfR- carrying receptors; as liver and brain **(Macedo and deSousa, 2008)** and rapidly dividing cells such as intestinal epithelium **(Parrish, 1995)**. The pathological findings in the spleen could be attributed to the lymphotropic nature of FPLV in naturally infected animals **(Ikeda *et al*., 1998)**. In the present study atrophy of lymphoid tissues of the spleen might be possible to the ability of FPLV to induce apoptosis as a key element of the pathophysiology of lymphoid tissues associated with FPLV infection **(Ikeda *et al*., 1998)**.

Parvovirus, small DNA- containing animal viruses, have an extremely limited genome, and therefore parvovirus replication is dependent on actively proliferating host cells **(Baranski *et al*., 1988)**. Intestinal epithelia and mucous

gland epithelia are continually proliferating tissues and highly active cells, respectively, which provides a good target for parvovirus replication. Virus replication results in the destruction of germinal epithelial cells in the intestinal crypts, leading to the demonstrated villi shortening and clinically to vomiting and diarrhea **(McKnight *et al*., 2007)**.

Predominantly, transferring receptors play a key role for virus invasion where erythropoiesis and active cell division are active **(Macedo and de Susa, 2008)**. Liver is the main source of transferring synthesis, but other sources, such as the brain, also produce it. FPLV bind to the human and feline transferring receptors [TfRs] and use those receptors to enter and infect cells. FPLV attracted to transferring receptors carrying cells and enter cells by a dynamin - dependent, clathrin- mediated endocytic pathway, then viral capsids colocalize with transferring in perinuclear veaicles of cells shortly after entry **(Parker *et al*., 2001)**. In the present study massive infection of the hepatocytes was indicated by inclusion bodies formation.

The detected cerebellar hypogranular layer was explained by **Re'Sibois *et al*. (2007)** to be a primary neural target of the viral attack. As a consequence of the external granular- cell destruction, feline parvovirus associated cerebellar hypoplasia is generally regarded as a granuloprival disease **(DeLahunta, 1971)**.

The tubular disorganization and modification of glomerular compartment was incorporated with the findings of **Aresu *et al*. (2009)**. The role of transferring in erythropoiesis **(Macedo and de Sousa, 2008)** could explain the presence of inclusion bodies in infected renal tubular epithelia.

The characteristic appearance of the inclusion bodies of FPLV infected cells could be explained by the mechanism of FPLV to condense chromatin along the inner aspect of nuclear membrane **(Ikeda *et al*., 1998)**.

In the current study parvoviruses infecting various wild carnivores were analyzed by DNA sequencing after PCR amplification. Specific primers were used to amplify sequences of the wild carnivore parvoviruses for comparison with typical sequences of other feline parvoviruses. The sequence analysis allowed classification based on conserved nucleotide differences with regarding for the FPL –virus specific region (a fragment of 700 = bp) in VP2 gene. The sequence described was either similar to sequences of typical FPV DNA retrieved from GenBank except two point mutations as descried in results.

Although several mutations were detected in the VPL –Vp2 sequence examined, accumulation of point mutations was not consistent with a clear temporal or geographical distribution. Due to the first- base changeT889G has been recently described for the antigenic variants of CPV-2 currently circulating throughout the world **(Meers *et al*., 2007)**. Residue 297 is located in the antigenic region close to epitope B and substitutions at this position may be responsible for changes in antigenicity ofCPV-2 variants **(Truyen, 2006)**. Analogously, a change at position 440 (T→A) has been described for Italian, Indian, Korean and American CPV- 2 **(Kapil *et al*., 2007)** and for a clone from a mixed CPV-2 population detected in a demostic cat from Italy **(Battilani *et al*., 2006 b)**. As for the FPL-v strains, that change is a consequence of a first - base mutation (A1318G) **(Decaro *et al*., 2008)**. Both residues 297 and 440 are present in the GH loop region of the VP- 2 protein, where the greatest variability among parvoviruses has been obsereved **(Battilani *et al*., 2002)**.

FPLV is highly significant pathogen for various domesticated and wild carnivore species **(Steinel *et al*., 2000)**. Although FLPV maintains certain degree of genetic stability (**Battilani *et al*., 2006b)**, it was incriminated to be the origin of CPV **(Hueffer *et al*., 2004)** with evidence it gave rise to several antigenic variants CPV2s (CPV2a, CPV2b and CPV2c) with a modified ability to naturally infect dogs and cats **(Martella *et al*., 2005)** with a wide range of geographic distribution **(Kapil *et al*., 2007)**.

The present study detected the FPLV infection using PCR and nucleic acid sequencing as a genetic diagnostic tools. Sequencing of the

PCR products and its comparison with reference strains showed low rate.

In the present study, close genome homology and little nucleotide mutation could be attributed to the FPLV character of slow rate of random genetic drift and low genomic substitution rates **(Hoelzer *et al*., 2008)**.

One should be always careful about the cross-contamination of the environment because of their highly resistant nature **(Mochizuki *et al*., 1996)**. FPLV amino acids 80,564 and 568 are required for replication in the cat. Meanwhile, amino acid changes at positions 323,564 of VP2 from FPLV may alter the host range of FLPV, gaining it the ability to infect monkey in captive **(Yang *et al*., 2010)**. The recombination between different FPLV subspecies in nature, which is considered as an element in the generation and evolution of parvoviruses **(Ohshima and Mochizuki, 2009)**, in addition to distribution of FPLV antigenic types in captive large cats suggested an interspecies transmission from domestic dogs **(Steinel *et al*., 2000)**. Furthermore, modified ability to infect primates which had been recorded by positive serosurvallance of CPV in free ranging- Namibina cheetahs **(Parker *et al*., 2001)** give a hypothesis that both of CPV and FPLV could be undergo mutual interspecies transmission between dogs and cats, and it is postulated that they may cause disease in some adventitious hosts **(Mochizuki *et al*., 1996)**. It is well established that certain important human infectious diseases, such as severe acute respiratory syndrome (SARS) and highly pathogenic avian influenza (H5N1, H1N1), have origins in non-human hosts. Gene mutation, deletion or arrangements of some key sites in these viruses have been responsible for changes of their host range and pathogenicity. The isolation of an FPV mutant from a primate, therefore, provides some cause for concern, and indicates the need to conduct further studies of its pathogenesis and mutations.

As molecular studies have not been carried out for several decades; therefore the antigenic properties of the currently circulating strains are not well known **(Decaro *et al*., 2008)**.

According to our knowledge this is the first record and genetic analysis of feline panleukopenia in Egypt.

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Figure legends

-Fig. (1): Photomicrograph of cerebrum showing activation of astocytes. **H&E X400**

-Fig. (2): Photomicrograph of cerebrum showing intraneural and extraneural vacuole formation.

H&E X400

-Fig. (3): Photomicrograph of cerebellum showing purkinje cells appeared pyknnotic, shrunken and randomly placed and oriented. **H&E X 200**

-Fig. (4): Photomicrograph of intestine showing hypertorphoid mucous gland distended with mucous**.**

H&E X400

-Fig. (5): Photomicrograph of colon mucous glands showing eosinophilic intranuclear inclusion bodies in the lining epithelia. **H&E X400**

-Fig. (6): Photomicrograph of spleen showing depletion of spleenic follicles with marked thickening of the trabeculae. **H&E X 100**

-Fig. (7): Photomicrograph of liver showing hepatic cords distortion, hepatocytes vacuolation in addition to eosinophilic intranuclear inclusion bodies. **H&E X400**

-Fig. (8): Photomicrograph of liver showing eosinophilic intranuclear inclusion bodies in the hepatocytes.

Phloxin &tartrazin X400

-Fig. (9): Photomicrograph of lungs showing serous exudate in the alveoli. **H&E X400**

-Fig. **(10):** Photomicrograph of lungs showing massive infiltration of alveolar macrophages in the bronchial lumen. **H&E X400**

-Fig. (11): Photomicrograph of kidneys showing hypertrophy of proximal convulated tubules with granular degeneration of the cytoplasm and even necrosis in addition to periglomerular edema. **H & E X400**

-Fig. **(12):** Photomicrograph of kidneys showing some intranuclear inclusion bodies in the renal lining epithelia. **H&E X400 (In set: Phloxin &tartrazin X400)**

-Fig. (13): Electrophotoretic analysis of PCR products of FPLV-VP2 gene isolates showing expected molecular weight: Molecular weight marker. Lane (2): PCR product = 700bp.

-Fig. (14): Aligment of JQ801345 EGY FPV1 sequences of the new isolate “Partial VP2” and nine reference sequences retrieved from GenBank.

-Fig. **(15):** Phylogenetic relationships among the different parvovirus isolates based on partial- legth VP2 nucleatide sequence. The tree was inferred by DNAstar using the neighour-joining method. For each strain, the country and the GenBank accession number were indicated.

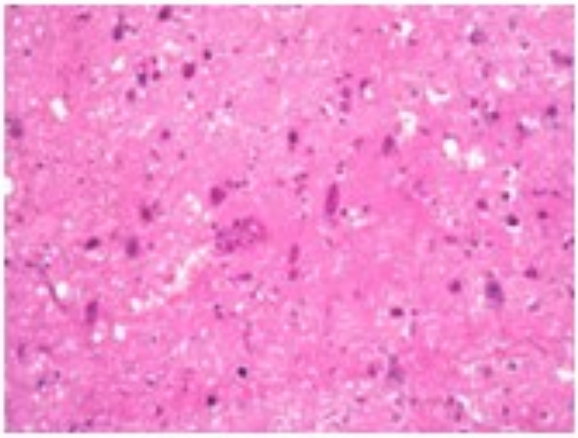
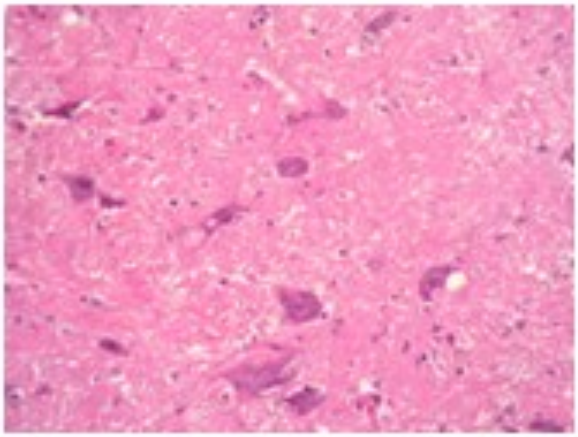


Fig. 1



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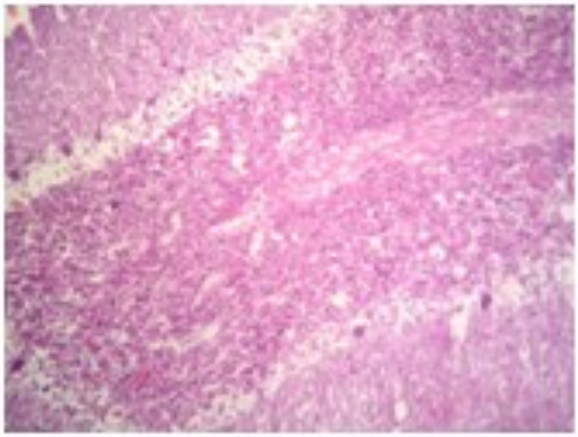

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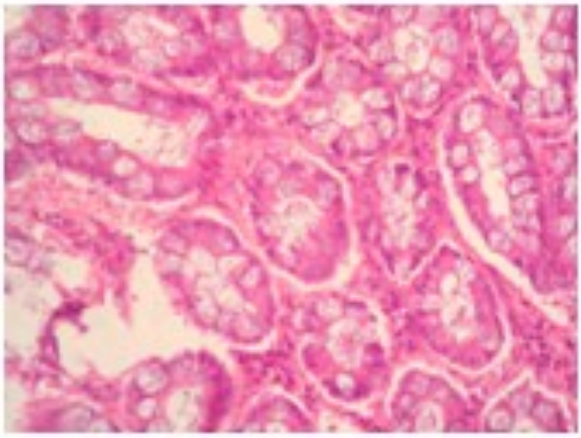

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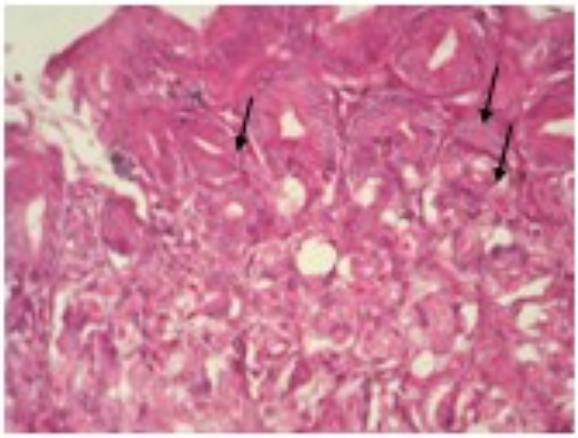

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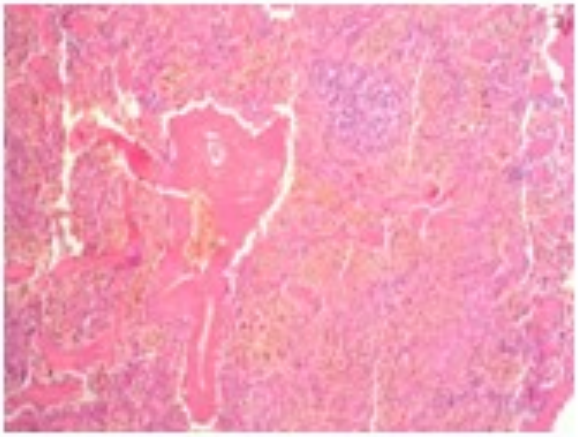

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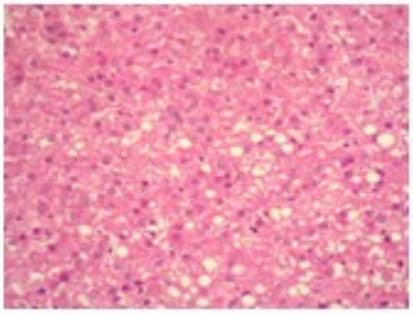

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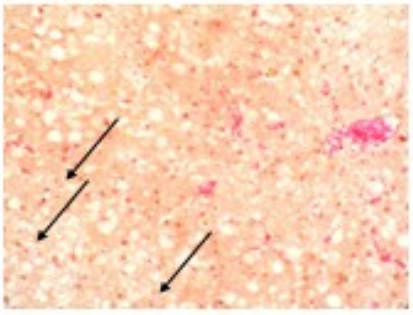


Fig. 8

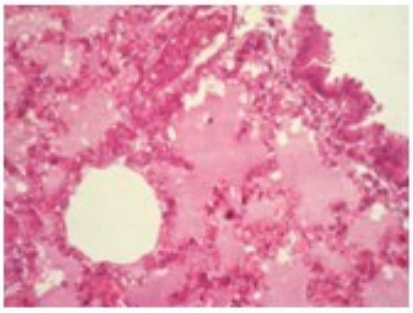


Fig. 9

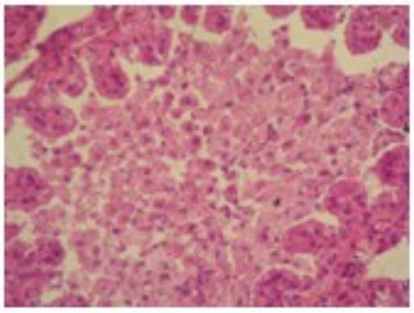


Fig. 10

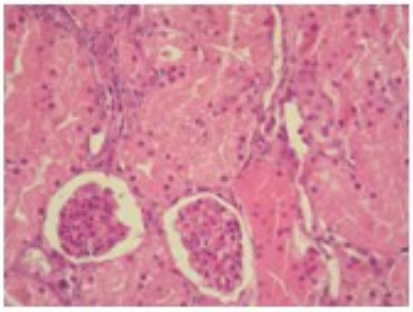


Fig. 11

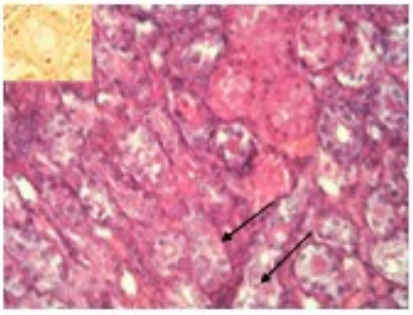


Fig. 12

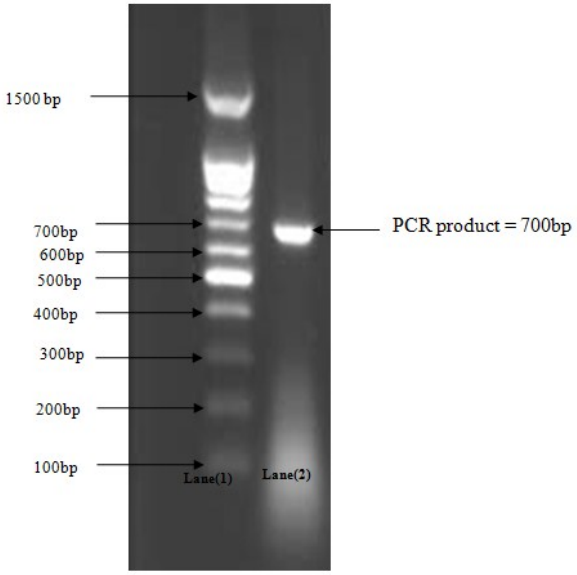
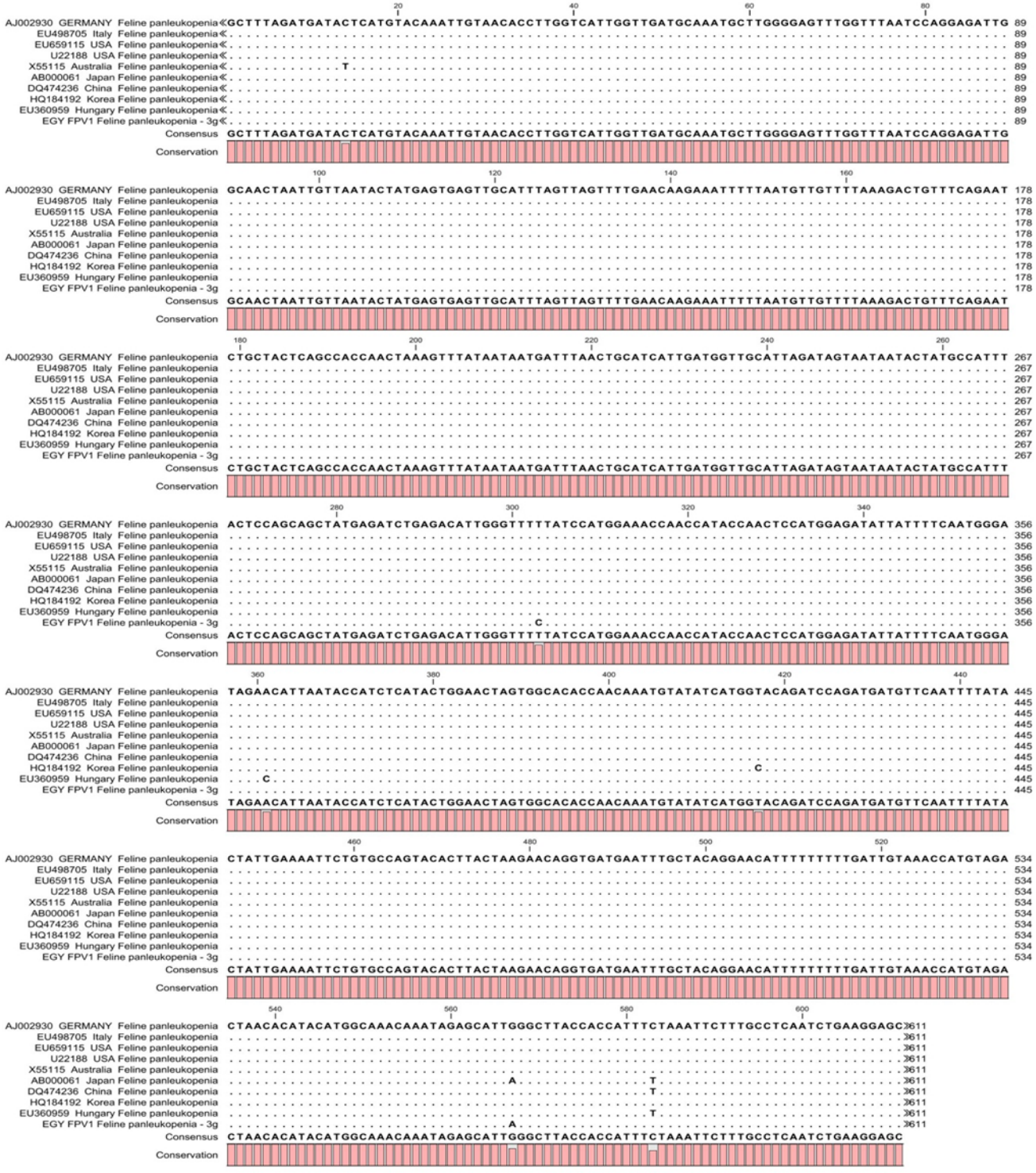


Fig. 13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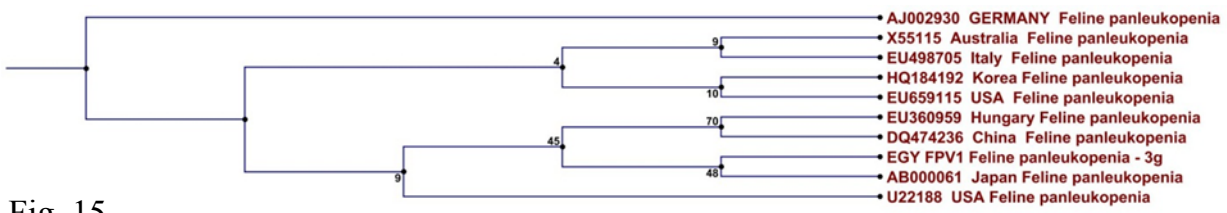
U22188 USA Feline panleukopenia
X55115 Australia Feline panleukopenia
AB000061 Japan Feline panleukopenia
DQ474236 China Feline panleukopenia
HQ184192 Korea Feline panleukopenia
EU360959 Hungary Feline panleukopenia
EGY FPV1 Feline panleukopenia - 3g
Consensus
Conservation

Fig. 14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EGY FPV1 Feline panleukopenia - 3g
AB000061 Japan Feline panleukopenia
U22188 USA Feline panleukopenia

Fig. 15

الخواص الباثولوجية و الجزيئية لمرض نقص الكريات البيضاء فى بعض القطط الشيرازى

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ابراهيم محمد عبد العزيز القلماوى – كيميائى – قسم بحوث البيوتكنولوجى – الشركة القابضة للأمصال و اللقاحات

الملخص العربى

فى ضوء أهمية القطط كمصدر محتمل للتنوع الجينى لفيروس البارفو و لأن مرض نقص الكريات البيضاء فى القطط قد ظهر مرة أخرى كسبب رئيسى للنفوق فى العائلة القططية حول العالم, فأن هذه الدراسة قد أوضحت لأول مرة الخواص الوراثية لفيروس نقص الكريات البيضاء الموجوود فى القطط الشيرازى الموجودة بالبيئة المصرية. وقد أوضحت الدراسة أن التشخيص المبدئى للأصابة بمرض نقص الكريات البيضاء كان من خلال التاريخ المرضى و العلامات الباثولوجية. التحورفى فيروس نقص الكريات البيضاء قد تحدد بواسطة تفاعل البلمرة المتسلسل على الأنسجة المجمدة من القطط الشيرازى النافقة. الأجزاء الخاصة تم الحصول عليه من تفاعل البلمرة المتسلسل بأستخدام بادئ توافقى لعائلة البارفو و بادئ خاص بفيروس نقص الكريات البيضاء بالقطط. التتابع الجينى الخاص بالجين الناتج من تفاعل البلمرة المتسلسل قد تحدد وتم مقارنته بالعزلات المسجلة لفيروس نقص الكريات البيضاء بالقطط و بارفو العائلة الكلبية. تحليل السلالة الجينية أشار الى أن المعزولة لفيروس نقص الكريات البيضاء تكون فرع وحيد المنشأ فى مجموعة فيروسات نقص الكريات البيضاء والتى تختلف عن ال 11 معزولة الأخرى من الدول الأخرى (وفقا لمعلومات بنك الجينات) . الفيروس المعزول سبب أعراض أكلينيكية مطابقة و تغيرات مرضية بالأنسجة فى القطط. من أهم التغيرات المرضية بالأنسجة كان وجود أجسام أحتوائية داخل أ بعض الخلايا وخاصة فى الأنسجة ذات معدل التكاثر العالى مثل الكبد و الأمعاء و الكلى .و تعتبر هذه الدراسة هى أول تسجيل علمى لفيروس نقص الكريات البيضاء فى القطط الشيرازى بمصر. الخلاصة: مما سبق يتبين لنا وجود معزولة ججديدة لمرض نقص الكريات البيضاء بالقطط ولكنه يحدث نفس التأثيرات المرضية