Molecular diagnosis of Feline Immunodeficiency Virus (FIV) in cerebrospinal fluid (CSF) and blood by RT-PCR and PCR methods among stray cats and relationships with clinical signs

H. MOMTAZ1*, M. H. SALJOOGHIAN ISFAHANI2, S. ADIBI3, M. MOMENI4, N. SOUOD5

1Department of Microbiology, Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, P.O. Box: 166, Shahrekord, IRAN.
2Young Researcher’s Club, Shahrekord Branch, Islamic Azad University, Shahrekord, IRAN.
3Arman Pajuhan Ave Sina (ARPA Sina) Cooperative Research Center, Isfahan, IRAN.
4Research Center of Biotechnology, Shahrekord Branch, Islamic Azad University, Shahrekord, IRAN.
5Young Researcher’s Club, Jahrom Branch, Islamic Azad University, Jahrom, IRAN.

*Corresponding author: hamomtaz@iaushk.ac.ir or hamomtaz@yahoo.com

SUMMARY

The purposes of this study were to detect the FIV (feline immunodeficiency virus) infection using PCR from blood and cerebrospinal fluid (CSF) samples and RT-PCR from CSF samples in a stray cat population (n = 100) from the Isfahan Province, Iran and to correlate the FIV status with clinical signs. The FIV prevalence evidenced by blood PCR was strong (64%), males and adults were significantly more often infected and the occurrence of clinical signs and particularly of oral diseases were significantly in FIV positive animals. Whereas PCR applied to CSF samples failed to detect the virus, the viral RNA fragments were positively detected by RT-PCR in 40.6% (26/64) of CSF samples from FIV positive cats but were not associated with neurological signs. These results show that PCR from blood samples is useful for detecting FIV infection in cats with unknown serological status and that the central nervous system contamination involves cell free virus particles and is not strictly associated to neurological disorders.

Keywords: Stray cat, Feline immunodeficiency virus, PCR, RT-PCR, Peripheral blood mononuclear cells, cerebrospinal fluid, risk factor, Iran.

RÉSUMÉ

Diagnostic moléculaire du virus de l’immunodéficience féline dans le liquide cérébrospinal et dans le sang par RT-PCR et PCR chez les chats errants et relations avec les signes cliniques observés

Les objectifs de cette étude étaient de détecter le virus de l’immunodéficience féline (FIV) par PCR à partir du sang et du liquide cérébrospinal (LCS) et par RT-PCR à partir des échantillons de LCS obtenus sur 100 chats errants de la province d’Isfahan (Iran) et de corrélérer le statut viral avec la survenue de différents signes cliniques. La prévalence du FIV déterminée par PCR sur les échantillons sanguins s’est avérée élevée (64 %), les chats mâles et les adultes étant le plus souvent infectés et l’apparition d’affections de la sphère buccale étant significativement plus fréquente chez les chats FIV positifs. Alors qu’aucun prélèvement de liquide cérébrospinal ne s’est révélé positif par PCR, l’ARN viral a été mis en évidence par RT-PCR dans 40.6 % (36/64) des échantillons issus de chats FIV positifs sans être associé avec l’existence de signes neurologiques. Ces résultats montrent que la PCR à partir des échantillons sanguins est utile dans le diagnostic de l’infection chez des animaux dont le statut sérologique est inconnu et que la contamination du système nerveux central implique des particules virales libres mais n’est pas associée à une quelconque atteinte neurologique.

Mots clés : Chat errant, virus de l’immunodéficience féline, PCR, RT-PCR, cellules mononucléées sanguines périphériques, liquide cérébrospinal, facteur de risque, Iran.

Introduction

Feline immunodeficiency virus (FIV), a T-lymphotropic lentivirus that causes an immunodeficiency syndrome in cats, was isolated in Davis, California in 1986 [9, 22]. FIV shares many properties characteristic of other lentiviruses such as human immunodeficiency virus (HIV), but it is antigenetically distinct. It is, therefore, considered as a useful viral toll for AIDS studies in small animals. In addition, it is a widespread threat to the health of domestic cats [14, 22]. FIV is a major pathogen of domestic cats in the world. As with HIV infection in humans, FIV infection in cat results in an acute transient illness, lymphadenopathy and an asymptomatic period characterized by down modulation of viral replication, progressive decline in CD4+ lymphocyte numbers, decline in the CD4+/CD8+ ratio, and progressive impairment of immune function [6]. The major route of transmission of FIV appears to be biting [35]. Free-roaming male cats engaged in territorial behaviour are exposed to the highest risk for infection in nature.

Neoplastic diseases, particularly lymphomas, are commonly observed in cats infected with FIV [12]. Lentiviruses are generally believed to play an indirect role in the development of tumours, but a recent study supported a direct role of FIV in B-lymphocyte transformation [6]. Naturally infected FIV-positive (FIV+) cats exhibit clinical signs of fever, haemopoietic
disorders, dermatitis, otitis, lymphadenopathy, stomatitis, gingivitis, neurological diseases, ocular diseases, weight loss, lethargy, anorexia, emaciation, vomiting, cystitis, nephritis, diarrhea, abscesses, skin diseases, renal insufficiency and failure, hepatic disease and upper respiratory tract infections [8, 23, 28]. Clinical signs are most often a reflection of opportunistic infections, neoplasia and/or myelosuppression, as the disease progresses [28]. Like in HIV and simian immunodeficiency virus (SIV) infections, FIV is likely to enter the brain via the blood-brain and blood-cerebrospinal fluid (CSF) barriers [4, 19, 26], and thus understanding the mechanisms of interactions of virus and cells at both of these barriers is important in the quest to design therapies to prevent or modify central nervous system (CNS) infections.

A diagnosis of infection is made by detection of specific antiviral antibodies in serum. Almost all infected cats develop antibodies to FIV within 2 to 6 weeks after infection. Several serological methods for detecting antibodies to feline immunodeficiency virus have been described, e.g., indirect fluorescence [24], virus neutralization [31], and different indirect ELISAs combined with immunoblot [3, 15]. Recently, recombinant viral proteins-based rapid immuno-chromatography tests and ELISA tests have found wide application in the detection of antiviral antibodies [25]. However, FIV isolation from cultured peripheral blood mononuclear cells (PBMC) of stray cats with no detectable antibody in the serum has been reported [11]. No evidence links FIV infection to any human disease, including AIDS. Investigations have failed to identify antibodies in people that have been bitten by infected cat or who have inadvertently injected themselves with virus-containing material [9]. The detection of proviral DNA in peripheral blood monocytes allows identification of the virus independently of the presence of antibodies or viremia. The PCR technique is extremely sensitive and is considered as a dependent method currently used in research cats. The method theoretically allows the detection of a single DNA molecule in a background of $10^5$ cells [27].

The aims of the present study were to identify the FIV by PCR in blood and CSF, and by RT-PCR in CSF, and to compare the efficiency of these two techniques for diagnosis of FIV infection in cats.

### Materials and Methods

#### ANIMALS AND SAMPLING

A total of 100 stray cats (40 females and 60 males) between 5000 stray cats were used in the study. They were stemming from different areas of the Isfahan province and 42 were less than 2 years old, 36 were 2-5 years old and 22 were older than 5 years old. They have been captured by trapping (baited cage-traps) and they were anaesthetized using 0.8-1.5 mL Ketamine 10% intramuscularly for blood and CSF sampling.

For CSF sampling, the atlanto-occipital area is clipped and surgically prepared under aseptic condition; the needle is inserted through the skin in the dorsal midline at level of the cranial border of the wings of the atlas. Once the skin has been penetrated, the stylet of the needle is removed. The needle is advanced very slowly, until cerebrospinal fluid is seen to flow into the hub. The cerebrospinal fluid (0.5-1 mL) was collected from each stray cat and stored at -70°C prior testing.

Whole blood samples (2-4 mL) were obtained from each cat by puncture of the jugular vein and cephalic vein into sterile microtubes containing EDTA as anticoagulant. After centrifugation (2 000g, 10 minutes, 4°C), the buffy coat layer was isolated and stored at -20°C prior testing.

### DIRECT FIV DIAGNOSTIC METHODS

#### Polymerase chain reaction (PCR)

DNA was isolated from the cerebrospinal fluid and blood samples using Genomic DNA purification kit (Fermentas) according to the manufacturer’s recommendations and the optical density was measured at 260 nm. Isolated DNA from CSF and blood samples were used for PCR.

The oligonucleotide primers used for PCR were from the p24 region of FIV. Their sequences were described by MATTEUCCI et al. [21] and were presented in Table I. The amplification reactions were performed in 50 µL reaction mixtures containing 0.1 mM of each deoxynucleotide, 15 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 9), 2 mM MgCl$_2$, 10% dimethyl sulfoxide (DMSO, Sigma), 1 U of Taq DNA polymerase (Fermentas) and 40 ng of template DNA. The PCR reaction was carried out in a PCR programmed thermocycler (Eppendorf, Master-cycler 5330, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) using the thermal profiles: initial cycle 94°C for 3 minutes, followed by further 40 cycles (with denaturation at 94°C for 60 seconds, annealing at 50°C for 60 seconds and extension by polymerase at 72°C for 120 seconds) and the final cycle was run at 72°C for 5 minutes. Finally 20 µL of final PCR products were run on a 1.5% agarose gel containing ethidium bromide in 1X Tris Borate EDTA (TBE) buffer along with 100 bp DNA ladder (Fermentas).

#### Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from CSF samples using the RNeasy Total RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Synthesis of cDNA was carried

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’-3’)</th>
<th>PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIV-F</td>
<td>GGCATATCCCTATTCACACAG</td>
<td>675 bp</td>
</tr>
<tr>
<td>FIV-R</td>
<td>AAGAGTTGCAATTATATCC</td>
<td></td>
</tr>
</tbody>
</table>

Table I: Primers used for PCR and RT-PCR analyses of FIV from CSF (cerebrospinal fluid) and blood samples in stray cats [21].
MOLECULAR DIAGNOSIS OF FELINE IMMUNODEFICIENCY VIRUS

out using Moloney murine leukaemia virus reverse transcriptase (MMLV-RT, Fermentas) and random hexamere primers (Fermentas). Reverse transcription of heat-denatured RNA (5 minutes at 70°C in 32 μL of reaction buffer for MMLV-RT in the presence of 0.1 mM of each dATP, dCTP, dGTP and dTTP) was performed after addition of 8 μL of reaction mixture (10 mM dithiothreitol, 0.4 μg of random hexamere primers, 5 U of RNase inhibitor (Fermentas) and 400 U of MMLV-RT) for 5 minutes at 22°C, 15 minutes at 37°C and 30 minutes at 42°C.

After reverse transcription, the reactions were heated to 99°C for 5 minutes in order to inactivate MMLV-RT. Amplification of cDNA by PCR was carried out in a total volume of 50 μL in the reaction buffer for Taq DNA polymerase containing 1 U of Taq DNA polymerase (Fermentas), 10 pmol of each primer (FIV-F, FIV-R), 1 mM MgCl₂, 0.1 mM dNTP, and 2.5 μL of cDNA. The thermal program was similar to the previous method.

STATISTICAL ANALYSIS

The association test x², software SPSS, ver. 16 was used to calculate p values. To calculate concordance level, P value was used with a confidence level of 95%.

Results

Using PCR in blood samples, the overall prevalence of the feline immunodeficiency virus (FIV) in the stray cat population was 64% whereas this technique failed to detect the virus from the CSF samples (prevalence: 0%).

By contrast, the RT-PCR applied to the CSF samples has allowed the FIV detection in 26 cats. As shown in Table II, all cats for which the RT-PCR in CSF sample was positive have also given positive results with PCR from blood samples and no CSF sample from blood PCR negative cats was positive for RT-PCR. Consequently, the relative RT-PCR sensitivity and specificity to PCR were 40.63% and 100.00% leading to an agreement score between the 2 methods of 62.00%.

As shown in Table III, whereas the proportions of positive cats by PCR in blood samples were not significantly affected by the gender, the proportion of males detected as positive using RT-PCR from CSF samples was significantly higher than that of females (P < 0.001). Furthermore, the proportions of FIV positive cats have increased with the age of animals whatever the molecular method used (P < 0.001).

Clinical signs encountered in the stray cat population according to their FIV status detected by PCR from blood samples and by RT-PCR from CSF samples were listed in Table IV. It

<table>
<thead>
<tr>
<th>PCR in blood samples</th>
<th>Positive (n = 64)</th>
<th>Negative (n = 36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR in CSF samples</td>
<td>Positive (n = 26)</td>
<td>0</td>
</tr>
<tr>
<td>Positive (n = 74)</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Relative Sensitivity</td>
<td>40.63%</td>
<td></td>
</tr>
<tr>
<td>Relative Specificity</td>
<td>100.00%</td>
<td></td>
</tr>
<tr>
<td>Agreement score</td>
<td>62.00%</td>
<td></td>
</tr>
</tbody>
</table>

Table II: Relative technical performances of the RT-PCR in CSF samples to the PCR in blood samples for the FIV detection in the stray cat population (n = 100).

<table>
<thead>
<tr>
<th>PCR in blood samples</th>
<th>Positive (n = 64)</th>
<th>P</th>
<th>RT-PCR in CSF samples</th>
<th>Positive (n = 26)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Positive (n = 40)</td>
<td>57.5% (23)</td>
<td>NS</td>
<td>Positive (n = 26)</td>
<td>17.5% (7)</td>
</tr>
<tr>
<td>Males (n = 60)</td>
<td>68.3% (41)</td>
<td></td>
<td></td>
<td>31.6% (19)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>Below 2 year (n = 42)</td>
<td>54.7% (23)</td>
<td></td>
<td></td>
<td>14.3% (6)</td>
</tr>
<tr>
<td>Between 2 -5 year (n = 36)</td>
<td>63.9% (23)</td>
<td></td>
<td></td>
<td>25.0% (9)</td>
<td></td>
</tr>
<tr>
<td>Above 5 year (n = 22)</td>
<td>81.8% (18)</td>
<td>&lt; 0.001</td>
<td></td>
<td>50.0% (11)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Table III: FIV status detected by PCR from blood samples and by RT-PCR from CSF samples of the stray cats (n = 100) according to the gender and to the age.
was firstly observed that the clinical signs whatever their nature were significantly ($P < 0.05$) more frequently observed in FIV positive cats than in FIV negative ones. Moreover, dental and oral (stomatitis and gingivitis) diseases, lymphadenopathy, ocular diseases and recent fight wounds were the most common clinical features found in the whole cat population, followed by respiratory diseases and fever while any neurological signs or mesenteric lymphadenomegaly detected throughout abdominal palpation were not evidenced. Nevertheless, only the frequency of oral diseases was significantly enhanced in FIV positive cats compared to negative ones ($P < 0.05$) and was associated to a high odds ratio (4.242). At a lesser extend, the odds ratios for bite wounds, respiratory and ocular diseases were also elevated in FIV positive cats. In addition, the FIV positive status detected specifically using RT-PCR in CSF samples was not significantly coupled to the occurrence of specific clinical signs.

**Discussion**

The overall prevalence rate of the FIV infection found among the 100 stray cats tested in this study was 64% which is markedly higher than the prevalence rates previously reported in other countries such as North America (2.5%) [16], Ontario (5.9%) [17], Atlantic Canada (7.6%) [10], Germany (10%) [33], Australia (20.8%) [20], Turkey (22.3%) [36], Italy (24%) [2] and Japan (43.9%) [13]. The greater prevalences observed in males and in adult cats (more than 6 years old) are in agreement with previous studies [13, 16, 33-35]. Because of the large population of stray cats in the studied country and probably the strong prevalence of feline immunodeficiency virus (FIV) among them, therefore the FIV infection appears as a treat for house cats as well. The main route of FIV transmission appears to be biting in this study, FIV positive cats presenting more often recent fight wounds than negative cats. Therefore, males and adults cats which frequently fight may be more susceptible to FIV infection. As a consequence, because of the large population of stray cats in Iran, territorial aggressiveness and courtship fighting, the FIV transmission risk to healthy stray and house cats that may have contacts with infected animals is increased. Clients and staff should be educated about disease prevention, and this information should be reinforced with pamphlets and written protocols. There is evidence that neutering and spaying do not have a statistically significant effect on FIV prevalence [10, 36]. In addition, although neutered cats do not indulge in courtship fighting, they still retain territorial aggressiveness [36].

The PCR is considered as a rapid diagnosis method for the FIV provirus detection in peripheral blood mononuclear cells of infected cats and could be useful as an additional method to confirm the FIV infection in animals with undetermined serological status [29]. However, CELER Jr et al. [3] have demonstrated that out of 17 FIV seropositive cats, the semi-nested PCR failed to detect the proviral DNA in 3 blood samples, indicating that the proportions of infected peripheral blood mononuclear cells were not sufficient for blood PCR in some infected cats. In this study, all the CSF samples were negative with the PCR method for FIV detection, while some CSF samples (26) from FIV positive cats (identified by blood PCR positivity) were also positive with RT-PCR. These results are in agreement with the findings of LIU et al. [18], the appearance of viral RNA in the cerebrospinal fluid suggesting an efficient route of virus transfer across the blood-CSF barrier. Brain capillary endothelial cells were found to be relatively resistant to infection with both cell-free and lymphocyte-associated FIV [5]. Therefore, direct infection of endothelium probably does not serve as an important route for FIV entry into the CNS [5], but in this study as shown by combined results from PCR and RT-PCR it seems that cell-free FIV entered into the CNS, while lymphocyte-associated FIV (FIV provirus) did not. This probable pattern of CNS contamination may explain not only the important discrepancy found between the PCR

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**TABLE IV: Associations between clinical signs and the FIV status detected by PCR from blood samples and by RT-PCR from CSF samples of the stray cats (n = 100).**

<table>
<thead>
<tr>
<th>Clinical Signs</th>
<th>PCR in blood samples (n = 64)</th>
<th>RT-PCR in CSF samples (n = 74)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With clinical signs</td>
<td>Positive &amp; Negative OR P</td>
<td>Positive &amp; Negative OR P</td>
</tr>
<tr>
<td>Dehydration</td>
<td>32 (n = 64) &amp; 9 (n = 36) 3.000 &lt; 0.05</td>
<td>19 (n = 26) &amp; 6 (n = 74) 30.762 &lt; 0.05</td>
</tr>
<tr>
<td>Fever</td>
<td>3 (n = 64) &amp; 2 (n = 36) 0.836 0.848</td>
<td>1 (n = 26) &amp; 0 (n = 74) - 0.90</td>
</tr>
<tr>
<td>Oral disease</td>
<td>8 (n = 64) &amp; 5 (n = 36) 0.886 0.843</td>
<td>4 (n = 26) &amp; 3 (n = 74) 4.303 0.051</td>
</tr>
<tr>
<td>Ocular disease</td>
<td>26 (n = 64) &amp; 5 (n = 36) 4.242 &lt; 0.05</td>
<td>17 (n = 26) &amp; 4 (n = 74) 33.056 &lt; 0.05</td>
</tr>
<tr>
<td>Respiratory disease</td>
<td>10 (n = 64) &amp; 5 (n = 36) 1.148 0.815</td>
<td>8 (n = 26) &amp; 3 (n = 74) 10.519 &lt; 0.05</td>
</tr>
<tr>
<td>Skin disease</td>
<td>7 (n = 64) &amp; 3 (n = 36) 1.351 0.677</td>
<td>4 (n = 26) &amp; 3 (n = 74) 4.303 0.051</td>
</tr>
<tr>
<td>Neurological signs</td>
<td>4 (n = 64) &amp; 2 (n = 36) 1.133 0.888</td>
<td>4 (n = 26) &amp; 0 (n = 74) - 0.001</td>
</tr>
<tr>
<td>Bite wound</td>
<td>0 (n = 64) &amp; 0 (n = 36) ND ND</td>
<td>0 (n = 26) &amp; 0 (n = 74) ND ND</td>
</tr>
<tr>
<td>Abscess formation</td>
<td>10 (n = 64) &amp; 3 (n = 36) 2.037 0.298</td>
<td>4 (n = 26) &amp; 1 (n = 74) 13.273 &lt; 0.05</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>0 (n = 64) &amp; 0 (n = 36) ND ND</td>
<td>0 (n = 26) &amp; 0 (n = 74) ND ND</td>
</tr>
<tr>
<td>MLA</td>
<td>11 (n = 64) &amp; 6 (n = 36) 1.038 0.947</td>
<td>5 (n = 26) &amp; 2 (n = 74) 8.571 &lt; 0.05</td>
</tr>
</tbody>
</table>

PCR: Polymerase Chain Reaction; RT-PCR: Reverse Transcription – Polymerase Chain Reaction; CSF: cerebrospinal fluid; MLA: mesenteric lymphadenomegaly; OR: Odds ratio; ND: not determined.
and RT-PCR methods applied to CSF samples, but also the mild agreement score (62%) between blood PCR and CSF RT-PCR for the FIV status establishment. It is documented that clinically apparent neurological signs may be present in up to 30% of infected cats and subclinical abnormalities would be detectable by electrophysiological evaluation in a greater percentage of cats [32]. However, in the present study, although FIV infects the CNS in the most naturally infected cats, none neurological sign (including twitching movements of the face and tongue, psychotic behaviour, compulsive roaming, dementia, loss of bladder control, disturbed sleep patterns, nystagmus, ataxia and seizures) was evidenced in the FIV positive cats, even in those with a RT-PCR positive cerebrospinal fluid.

There are 5 subtypes (A to E) of FIV isolates based on the sequence diversity in variable regions (V3 to V5). Despite the heterogeneous geographic distribution, most FIV isolates belong to either subtype A or B. Studies have hypothesized that subtype B is in a more advanced state of host adaptation and may, therefore, be less pathogen than the subtype A [1, 29]. Cats infected with the subtype A virus developed progressive immunological abnormalities and severe clinical signs of immunodeficiency syndrome for 8 months to 8 years after infection, whereas cats infected with the subtype B did not show any significant clinical signs of immunodeficiency syndrome during the same time period [27]. No study has been still performed to evaluate the prevalence of various FIV strains in Iran and this warrants further investigations. Nevertheless, as it is found in the present study that oral diseases were significantly more common in FIV positive cats than in negative ones, systemic immunosuppressive viral effects which would allow microbial invasion of the oral cavity [7, 30, 34] may be evoked and suggested at least a marked incidence of the subtype A.

As a conclusion, the FIV infection is largely present in the stray cat population in the Isfahan Province, Iran and is become endemic probably because of the frequent fights involving mainly males and adult animals. The viral infection can be directly and rapidly detected using PCR from blood samples, particularly in cats with an unknown serological status, but not from CSF samples because infected peripheral mononuclear cells cannot pass across the blood-CSF barrier. Only viral RNA fragments can be detected with RT-PCR in CSF samples but their presence was not systematic in FIV positive cats and was not obligatory linked to neurological anomalies.

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References


