

**HIGH PREVALENCE OF FELINE IMMUNODEFICIENCY VIRUS (FIV) AND FELINE LEUKEMIA VIRUS (FeLV) IN SLOVENIA**

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*The purpose of this study was to determine the prevalences of FIV and FeLV infections in domestic cats by the enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR). Of the 42 cats examined by the ELISA test, 14 (33.3 %) were seropositive for FIV antibody, 10 (23.8 %) for FeLV antigen, 1 (2.4 %) was positive for both viruses and 17 were negative for infection with either virus. In the PCR assay, 17 cats (40.5 %) were positive for FeLV, 11 (26.2%) for FIV, 9 (21.4%) for both viruses and 5 cats (11.9%) were negative for the presence of viral DNA. The average age of FIV positive cats was 6 and of FeLV 7 years. Infections were more common in outdoor cats (65% FIV, 61.1% FeLV positive) and in male cats (71.4% FIV, 80% FeLV). The results of both tests used in this study have shown high prevalence of both infections among cats in Slovenia. The ELISA method was found useful as a screening test for fast detection of FIV and FeLV infections, but additional testing with PCR was shown to be more effective in the characterization and progression of both infections.*

*Keywords: FIV, FeLV, ELISA, PCR assays*

INTRODUCTION

The feline immunodeficiency virus (FIV) is a retrovirus in the subgroup of lentiviruses distantly related to the feline leukaemia virus (FeLV), an oncogenic retrovirus of cats (Goff, 2001). FIV is transmitted primarily by parenteral inoculation of the virus present in the saliva or blood, presumably by bite and fight wounds. Vertical *in utero* transmission (Jordan *et al.*, 1999), mucosal infection through sexual contact (Stokes *et al.*, 1999), and infections of neonates through suckling have also been reported (Allison and Hoover, 2003). Soon after infection, there is a marked viraemia, during which the virus disperses throughout the body from the site of entry (Pedersen *et al.*, 1989). Two to eight weeks after infection, specific antiviral antibodies appear, and the virus becomes difficult to be detected

in the blood. Infected cats enter a period spanning several years with few clinical abnormalities. Most FIV-infected cats developed overt disease from secondary infections, neoplasia, or persistent neurological abnormalities (Hartmann, 1996).

Feline leukaemia virus (FeLV) is a horizontally transmitted, oncogenic, myelosuppressive and immunosuppressive retrovirus (Reinacher, 1989). In contrast to infections with FIV, the different outcome of FeLV infection depends on the interplay of the viral dose and strain and the cat's immunological response. The immunological response is affected by the cat's age, immune status and genetic make up (Hoover and Mullins, 1991; Lutz *et al.*, 1980; Rojko and Kociba, 1991). Most cats infected with FeLV develop a regressive infection characterized by undetectable or transient viraemia and an effective immune response (Hoover and Mullins, 1991; Flynn, 2002). Cats that become persistently infected are viraemic and are diagnosed by the detection of the virus or viral antigens in the blood. These cats have a very high risk of developing a serious disease, and transmit the virus in the saliva to other cats. FeLV can also persist more insidiously, as atypical, latent or sequestered infection. In these situations the detectable virus is absent from the circulation (Hoover and Mullins, 1991).

Most available FIV and FeLV tests for commercial or in-practice laboratories are based on an enzyme-linked immunosorbent assay (ELISA). They enable the detection of FIV antibodies and therefore only works in infections that have stimulated detectable antibodies. The FeLV ELISA test detects FeLV group-specific antigens in the plasma, serum or saliva of cats expressing the viral antigen.

In recent years very sensitive molecular methods enabling the amplification of viral genetic material have been developed and used to detect retroviruses, such as FIV and FeLV (Hohdatsu *et al.*, 1992; Rimstad *et al.*, 1992; Lawson *et al.*, 1993; Matteucci *et al.*, 1993; Momoi *et al.*, 1993; Inoshima *et al.*, 1995; Vahlenkamp *et al.*, 1995; Jackson *et al.*, 1996; Miyazawa and Jarrett, 1997; Ohkura *et al.*, 1997; Leutenegger *et al.*, 1999; Hofmann-Lehmann *et al.*, 2001; Tandon *et al.*, 2005). The polymerase chain reaction (PCR) detects the DNA of FIV after it was integrated into the host's genome. This test confirms that the cat has not only been exposed to FIV, but has become infected. Cats with positive PCR tests should be considered a source of the virus. The PCR has also greatly enhanced the ability to detect FeLV in blood samples particularly in instances where infectious virus particles are non-replicating or are present in very low amounts.

The aim of our study was to determine the prevalence of FIV and FeLV infections in Slovenia as detected by ELISA versus PCR method. Some epidemiological parameters of infected cats such as health status, sex, age and lifestyle were also studied.

#### MATERIAL AND METHODS

Forty-two random source cats of various ages (range 1-11 years) and sexes (28 males and 14 females) were tested in this study. Cats were chosen from a wide region of Ljubljana city. Each cat's sex, body weight, lifestyle and clinical histories

were recorded by the veterinarian in charge by filling in a detailed questionnaire at the time of sample collection. Blood samples were taken by jugular venipuncture by use of vacuum tubes (Mictrotainer™, Beckton and Dickinson, Franklin Lakes, USA) containing K3 EDTA as anticoagulant. Blood was also collected into plain tubes, which stood for 30 minutes prior centrifugation at 1200 rpm for 10 min. Serum was harvested and used for serological tests.

A commercial ELISA kit (Feline Leukemia Virus Antigen/Feline Immunodeficiency Virus Antibody Test Kit, IDEXX Laboratories, Westbrook Maine, USA) was used to determine the presence of antibodies against FIV and FeLV antigen in the serum. This kit detects the FeLV p27 group specific core antigen by using monoclonal antibodies conjugated to an enzyme, and specific antibodies directed to FIV gag or env(gp40) proteins, which indicate that the animal has been exposed to the FIV virus and is indicative of an active FIV infection. Tests were conducted according to the manufacturer's directions and were described as positive or negative.

PCR was performed with the DNA extracted from the cat's whole blood by QIAamp DNA Blood Kit (QIAGEN GmbH, Germany). FIV specific primers based on the nucleotide sequences of the *gag* gene generated a 228 bp in the first and a 168 bp in the second round of amplification (Hofmann-Lehmann *et al.*, 1997). The primers for FeLV detection were selected from a conserved region in the U3 long terminal repeat (LTR). Two specific pairs of primers amplified a 235 bp product in the first round and a 166 bp product in the second round of PCR, respectively (Hofmann-Lehmann *et al.*, 2001; Jackson *et al.*, 1993).

For the first round of DNA amplification 4.0  $\mu\text{L}$  of the isolated DNA was added to a mixture containing 36.0  $\mu\text{L}$  of  $\text{H}_2\text{O}$ , 5.0  $\mu\text{L}$  10x PCR buffer, 1.0  $\mu\text{L}$  dNTP mix (10 mM), 1.0  $\mu\text{L}$  of each forward and reverse primer (20 pmol/ $\mu\text{L}$ ), 1.5  $\mu\text{L}$   $\text{MgCl}_2$  (50 mM) and 0.5  $\mu\text{L}$  Platinum *Taq* DNA polymerase (5 U/ $\mu\text{L}$ ) (Invitrogen, California, USA). After initial denaturation at 94°C for 2 min, the amplification was carried out for 40 cycles (30 sec at 94°C for denaturation, 30 sec at 56°C for annealing and 1 min at 72°C for elongation) in a programmed thermal cycler (T3 Thermocycler, Whatman Biometra, Germany). The appropriate annealing temperature was selected after testing three different temperatures (54°C, 56°C and 58°C) and the optimal  $\text{Mg}^{2+}$  concentration was determined by testing different concentrations of  $\text{MgCl}_2$  in the reaction mixtures (1 to 2.5 mM). For nested-PCR amplification 1.0  $\mu\text{L}$  of the first round PCR product was added to a mixture containing 39.0  $\mu\text{L}$  dd  $\text{H}_2\text{O}$ , 5.0  $\mu\text{L}$  10x PCR pufer, 1.0  $\mu\text{L}$  dNTP mix (10 mM), 1.0  $\mu\text{L}$  of each forward and reverse primer (20 pmol/ $\mu\text{L}$ ), 1.5  $\mu\text{L}$   $\text{MgCl}_2$  (50 mM) and 0.5  $\mu\text{L}$  Platinum *Taq* DNA polymerase (5 U/ $\mu\text{L}$ ) (Invitrogen, California, USA). The cycling conditions were the same as for the first amplification round. PCR products were separated by electrophoresis in 1.8 % ethidium bromide stained agarose gel and documented (Bio Imaging System, GeneGenius Syngene, UK).

## RESULTS

The FIV and FeLV test results obtained by ELISA and PCR assays are presented in Table 1. Of the 42 cats examined by ELISA test, 14 (33.3 %) were seropositive for FIV antibody, 10 (23.8 %) were seropositive for FeLV antigen, 1 (2.4 %) animal was seropositive and 17 (40.5 %) were seronegative for infection with both viruses.

Among 42 cats included in the present investigation 27 cats showed different and non-specific clinical signs of the disease, while others were healthy at the time of diagnosis (Table 1). More outdoor than indoor living cats were determined positive, 65 % FIV- and 61.1 % FeLV-positive cats. In our study both infections were more common in male cats, 71.4 % FIV- and 80 % FeLV-positive. The average age of cats infected with FIV was 6 and those infected with FeLV 7 years.

The confirmation of ELISA test results was done by PCR assay, a more specific and sensitive DNA test, which detects the genetic material of FIV and FeLV. The results are presented in Table 1.

Table 1. FIV and FeLV testing of cats: results of clinical examination and the ELISA and the PCR assays

Sample	Clinical examination*	FIV			FeLV		
		ELISA	PCR	nPCR	ELISA	PCR	nPCR
1	+	-	-	-	-	-	+
2	+	-	-	-	-	-	+
3	-	-	-	+	-	+	+
4	-	-	-	-	-	-	-
5	-	-	-	+	-	-	-
6	-	-	-	+	-	+	+
7	-	-	-	-	-	+	+
8	-	-	-	-	-	+	+
9	-	-	-	-	-	+	+
10	-	-	-	-	-	+	+
11	+	-	-	+	-	+	+
12	-	-	-	-	-	+	+
13	+	-	-	+	-	+	+
14	-	-	-	-	-	+	+
15	-	-	-	-	-	-	-
16	-	-	-	+	-	+	+
17	-	-	-	-	-	-	-
18	+	+	-	+	-	-	-
19	+	+	-	+	-	-	-

Cont. Table 1.							
20	+	+	-	+	-	-	-
21	+	+	-	+	-	-	-
22	+	+	-	+	-	-	-
23	+	+	-	+	-	+	+
24	+	+	-	+	-	-	-
25	+	+	-	+	-	-	-
26	+	+	-	+	-	-	-
27	+	+	-	-	-	-	-
28	+	+	-	+	-	-	-
29	+	+	-	+	-	-	+
30	+	+	-	+	-	+	+
31	+	+	-	+	-	-	-
32	+	-	-	-	+	+	+
33	+	-	-	-	+	+	+
34	+	-	-	-	+	+	+
35	+	-	-	-	+	+	+
36	-	-	-	-	+	+	+
37	+	-	-	-	+	+	+
38	+	-	-	-	+	+	+
39	+	-	-	-	+	+	+
40	+	-	-	-	+	-	+
41	+	-	-	-	+	-	-
42	-	+	-	+	+	+	+
Σ (+)	27	15	0	20	11	22	26

\*Sick....+ / Healthy...-

In total, 20 (47.6 %) cats were FIV positive as determined by PCR. In the performed PCR analysis, additional 6 blood samples of seronegative cats were found FIV-positive in nested-PCR. In all tested animals the level of FIV DNA was very low (detected only in the nested-PCR step). In one cat with positive FIV ELISA test viral DNA was not detected.

Among 42 cats tested also for FeLV by PCR assay 26 (61.9 %) were found positive (Table 1). Another 16 FeLV ELISA-negative cats were determined to have viral DNA in blood samples by the first (13 cats) or at least by the second round (3 cats) of amplification. Most PCR-positive and ELISA-negative cats determined in our study were about 7 years of age with no or unspecific clinical signs of disease. Among all cats tested by PCR assay 9 (21.4%) were found to be co-infected with FIV and FeLV and only 5 (11.9%) were free of infection with either virus.

## DISCUSSION

Serosurveillance already performed in Slovenia ten years ago had shown that FIV and FeLV infected a large range of pet cats; 32.56 % cats had antibodies against FIV and 45.45 % were positive for FeLV antigen, as determined by commercially available ELISA tests (Tozon, 1995). All over the world, the prevalence between 1.4 % and 30.4 % had been reported for FeLV and up to 30.9 % for FIV, depending on the geographical location, life style according outdoor and indoor living cats, and their clinical status (Bandedecchi *et al.*, 2006). The prevalence depends on the type of animal and would be expected to be much higher in sick cats. Four studies from Italy showed marked differences in prevalence between sick and healthy cats. The prevalence of FeLV positive sick cats was found to be 18 % (Bandedecchi *et al.*, 1992) and 15.3 % (Pennisi, 1994), respectively. On the contrary, in healthy cats the prevalence was only 4.7 % (Pennisi, 1994) or 10.6 % (Magi *et al.*, 2002). In the same studies much higher percentages of FIV positive sick cats were found, 24 % (Bandedecchi *et al.*, 1992) and 30.9 % (Pennisi, 1994), respectively, compared with healthy cats in which only 10.7 % (Pennisi, 1994) or 14.1 % (Magi *et al.*, 2002) cats were positive. Similar results were obtained in Spain, 30.4 % FeLV- and 13.9 % FIV-positive sick cats, compared to healthy cats with the prevalence of 15.6 % for FeLV and 8.3 % for FIV (Arjona *et al.*, 2000). In a nationwide study in Germany 13.4 % of cats were found to be FeLV carriers, while 8.4 % showed evidence of FIV infection and 2.1 % were infected with both viruses (Fuchs *et al.*, 1994). Other studies performed in Europe on samples from stray cats have shown 13.2 % FeLV-positive, 5.8 % FIV-positive and 1.0 % FeLV- and FIV-positive cats in Czech Republic (Knotek *et al.*, 1999); 3.8 % FeLV- and 11.3 % FIV-positive cats in Belgium (Dorny *et al.*, 2002) and 3.5 % FeLV- and 10.4 % FIV-positive cats in England (Muirden, 2002). The prevalence in stray cats in USA was only 4.3 % for FeLV and 3.5 % for FIV (Lee *et al.*, 2002). In the population of healthy cats the prevalence of both infections showed mark differences in different parts of the world: 6.5-7.5 % FeLV- and 0.2 % FIV-positive in Australia (Malik *et al.*, 1997), 2.9 % FeLV- and 9.8 % FIV-positive in Japan (Maruyama *et al.*, 2003) and 2.7 % FeLV- and 16.8 % FIV-positive in Turkey (Yilmaz *et al.*, 2000).

Our study showed that more outdoor living cats were determined positive (65 % FIV- and 61.1 % FeLV-positive cats), which is in accordance with previous studies that have also shown that outdoor cats are at a higher risk of infection (O'Connor *et al.*, 1991; Pennisi, 1994; Tozon, 1995; Arjona *et al.*, 2000; Lee *et al.*, 2002) due to their more frequent exposure to these and other infectious agents.

In our study both infections were more common in male cats (71.4 % FIV- and 80 % FeLV-positive). This finding is also in agreement with the study results presented by Arjona *et al.*, (2000), Yilmaz *et al.*, (2000), Lee *et al.*, (2002), Maruyama *et al.*, (2003). Bites appear to be one of the most efficient modes of transmission and are more frequently expressed among more aggressive male cats (Yamamoto *et al.*, 1989). On the other hand, Bandedecchi *et al.*, (2006) found no differences in prevalence between sexes of cats. However, the differences in the rate of FIV positivity among cats have been explained especially in the sense that

estradiol may protect peripheral lymphocytes from apoptosis after stimulation (Hofmann-Lehmann *et al.*, 1998), which makes female cats more efficiently protected from FIV infection.

The average age of cats infected with FIV was 6 and those infected with FeLV 7 years. We could not confirm the observation of previous studies in which the tendency for the prevalence of FeLV decreases with years, because FeLV positive animals often die at a younger age (Arjona *et al.*, 2000).

In our study, FIV and FeLV ELISA test results were confirmed by PCR, a more specific and sensitive DNA test, which detects the genetic material of FIV and FeLV. FIV PCR detects the genetic material in the early stage of infection prior to seroconversion and after it has been integrated into the host's genome. This is the only test available, which confirms infection, rather than exposure.

High prevalence of FIV infection, 47.6% positive cats determined by PCR, in this study confirmed the previous finding concerning high prevalence of FIV infected cats in Slovenia (Tozon, 1995). In FIV PCR analysis, additional 6 blood samples of seronegative cats were found FIV-positive in nested-PCR. We may speculate that these cats were in the early stage of infection when the proviral DNA could be detected in the peripheral blood prior to serum antibodies (Ohkura *et al.*, 1997). In all tested animals the level of FIV DNA was very low (detected only in the nested-PCR step) confirming that the amount of the virus in the blood is low during the clinically silent phase of infection (Pedersen *et al.*, 1989). In one cat with positive FIV ELISA test viral DNA was not detected. However, false positive results obtained by the in-practice ELISA test are not uncommon. It was recommended that these results have to be confirmed with a Western blot analysis (Barr, 1996).

As tested by PCR assay, 61.9 % of all cats tested for FeLV were found positive, which confirmed high prevalence of FeLV infection. The PCR method used in our study detects all three subgroups of FeLV (Miyazawa and Jarrett, 1997). It enables the confirmation of the presence of exogenous FeLV proviral DNA in blood cells. Among 11 FeLV antigen positive cats, 10 of them were also positive by PCR analysis. The ELISA test, whether in-practice or laboratory-based, is best considered as a screening test. In a proportion of samples, the screening test might give a false positive result for the antigen (Kerr and Smith, 1995). Most experts recommend that when a cat is found to be FeLV-positive by ELISA, the diagnosis should be confirmed by a second, different type of test.

Another 16 FeLV ELISA-negative cats were determined to have viral DNA in blood samples by the first (13 cats) or at least by the second round (3 cats) of amplification. Recently, it has been shown that the PCR assay was more sensitive than a FeLV antigen detecting ELISA in very early infections (Tandon *et al.*, 2005). Cats became provirus positive 1-2 weeks before the antigen was detectable. PCR positive and ELISA negative cats were described in experimentally, as well as in naturally FeLV-infected cats (Hofmann-Lehmann *et al.*, 2001). In addition, experimentally FeLV-infected cats that were transiently infected remained positive for viral DNA, even after clearing the antigen from their blood. These animals stay healthy longer and have a significantly longer survival time than cats with persistent FeLV viraemia (Hofmann-Lehmann *et al.*, 1997). Similarly, most PCR-positive and ELISA-negative cats determined in our study were about 7 years of



age with no or unspecific clinical signs of disease. However, the provirus positive status might also contribute to the long-lasting maintenance of protective immunity (Hofmann-Lehmann *et al.*, 2006). Among all cats tested by PCR assay only 5 (11.9%) were free of infection with either virus.

From the results of this study, we may conclude that in Slovenia an important percentage of cats with varied and nonspecific clinical signs are infected with FIV and FeLV. A significantly higher percentage of infected animals was determined by PCR test in comparison to the commercially available ELISA test. The ELISA screening test used in veterinary practice for the detection of FIV and FeLV was shown to be insufficient when the infection had to be determined in apparently healthy cats. Moreover, the PCR test may provide a valuable information of the disease status and progression in FIV and FeLV infected cats.

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**VELIKA UČESTALOST INFEKCIJA VIRUSIMA IMUNODEFICIJENCIJE MAČAKA (FIV) I VIRUSOM MAČIJE LEUKEMIJE (FELV) U SLOVENIJI**

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SADRŽAJ

Cilj ovog rada je bio da se ustanovi učestalost pojave FIV i FeLV zaraza kod domaćih mačaka enzimskim imunoapsorbicionim testom (ELISA) i polimeraznom lančanom reakcijom (PCR). Od ukupno 42 mačke, kod 14 jedinki (33,3 %) potvrdili smo prisutnost specifičnih protutijela protiv FIV u serumu primenom ELISA testa, a kod 10 jedinki (23,8 %) smo potvrdili prisutnost FeLV antigena. Samo jedna mačka (2,4 %) je bila pozitivna, a ostalih 17 su bile negativne na oba virusa. Metodom PCR, 17 (40,5 %) mačaka je bilo pozitivno na FeLV, 11 (26,2%) na FIV, 9 (21,4%) na oba virusa dok je 5 jedinki (11,9%) bilo negativno na prisutnost virusnih DNA. Prosječna starost FIV pozitivnih mačaka je bila 6, a FeLV pozitivnih, 7 godina. Zaraza je bila češća kod mačaka, koje žive u prirodi (65% FIV, 61,1% FeLV pozitivne) i kod muških životinja (71,4% FIV, 80% FeLV). Rezultati testiranja sa obe metode potvrđuju visoku pojavnost ovih zaraza kod mačaka u Sloveniji. ELISA metoda se pokazala korisnom za brzu dijagnostiku FIV i FeLV, a dodatna PCR metoda ima veću osjetljivost.