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# The prevalence of feline leukemia virus and feline immunodeficiency virus in domestic cats in Hungary

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# Abbreviations

| | |
|---|---|
| AIDS | Acquired Immune Deficiency Syndrome |
| bp | Base pair |
| cDNA | Complementary deoxyribonucleic acid |
| CI | Confidence interval |
| DNA | Deoxyribonucleic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| FeLV | Feline leukemia virus |
| FIV | Feline immunodeficiency virus |
| HIV | Human immunodeficiency virus |
| iIFA | Indirect immunofluorescence assay |
| IFN-γ | Interferon gamma |
| IL-2 | Interleukin-2 |
| IL-10 | Interleukin-10 |
| IL-12 | Interleukin-12 |
| LTR | Long terminal repeat |
| PCR | Polymerase chain reaction |
| RNA | Ribonucleic acid |

# 1. Introduction

Feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) both belong to the most common feline diseases worldwide affecting both domestic cats as well as wild felids. Both of them are RNA viruses classified into the family of retroviruses.

FeLV was first described in 1964 and is a highly transmissible enveloped single stranded RNA virus that belongs to the genus of gammaretroviruses ( Jarrett et al., 1964; Willett and Hosie, 2013). The infection with FeLV leads to different often neoplastic related outcomes and in case of progressive infection it can even lead to a fatal disease (Hartmann, 2012). Especially kittens tend to develop a progressive infection if they get infected by the FeLV (Levy et al., 2008).

FIV is allocated to the genus of lentiviruses (Hartmann, 2012). It was initially detected in the mid 1980s in Northern California (Tompkins and Tompkins, 2008). Once a cat gets in contact with the FIV the cat stays infected for life since the virus integrates a DNA copy of its RNA into the cat's healthy genome (Westman et al., 2019).

To check if a cat is infected with one of the two viruses there are in-clinic test kits available that detect the core viral antigen p27 in case of FeLV and antibodies against the protein p24 in case of FIV (Levy et al., 2008).

Worldwide there is a relatively low prevalence of these viruses. FeLV prevalence in Australia is around 2% (Westman et al., 2019) and in the United States and Canada the seroprevalence for FeLV is around 3.1% (Burling et al., 2017). In Germany the prevalence of FeLV is 3.6% (Gleich et al., 2009) and 5.6% in eastern Austria (Firth and Möstl, 2015). The FIV prevalence in Australia is 15% (Westman et al., 2019) and 3.6% in the United States and Canada (Burling et al., 2017). In Germany the prevalence of FIV is 3.2% (Gleich et al., 2009).

The aim of my thesis is to determine the prevalence of these viruses in Hungary in domestic cats and to examine the phylogenetic relationships of the different FIV strains.

# 2. Literature review

## 2.1. FeLV – Feline leukemia virus

FeLV is highly transmissible. Several routes how cats acquire FeLV have been described. The most common ways of viral transmission are: (i) horizontal transmission if the cats live together and therefore get in contact with infected saliva or nasal discharge due to grooming or (ii) vertically (transplacental) from the queen to the kittens. In addition, there are some other routes of infection like infection via drinking of the colostrum of an infected mother, by licking of a queen of her young kittens, by a bite of an FeLV positive cat during fighting or via venereal transmission since virus particles were isolated from epithelia cells of vaginas and semen from tomcats (Levy et al., 2008; Rand, 2009). Bite injuries are highly infective since infectious saliva will enter directly the bloodstream. In fact, any body fluid like nasal discharge, saliva, milk, semen, urine or faeces is a putative source for an infection with this virus (Levy et al., 2008).

After initial infection the virus is first found in local lymphoid tissues, e.g. tonsils and retropharyngeal lymph nodes. Via infected monocytes and lymphocytes the virus enters the bloodstream. After about 12 days it can then be detected also in the bone (Levy et al., 2008). Nowadays it's considered that cats stay infected lifelong even if no acute viremia occurs (Levy et al., 2008). In about 30% of FeLV infections there is a strong enough immune response to prevent virus replication. These cats are antigen negative, but provirus positive and considered as carriers of the virus (Hartmann, 2012). In these cases, FeLV provirus can be detected in the blood by the polymerase chain reaction (PCR) method. Since the provirus is permanently integrated into the genome of infected cells at random sites there is currently no established technique available which can exert viral DNA from the host cell genome leading to a complete viral elimination. These cats are normally not infectious however, their blood should not be used for transfusions (Levy et al., 2008). Severe infectious diseases, cancers or stress can compromise the immune system and reactivate the virus leading to another viremia (Hartmann, 2012). In a cell-free environment the virus is unstable and can survive only for a short period of time (Rand, 2009).

FeLV belongs to the simpler retroviruses which means it encodes only for the three common genes: *gag*, *pol* and *env*. But it does not encode for any additional genes found in other more complex retroviruses like HIV. The *gag* genes encode for structural proteins, the *pol* genes for enzymatic proteins and *env* for the envelope proteins. The *pol* genes are needed for processing all the viral proteins and most important the reverse transcription of the FeLV and

the integration of its DNA into the host genome. The two most important envelope proteins encoded by the *env* genes are the transmembrane protein p15E and the external envelope protein gp70 (Greggs et al., 2011).

After the virus penetrates the membrane of a host cell it matures by reverse transcription of its RNA genome into cDNA (complementary DNA) followed by integration of the DNA into the host cell genome. The transcription machinery of the host cell than will start to synthesize several viral proteins like p15 matrix, p27 capsid, and p10 nucleocapsid (Willett and Hosie, 2013). When the virus leaves the target cell it acquires the envelope glycoprotein Env and compromises the surface glycoprotein gp70 and the transmembrane protein p15E. Antibodies neutralise the viral gp70 therefore this protein is an important part of vaccines (Willett and Hosie, 2013).

Gp70 contains the three major subgroup antigens A, B or C of FeLV. Those subgroups determine the virulence and the severity of the path of infection later on (Willett and Hosie, 2013). For example FeLV-B is often seen in tumour cases while FeLV-C is connected to non-regenerative anemia (Hartmann, 2012). The most common subtype is FeLV-A. It can be found in every infected cell either alone or in combination with the other two subtypes (Rand, 2009). FeLV-A subtype is important for the transmission of the virus between animals. FeLV-B and FeLV-C subtypes develop in cats with viremia (Willett and Hosie, 2013). FeLV-T arises *de novo* during the course of infection (Chiu et al., 2018).

All subtypes attach to different cellular receptors. FeLV-A subtype attaches the thiamine transporter THTR1, FeLV-B binds to the phosphate symporter Pit-1 or Pit-2 and FeLV-C uses the haem transporter FLVCR1 to enter the host cell. In tissue of cats THTR1 is expressed at a lot (Willett and Hosie, 2013).

The outcome of the viral infection is controversial (Levy et al., 2008). Newly infected cats can recover from the infection and become immune. In the other cases the newly infected cat develops a persistent infection. In this case a viremia occurs and it is likely for the cat to establish other fatal diseases (Willett and Hosie, 2013).

There are different stages in the FeLV infection: abortive infection, regressive infection and progressive infection (Hartmann, 2012).

Abortive infection occurs when the cat is exposed to a low dose of FeLV. The virus begins to replicate in the local lymphoid tissue. The replicating virus can be stopped and eliminated because of an effective immune response and the cat never suffers of viremia (Hartmann, 2012). These cats have high levels of antibodies in their blood. Proviral DNA, viral RNA or FeLV antigens cannot be detected in the blood. It is not known how often this situation

really occurs because it is unlikely for a cat to completely clear the virus from all cells (Hartmann, 2012).

Regressive infection occurs when the immune system stops the virus replication and viremia before or after the bone marrow is affected. Around after 3 weeks of viremia the bone marrow gets affected and the cat cannot eliminate the virus completely. When the viremia is terminated there is no active virus replication but there are still proviruses in the cellular genome. In cats that are in regressive stage of infection no FeLV antigen can be detected and these cats are not infective (Hartmann, 2012).

Progressive infection occurs when the infection with FeLV is not limited early during the infection. Massive virus replication is seen in those cats. First in the lymphoid tissues then in the bone marrow and in mucosal and glandular epithelial tissues. They cannot limit the viremia and are able to shed the virus from infected cells and infect other cats. Cats in the progressive infection stage often develop severe disease and die early (Hartmann, 2012).

FeLV is more pathogenic than FIV. FeLV is the causative agent of tumours, bone marrow suppression syndromes like anemia and can lead to severe secondary infections because of the weak immune system of the cat. In regular, FeLV-positive cats are presented to the vet with anemia or immunosuppression and only rarely with tumours (Hartmann, 2012). Young kittens infected with the virus are most likely to develop pathogenic effects (Grant et al., 1980). New born kittens for example can acquire the “fading kitten syndrome” where their thymus atrophies. This leads to severe immunosuppression and early death. If cats get infected during an older age they usually develop regressive infections or progressive infection with milder symptoms than young cats (Hartmann, 2012).

### 2.1.1 Diagnosis of FeLV

The majority of in-practice tests or test kits used for FeLV detect viral antigens in the blood (either whole blood, plasma or serum is used; Willett and Hosie, 2013). The viral target for those tests is the p27 protein of the capsid which is enriched in the cytoplasm of infected cells and shed into the blood of viraemic cats. It can be detected with the help of immunofluorescence tests or enzyme linked immunosorbent assay (ELISA; Rand, 2009).

In principle, highly specific monoclonal antibodies are utilized for detection by ELISA kits. These tests are highly sensitive but with varying specificity. False negative results might be e.g. obtained in cats in stage of a regressive infection and therefore low viremia. As a second method PCR (polymerase chain reaction) is utilized. With PCR even minute amounts of

proviral DNA or viral RNA are multiplicated exponentially and then size-fractionated and detected on agarose gels (Willett and Hosie, 2013). By PCR the provirus DNA and the plasma viral RNA can be detected as early as one week after infection (Levy et al., 2008).

### 2.1.2 FeLV vaccination

There are different types of vaccines against FeLV. There are injectable inactivated adjuvanted vaccines and non-adjuvanted recombinant injectable vaccines. The vaccination provides immunity for at least 12 months post vaccination. Before the first vaccination the cats should be tested for the FeLV. The vaccine does not provide sufficient protection in all cases that's why it is still important also in vaccinated cats to determine the infection status and perform tests for FeLV (Levy et al., 2008).

In kittens two doses are recommended for the initial immunisation. The first shot should be done around 8 weeks of age and the second one 3–4 weeks after the first dose. After the initial immunisation the vaccine should be given annually (Levy et al., 2008).

## 2.2. FIV – Feline immunodeficiency virus

The FIV proviral genome contains three main structural genes like other related retroviruses: *gag*, *pol* and *env*. The *gag* gene encodes structural proteins, i.e. the capsid, the nucleocapsid and the matrix. The *pol* gene encodes enzymatic proteins like the reverse transcriptase transcribing single stranded RNA into RNA/DNA double strands and enzymes important for the integration and the genomic repair of the virus. The *env* genes encode for envelope-integrated surface protein (gp120) and a transmembrane protein (pg41).

So far, seven FIV variants (subtypes A–F, U-NZenv) have been described. Figure 1 shows FIV subtypes A, B and F circulate in Europe (Huguet et al., 2019). Also, individual isolations of other subtypes were published.

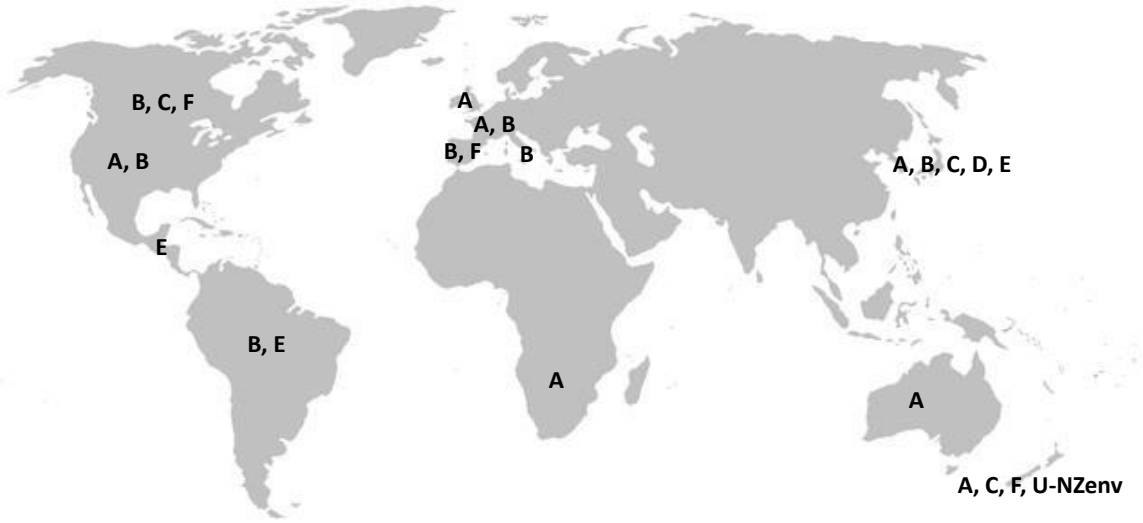



**Figure 1:** Distribution of the different FIV subtype strains (map scheme: www.outline-world-map.com).

Subtype A and B are spread worldwide.

FIV can be transmitted via blood and saliva of infected cats. The most common way of transmission is via cat bites. (Rand, 2009). Although the virus has been detected also in the semen of infected cats, sexual transmission is rare in FIV in contrary to HIV where sexual transmission is the most common route of infection (Levy et al., 2008).

FIV infection undergoes three phases (Table 1): an acute phase, an asymptomatic phase and a terminal phase (Acquired Immune Deficiency Syndrome - AIDS). It is difficult to discriminate these phases clearly. Furthermore, some cats that have been diagnosed with feline AIDS could recover and go back to an asymptomatic phase (Hartmann, 2012).

In most cases the acute phase of the infection is unnoticed by the cat owners (Levy et al., 2008). The acute phase starts a few days to weeks after initial infection and can last until up to 4 months (Rand, 2009). The cats can develop fever, lymphadenopathy and leukemia during this phase (Levy et al., 2008). FIV infection spreads quickly to different organs of the cat like the lymphoid cells, the gastrointestinal tract and the brain (Eckstrand et al., 2017).

During the acute phase FIV infects leukocytes like CD4+ T cells, CD8+ T cells, B-cells, monocytes/macrophages, dendritic cells and megakaryocytes (Eckstrand et al., 2017). During the first two weeks of infection CD4+ (T-helper cells) and CD8+ (cytotoxic-suppressor) the amount of T-lymphocytes shrinks. After the lymphopenia the cat activates a mature immune

response where FIV antibodies are produced and the CD8+ T-lymphocytes are restored in higher levels than before infection. This indicates an inversion of the CD4+ : CD8+ T-lymphocyte ratio which lasts for lifetime. Unfortunately, the immune response is not sufficient to eliminate the virus and thus the number CD4+ and CD8+ T-lymphocytes will drop continuously (Levy et al., 2008).

After the acute phase an asymptomatic period follows. This period can last from months to years. Usually, no clinical symptoms are present but there is a dysfunction of the immune system which can lead to an acquired immunodeficiency syndrome (feline AIDS) the terminal stage of infection (Rand, 2009), (Hartmann, 2012). The virus will affect the cellular immunity more than the humoral part of the immune system. Hence chronic inflammations, infection with intracellular organisms and neoplasia are more common in FIV-infected cats than infections that can be controlled by antibodies (Levy et al., 2008).

The progressive immunosuppression can result in feline AIDS. Cytokine dysregulation, immunologic apoptosis and inappropriate activation of immune regulatory cells are mechanisms that lead to the immunosuppression. Cytokine dysregulation in infected cats results in a drop of the production of interleukin-2 (IL-2) and interleukin-12 (IL-12) and an increase of interferon gamma (IFN-γ) and interleukin-10 (IL-10). That leads to an elevation of the IL10:IL12 ratio which is in connection with the inability of the FIV-positive cat to produce a sufficient immune response to secondary pathogens (Tompkins and Tompkins, 2008).

The immunologic apoptosis is linked to the CD4+ T cells that are unable to produce IL2 and therefore in case of re-stimulation cannot proliferate. The lack of IL2 induces apoptosis of the stimulated cells. The apoptosis is not only limited to CD4+ cells it can also affect CD8+ cells (Tompkins and Tompkins, 2008).

FIV uses two cell surface receptors to attach and enter their target cells: The costimulatory molecule (CD134) and the chemokine receptor CXCR4 (Eckstrand et al., 2017). CD134 is found on the surface of CD4+ T cells and the *env*-SU protein of FIV can bind to it. This binding induces a conformational change in the *env* protein which leads to binding of the V3 region of the *env*-SU to the chemokine receptor CXCR4 (Eckstrand et al., 2017).

In most of the FIV-positive cats the virus does not cause severe clinical signs and with good care the cats can live for years without problems (Hartmann, 2012). Although infected cats are more prone to develop secondary diseases than healthy cats like neoplasia or infections. The most common clinical sign in FIV-infected cats are stomatitis and gingivitis. These infections are painful for the cats and tooth loss happens frequently (Hartmann, 2012).

Clinical abnormalities that can be found in the blood of a FIV positive cat are: leukopenia, neutropenia, lymphopenia and anemia. In the bone marrow changes like hyperplasia, dysplasia and a surplus in eosinophils and plasma cells occurs (Beebe et al., 1992). In the last stage of the infection so-called feline AIDS the cats show neoplasia, myelosuppression, neurological diseases and increased opportunistic infections (Hartmann, 2012).

**Table 1:** Different stages of the FIV infection, their clinical signs and duration

| Stage of infection | Clinical signs | Duration |
|---|---|---|
| **Acute phase** | Fever, lymphadenopathy, neutropenia, leukemia (Levy et al., 2008) | A few days to months (Rand, 2009) |
| **Asymptomatic phase** | No clinical signs (Rand, 2009) (Hartmann, 2012) | Months to years (Rand, 2009) |
| **Terminal phase (feline AIDS)** | Dysfunction of the immune system, opportunistic and chronic infections, neoplasia, myelosuppression, neurological problems (Hartmann, 2012) | Several months (Rand, 2009) |

### 2.2.1 Diagnosis of FIV

Infections with FIV are usually established by detection of antibodies against the virus in the blood of cats with methods like indirect immunofluorescence assay (iIFA) or ELISA (Hohdatsu et al., 1992).

The commercially available in-clinic test kits detect antibodies against the viral protein p24. One aspect to consider is that it will take up to 60 days or in some cats even longer after exposure until antibodies are generated *in vivo* by the immune system. Thus, if the in-clinic test kit is negative but the vet suspects a recent infection the test should be repeated in a minimum of 60 days after viral exposure (Levy et al., 2008). Another problem arose since the vaccination against FIV was introduced. The antibodies of vaccinated cats cannot be distinguished from the antibodies of naturally infected cats. Vaccine-induced antibodies can be detected up to 4 years after vaccination and usually appear in the blood within a few weeks post vaccination (Levy et al., 2008). In general, a positively tested cat might i) be just vaccinated against FIV without any real infection or ii) be vaccinated and infected or iii) not vaccinated and truly infected with FIV.

To determine the actual current infection status of a cat PCR tests are done. With PCR the presence of the provirus of FIV in the host genome can be established (Hohdatsu et al., 1992) suggesting a previous infection. In addition, if viral RNA is detectable in the blood by PCR an ongoing infection is occurring (Levy et al., 2008).

### 2.2.2 FIV vaccination

In 2002 the first vaccine against FIV was introduced (Levy et al., 2008). There is no cross-protective immunity against viruses from other strains. It is a whole virus vaccine comprising an inactivated virus conjugated to an immune-stimulating adjuvant. The cats should be aged 8 weeks or older when being vaccinated. In a period of 2–3 weeks three initial doses are injected subcutaneously and then the cat should be revaccinated on a yearly basis. The vaccine should be considered for cats which are of high risk to get infected like cats that are held together with other FIV-positive cats or outdoor cats that get in fights often. Before administering the vaccination the owner must be informed that after the vaccination the cat will test positive for FIV infection with some test methods (Levy et al., 2008).

The Fel-O-Vax FIV (Boehringer Ingelheim) dual subtype vaccine contains whole inactivated virus subtypes A and D, therefore it does not trigger a sufficient protection against FIV subtype B (or others than A and D) (Yamamoto et al., 2007).

# 3. Materials and methods

For this study no sheltered or free-roaming cats were included, all cats included had rightful owners. 335 domestic cats from all over Hungary were tested in a total of 24 clinics (Figure 2) over the period of 3 years (2016–2018). We continued a study formerly done on 183 specimens by the same institution. Patient history, age, sex and vaccination status of the cats were registered.

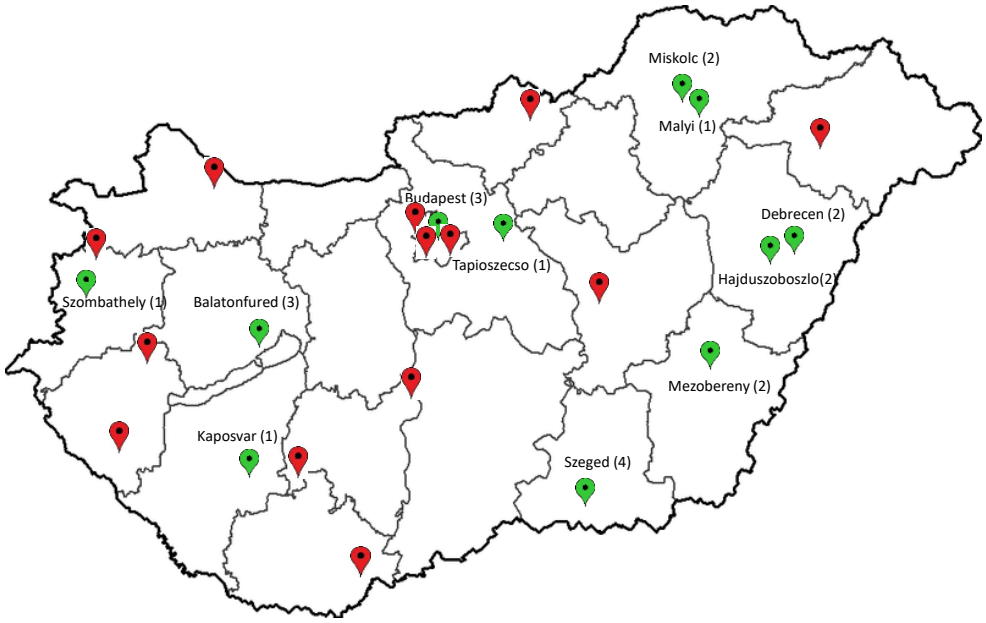



**Figure 2:** Locations of the origins of our samples (map scheme: www.terkepek.net).

The locations of the 24 clinics are highlighted in this map of Hungary with red and green pins. The green pins indicate that we used the samples from that city for the sequencing of the FIV strains. The number given in brackets indicates the number of sequences we obtained from that location.

After a thorough general examination in a cooperating clinic EDTA-anticoagulated blood samples were drawn. From the individual samples a Witness FeLV-FIV ELISA test (Zoetis) was performed in the animal clinic. The rest of the blood samples was sent to the Department of Pathology at the University of Veterinary Medicine Budapest and stored there at -80°C for further examination.

## 3.1. Serology testing

The serological testing was performed with the help of in clinic ELISA tests (Witness FeLV/FIV test kit from Zoetis). ELISA is a plate-based assay that is commonly used to detect enzymes, peptidergic hormones as well as antibodies and antigens from blood serum, milk or urine samples. The ELISA test is based on a specific antigen-antibody reaction. The antigen or antibody is coupled to a solid surface (typically to a micro-well plate) where it reacts with the counterpart antigen or antibody in the solution to be tested. The counterpart antigen or antibody is coupled to a so-called reporter molecule like an enzyme or a second antibody marked with an enzyme generating a coloured end product. The coloured end product absorbs at a specific wavelength and is quantitated in a spectrophotometer. The intensity of the colour generated linearly correlated to the quantity of antibodies or antigens in the sample.

For this study point-of-care tests detecting the p27 antigen in case of FeLV (92.2% sensitivity and 96,5% specificity) and antibodies against p24 antigen in case of FIV (93,8% sensitivity and 93,4% specificity) respectively, were used (Zoetis Inc., 2020). The values for sensitivity and specificity were taken from data provided by Zoetis. For these tests one drop of EDTA-anticoagulated whole blood was used.

## 3.2. PCR testing

The polymerase chain reaction (PCR) is the standard technique for an exponential amplification of low amounts of DNA. It finally produces millions of copies of a specific DNA sample which than can be detected by various means, i.e. ethidium staining of size-fractioned bands on DNA gels or by spectrophotometric techniques. The basic steps of a PCR are: initialization, followed by 20–40 cycles of denaturation, annealing and extension followed by a final elongation step. For each step its characteristic temperature is applied.

In case of FIV nucleic acid extraction from blood samples was done according to the manufacturer's protocol in a QIAcube instrument (Qiagen) with the QIAmp cador Pathogen Mini Kit (Qiagen). Nucleic acids were extracted into 60µl RNase-free distilled water (Qiagen). For the endpoint PCR for FIV, TopTaq Master Mix Kit (Qiagen) was used in accordance to the manufacturer's instructions: 25µl master mix containing 0.5µl forward primer (40µM), 0.5µl reverse primer (40 µM), 5 µl CoralLoad Concentrate, 18 µl RNase-free water and 1µl template DNA. The hot-start PCR amplification was carried out according to the protocol provided by the manufacturer. First, initialization step is done at 95°C for 15 mins. Followed by the

denaturation step at 95°C for 45 seconds to separate double stranded DNA. Then annealing of the primers to the single-stranded DNA templates is done at 60°C for 45 seconds. Next is the extension step where the DNA polymerase synthetizes a new DNA strand complementary to the DNA template at 72°C for 1 min. In total 40 thermal cycles were done. After that the incubation chamber is heated to 72°C for 15 mins for the final elongation of cDNA.

The FIV primers used in the study amplify early reverse transcription products (process of reverse transcription of RNA to DNA). The early reverse transcription products are indicators of a viral infection. The primers bind in the long terminal repeat (LTR) region: the sequence of the LTR-sense primer used was 5'-GCG CTA GCA GCT GCC TAA CCG CAA AAC CAC-3' and that of the LTR-antisense primer was 5'-GTA TCT GTG GGA GCC TCA AGG GAG AAC TC-3' (Sutton, 2007).

One Step RT-PCR Kit (Qiagen) was used for the detection of FeLV. In case of FeLV the blood samples were analysed for RNA products. A master mix containing 5.7 µl RNase-free water, 2 µl 5× buffer, 0.4 µl dNTP, 0.4 µl enzyme mix, 0.1 µl RNase inhibitor, 0.2 µl forward primer (40 µM) and 0.2 µl reverse primer (40 µM) was pipetted into tubes for each sample (0.5 µl each). The first step was 50°C for 30 mins and 94°C for 3 mins (reverse transcription and initial denaturation), then the RNA/DNA template was multiplied by 95°C for 15s, 60°C for 1 min and 72°C for 1 min (45 cycles); and final elongation was at 72°C for 10 mins. FeLV primers with a length of 21 nucleotide each were taken from a publication: the sequence of the forward primer FeLV U3-exoR used was 5'-AAC AGC AGA AGT TTC AAG GCC-3' and that of the reverse primer FeLV U3-exoF was 5'-TTA TAG CAG AAA GCG CGC G-3' (Tandon et al., 2005).

The 163 base pair (bp) (FIV) and 150 bp (FeLV) long amplicons were size-fractionated by gel-electrophoresis in a 1.5% agarose gel and visualized by blue light (Figure 3).

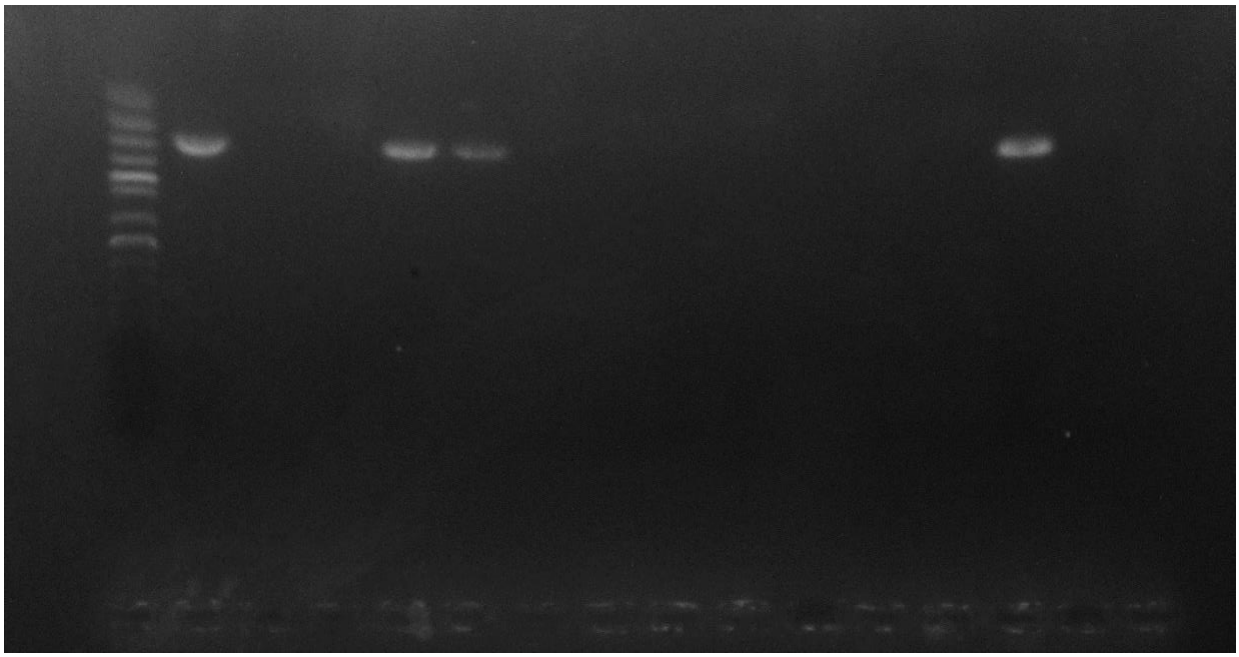


**Figure 3:** Agarose gel-electrophoresis of FeLV 150 bp long PCR products.

PCR products were size-fractionated on an agarose gel and visualized by blue light. Left lane: DNA size markers, followed by a positive and negative control and 13 PCR samples (3 positive and 10 negative).

## 3.3 Statistical analysis

The statistical calculations were performed with the R program (R Core Team, Vienna, Austria). To determine the correlation between the examined variables and the FeLV and/or FIV infection status a logistical regression model was used (R Core Team, 2016). The Epi.prev function was used to calculate prevalences (Sullivan et al., 2009). The correlation between the sex of the cats and the infection rate was calculated with a contingency table and the Fisher's exact test using two-tailed p-values. Cohen's kappa was counted to show possible cross-compliance between the ELISA and PCR methods (Dinya E et al., 2016).

## 3.4 Phylogenetic analysis

If there was a FIV-positive sample detected the partial proviral *pol* genes were amplified. The PCR products were put into the gel electrophoresis, amplicons of 576 bp length were cut manually and purified with the help of a Qiagen Gel Extraction Kit (Qiagen). The next step was the bidirectional Sanger sequencing reaction with corresponding primers. Capillary

electrophoresis was made by a commercial provider (Microsynth.). In two cases (I/14/16 and II/7/8) repeated sequencing did not result in clean sequences. Therefore these PCR products were cloned into a pJET1.2 vector and then subsequently transformed into *Escherichia coli*. This was done with the help of a CloneJET PCR Cloning Kit (ThermoFisherScientific).

After checking the obtained sequences, they were assembled using the E-INS-I method of the online software MAFFT version 7. The sequences were compared with available FIV genomes downloaded from the GenBank that represents the overall diversity of the virus. Analysis to find the maximum likelihood were done and the trees were visualized and edited with MEGA7 using the Tamura Nei model with 1000 bootstrap replicates and gamma distribution.

# 4. Results

The mean age of the cats used for this survey was 4.9 years (range 5 months to 18 years). A total of 155 females (46.3%) and 180 males (53.7%) were included in the study. Ninety (58.1%) of the female cats were intact and 65 (41.9%) were spayed. Out of the males 119 (66.1%) were intact and 61 (33.9%) were castrated. Seventy-eight (23.28%) of the cats included in this study were kept inside, whereas 257 (76.72%) had access to outside as seen in Figure 4. Out of the 335 cats examined in this study 136 appeared clinically healthy during the physical examination. We found that only a small number of cats were ever vaccinated during life time. Ninety-eight (29.3%) cats out of the 335 were vaccinated (usually by a combined vaccination) whereas only 39 (11.6%) cats were vaccinated against FeLV. It is interesting to note that 2 of them were actually FeLV-infected.

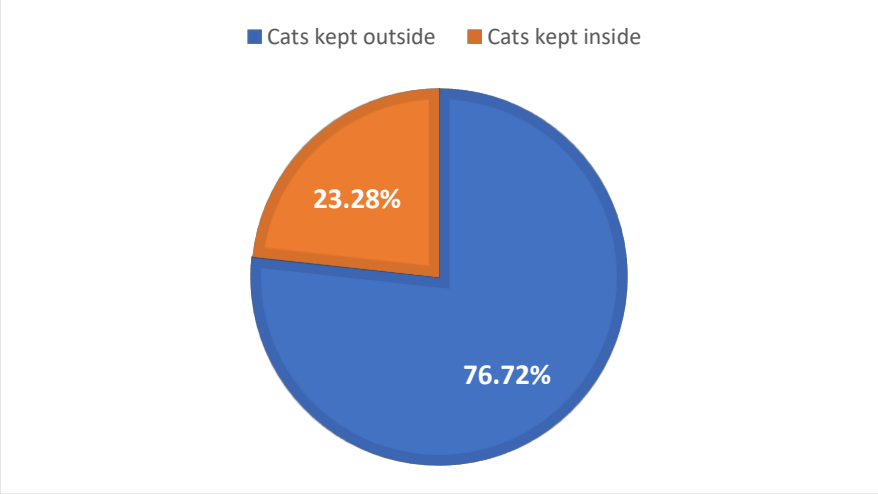



**Figure 4:** Ratio of cats that were kept inside (orange) versus those kept outside (blue). In total, 78 cats were kept inside and 257 had outdoor access.

From the 335 samples tested with the ELISA test kit 47 (14.0%) were positive for FeLV and 51 (15.2%) were positive for FIV. The subsequent PCR test showed that 58 (17.3%) cats tested positive for FeLV and 44 (13.1%) for FIV.

Some cats tested positive by only one of the two methods performed, i.e PCR or ELISA. In case of FeLV 4 out of 47 ELISA test results and 15 out of 58 PCR results were single positive

for one of the two test but not for the other. In case of FIV 11 out of 51 ELISA tests and 4 out of 44 PCR tests were single positive (Table 2).

**Table 2:** ELISA and PCR test results.

This table shows the total number of the positive tested cats for each retrovirus and each test method, but also the single positive results that could have been missed otherwise. The difference between the total number of positive FIV ELISA results and total number of FIV PCR results is 7. The difference between the total number of positive FeLV ELISA results and total number of FeLV PCR results is 11.

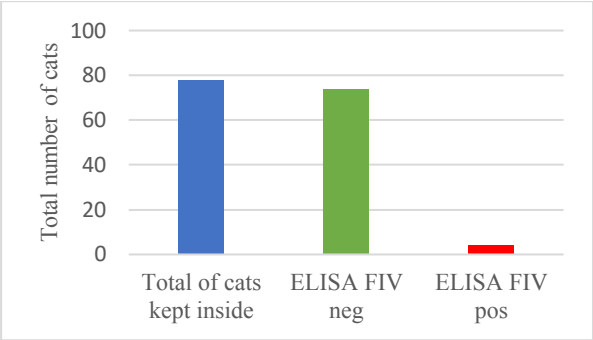
| **Total number of cats tested** | **335** |
|---|---|
| FIV | |
| Total number of cats positive by ELISA | 51 (15.2%) |
| Number of cats positive by ELISA with a negative PCR test | 11 |
| Total number of cats positive by PCR | 44 (13.1%) |
| Number of cats positive by PCR with a negative ELISA test | 4 |
| FeLV | |
| Total number of cats positive by ELISA | 47 (14.0%) |
| Number of cats positive by ELISA with a negative PCR test | 4 |
| Total number of cats positive by PCR | 58 (17.3%) |
| Number of cats positive by PCR with a negative ELISA test | 15 |

14.03% was the total apparent prevalence of FeLV counted with the ELISA test (95% confidence interval 10.72–18.16) and in the case of FIV the total apparent prevalence was 15.22% (95% CI 11.77–19.46). True prevalence also counted from the Witness ELISA test was 11.78% (95% CI 8.08–16.4) in case of FeLV and 9.89 (95% CI 5.93–14.75) for FIV.

The apparent prevalence calculated from the subsequent PCR results for FeLV were 17.31% (95% CI 13.65–21.73) and for FIV were 13.13% (95% CI 9.93–17.17). Cross-compliance was analysed by calculating Cohen's κ coefficient. The coefficient was 0.786 (95%

CI 0.68–0.87) for FeLV (P<0.05) and 0.816 (95% CI 0.72–0.9) for FIV (P<0.05). These results indicate a coherence between the Witness ELISA and PCR results.

The results from the ELISA test used in a logistic regression model show that cats with outdoor access have a 2.8 (95% CI 1.19–8.49; P=0.033) times higher chance of infection with FeLV and a 4.1 (95% CI 1.62–14.07; P=0.0083) times higher chance of infection with FIV (Figures 5–8).



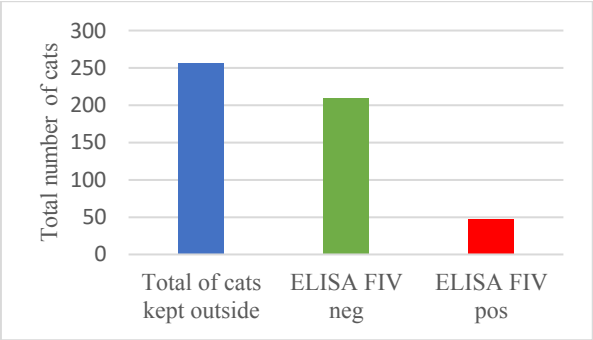
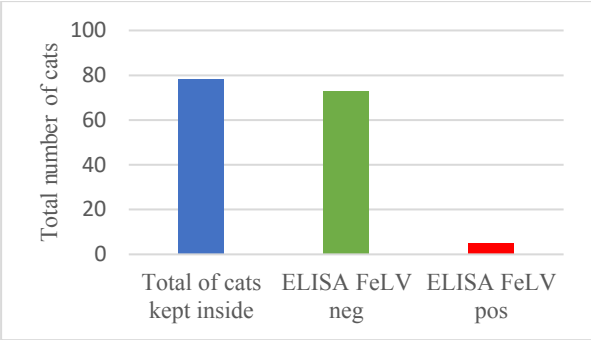




**Figure 5** (left) and **Figure 6** (right): FIV ELISA test results from cats kept inside (left) and outside (right).

The total number of inside kept cats was 78. Out of the 78 cats only 4 (5.1%) tested positive for FIV with the ELISA in the clinic test whereas 74 tested negative. There were 47 (18.2%) positive ELISA test results out of 257 cats kept outside. These results showed that the chance of infection with FIV is 4.1 times higher for outside kept cats.



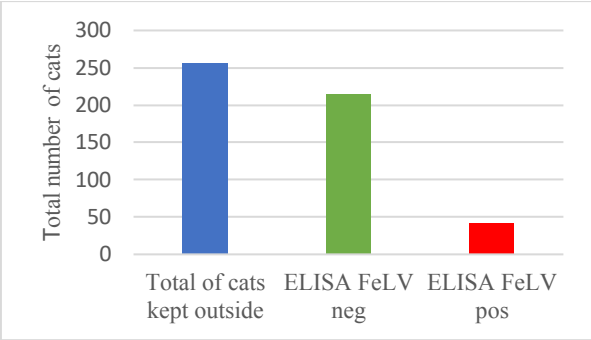
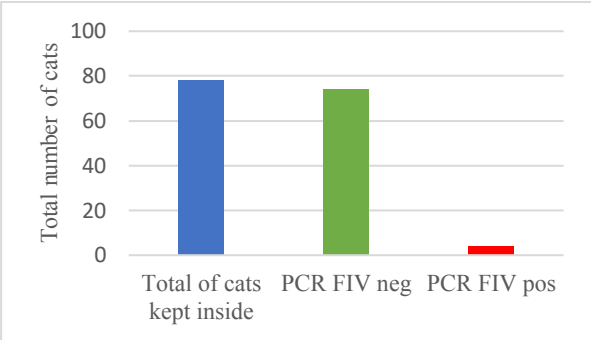




**Figure 7** (left) and **Figure 8** (right): FeLV ELISA in the clinic test from inside (left) and outside (right) kept cats.

Only 5 (6.4%) of the 78 inside kept cats tested positive for FeLV with by ELISA while 42 (16.3%) of the outside kept cats tested positive for FeLV.

Using the subsequent PCR results for the calculation there is a 2.5 (95% CI 1.156–6.29; P=0.0306) and a 3.4 (95% CI 1.32–11.64; P=0.0235) higher chance of infection with FeLV and FIV in cats with outdoor access (Figures 9–12).



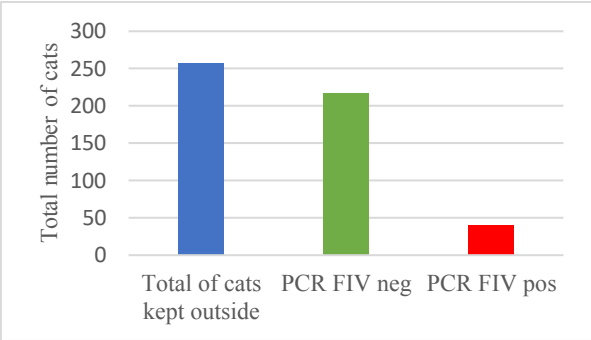
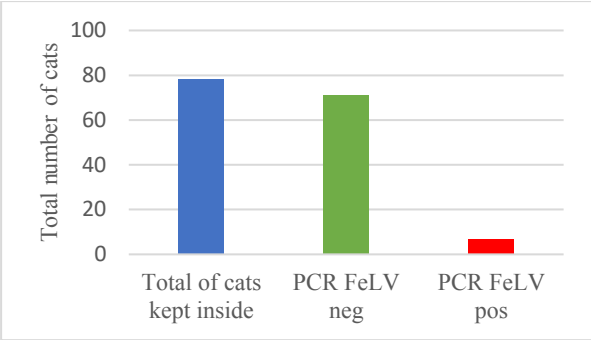




**Figure 9** (left) and **Figure 10** (right): FIV PCR test results from inside (left) and outside (right) kept cats.

Out of 78 inside kept cats 4 (5.1%) tested positive for FIV. From the 257 cats kept outside 40 (15.5%) yielded a positive PCR test result. According to our data gained by PCR there is a 3.4 higher chance of infection with FIV if the cats that were kept outside.



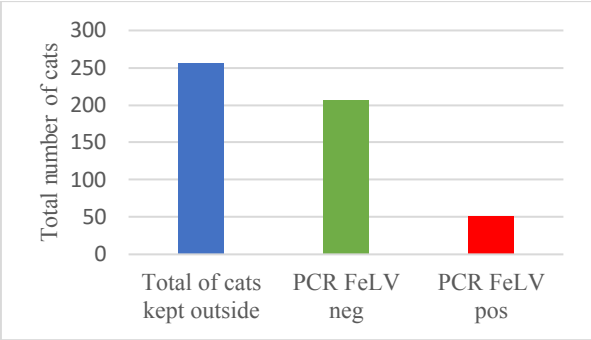




**Figure 11** (left) and **Figure 12** (right): FeLV PCR test results from cats kept inside (left) and outside (right).

From the cats kept inside 7 (8.9%) and from the cats kept outside 51 (19.8%) tested positive by the PCR method. Cats kept outside showed a 2.5 higher chance of infection compared to thoses kept inside.

There was no significant correlation found between the animal's age and the FeLV infection using both the Witness ELISA or PCR results. On other hand in case of FIV infection both Witness ELISA (95% CI 1.04–1.19; P=0.0012) and PCR (95% CI 1.04–1.19; P=0.0014) results showed that there is a 1.12 times higher chance of infection for every year the cat was older.

For FeLV no significant correlation between sex and infection rate was evident in our study (Figures 13, 14 and Table 3). Out of 155 females 27 (17.4%) tested positive by PCR and 19 (12.3%) were positive with the ELISA test. Out of the 180 males 31 (17.2%) were positive by PCR testing and 28 (18.8%) showed a positive ELISA test result. In total, male cats were 1.32 times more likely to be FeLV-positive than female cats (95% CI 0.71–2.5, P=0.3872), however, this small difference did not reach statistical significance by the Fisher's exact test (P=1,000 for PCR and P=0,532 ELISA).

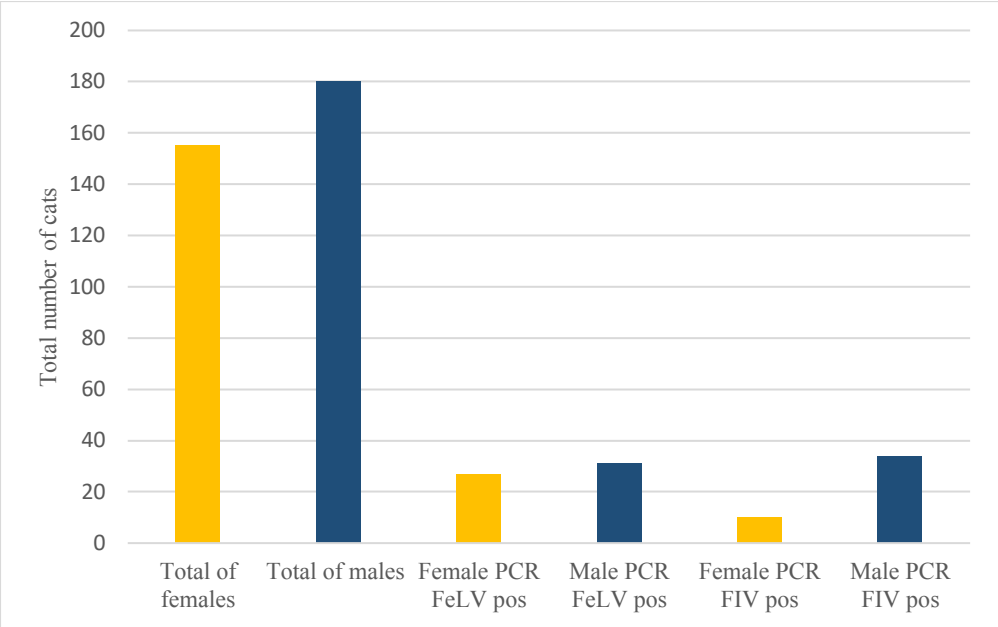



**Figure 13:** Comparison of the positive PCR test results versus gender of the cats included in this survey.

On the other hand for FIV a 4.3-fold higher rate of infection was found. Out of 155 females 10 (6.5%) tested positive by PCR and 9 (5.8%) were positive with the ELISA test. Out of the 180 males 33 (18.9%) were positive by PCR testing and 42 (33.8%) showed a positive ELISA test result. Analysis of the data by the Fisher exact test demonstrated a significant correlation between male sex and infection rate for both the ELISA (P=0.0001) and the PCR (P=0.0033) test.

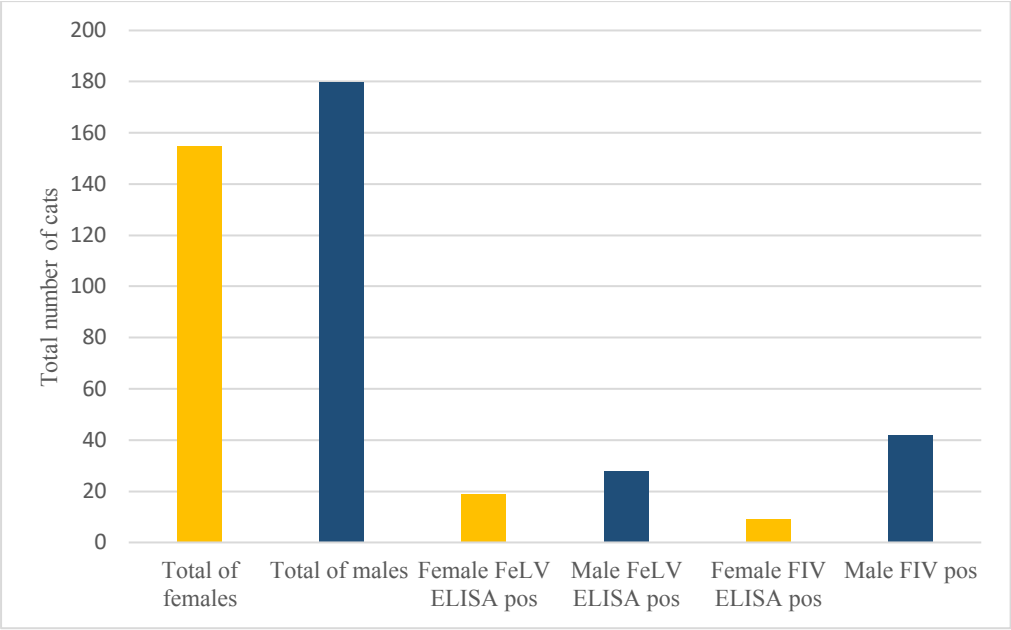



**Figure 14:** Comparison of the positive ELISA test result and the gender of the cats included in this survey.

**Table 3:** ELISA and PCR test results versus gender.

This table shows infection rates of male and female cats in percent plus the ratio obtained by PCR and ELISA testing.

| % of positive tests | male | female | ratio m/f |
|---|---|---|---|
| **FIV** | | | |
| cats positive by ELISA | 33.8% | 5.8% | 5.8× |
| cats positive by PCR | 18.9% | 6.5% | 2.9× |
| **FeLV** | | | |
| cats positive by ELISA | 18.8% | 12.3% | 1.5× |
| cats positive by PCR | 17.2% | 17.4% | 0.99× |

There was also no statistically significant correlation between the state of infection and the neutering status of the cats. Intact males showed a 1.34 times higher risk of infection (95%

CI 0.57–3.42; P=0.5187) and intact females showed a 0.78 times higher risk of infection (95% CI 0.3–2.8; P=0.609).

There were 44 PCR-positive FIV cases and with the help of Sanger sequencing we managed to obtain 22 partial *pol* gene sequences. The other 22 samples probably did not contain enough amount of nucleic acids or contained only poor quality nucleic acids. Out of the functioning 22 partial *pol* gene sequences only two came from indoor cats all the other strains originated from cats with outdoor access. 16 strains were sampled from male cats and 6 from female cats. The samples came from all over Hungary.

All of the 22 strains belonged to the FIV subtype B. Within the subtype B clade the Hungarian sequences were put into a monophyletic group. The group also includes a sequence originating from Joinville, Brazil, 2017 (acc. number: KY629414.1). Within the Hungarian clade the sequences can be aligned to several subgroups that reflect the geographical origin. The subgroups are supported by relatively high bootstrap values (Figure 15). The overall mean genetic similarity between the strains analysed by pairwise genetic analysis was 98.2% (the lowest value was 88.1% and the highest was 99.8%). The GenBank accession numbers for the sequences are MN401525–MN401446.


**Figure 15:** Phylogenetic tree reconstructed from partial *pol* gene sequences of Hungarian FIV strains and selected reference sequences from GenBank.

Maximum likelihood bootstrap support values (⩾70) are shown as percentages above the branches. Two indoor cats are marked with red dots; all the others had outdoor access. Strains displayed on the phylogenetic tree are coded differently in the case of Hungarian strains, the code of sample and city of the cat's origin are shown. In case of reference sequences obtained from GenBank, accession number, year of publication and country of collection are displayed.

# 5. Discussion

Our data show a relatively high rate of infection for FeLV and FIV of domestic cats in Hungary compared to infection rates published for several other countries. The true prevalence for FeLV was 11.78% with ELISA results and 9.89% for FIV. Apparent prevalences obtained from PCR results were also high compared to surrounding countries data.

FeLV prevalence in domestic cats in Australia is around 2% (Westman et al., 2019) and in the United States plus Canada the seroprevalence for FeLV is around 3.1% (Burling et al., 2017). In countries closer to Hungary the FeLV prevalence is still lower than in Hungary: In Germany the prevalence of FeLV is 3.6% (Gleich et al., 2009) and in eastern Austria the prevalence of FeLV is 5.6% (Firth and Möstl, 2015).

The FIV prevalence in Australia is 15% (Westman et al., 2019) and 3.6% in the United States and Canada (Burling et al., 2017). The prevalence of FIV in Hungary compared to Australia does not show much difference. In Germany the prevalence of FIV is 3.2% (Gleich et al., 2009) which is lower than in Hungary. However, it should be note that this statement is only true if we exclude the prevalence of FeLV and FIV in strays or sheltered cats of other countries. Due to both retroviruses being highly contagious they spread faster if cats are kept in higher densities like in shelters.

According to our data the vaccination program in Hungary needs to be improved significantly. Only a small fraction of the cats (29.3%) were vaccinated and an even smaller one was vaccinated against FeLV (11.6%). As mentioned before, two of the cats vaccinated against FeLV even were positive for the virus (ELISA and PCR tests showed positive results). This might be explained by the fact that the cats were immunized only on an irregular basis thus leading to time periods where the cats might remain unprotected and are prone to infection.

The high prevalence rates of both retroviruses can be explained by the fact that 77% of the cats included had the opportunity to go outside and get in contact with other cats (also most of them were not neutered). These circumstances increase the risk of the cats to get infected. On the other hand, high prevalence rates could also be explained by the false positive paradoxon. Diagnostic test tools can never have a 100% specificity and especially in circumstances of low case prevalence rates (like in our study) there can a high number of false positive test results be expected, sometimes even higher than the true positive test results.

In some cats we could only detect one positive result either with ELISA or PCR method (Table 2). That can be due to several reasons. One for example is that the ELISA in the clinic test in case of FIV detects antibodies while the PCR method detects the LTR region of the

genome of the virus. Especially during early infection there will be no detectable antibodies in the blood of infected cats. As discussed in the literature review it can take up to 60 days or even longer until cats produce a level of antibodies detectable by ELISA (Levy et al., 2008), thus yielding a positive PCR result in combination with a negative ELISA test result. Another situation leading to a false negative ELISA result might be the terminal phase of FIV (feline AIDS) where the immune system of the cat generating circulating antibodies is greatly suppressed but the viral load detected by PCR is typically high.

A true positive FIV ELISA result but a false negative PCR result can be obtained in cases where only a low number of proviral DNA or viral RNA is circulating in the bloodstream of the cats like in the asymptomatic phase of FIV infection. False negative PCR results can also occur in case of viral genetic diversity where the primers of the PCR cannot bind to the DNA or RNA template because of a mutation in the nucleotide sequence in the region of one of the two primers.

In theory, there is also the possibility of maternal antibodies interfering with the test results. However, that does not apply to our study since any of the younger cats aged 5–6 months included tested negative for FIV by both the ELISA and the PCR method, respectively.

In case of FeLV the ELISA detects the p27 antigen whereas the PCR detects the U3 part of the LTR region of the viral genome. A false negative ELISA result but a true PCR result can be found in similar cases as with the FIV: Either during the early stage of FeLV infection or during the regressive phase of the FeLV infection where no viremia occurs. In these cases, only minute amounts of proviral DNA or RNA can be detected in the bloodstream but the amount of the antigen is too low to detect it with ELISA.

Our study shows an increased risk of infection for both retroviruses if the cat has the opportuntity to go outside or is strictly kept outside. According to the PCR results outdoor cats have a 2.5 and a 3.4 higher chance of getting infected with FeLV and FIV. This makes sense since both of them are highly contagious viruses. In case of FeLV its inevitable for cats which are allowed outside access to get in contact with other cats. Since FeLV can be transmitted via any body fluid healthy cats can easily get infected just by getting in contact with infected cats and also during mating (Rand, 2009). The risk of infection in case of indoor kept cats is lower. Indoor kept kittens could still get infected vertically or by drinking the colostrum of their mother (Levy et al., 2008). Adult indoor kept cats usually get infected during grooming an infected cat that's why it is advisable before introducing any new cat into your home to test the cat for FeLV. In our study we did not check if the positive indoor cats were kept alone or in a group

of cats. It would be interesting to see if the majorty of positive indoor cats were kept alone or in a group.

FIV transmission occurs via blood or saliva and rarely happens during mating (Levy et al., 2008). So also in case of FIV outdoor cats are exposed to a higher risk of fighting with infected cats than indoor cats which could explain they high number of FIV positive outdoor kept cats in our study.

According to the PCR and ELISA test results of our survey male cats were more likely to get infected with FIV than females (according to PCR around three times more likely and according to ELISA almos six times more likely). Intrestingly, for FeLV no statistically significant difference between gender and infection status was seen. It is well established that FeLV is more likely transmitted via a so-called friendly contact like grooming each other whereas FIV is typically transmitted via fights by bite wounds. Thus, this difference can plausibly be explained by the fact that not-neutred males stray further away from their homes, cross more territories on their way and get in contact with more cats than females and tomcats get more often involved into fights receiving more frequently biting wounds than their female counterparts.

In case of the phylogenetic analysis it must be mentioned that the 22 samples we examined are not enough to give an overall picture over FIV strains that are currently found in Hungary. Any of the FIV sequences we obtained belonged to the subtype B of FIV. That's why we can postulate that subtype B is the most prevalent in Hungary currently. Further examination of more FIV strains all over Hungary could be useful in case of FIV vaccine development, since the currently available vaccine (Fel-O-Vax FIV) does not produce enough immunity against the FIV subtype B to protect the cats from the infection.

The sequences we found form a unique cluster that includes all Hungarian sequences (and one Brazilian sequence). Due to this monophyletic pattern there is a possibility of a common ancestor for the Hungarian (and the one Brazilian) FIV sequences. Based on this we hypothesize that there was only a single virus introduction event. The genetic diversity found could be explained due to local evolution of the strains.

In conclusion this was the first thorough study beside other, more regional ones, dealing with the prevalence of FeLV and FIV and the phylogenetic analysis of FIV strains in Hungary.

# 6. Summary

Feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) both belong to the most common retroviral diseases worldwide affecting domestic cats as well as wild felids. Both viruses can cause diseases with fatal outcomes in cats. The aim of this thesis was to further determine the prevalence of both retroviruses in domestic cats in Hungary, continuing a previous study. In total 335 whole blood samples were obtained from domestic cats from 24 animal clinics over a time period of three years (2016–2018). Sheltered or free-roaming cats were excluded from this survey. The blood samples were analysed by two established methods, e.g. polymerase chain rection (PCR) and enzyme-linked immunosorbent assay (ELISA). By reverse transcription PCR (RT-PCR) a specific region of the RNA genome was amplified for detection. The commercially available ELISA kits used were directed against the p27 protein (FeLV) or antibodies recognizing the p24 antigen (FIV), respectively. For a statistical analysis the R-project was used. The true prevalence of FeLV calculated from the ELISA test results in Hungary was 11.8% and for FIV it was 9.9%. The apparent prevalence obtained from the PCR results for FeLV was 17.3% and for FIV it was 13.1% indicating a reasonable agreement between both methods (Cohen's kappa value was 0.79). Our data show that compared to other countries, the prevalence of FeLV and FIV in Hungary is relatively high. Further examining 47 FIV-positive samples by Sanger sequencing, we obtained 22 partial *pol* gene sequences. Comparing to sequences with multiple alignment to those found in the GenBank, the 22 Hungarian strains belong to FIV subtype B. GenBank accession numbers are MN401425–MN401446. the overall mean genetic similarity between the analysed strains was 98.2%.

# 7. Összefoglaló


**A macska leukemia vírus és a macska immundeficiencia vírus prevalenciája a házimacskákban Magyarországon**

A macska leukemia vírusa (FeLV) és a macska immunodeficiencia vírusa (FIV) okozta kórképek az egyik leggyakoribb házi macskákat és vadon élő macskaféléket érintő retrovírusos betegségek közé tartoznak. Mindkét vírus halálos kimenetelű betegségeket okozhat. Dolgozatom célja az volt, hogy egy korábbi vizsgálatot folytatva, meghatározzam a retrovírusok prevalenciáját magyarországi macskapopulációkban. Három év alatt (2016–2018) 24 állatklinikán összesen 335 teljes vérmintát sikerült nyerni házi macskákból. A menhelyen élő, illetve kóbor macskák nem kerültek be a kutatásba. A vérmintákat két módszerrel vizsgáltuk, ezek a polimeráz láncreakció (PCR) és az ezyme-linked immunosorbent assay (ELISA). Reverz transzkripciós PCR (RT-PCR) segítségével az RNS genom egy specifikus régióját amplifikáltuk a kimutatáshoz. A kereskedelemben kapható ELISA kitek a p27 antigént (FeLV) vagy a p24 antigént felismerő ellenanyagokat (FIV) mutatják ki. Statisztikai elemzéshez az R-programot használtuk. Az ELISA teszt eredményeiből számítva a FeLV valódi prevalenciája Magyarországon 11,8%, a FIV esetében pedig 9,9% volt. A FeLV PCR eredményeiből kapott látszólagos prevalencia 17,3%, a FIV esetében pedig 13,1% volt, ami azt jelenti, a két módszer jól megfeleltethető egymásnak (Cohen-féle kappa értéke 0,79). Adataink azt mutatják, hogy más országokkal összehasonlítva, a FeLV és FIV prevalenciája Magyarországon viszonylag magas. A 47 db FIV-pozitív minta további vizsgálata során Sanger szekvenálással összesen 22 részleges *pol* génszekvenciát nyertünk, melyek megfelelő minőségűek voltak a további vizsgálatokhoz. Összehasonlítva a GenBank-ban talált szekvenciákkal, a 22 magyar törzs a FIV B altípusba tartozik. A GenBank leltári számok MN401425 – MN401446. A vizsgált törzsek átlagos genetikai hasonlósága 98,2% volt.

# 8. Acknowledgments

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