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Statistical analysis of the prevalence of Feline Immunodeficiency Virus (FIV) in domestic cats in Hungary

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List of abbreviations

AIDS: Acquired Immune Deficiency Syndrome

AZT: Azidothymidin

CA: Capsid

dsDNA: Double stranded deoxyribonucleic acid

DU: Deoxyuridine pyrophosphatase

dUMP: Deoxyuridine monophosphate

dUTP: Deoxyuridine triphosphate

dUTPase: Deoxyuridine triphostphatase

ELISA: Enzyme-linked immunosorbent assay

env*:* envelope

FeLV: Feline Leukemia Virus

FIV: Feline Immunodeficiency Virus

FORL: feline odontoclastic resorptive syndrome

gag: group specific antigen

HIV: Human Immunodeficiency Virus

IFN: Interferon

MA: Matrix

MPC: Multiple viral protein chain

NCA: Nucleic capsid

NPV: negative predictive value

PBMC: Peripheral blood mononuclear cells

PCR: Polymerase chain reaction

PMEA: as 9-2-(phosphonylmethoxyethyl) adenine

pol: polymerase

PPV: positive predictive value

pro: protease

RER: Rough endoplasmic reticulum

RT-PCR: reverse transcription polymerase chain reaction

ssDNA: Single stranded deoxyribonucleic acid

ssRNA: Single stranded ribonucleic acid

Vif: Viral infective factor

3TC: Lamivudine

1 Introduction

Feline Immunodeficiency Virus (FIV) is an enveloped single stranded ribonucleic acid (ssRNA) virus of the genera lentivirus. It is of the *Retroviridae* family and it infects animals of two different families: *Felidae* and *Hyaenidae* (Perharić *et al.*, 2016). The domestic cat, *Felis catus*, gets infected by the strain FIV-Fc. This strain is divided into five phylogenetic subtypes which are described as A to E (Sodora *et al.*, 1994; Kakinuma *et al.*, 1995; Pecoraro *et al.*, 1996). There is a putative subtype F, which so far has only been reported in Portugal (Marçola *et al.*, 2013). The subtypes are differentiated by the genetic sequence of V3 and V5 region of the envelope (*env*) gene. The occurrence of the different subtypes is geographically different. Some subtypes such as A, B and C, have a more widely spread, than subtypes D and E. Subtype A can be found worldwide with the sole exception of South-America, subtype B has been documented in North-America, South-America, Europe and Japan. Subtypes D and E have only been found regionally (Sodora *et al.*, 1994; Steinrigl and Klein, 2003; Kann *et al.*, 2007; Bęczkowski *et al.*, 2014): subtype D in Japan and E in Argentina and Japan. Some subtypes are evolutionarily older than other ones and the pathogens have been found to be on different levels of pathogenicity. In this light subtype B is understood to be older and less pathogenic, than subtype A (Bęczkowski *et al.*, 2014). Infection by multiple subtypes is possible and may lead to the so called superinfection. This makes the virus prone to higher virulence and pathogenicity due to the opportunity of recombination (Kann *et al.*, 2007; Bęczkowski *et al.*, 2014).

Discovered for the first time in 1986 FIV is known to cause an acquired immune deficiency syndrome (AIDS) in cats, just as the Human Immunodeficiency Virus (HIV) does in humans (Pedersen *et al.*, 1987).

The infection is clinically divided into three stages, frequently ending in the final stage characterized by the typical acquired immune deficiency syndrome as in HIV (Ishida and Tomoda, 1990). FIV morbidity and mortality is very low, making the pathogen hard to detect if no specific tests are taken, and giving FIV-positive cats a chance for a normal life span. Clinical signs are very broad and symptoms as immunosuppression, neoplasia, hematopoietic changes or neurological signs can occur (Hartmann, 2011, 2012; Bęczkowski *et al.*, 2015). Besides unspecific clinical signs, FIV makes the cat prone to secondary

infections e.g. Feline Leukemia Virus (FeLV) or bacterial, parasitological infections. Pathology is very unspecific, since cats rarely die only because of the FIV-infection. Transmission of the virus theoretically has many ways, but in reality it mostly occurs via salivary route. FIV has a large cell tropism unlike HIV, and works with different receptors and co-receptors. As in HIV, mutation plays an important role in the survival of the virus. Laboratory diagnostic methods are the only way to detect FIV, such as enzyme linked immunosorbent assay (ELISA), western blot or polymerase chain reaction (PCR). Treatment of FIV is impossible until now, we can only deal with secondary infections and are able to modify FIV pathogenesis in some ways discussed later. Vaccination exists in the USA, Australia and New-Zealand, but it is not used in the EU. Prevention of the virus is the best way to decrease the spread of FIV, but sadly only a minority of cats are getting screened and tested, making it hard to stop the transmission of FIV.

The main goal of our research was to map the spread of different subtypes of FIV viruses in Hungary, as the most significant retroviral causative agent of domesticated cats. The collected results and data was evaluated for geographical distribution and other aspects (such as whether the samples collected were from asymptomatic or symptomatic cats), and we prepared statistical reports.

2 Review of the literature

2.1 Etiology of FIV

Feline Immunodeficiency Virus is a lentivirus, a complex retrovirus as HIV (Coffin, Hughes and Varmus, 1997). *Retroviridae* family is divided into 7 genera, which are classified under "simple" and "complex" retroviruses. This classification is based on the genetic coding of the viruses. Simple retroviruses are only able to encode the genes *env*, group specific antigen (*gag*), polymerase (*pol*), while complex retroviruses are able to encode further (Coffin, Hughes and Varmus, 1997).

FIV is an enveloped ssRNA virus. Its size is around 100–125 nm in diameter and it has mostly a spherical or ellipsoid form (Bendinelli *et al.*, 1995). The membrane of the virus is formed by a double lipid layer, where we find proteins formed by the *env* gene, divided into transmembrane (TM) protein, also called gp95, and the surface protein (SU), also known as gp120. The SU protein has an N-terminal facing the exterior and a C-terminal region facing inwards. On the N-terminal we find 5 variable regions, labelled V1–V5, with V3 playing the most important role for binding to the host cell. The TM has 3 variable regions, labelled V6–V8 (Phillips *et al.*, 1990; Pancino *et al.*, 1993). The inside of the virus is formed by different layers of proteins described as the matrix (MA), capsid (CA) and nucleocapsid (NCA). These proteins are encoded by the polyprotein *gag*, with the MA forming the outer ring layer and the CA the inner ring layer (Elder *et al.*, 1992). Inside the CA, the ssRNA is situated with the NCA around and some enzymes, as the integrase (IN), protease (pro) and reverse transcriptase (RT) inside. These enzymes play a crucial role in the replication of the virus and are encoded by the polyprotein *pol* gene. Furthermore, *pol* contains a gene, which encodes deoxyuridine pyrophosphatase (DU) among RT and IN (Elder *et al.*, 1993; Lerner *et al.*, 1995).

Infecting domestic cats, FIV attacks mostly CD4+ cells (Kenyon and Lever, 2011). Unlike HIV, CD134 receptor of T-cells is the main receptor binding FIV, and with the help of the co-receptor CXCR4, the SU is binding to the host cell without being detected (Shimojima *et al.*, 2004). It also infects other cells like CD8+, B-cells, macrophages, monocytes, astrocytes and microglia cells (Pedersen *et al.*, 1987; Brunner and Pedersen, 1989; Dow, Poss and Hoover, 1990; Danave *et al.*, 1994; Dean *et al.*, 1996). The cysteine rich domain 1 (CRD1) and 2 (CRD2) of the CD134 receptor are important for the fusion of FIV. When *env* of FIV

and CD134 of the host cell fuses, the virus exposes an epitope on the V3 region of *env*, which will be able to bind with the co-receptor CXCR4 (de Parseval *et al.*, 2005, 2006). Since FIV also infects other cells in the course of infection, which do not have CD134 receptor, it binds these cells by a change in the *env* gene, so the virus can fuse with CXCR4 and has a decreased dependency on the number of CD134 receptors (English *et al.*, 1993; Dean *et al.*, 1996; Willett and Hosie, 2008). After having bound with the host cell, the virus membrane fuses with the host cell and liberates the proteins and enzymes into the cytoplasm of the host cell.

The replication of the virus takes place in the host cell. The two strains of ssRNA will be copied into double stranded deoxyribonucleic acid (dsDNA) by RT. The dsDNA helix gets connected to the enzyme integrase, and integrates into the host cell's nucleus (Kenyon and Lever, 2011). There the polymerase replicates the DNA into messenger ribonucleic acid (mRNA), and sends it to the ribosomes in the rough endoplasmic reticulum (RER), which will decode the strand and form multiple viral protein chains (MPC). The MPC goes with the RNA to the host cell's membrane, where they fuse and form immature virions. These immature virions are not able to infect and enter new host cells yet. For being able to do this, the MPC will be cut by the enzyme protease into single proteins. By then the virion is able to develop into a mature virus, ready to attack new cells.

Mutation plays an important role in FIV, as it does in HIV. Although it is known that the rate is not that high, mutations and recombinations regularly occur. They are very important for the survival of the virus, since it needs these mutations to escape from the host organism's immune system. Mutations can occur on the variable region of envelope surface protein e.g. V2, V3 and V4, as well as on the constant regions (Lerner and Elder, 2000).The reverse transcriptase plays a crucial role and is one of the main areas, where point mutation and recombination happens.

The host cell tries to defend itself from infection by the action of APOBEC3 protein. These are cytidine deaminases, which are responsible for a G to A mutation during reverse transcription (Münk *et al.*, 2008). Thus the virus has the viral infectivity factor (Vif), which tries to block the APOBEC3 protein. Another defence mechanism of the virus is the DU, which tries to limit uracil misincorporation, so as G to A transitions by transforming deoxyuridine triphosphate (dUTP) into deoxyuridine monophosphate (dUMP) in the viral DNA (Wagaman *et al.*, 1993; Lerner *et al.*, 1995). The RT is also the access point of nowadays treatment. Inhibitors, which block the RT or IN, are the ways of blocking the virus replication as it will be explained later.

2.2 Transmission

FIV spreads by direct contact. Vertical (from queen to kitten) and horizontal (from cat to another cat) transmission is possible, but the main route is through bite wounds (Shelton *et al.*, 1989). Furthermore, there is a big difference in natural infection and iatrogenic/laboratory infection. While tests in laboratory have shown, that the infection may occur via parenteral routes, such as intravenous administration as well as over the mucosal membranes (e.g. rectal or vaginal route), these transmissions are rare, especially compared to HIV, where the mucosal transmission is the main mode of transmission (Bishop *et al.*, 1996; Burkhard and Dean, 2003). Outdoor unneutered cats, especially male cats who are aggressive and fight for territories, are the main transmitter of FIV, making the control of the virus challenging.

Queens being chronically or acutely infected may transmit the virus transplacentarly, intrapartum and via milk-borne routes (Sellon *et al.*, 1994; O'Neil, Burkhard and Hoover, 1996; Rogers and Hoover, 1998). Kittens of infected or vaccinated queens may show seropositivity after birth for up to 12 weeks (MacDonald *et al.*, 2004). It is important, that these kittens have to be tested after 6 months, to find out whether they are infected or were just immunised (this is a problem in those countries, where vaccination is used). Studies have proven, that the FIV-positive and FIV-negative cats, which are held together indoors, share litters and are neutered, are very unlikely to infect each other, as long as they do not show any aggression towards the other (Litster, 2014).

2.3 Pathogenesis

As already mentioned above, FIV is infecting a broad range of cells. Not every strain infects the same cells at the same amount. Some strains are entering mainly lymphocytes, while others are either more prone to attack macrophages, or both equally (Hartmann, 1998). The process of the disease is characterized by three main phases. The first stage is the acute viremic stage, in which the virus replicates in large amounts and scatters through the body infecting all organs. Lymph nodes play a crucial role as the main sites of replication for FIV. Isolation of the virus is possible one week post infection (p.i.) from peripheral blood

mononuclear cells (PBMC) and lymph nodes, lymphoid organs. Other non-lymphoid organs showed positivity 3 weeks p.i. (Matteucci *et al.*, 1993). During this phase, the cats undergo seroconversion by having a humoral response producing antibodies and cytotoxic lymphocytes, which takes place at the earliest 2 weeks after infection (Yamamoto *et al.*, 1988; Flynn *et al.*, 1994; Reubel *et al.*, 1994).

The viremic stage is usually followed by a long asymptomatic phase, when the cat shows no clinical signs. This stage can take up to 6 years or longer, giving infected cats actually the chance of a normal life span and of dying without even reaching to the final stage (Hofmann-Lehmann *et al.*, 1997). During the asymptomatic phase a slow regression of CD4+ cells and an increase of CD8+ values leads to an CD4+/CD8 inversion ratio (Tompkins *et al.*, 1991; Murphy *et al.*, 2012). Nevertheless, the progressive decline of the cells continues, leading slowly to the final stage: the terminal immunodeficiency phase.

The final stage is very similar to the human AIDS. At the beginning of this stage, the number of CD4+ cells is already very low (Eckstrand *et al.*, 2016). A rapid decline of CD4+ cells is observable, which can not only be explained by the effect of FIV. Apoptosis (active programmed cell death) happens similarly to the process in HIV, leading to the heavy fall of CD4+ cells (Bishop *et al.*, 1993). Hence the immune system breaks down, and makes the cat prone to secondary infections. The main site for detection of the infected cells is lymphoid tissue, but we can also detect infected cells in a vast majority of different organs such as intestines, kidney, bone marrow and especially salivary gland, since this is the main site of transmission (Beebe *et al.*, 1994; Park *et al.*, 1995; Obert and Hoover, 2000).

Here needs to be pointed out, that FIV does not necessarily follow these three stages. The pathogenesis of the disease is not easily distinguishable and not every cat shows all three stages. It is even possible that cats that have reached the immunodeficiency stage, return to an asymptomatic stage, if they are taken good care of (Hartmann, 2012).

2.4 Clinical signs

Clinical signs of FIV are very unspecific, ranging from fever to neurological signs. There are some symptoms that are more common in cats being FIV-positive such as for example anorexia, enlarged lymph nodes, enteritis, gingivitis, feline odontoclastic resorptive syndrome (FORL), pneumonia and ocular symptoms (glaucoma, anterior uveitis, pars

planitis, retina degeneration and retinal bleedings). Neurological signs such as behavioural changes, ataxia, seizures and nystagmus may occur as well (Phillips *et al.*, 1994). Blood samples will show mostly a non-regenerative anaemia (**fig. 1**), neutropenia or thrombocytopenia (Nelson and Couto, 2014). During the acute phase of the disease, symptoms such as fever, depression, anorexia and generalized enlarged lymph nodes can show up (Hopper *et al.*, 1989; Yamamoto *et al.*, 1989; Obert and Hoover, 2000). The symptoms can last for a few weeks, but are often unnoticed by the owners. FIV-infected cats have a five time higher chance to develop lymphomas (**fig. 6**) originating mostly from B-cells and leukemia, than FIV-negative cats (Poli *et al.*, 1994; Magden, Quackenbush and VandeWoude, 2011). Neurological symptoms in FIV-positive cats are strain dependant (Power *et al.*, 1998) and can occur at a rate of up to 5% in natural infection. The most common symptom in FIV is stomatitis (**fig. 2**), affecting mostly maxillary teeth, starting caudally and progressing rostral, but this can happen due to other viral infections as well.

Due to severe immunosuppression, the infected cats are highly prone to secondary infections. Frequent opportunistic secondary infections are FeLV, calicivirus, *Mycoplasma haemofelis*, dermatophytes, *Babesia felis*, *Toxoplasma gondii*, and cryptosporidiosis (Sparger, 2012). This plays an important role in the diagnosis and treatment of the cat. To diagnose the disease only by clinical signs, is however very unlikely. Clinical signs in an FIV-positive cat can occur either primarly from FIV-infection or from a secondary infection. Furthermore due to the long asymptomatic phase, the symptoms could also just be age related, regardless of the fact that the cat may be FIV-infected.

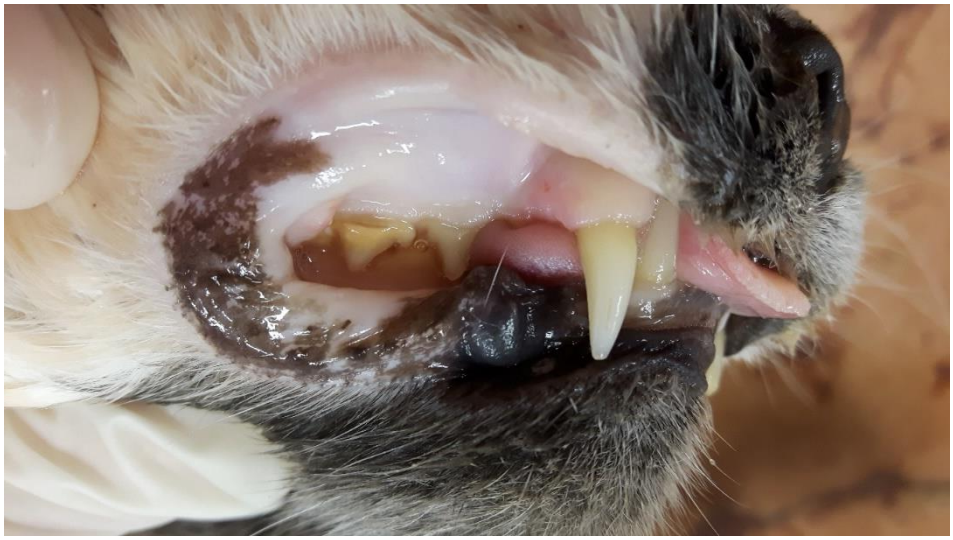

Figure 1: Necropsy picture of a cat with anaemia due to FIV-infection

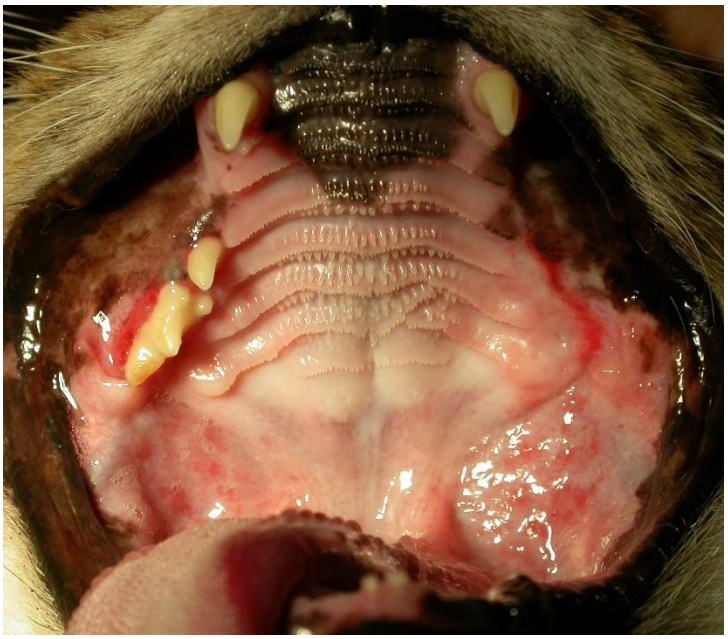

Figure 2: Mild stomatitis in a FIV-positive cat (courtesy of Dr. Csaba Jakab)

2.5 *Pathology*

FIV-positive cats may show multiple pathological changes. Lymph nodes and the thymus may be hyperplastic (**fig. 5**) or have follicular depletion, while the thymus can also be atrophic. Bone marrow dysplasia or myeloid hyperplasia can be observed frequently as well. Additionally, respiratory changes, such as interstitial pneumonia or gastrointestinal changes, (e.g. lymphoplasmocytic stomatitis, hepatitis and colitis (**fig. 3 and 4**)) can occur. Neurological changes can be perivascular lymphocytic infiltration, gliosis, myelitis and loss of neurons (Greene 2006). Hypergammaglobulinaemia can appear due to the excessive amount of antibodies. In the chronic stage, the cats produce antibodies that will form complex structures with the antigens. These immunocomplexes are deposited in narrow capillary beds and lead to vasculitis, and in the case of the kidney, to glomerulonephritis (Miró *et al.*, 2007; Gleich and Hartmann, 2009). These pathological alterations are however not specific for FIV and no diagnosis can be done based only on pathological findings.

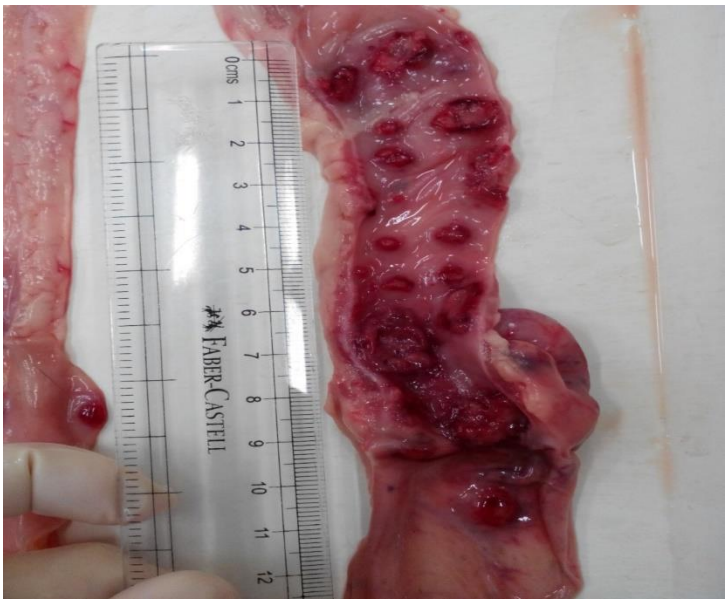

Figure 3: Necropsy picture of colitis ulcerosa in a FIV-positive cat.

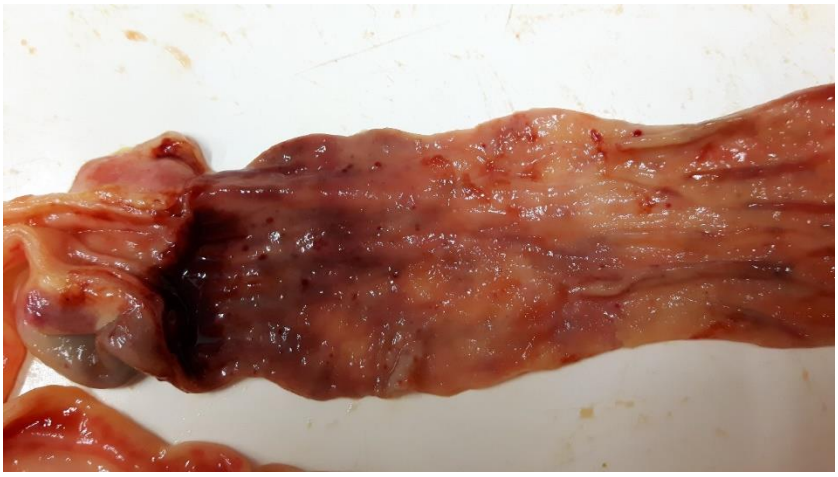

Figure 4: Necropsy picture of colitis haemorrhagica in a FIV-infected cat

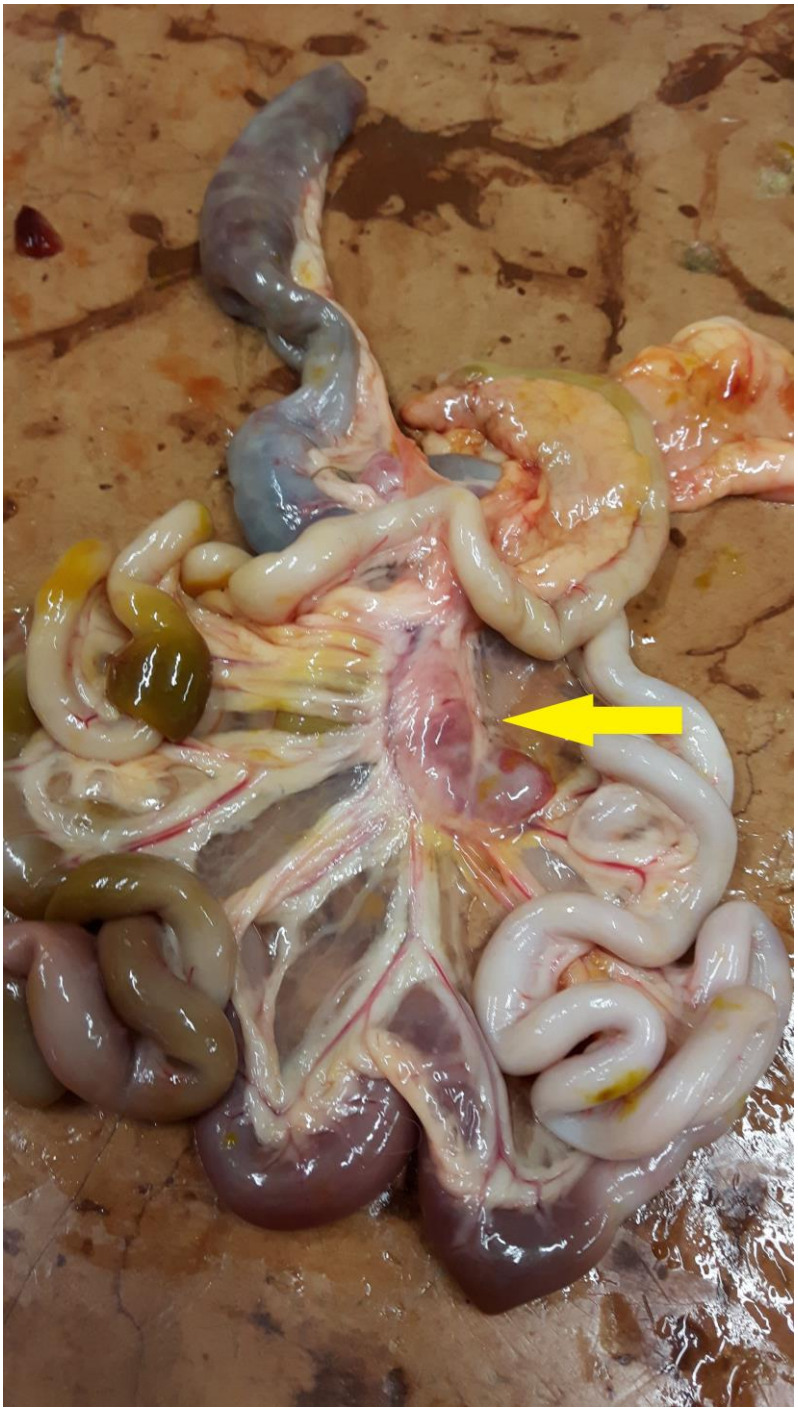

Figure 5: Necropsy picture of enlarged mesenteric lymph nodes (arrow) in a FIV-positive cat.

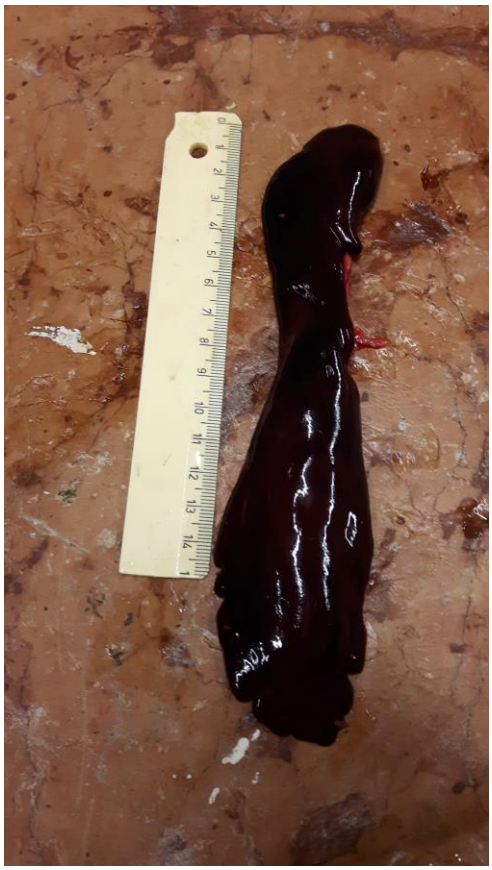

Figure 6: Necropsy picture of splenomegaly in a FIV-positive cat.

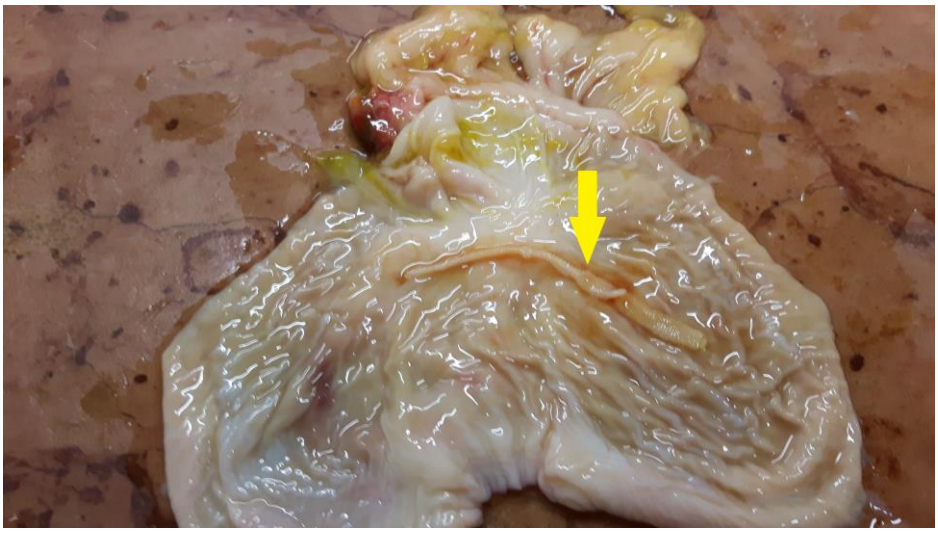

Figure 7: Necropsy picture of *Taenia taeniaeformis* in stomach of a FIV-infected cat.

2.6 Diagnosis

Diagnosing FIV based on clinical signs and anamnesis is not likely. The only way to diagnose the infection is through laboratory testing. There are two main approach areas allowing diagnosis of FIV with laboratory examinations: serology and virus isolation. Below I will expand on both techniques individually

Serology:

The easiest and quickest diagnostic test is by ELISA or rapid immunomigration-type assays. They are based on antibody detection. Although they have a good accuracy, sensitivity and specificity, none of the tests are 100% efficient. The mistakes can be based on technical errors by the use of whole blood instead of serum in case of ELISA.

A different method to use is western blot. In former days it was believed, that western blot is more accurate than ELISA, however recent tests have proven the opposite (Levy, Crawford and Slater, 2004). Furthermore, western blot has further disadvantages as it is more expensive, time consuming and requires a very specific knowledge of the person performing it, which makes it prone to errors.

Antibodies are detectable 8 weeks after infection at the earliest, but it may take longer. It is very likely, that in the acute viremic stage of infection serological tests are negative, and therefore it is recommended to repeat it a few weeks later (Levy *et al.*, 2009).

There is a vaccine available on the market against FIV in the USA, Australia and New-Zealand. Kittens born from infected or vaccinated queens may also have anti-FIV antibodies till 12 weeks of age. This may lead to false positivity of tests, if the owner or veterinarian does not know, whether the cat has been vaccinated or not. For kittens, it is recommended to test them a second time after 6 months to double-check the first test results (MacDonald *et al.*, 2004). In general it is advisable not to test before 6-month of age due to possible presence of maternal antibodies.

Virus isolation:

Cats entering the final stage may not be able to produce any antibodies, making FIV undetectable by ELISA, rapid immunomigration or western blot. In that case, PCR is an option of diagnosis. PCR is not done routinely due to lack of standardisation and time in hospitals. Furthermore, PCR's sensitivity and specificity is lacking accuracy which advises that one should remain sceptical with the final diagnosis (Bienzle *et al.*, 2004).

It has to be pointed out, that to this day, there is no 100% accurate test on the market. If ELISA or other serology tests are positive, they should be confirmed by a second test such as western blot or PCR, to ensure the result.

2.7 Treatment

Anti-retroviral drugs will always be in the main field of study for HIV. Therefore large progresses have been made in HIV therapy, while in FIV therapy less studies and improvement in treatment has occurred. Retroviral therapy is targeting six different steps in the lifecycle of the virus. These are the entry of the virus into the host cell, the fusion of the membranes, the reverse transcription, the integration of viral DNA into host genome, viral transcription and the production and maturation of virions (Mohammadi and Bienzle, 2012). Treatment of FIV is not possible (we cannot eliminate the virus entirely from the body), but we are able to give the cat an improved quality of life and increased life expectancy. Medications such as azidothymidin (AZT), interferons (IFN) and human erythropoietin have shown positive effects in health of the patient.

Anti-retroviral drugs:

AZT is a nucleoside-analogue and blocks the reverse-transcriptase of lentivirus. It is able to inhibit the infection of new cells, but does not prevent the replication of the virus in already infected cells. So far it stands as the best tested anti-FIV medication on the market. It is proven to improve clinical signs (Hartmann *et al.*, 1992) and even prophylactically protect against FIV, but it does not reduce virus loads in chronically infected cats (Arai, Earl and Yamamoto, 2002). Many studies have been performed on the prophylactic performance of AZT. Cats treated prophylactically with a moderate AZT dose (20mg/kg/day for 4 weeks) starting one day before inoculation of FIV, showed a non-detectable viral plasma load, however the peripheral blood mononuclear cells virus load was the same or even higher, than in the infected control group (Meers *et al.*, 1993). Another study with a dose of 30mg/kg/day for 4 weeks showed no detectable viral plasma load, had delayed PBMC viral load or delayed loss of CD4+ cells (Hayes *et al.*, 1993, 1995). In combination with lamivudine (3TC) it has shown very good results in prophylactic treatment (Arai, Earl and Yamamoto, 2002). Due to the side effects (non-regenerative anaemia), cats treated with AZT

should have regularly blood tests and cats with bone marrow suppression are not supposed to be treated with it (Hartmann *et al.*, 1992).

Other antiretroviral drugs such as 9-2-(phosphonylmethoxyethyl) adenine (PMEA) also showed an increase in the CD4+/CD8+ ratio and improvement in immunological status (Hartmann *et al.*, 1992). It also kept viral DNA level low during treatment, but did not manage to prevent from infection (Philpott *et al*, 1992).

Interferons (INF) are antiviral glycoproteins leading to the production of antibodies. They are cytokines, that actually have no specific antiviral effect (Doménech *et al.*, 2011). Low dose administration of human IFN-alpha has shown improvements in the clinical image as well as a longer survival of CD4+ cells and a slow increase in CD8+ cells (Pedretti *et al.*, 2006). It also can be given in high dosage for several weeks by oral administration. Feline IFN-omega has also shown benefits in clinical signs. Both are though still lacking on proper studies.

Immunomodulatory therapy:

Besides specific antiviral treatment, immunomodulatory treatment can be performed to enhance immune function of the infected cat, leading to a better control of the viral burden and a better healing of lesions. These are however more theoretical, than actually proven scientifically (Greene 2006). One of the medication is human erythropoietin. Human erythropoietin can be given to improve red and white blood cell count without any adverse effects (Arai *et al.*, 2000). Administration of *Staphylococcus* protein A or *Propionibacterium acnes* are also two medications often given to FIV-positive cats to enhance the immune system (Ettinger & Feldman 2005). Bovine lactoferrin (40mg/kg) may be given as well. In tests it revealed, that it has improved stomatitis in FIV-infected cats by oral administration (Sato *et al.*, 1996).

Other medications:

The treatment of FIV-positive cats should always be based on health status of the patient. Since age and secondary infections play important roles, treatment with antibiotics and antiparasitics, e.g. metronidazole (5mg/kg) and clindamycin (5-11mg/kg), vitamins and other medications are necessary and will improve the prognosis of infection.

2.8 Prevention

To prevent the spread of the virus, some measures can and should be taken. When taking in a new cat into a FIV seronegative multiple cat household, testing of the cat should be performed. If a cat is positive, it should be kept indoors, isolated from other cats to prevent the spread of the disease. Regular cleaning of the litters and dishes also helps preventing the spread of FIV. Furthermore a regular testing of outdoor cats should be enhanced. If a cat has been exposed to a possible infection, it is recommended to test the cat 60 days later (Goldkamp *et al.*, 2008).

Kittens of an infected queen should not be nursed by that queen, and should be tested after 6 months of birth, since antibodies might be present till that age due to maternal immunity (Nelson and Couto, 2014).

In the USA, Australia and New-Zealand there is a vaccine against FIV, but it is not used in Europe, because of the lack of safety proof and because laboratory diagnostic tests can not differentiate well between the virus- and vaccine-induced antibodies, leading to false positivity. This inactivated vaccine consists of FIV subtype A and D, but its efficacy is approximately 60–70%, and it does not defend against other subtypes (Crawford and Levy, 2007).

Finally, humans cannot be infected, but since FIV leads to secondary infections, other zoonotic diseases may be spread by immunosuppressed cats.

3 Materials and Methods

A total of 184 (n=184) client-owned domestic cats were tested from all over Hungary, presented in 13 clinics (**fig. 8**), over a period of 1 year (2015–2016). No free-roaming or sheltered cats were included in the survey. The samples were encoded and age, sex and house holding status of cats were registered. Anamnesis was also taken of each cat looking for any clinical symptom in its earlier life and time of presentation. General physical examination has been performed. From 184 cats, 72 were healthy and 112 showed clinical signs. Out of the 112 sick cats, 36 had oral cavity problems, 27 haematological disorders, 11 respiratory symptoms, 2 respiratory and haematological signs and 36 cats with other symptoms (e.g. anorexia, lethargy, fever). Gender distribution was 103 males and 81 queens. 41 of them live only indoors, and 143 have access to outside. The average age of the cats is 5 years.

EDTA-anticoagulated blood samples were drawn from cats presented in clinics (either asymptomatic or ill) and WITNESS® FeLV-FIV test (Zoetis®) was performed immediately as per manufacturer's instructions. The rest of the samples were sent to the Pathology Department (University of Veterinary Medicine, Budapest) for further examination, samples were stored at -80°C until PCR testing. Conventional PCR tests were performed from these samples, then statistical analysis of collected data was carried out.

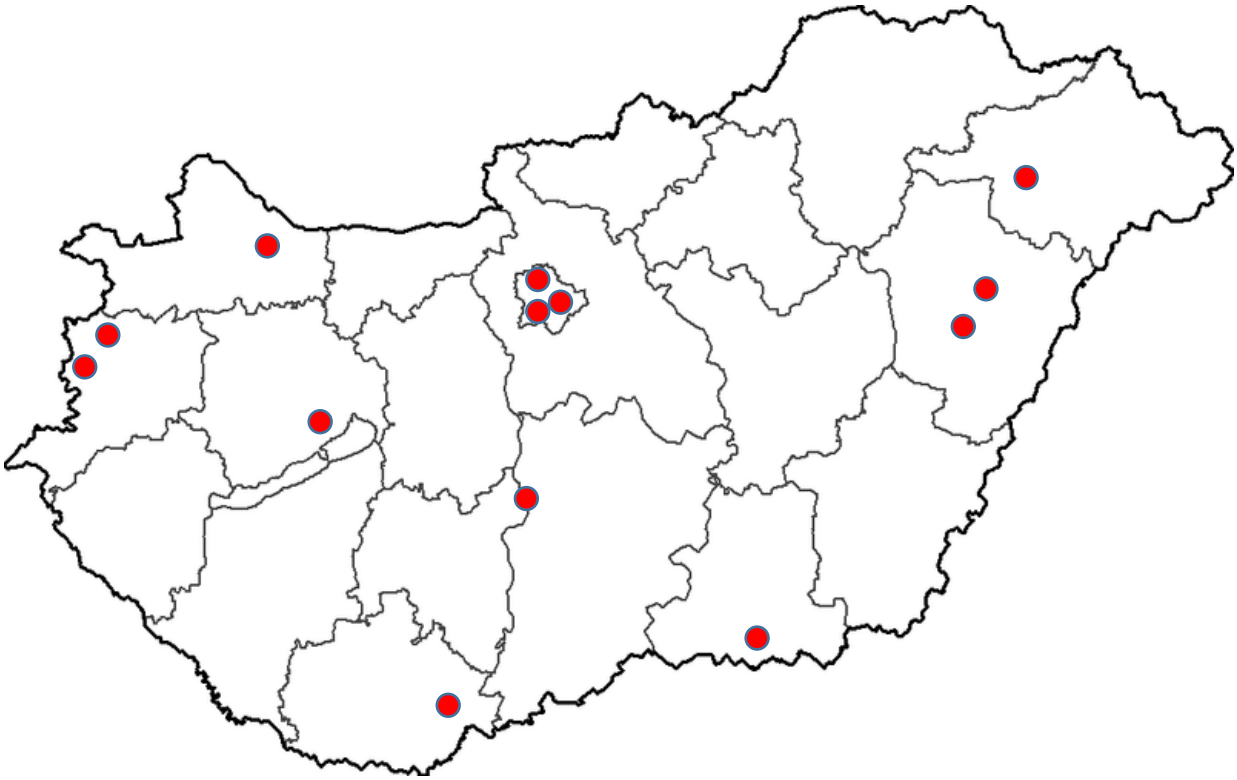

Figure 8: The graphic shows the clinics, where the samples were taken in Hungary.

3.1 Serology testing

Witness® FeLV-FIV (Feline Leukemia Virus Antigen – Feline Immunodeficiency Virus Antibody) Test Kit (Zoetis®) is an accurate, affordable and simple in-clinic test that detects the presence of FeLV antigen and FIV antibodies.

The accuracy of Witness® FeLV-FIV test in the results is 93.8% sensitivity and 93.4% specificity. Sensitivity represents the ability to correctly identify positive samples. Specificity represents the ability to correctly identify negative samples. The test is based on rapid immunomigration. A test result should always be interpreted in the context of all available clinical information and history of the cat. One drop (0.05 ml) of EDTA-anticoagulated whole blood was used as per manufacturer's instructions.

3.2 Polymerase chain reaction (PCR)

PCR is a method based on the DNA polymerase to produce new strands of DNA complementary to the template strand. As a result, we can amplify low numbers of nucleic acid from infected cells and detect them with electrophoresis.

Template DNA is the extracted product that contains the target sequence in case of infection. Nucleic acid extraction was made in QIAcube machine (Qiagen®) with QIAmp® Viral RNA Isolation Kit (Qiagen®) as per manufacturer's instructions. After extraction preparation for standard PCR, TopTaq Master Mix Kit (Qiagen®) was used as per manufacturer's instructions: 25 µl master mix, 0.5 µl forward primer, 0.5 µl reverse primer, 5 µl CoralLoad Concentrate®, 18 µl RNase-free water, 1 µl template DNA were mixed together in the case of each sample. Genesy PCR Thermal Cycler (Tianlong®) was used for hot-start PCR amplification with the given protocol: 95°C for 15 minutes in stage 1; 95°C for 45 seconds and 60°C for 45 seconds and 72°C for 1 minute in stage 2 (40 cycles); 72°C for 15 minutes. After amplification, products were stored at 4°C until electrophoresis.

DNA polymerase is a type of enzyme that synthesizes new strands of DNA, complementary to the target sequence. The first and most commonly used of these enzymes is *Taq* DNA polymerase (from *Thermus aquaticus*), whereas *Pfu* DNA polymerase (from *Pyrococcus furiosus*) is used widely because of its higher fidelity when copying DNA. Both are heat resistant. Nucleotides (dNTPs or deoxynucleotide triphosphates) are single units of the bases adenine (A), thymine (T), guanine (G) and cytosine (C), which are essential for composing new DNA strands together. Reverse transcription PCR (RT-PCR) is PCR preceded with conversion of sample RNA into cDNA with the enzyme reverse transcriptase.

Primers are short pieces of single-stranded DNA that are complementary to the target sequence. The polymerase begins synthesizing new DNA from the end of the primer. Our primers were provided by Dr. Ákos Hornyák (National Food Chain Safety Office, Veterinary Diagnostic Directorate): LTR-sense 5'-GCG CTA GCA GCT GCC TAA CCG CAA AAC CAC-3' and LTR-antisense 5'-GTA TCT GTG GGA GCC TCA AGG GAG AAC TC-3'. These primers are amplify early stage products of reverse transcription, therefore we can use it as a diagnostic primer for FIV infection(Sutton, 2007). The amplicon is 163 base pair long. PCR products were separated according to size on 1.3% agarose gel for analysis (**fig. 9**).

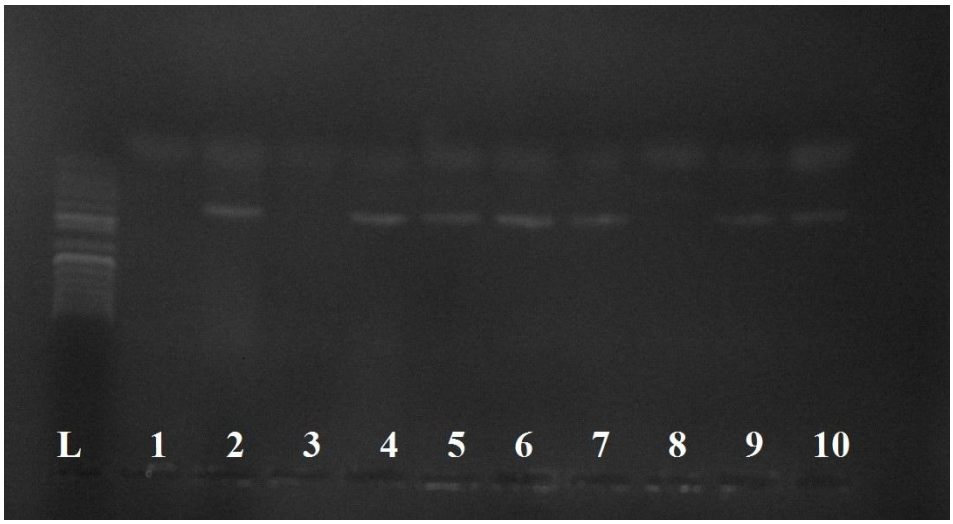

Figure 9: Picture of agarose gel electrophoresis. (From left: L: ladder, 1: negative control, 2: positive control, 3: negative sample, positive samples [4–7], 8: negative sample, positive samples [9–10]).

4 Results

Out of 184 cats (n=184), 30 cats (16.7%) were tested positive by the Witness® FeLV-FIV test kit. Among those cats 12 (40%) showed symptoms of the oral cavity as gingivitis, stomatitis, FORL etc. 7 cats (23%) showed haematological disorders as anaemia, leukopenia, *Mycoplasma haemofelis* infection, lymphopenia, decreased total protein (TP), azotaemia. 1 cat showed respiratory symptoms and 1 cat had respiratory and haematological symptoms (in total 7%). 7 cats (23%) showed other clinical signs as fever, lethargy, anorexia, parasite infection e.g. *Otodectes cynotis*. Only 2 cats (7%) showed no symptoms at all (**fig. 10**).
From the 30 cats that tested positive with the serology test, 22 cats (11.95% of the 184) cats were confirmed FIV-positive by PCR. 9 (40.9% of the 22) showed oral cavity problems, 5 (22.7%) had haematological disorders, 1 (4 %) had respiratory and haematological disorders and 5 (23%) had other symptoms. The 2 asymptomatic cats (9%) tested positive by serology, were also positive with PCR (**fig. 11**).

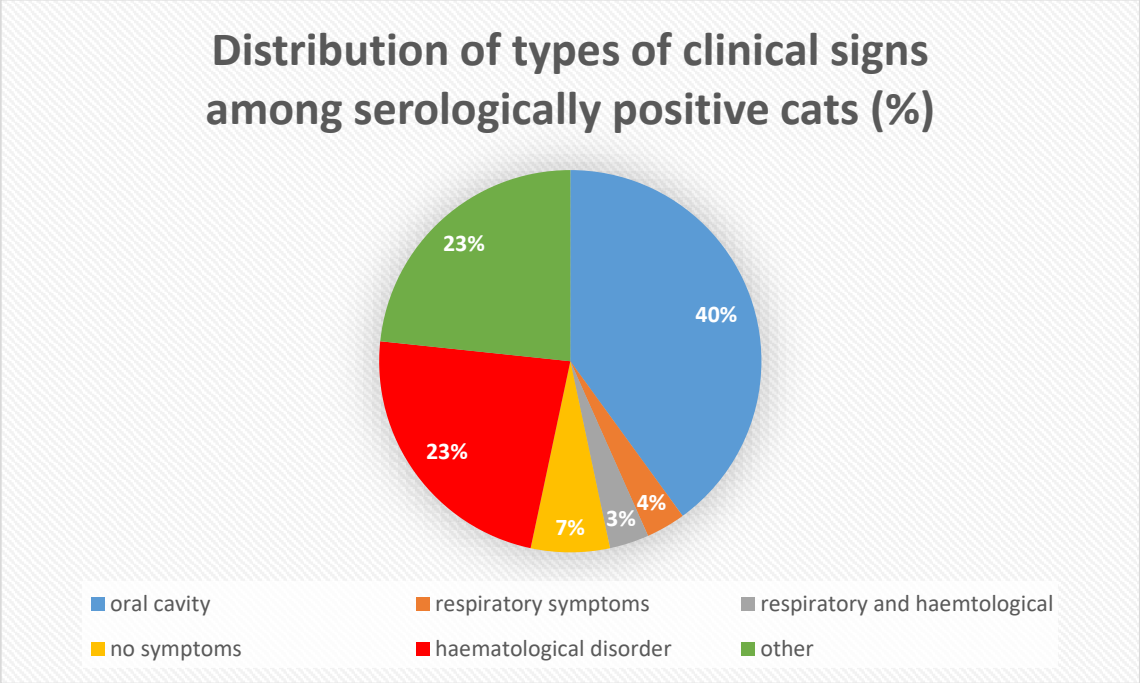

Figure 10: The graph shows what type of symptoms were experienced in FIV-positive cats. Oral cavity symptoms have to be found the most frequent clinical signs.

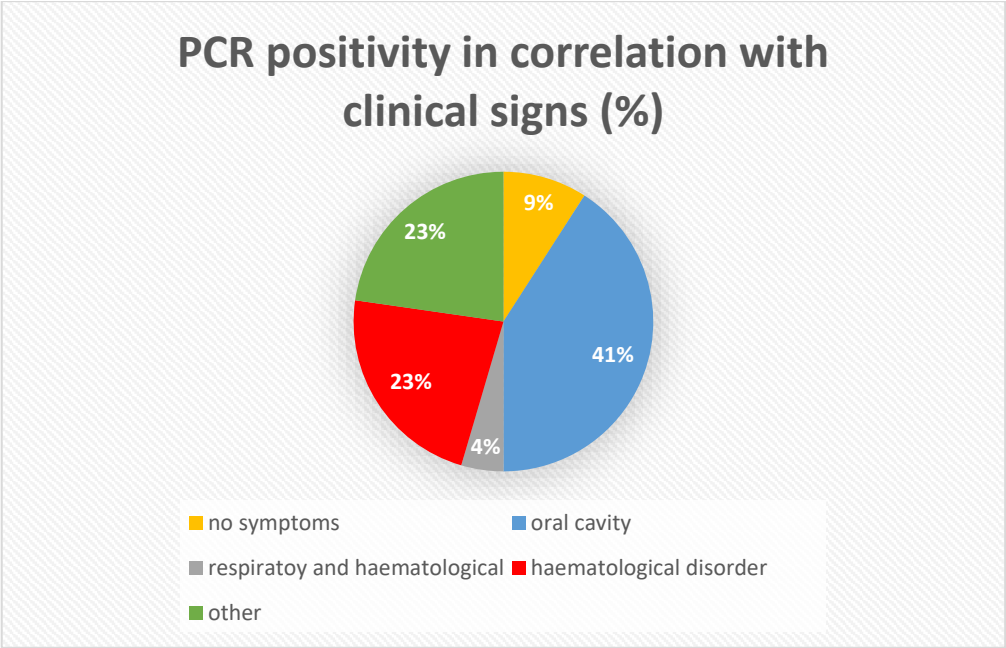

Figure 11: The graph shows what type of symptoms were experienced in FIV-infected cats. Oral cavity symptoms have to be found the most frequent clinical signs.

In total, from 72 asymptomatic cats, 2 were FIV-positive (2.7%) and from 112 unhealthy cats, 20 (17.9 %) were FIV-infected according to PCR (**fig. 12**).

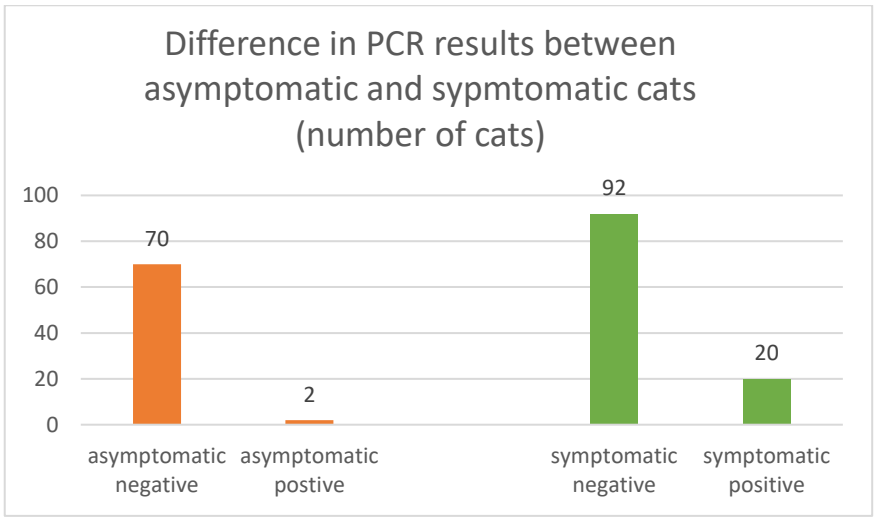

Figure 12: This graph reflects on the higher prevalence of FIV-positivity, if a cat shows symptoms. The graph show, that cats with symptoms have higher chance to be FIV-positive than asymptomatic cats.

Statistical evaluation was carried out with R 3.3.2. Software, p-value <0.05. The logistic regression has revealed that out of 108 male cats tested with Witness® FeLV-FIV, 17 (16.5%) were positive, while for the queens, 13 (16%) out of 69 were positive (**fig. 13**). It shows, that there is a 4.25 (95% confidence interval is 1.70–12.28) times higher chance of infection among male cats (p=0.0036) which is a significant difference. With PCR results, we have similar tendencies: 3.24 (95% confidence interval is 1.19–10.39) times higher chance with p=0.0303 significance (**fig. 14**). Of the 143 cats which had access to outdoors, 16 cats (25%) were seropositive, while only 1 cat (2.5%) of 41 indoor cats was tested positive with serology (**fig. 15**). Again, we experienced the same tendencies with PCR. The difference between indoor and outdoor kept cats however was not significant (p>0.05).

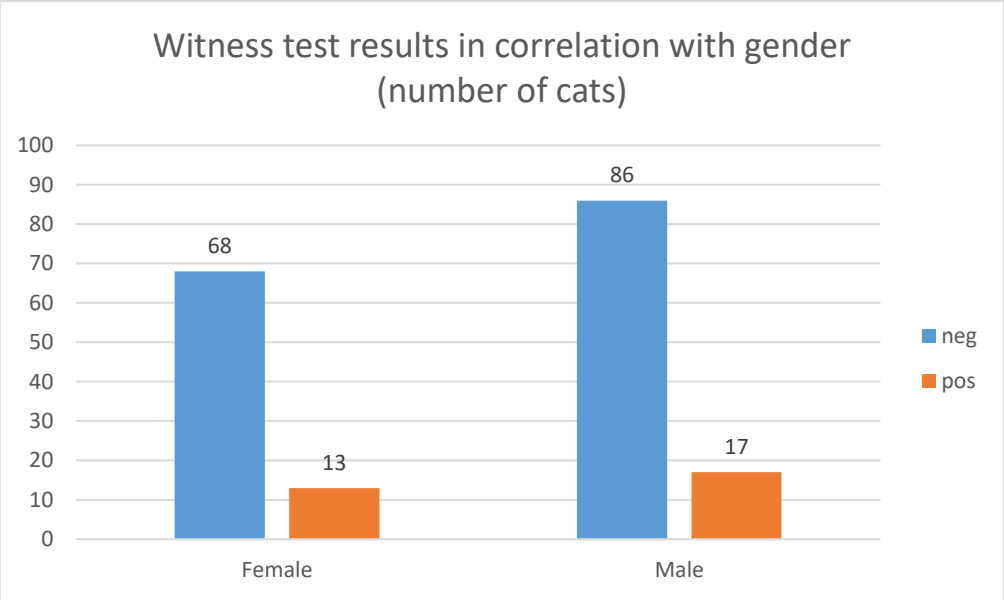

Figure 13: The graph shows us, if males or females are more prone to FIV. While there is no big difference with Witness test, PCR (fig. 14) reveals, that males are more prone to FIV infection.

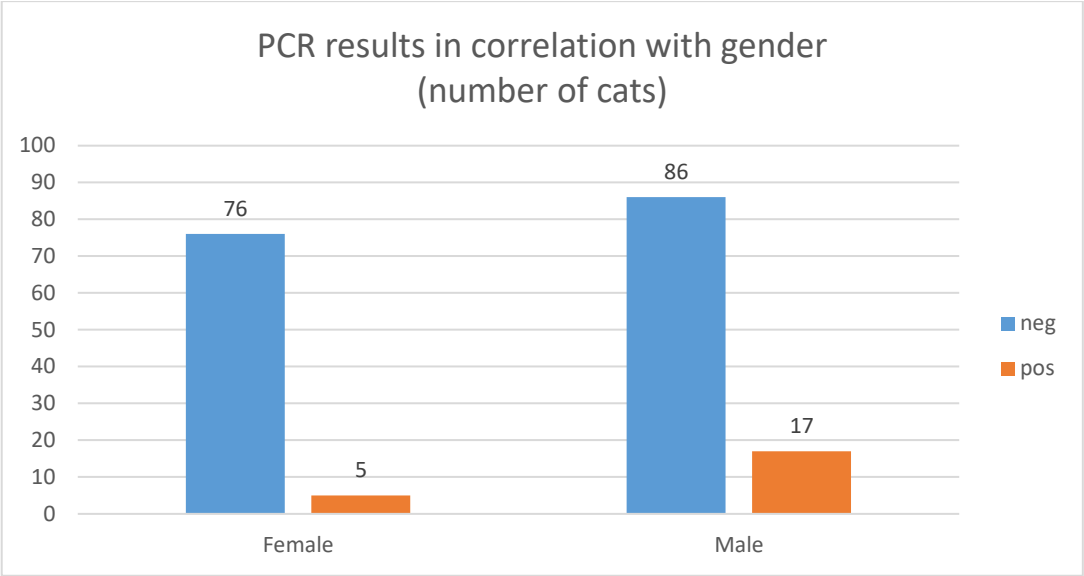

Figure 14: The graph shows us, if males or females are more prone to FIV. While there is no big difference with Witness test (fig. 13), PCR reveals, that males are more prone to FIV infection.

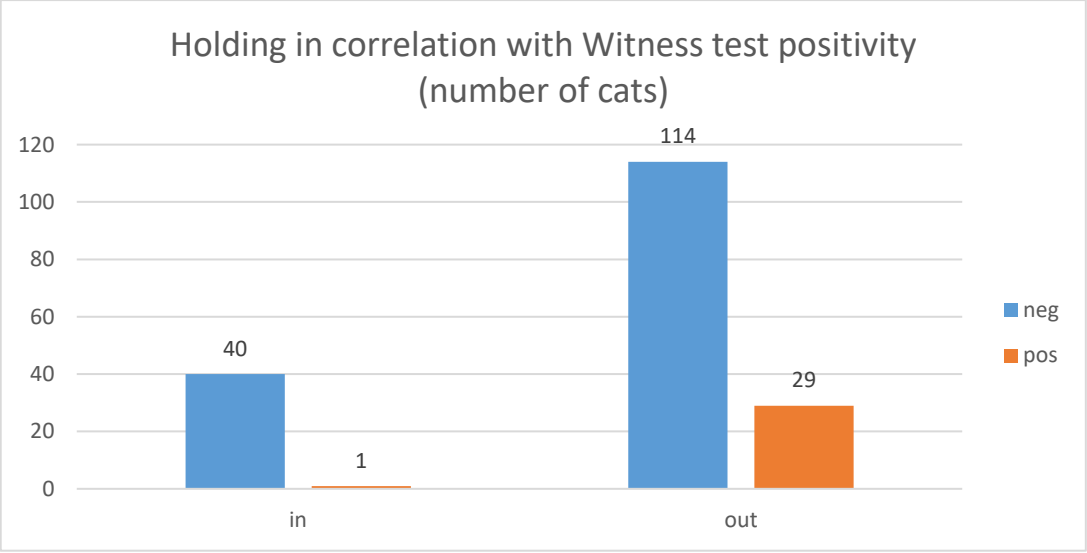

Figure 15: Based on our results, in this graph it can be seen, that cats with outdoor access are more endangered for FIV infection, than cats which live strictly inside.

Logistic regression was made in correlation with age and FIV-positivity as well. Results showed, that there was a 6.16 times higher chance among cats older than 3 years to be positive with the Witness® FeLV-FIV test (p=0.0014) and a 5.38 times higher chance to be positive with a PCR test (p=0.0093) (**fig. 16, 17**).

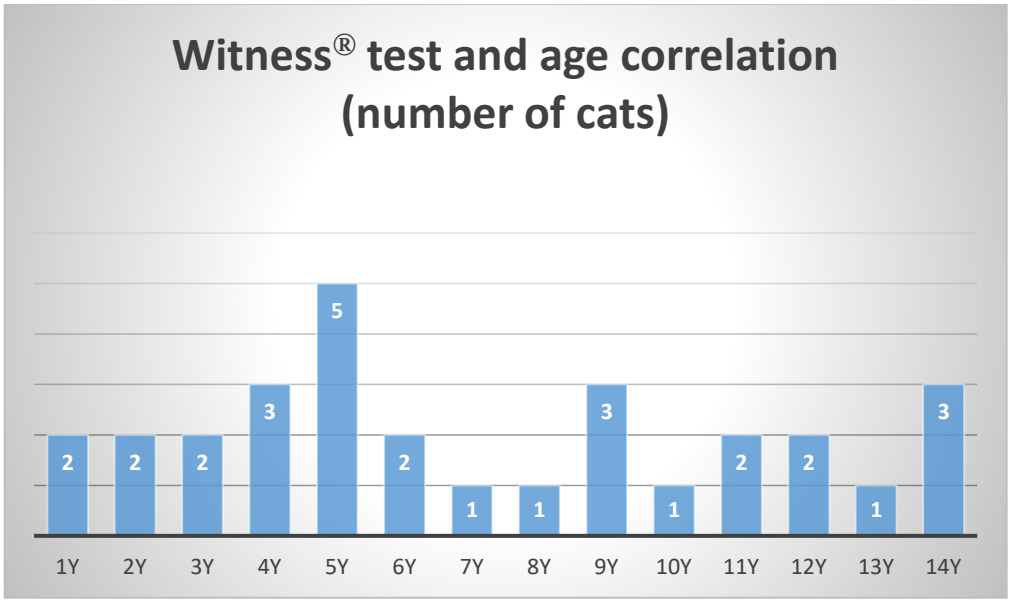

Figure 16: Age in relation with FIV-positivity, serology results.

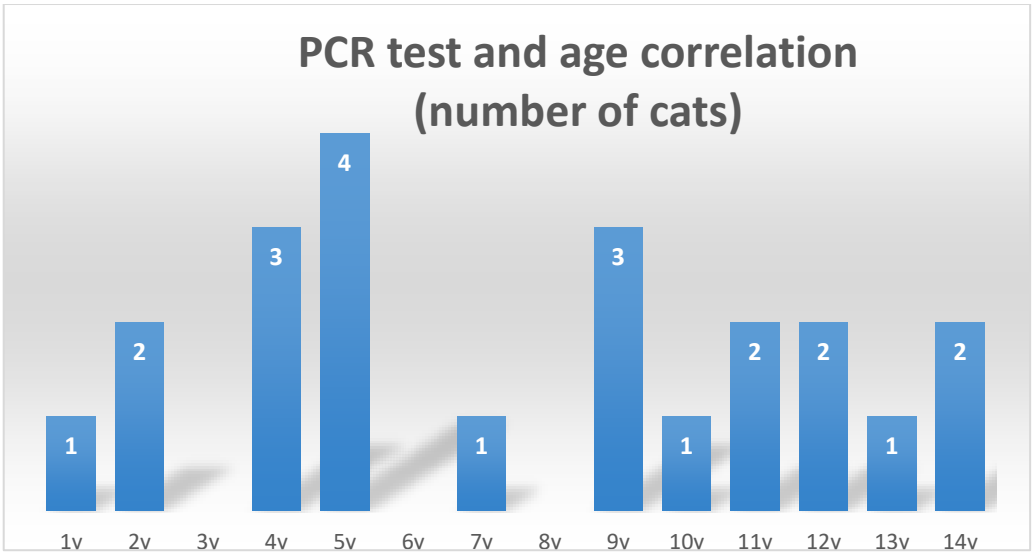

Figure 17: The graph shows, that FIV can be detected at any age, but there is a higher chance of FIV-infection, if the cat is older than 3 years.

As we can see, the calculated true prevalence (tp) of FIV is quite high in Hungary (**table 1**) compared to other European countries. The calculation was made with Rogan–Gladen formula (Rogan and Gladen, 1978), the confidence intervals are calculated with the Blaker–Reiczigel formula (Reiczigel *et al*, 2010).

With serology testing, true prevalence is 8.5% with 56.9% positive predictive value (PPV), which means if a Witness test is positive, the chance is only 56.9% that the cat is truly infected with FIV. With PCR analysis we could only calculate with approximate sensitivities and specificities taken from literature (Westman et al. 2016), because we don't have exact values for our diagnostic primers. Therefore there is a tp value calculated for a lower (93.0%) and a higher (96.0%) sensitivity and specificity, 4.3% and 7.3% respectively. The PPVs are 37.3% and 65.3% respectively. The negative predictive values (NPV) are above 99% with both tests, which means if a test is negative, the examined cat is presumably not infected.

	Sens.	Spec.	tp	PPV	NPV
PCR FIV	**93.0***	**93.0***	**4.3 (0.0; 10.0)**	**37.3 (0.0; 59.7)**	**99.7 (99.2; 100.0)**
PCR FIV	**96.0***	**96.0***	**7.3 (3.1; 12.6)**	**65.3 (43.3; 77.6)**	**99.7 (99.4; 99.9)**
Witness® FIV	**93.8**	**93.4**	**8.5 (3.7; 14.5)**	**56.9 (35.4; 70.7)**	**99.4 (98.9; 99.7)**

Table 1: Sens.: sensitivity, spec.: specificity, tp: true prevalence, PPV: positive predictive value, NPV: negative predictive value. In brackets we can see the 95% confidence intervals. *(Westman *et al.*, 2016)

5 Discussion

The aim of this study was to get an overview of the prevalence and occurrence of FIV in Hungary. Samples of 184 client-owned cats were taken and all possible data was collected. Furthermore, rapid immunomigration serology and conventional PCR tests were performed on all the samples.

30 cats (16.9%) were tested positive with serology test and 22 (12%) from these confirmed positive by PCR. From asymptomatic cats 2.7%, from symptomatic cats 17.9% were FIV-positive. These numbers are very high compared to our neighbouring countries. The average age of Witness-positive cats is 6.5 years and 8 years in the case of PCR-positive cats. PCR data has shown that male cats have a higher infection rate than females. Moreover, outdoor cats have a higher chance of infection than indoor cats. On one hand, these findings for age and holding agree with other experiments and data, and prove that male outdoor cats are the main carrier and transmitter of the virus due to their behaviour. On the other hand, the high prevalence of FIV is not corresponding to results in other countries in Europe. Although prevalence surveys show, that the infection rate can be as high as 30%, those studies however included free-roaming and sheltered cats. The prevalence among client-owned cats are significantly lower (2–5%) (Gleich, Krieger and Hartmann, 2009).

There are several explanations for this high prevalence in Hungary. The first is that the tests had correct (not false) results and we have a quite high prevalence in Hungary. The second reason may be the phenomenon of false positive paradox. The false positive paradox is a statistical result, in which false positive tests occur more often than true positive tests due to a low incidence rate of a condition (here: FIV infection) and the incidence rate of true positive rate was indeed lower than false positive rate. The probability of a positive test result is not only determined by the accuracy of the test, but also by the characteristics of the sampled population. The main factors in the case of this study are the experienced 7.3–8.5% of prevalence (**table 1**) and the sensitivity of the Witness® FeLV-FIV test, which is 93.8% (Rheinfurth, M. H.; Howell, L. W. March 1998). In a population with a low number of infected cats, there will be more cats that test positive for FIV incorrectly. To clarify this and have more correct statistics in the case of Hungary, we are planning to run a further survey with a higher sample number (n=200).

Another explanation could be the low screening level of client-owned and stray cats in general. This may be caused by either a lack of information of testing possibilities among owners, or the unwillingness to invest in regular screenings. The veterinarians' experience shows that owners seem to be willing to test only in the case of an illness.

Our testing methods may be an explanation for the high prevalence. Witness® FeLV-FIV test kits detect FIV based on anti-FIV antibody production. It is a simple and quick test, which can and should be performed in every clinic either for diagnostic purposes or for screening. Even though it is a good test, false positivity and false negativity are possible nevertheless: it can never reach a 100% accuracy rate. Since it is based on antibody production, it can only confirm an infection, if antibodies are present in the blood circulation. Unfortunately in the case of chronically ill cats, immunosuppression may be enhanced and bone marrow may not be able of providing a proper immune system response anymore. Another occurring problem in countries, where a commercial vaccine is available and is in use, is that serology tests cannot differentiate between antibodies induced through vaccinations and viral infection induced antibodies, which leads to false results. However, this is not relevant in our country, as there are no vaccines on the market.

PCR testing is currently the best way to confirm possibly false or unverified results. As mentioned above, serology testing can be false negative, either if the infection is in an early stage when antibodies are not yet present in the circulation, or in the end-stage, when the organism is unable to produce antibodies. The PCR method is able to amplify small quantities of viral RNA/proviral DNA in the specimen (in our case, EDTA-anticoagulated whole blood). With specific primers on the virus' constant region (to avoid false negative results because of primers' inability to bind in the case of mutations in the genome's more variable regions), we are able to detect viral nucleic acid in samples, and clarify the patients' infection status.

6 Conclusion

This research has highlighted the importance of learning more about retroviruses, and especially about FIV. Being the major non-primate model-virus of HIV and a relevant pathogen of wild and domestic cats, we have to raise the awareness of veterinarians, why it is important to test their patients, how they can deal with an already infected cat, and what the main points of care are (e.g. not to treat patients with steroids without knowing the FIV/FeLV status of the cat). However, we also should not forget how important it is to educate the clients. There is still a lot to achieve in this field in Hungary, as we have a high level of prevalence of FIV in the country.

Further examinations are planned: we start a second sample collecting trial with performing the same tests, and also genetic sequencing of FIV-positive specimens is in progress already.

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