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PREVALENCE OF FELINE IMMUNODEFICIENCY VIRUS AND FELINE LEUKEMIA VIRUS INFECTION IN SHELTER CATS IN TARTU: ASSOCIATION WITH AGE, GENDER, RHINITIS AND SERUM HAPTOGLOBIN CONCENTRATION

KASSIDE IMMUUNPUUDULIKKUSE VIIRUSE JA KASSIDE LEUKEEMIAVIIRUSE LEVIMUS TARTU VARJUPAIGA KASSIDEL: SEOSED VANUSE, SOO, RINIIDI JA HAPTOGLOBIINI KONTSENTRATSIOONIGA SEERUMIS

Final Thesis in Veterinary Medicine

Curriculum in Veterinary Medicine

Supervisors: Toomas Orro, DVM, *PhD*

Elisabeth Dorbek-Kolin, *DVM*

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ABSTRACT

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Title: Prevalence of feline immunodeficiency virus and feline leukemia virus infection in shelter cats in Tartu: association with age, gender, rhinitis and serum haptoglobin concentration

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Feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) are important infectious diseases of cats with a worldwide prevalence. Prevalence or risk factor studies in Estonia have not previously been conducted. Haptoglobin is an acute phase protein which increases in serum in case of infection and inflammation and should therefore increase in case of retroviral infection. This study was conducted to find the prevalence and risk factors of FIV and FeLV infection in Estonia and to assess the relation between haptoglobin concentration, FIV/FeLV infection and the risk factors for infection. Study population included 173 cats from Tartu animal shelter. Blood was collected and FIV/FeLV immunochromatographic Speed Duo tests conducted in Janne Orro animal clinic. Haptoglobin concentration was measured using a spectrophotometric assay developed for pigs. Statistical analysis was conducted using STATA 14.2. Results show the apparent prevalence of FIV to be 13.3% and the risk factor for infection to be male gender. There were too few FeLV positive samples to make estimations. Haptoglobin concentration increase was found to be associated with male gender and rhinitis, but not with FIV or FeLV infection. FIV prevalence and risk factors seem in accordance with other studies, but a larger and more versatile study population is needed for further studies. Haptoglobin measurement is a promising diagnostic method, but further study is required for establishing reference values.

Keywords: retrovirus, risk factor, acute phase protein

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LIST OF ABBREVIATIONS

AAFP – American Association of Feline Practitioners

AGP - alfa-1-acid glycoprotein

AIDS – acquired immunodeficiency syndrome

APP – acute phase protein

ARC – AIDS related complex

DNA - deoxyribonucleic acid

e. g. - *exempli gratia* (for example)

ELISA – enzyme - linked immunosorbent assay

et al. – *et alii* (and others)

FAIDS – feline acquired immunodeficiency syndrome

FeLV – feline leukemia virus

FISS – feline injection site sarcoma

FIV – feline immunodeficiency virus

FOCMA – feline oncornavirus associated cell membrane antigen

Hgb – hemoglobin

HIV – human immunodeficiency virus

IFA – indirect immunofluorescent antibody assay

IMHA – immune mediated hemolytic anemia

PCR – polymerase chain reaction

RNA – ribonucleic acid

RT – reverse transcriptase

SAA – serum amyloid A

TMB - tetramethylbenzidine

WSAVA – World Small Animal Veterinary Association

INTRODUCTION

Feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) are retroviruses of cats causing considerable morbidity and mortality all around the world. Infections cause immunosuppression and a myriad of other clinical symptoms, which include hematological, neurological and reproductive disorders; respiratory, enteric and ocular diseases; and tumors. FeLV infection is considered to shorten life expectancy more severely than FIV infection.

The infections are spread from cat to cat mostly via saliva and infection risk is considerably affected by certain risk factors (Gleich *et al.* 2009). Hypothetically the infection risk is influenced by the cats' age, gender, lifestyle (domestic vs stray) and health status. It can be expected that there are more FIV and FeLV positive cats among adult cats rather than juveniles, among intact males rather than sterilized females and among cats who have a concurring illness at the time of sampling (Levy *et al*. 2006). It is also to be expected that prevalences among shelter cats are somewhat higher than in domestic cats (Gleich *et al*. 2009). Considering FIV and FeLV are very serious and often eventually fatal diseases, it would be beneficial to know how prevalent they might be here in Estonia and also what the qualities associated with a higher risk of infection are.

Haptoglobin is a positive moderate acute phase protein in cats, which means haptoglobin blood concentrations should increase in case of infection or inflammation (Silvestre-Ferreira *et al.* 2016). Haptoglobin measurement can be a useful tool in disease diagnosis and management if not for the lack of consensus regarding reference values for cats (Kann *et al*. 2012). It is therefore important to develop reference ranges for healthy cats and to take into consideration which factors and how may influence these values. Theoretically haptoglobin concentrations should be higher in sick cats (FIV, FeLV, rhinitis) and older cats (Ceron *et al.* 2005; Kann et al. 2012).

The aim of this thesis was to investigate the prevalence and possible risk factors for FIV and FeLV infection in Estonia and to assess a correlation between infection with these viruses and changes in serum haptoglobin concentrations. This is based on the data collected from cats from Tartu animal shelter, laboratory haptoglobin concentration measurements from the cats' sera and statistical analysis using STATA 14.2. We wanted to investigate the correlation between haptoglobin serum concentration and infection with FIV and FeLV,

since it could be hypothesized that infection causes haptoglobin increase. Association between haptoglobin concentration and possible risk factors (gender, age, concurring rhinitis) was also assessed to see how these factors effect haptoglobin levels.

ACKNOWLEDGEMENTS

I would like to thank my supervisors Toomas Orro and Elisabeth Dorbek-Kolin for all the help and guidance, hours spent in the laboratory and last but not least, calming my nerves. A special thanks goes to Janne Orro animal clinic, the wonderful veterinarians, assistants and students there, who collect samples and data from shelter cats and give good care to them. I am also grateful to Tartu animal shelter for providing medical care to their inhabitants and therefore providing me with material for my thesis.

1. FELINE IMMUNODEFICIENCY VIRUS

FIV is a lentivirus belonging to the retrovirus family. It is a lymphotropic virus characterized by a long clinical latency period during which the immune function of infected cats deteriorates (Hartmann 2005). The immunodeficiency resulting from infection can lead to secondary infections, immune-mediated diseases, neurological disorders, increase tumor-risk and is ultimately fatal, but does not shorten life expectancy as much as FeLV infection (Gleich *et al*. 2009). In fact, many infected cats die of unrelated reasons before immunodeficiency is acquired and never develop clinical symptoms related to feline acquired immunodeficiency syndrome (FAIDS). Therefore, FIV does not seem to have a significant impact on the size of the cat population (Hartmann 2015).

FI virus has a diploid genome consisting of two identical single strands of RNA. The virion is spherical to ellipsoid with a diameter of about 100 nm (see figure 1). The central nucleoid is enclosed in a conical shell which is surrounded by an outer envelope with evenly distributed short (8 nm) projections.

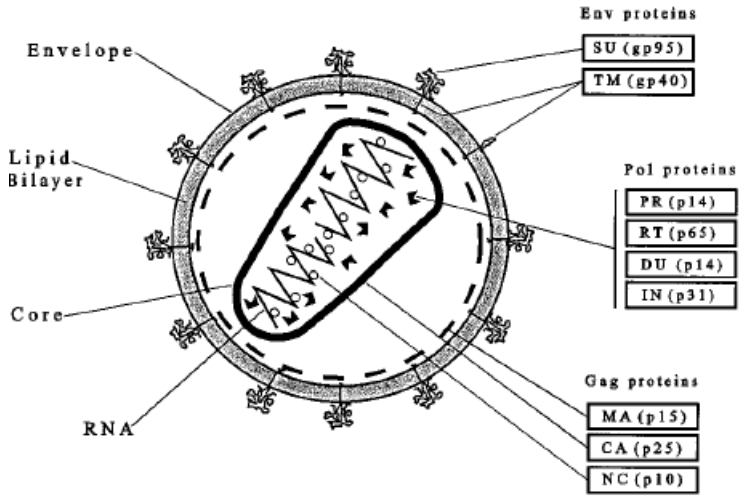

Figure 1. FIV virion molecular anatomy. *Source*: Bendinelli *et al*. 1995

It is considered a good animal model for studying human immunodeficiency virus pathogenesis due to its similarity to human immunodeficiency virus type 1 (HIV-1).

Research into HIV pathogenesis, antiviral therapy and vaccination are considered to benefit from FIV research (Sodora *et al*. 1994). FIV is morphologically indistinguishable from other lentiviruses (Bendinelli *et al*. 1995), although apparently less pathogenic than HIV (Hartmann 2005).

FI virus is genetically diverse and has several subtypes, recombinants of the subtypes are also possible. Overall 6 major subtypes are described: A, B, C, D, E (Hartmann, Sellon 2012) and F (Westman 2016). Subtype distribution varies geographically. Subtypes A, B and C are found in North-America (Little 2005). Subtypes A, B, C and D can be found in Europe with subtype A being more prevalent in Northern Europe and B in Southern Europe. All subtypes are found in Asia with B, C and D being more common. Subtypes A and B are represented in Australia and subtype A in Africa (Hartmann, Sellon 2012). Subtyping is important since commercial vaccines only contain subtypes A and D (Westman 2016).

The virus is inactivated by common disinfectants such as ethanol but can be viable in biological material in the environment for several weeks. Transmission in a hospital environment or a multicat household or shelter can therefore be eliminated with good hygiene practices. This includes disinfecting contaminated material as quickly as possible since virus infectivity increases on dried surfaces (Bendinelli *et al.* 1995; Kramer *et al.* 2006; Terpstra *et al.* 2007).

FI virus replicates in CD4+ and CD8+ lymphocytes, B lymphocytes, macrophages, microglia cells and astrocytes causing their destruction and concurring lesions (Bendinelli *et al*. 1995; Hartmann 2005). The virus can be isolated from bodily fluids – blood, saliva, cerebrospinal fluid and milk. The main route of infection is via bite wounds (Jordan *et al*. 1995; Little 2005). The virus can be found in the saliva in high concentrations; the saliva also contains infected leukocytes. Blood transmission occurs iatrogenically rather than during cat fights (Levy *et al*. 2008). Transmission is also possible in utero, during birth or through colostrum and milk (Little 2005; O'Neil *et al*. 1996) or venerally (Jordan *et al*. 1995), although these routes are uncommon in nature. FIV infection in pregnant queens can result in stillbirths, intrauterine growth retardation, premature delivery and increased neonatal death rate. Birth weights of FIV positive kittens are lower than those of non-infected kittens (O'Neil *et al*. 1996).

FIV antibodies can be isolated from blood serum 2-6 weeks post infection depending on the

virus dose and host immunity (Bendinelli *et al*. 1995; Levy *et al.* 2008). Bendinelli *et al*. 1995 have suggested that FIV has a synergistic effect with FeLV. FeLV infected cats that contract FIV have an exacerbated progression of FIV. This is bidirectional, FIV and FeLV positive cats also develop FeLV related tumors more frequently than cats infected with either virus alone.

1.1. Feline acquired immunodeficiency syndrome

Pathological effects observed in FIV-positive cats are both a direct result of the infection and indirect secondary to immunosuppression. Most common signs associated with feline retrovirus infections include anorexia, depression, fever, lymphomegaly, conjunctivitis, stomatitis, rhinotracheitis, enteritis, generalized skin infections and tumors. Anemia, leukopenia, neutropenia and lymphopenia might be seen in the blood panel (Arjona *et al*. 2000; Bendinelli *et al*. 1995; Hartmann 2015). Staging of the infection course and prognosis estimation are carried out based on patient history and clinical signs. Viral RNA load has also been proven to be associated with clinical stage of infection (Kann *et al*. 2014).

Bendinelli *et al*. 1995 have proposed a staging for the disease based on the severity of clinical symptoms. The primary infection or acute phase can be asymptomatic, but is more commonly characterized by transient and mild illness lasting for about 1-4 weeks with symptoms such as depression, anorexia, fever, generalized lymphadenopathy and neutropenia which may lead to secondary bacterial infections (Bendinelli *et al.* 1995). In animals with a more severe infection diarrhea, upper respiratory tract infections, dermatitis, conjunctivitis, gingivitis and stomatitis can occur. Since at this stage these symptoms are usually easily treated, they are not usually connected with FIV infection or go unnoticed by the owner. Hematological change at this stage is transient leukopenia due to neutropenia. CD4+ and CD8+ T-lymphocytes decline within the first few weeks of infection. When viral antibodies begin to form, the circulating viral load is suppressed and CD8+ T-lymphocytes are produced in a level exceeding pre-infection levels also reducing viremia for a while. This alters CD4+:CD8+ ratio as well (normal ratio >2), CD8+ T-lymphocytes are now in excess and will remain so through the infected animals' life. The immune system is however still unable to eliminate the virus and both CD4+ and CD8+ levels decrease in time (Flynn *et al*. 2000; Hartmann 2005; Hartmann 2015; Levy *et al.* 2008). Low CD4+ cell levels are

associated with chronic inflammation and opportunistic infections (Hartmann 2005). Histopathological changes usually comprise of hyperplastic processes in the lymph nodes (Bendinelli *et al*. 1995).

Clinical illness is followed by an asymptomatic stage of variable length while which a progressive dysfunction of the immune system occurs. According to Hartmann (2015) the animals are usually asymptomatic many years. It depends on the pathogenicity of the infecting FIV subtype, on other infections and the age of the host. Immunodeficiency may become apparent several years post infection although anti- FIV antibodies can be isolated from blood and saliva just as from a clinically ill cat during this period. Follicular hyperplasia of the lymph nodes is reduced in this stage. Some cats may skip the asymptomatic stage entirely. Length of the asymptomatic period does not reflect the prognosis in any way (Bendinelli *et al.* 1995).

ARC (AIDS-related complex) is an acronym borrowed from human medicine representing many symptoms prodromic to AIDS but not yet meeting the definition of AIDS. These include chronic secondary infections of the upper respiratory tract and oral cavity. There is a myriad of possible pathogens causing the infections. Common bacterial pathogens include *Staphylococcus, Pseudomonas, Streptococcus*, mycobacteria; viruses e.g. FeLV, calicivirus, feline parvo virus, herpesvirus, papillomavirus and poxvirus; fungi such as *Candida albicans, Microsporum canis*; protozoa including *Cryptosporidium* and parasites such as *Toxoplasma gondii, Dirofilaria immitis, Demodex canis, Notoedres cati, Otodectes cynotis.* Infection in this stage progresses into FAIDS in an unknown period of time (Bendinelli *et al.* 1995).

FAIDS develops after a variable time interval. It is characterized by severe secondary infections and to a lesser extent by neoplastic formations and rarely neurologic disorders. The animals are usually emaciated and anemic. Infections are often resistant to treatment and caused by opportunistic pathogens. About 50% of FIV positive cats have chronic ulceroproliferative stomatitis. This is the commonest disease syndrome associated with FIV and might occur due to chronic antigenic stimulation causing an immune response. Feline calicivirus coinfection is often present.

Neoplastic disorders are most often lymphomas, squamous cell carcinoma and leukemia (Hartmann 2005). FIV-positive cats are about five times more likely to develop lymphoma or leukemia than healthy cats. They develop mostly B-cell type lymphomas, whereas FeLV

positive cats usually have T-cell lymphomas. Tumor formation can be contributed to indirect FIV influence causing chronic B-cell hyperplasia or by inhibiting tumor surveillance mechanisms. The exact mechanism is unfortunately unknown. Occasional direct oncogenic activity of FI-virus is also possible. Other neoplasias FIV infected cats may develop include fibrosarcomas, mast cell tumors, oligodendrogliomas and meningiomas (Bendinelli *et al*. 1995; Hartmann 2005; Hartmann 2015).

About 5% of clinically ill FIV positive cats have neurological signs as a predominant complaint. Many more have microscopical central nervous system lesions, but this is in poor coordination with neurological symptoms. Neurological disorders include ataxia, nystagmus, convulsions, repetitive movements, tremors, changes in sleep patterns and dementia. Dementia might go unnoticed in cats since the change is subtle (Hartmann 2005).

Changes in laboratory results both in ARC and FAIDS stages mostly include neutropenia and occasionally lymphopenia, anemia, hyperglobulinemia, thrombocytopenia. Changes in cerebrospinal fluid include pleocytosis, anti-FIV antibody and IgG concentration increases. Immunodeficiency is mostly contributed to CD4+ cell decrease and also changes in cytokine pattern. Hyperglobulinemia often occurs with immune complex deposition and can therefore cause uveitis and glomerulonephritis (Bendinelli *et al.* 1995; Hartmann 2015). Lymph nodes become involuted as the disease progresses. Follicular atrophy, segmentation and hyalinization develop, cortical follicules are depleted of cells. Other possible histopathological changes include hypercellularity of the bone marrow and brain tissue gliosis, white matter pallor and perivascular infiltrates. This can be accompanied by signs of meningitis. Kidney abnormalities may include glomerulosclerosis, tubulo-interstitial lesions (lymphocytic-plasmocytic infiltration, fibrosis, degeneration) and amyloid deposits. Oral lesions are often characterized by gingivitis and periodontitis with lymphocyte and plasma cell infiltration. Animals with chronic diarrhea have villous atrophy especially marked in the locations of mural necrosis and fibrosis. There may be lesions of many other organs as well, such as cardiomyopathy, cholangiohepatitis or pneumonia. Survival time after entering this stage of the infection tends to be less than a year (Bendinelli *et al.* 1995).

1.2. Diagnostics

FIV positive cats have a persistent lifelong infection but a low viral load throughout their lives making antibody detection from peripheral blood the best in-clinic test for FIV infection. Serum antibodies are detectable 2 to 6 weeks (60 days) post infection. The most common antibody measured is p24. If infection is suspected but the test is negative, retesting should be done 60 days later to be certain that enough time has passed for sufficient antibody formation. False negative results are unlikely due to high sensitivity of most test kits. Positive results especially in clinically sound cats should be confirmed due to possibly severe consequences of a positive diagnosis. Kittens testing positive should be retested at the age of more than 6 months to rule out effect of maternal antibodies (Levy *et al.* 2008). Positive results may be confirmed by retesting with a second soluble antibody test, Western blotting, neutralization reaction, immunofluorescence assay or virus isolation. PCR has shown variable sensitivity and specificity depending on study so it is not the best method but does offer an alternative when there is suspicion of the animal being vaccinated (Bendinelli *et al.* 1995; Hartmann 2005; Levy *et al*. 2008). Methods based on detecting virus antigen can give a false negative result after the acute stage of infection when circulating virus is too low for detection and should not be routinely used (Little 2005).

1.3. Treatment

Developing an efficient vaccine is complicated for the same reasons as HIV vaccination development. Retroviruses have a high mutation rate and genetic diversity; also, lentiviral antigens have poor immunogenity in inducing vaccinal immunity. HIV and FIV might be able to use antibodies for enhancing pathogenicity or for entering cells (Hartmann 2005).

A vaccine for FIV was introduced in 2002. The only available vaccine so far is an inactivated adjuvanted product containing subtypes A and D. Vaccination is however not recommended as postulated in World Small Animal Veterinary Association (WSAVA) 2016 vaccination guidelines (Day *et al*. 2016). This is due to firstly the inability to quickly differentiate between vaccinated and FIV-infected cats. Preferably a validated PCR test would then be needed for virus detection, this is however expensive and time consuming. Secondly the subtypes occurring in a region might be different from those in the vaccine and cross-

protection might be questionable. Thirdly, the vaccine is also adjuvanted and must be given repeatedly. It requires 3 primary injections and a revaccination annually. This is something to be considered since the cat is a species prone to feline injection site sarcoma (FISS). Vaccination can be beneficial to high risk cats such as those living with FIV positive cats or aggressive cats going outdoors. FIV test should be done immediately prior to vaccination to rule out infection. Initial vaccination age is not younger than 8 weeks (Day *et al.* 2016; Levy *et al*. 2008). According to register of medicinal products authorised in Estonia, we do not have an authorised vaccine available at this time.

When considering vaccination against other diseases modified live virus vaccines should not be administered to FIV positive cats since due to immunosuppression this might cause illness. Killed core vaccines can be used in cats without clinical symptoms. Cats developing clinical symptoms should not be vaccinated (Day *et al.* 2016). It would be best to keep FIV positive cats indoors at all times and limit contact with other cats thus reducing the need for vaccination. Neutering leading to reduced aggressive behavior between cats and stress associated with mating behavior is another major contributor to reducing virus prevalence. Although vertical virus transmission is rare the possibility should be eliminated by spaying FIV positive queens provided their condition is stable enough (Levy *et al*. 2008).

Provided that husbandry conditions of cats are good, Levy *et al.* (2006) among other researchers have found the mean survival time of infected cats compared to non-infected cats to be not much shorter. In their 2006 study for example the mean survival times were 4.9 and 6.0 years, respectively. In disease management, the importance of nutrition should also be kept in mind. A balanced diet without raw meat and dairy products which have a higher risk of bacterial and parasitic infection is essential. Infected cats should be subjected to regular check-ups to determine a deterioration in their health status.

Antiviral therapy for feline patients is limited at this time. For retrovirus infection zidovudine may be used. It is a nucleoside analog that blocks viral reverse transcriptase leading to inhibition of virus replication. Side effects include non-regenerative anemia and to avoid this, minimal dosages are used (Leal *et al*. 2014; Levy *et al.* 2008). Long term treatment is a risk factor for developing resistant FIV mutations (Hartmann 2005). Interferon therapy includes feline interferon omega and human interferon alpha both of which have conflicting results. Exact mechanism of action is unknown but interferon seems to be involved in modulation of the pro-inflammatory innate immune response. Although evidence of efficacy

is scarce (Leal *et al.* 2014), there are few alternatives regarding antiviral therapy and interferon treatment should certainly be attempted in retrovirus infected cats (personal communication with dr. med. vet Matko Perharić, Zagreb University).

1.4. Risk factors

Most commonly reported factors increasing the risk of FIV infection are male gender, adulthood and outdoor access (Gleich *et al*. 2009; Levy *et al*. 2006; Levy *et al*. 2008). Male cats are 2-4 times more likely to be infected than females. The major risk group is composed of male intact adult cats with outdoor access (Hartmann 2005). Other factors associated with increased FIV infection include aggressive behavior, concurring illness and breed (purebreds versus domestic short hairs and domestic long hairs) (Gleich *et al*. 2009; Levy *et al*. 2006; Little 2005).

1.5. Distribution

FIV and FeLV can be considered among the commonest infectious pathogens of cats and have worldwide distributions (Arjona *et al*. 2000; Levy *et al*. 2006; Levy *et al.* 2008). The prevalence of FIV can be quite variable depending on geographical region and the pet-keeping traditions there ranging from about 2% in North - America (Levy *et al.* 2006) to more than 31% in Malaysia (Bande *et al*. 2012). FIV prevalence has not changed much since the discovery of the disease in 1986 and it can be considered enzootic worldwide. Prevalence can also vary depending on whether ill or healthy cats are tested, being higher among ill felines (Bendinelli *et al*. 1995; Hartmann 2005; Levy *et al*. 2008).

1.5.1. Worldwide

FIV is one of the most common viral diseases of cats in Europe. The prevalence appears to be somewhat lower in northern Europe (approximately 2-6%) than in southern Europe (approximately 8-11%, even 30% in Italy). This might be due to a larger number of free-roaming felines in southern Europe (Arjona *et al*. 2000; Gleich *et al*. 2009; Hartmann 2005).

Examples of prevalence studies are shown in the table below.

Table 1. The prevalence of feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) infection

Region	FIV %	FeLV %	Coinfection %	Reference
North-America	2.5	2.3	0.3	Levy *et al.* 2006
Canada	11	2.8	0.8	Little 2005
UK	10.4	3.5	-	Muirden 2002
Germany	3.2	3.6	-	Gleich *et al.* 2009
Spain	10.5	21.4	1.7	Arjona *et al*. 2000
Italy	11.3	8.4	1	Bandecchi *et al.* 2006
Finland	6.6	1	-	Sukura *et al.* 1992
Russia	16	16	-	Pavlova *et al*. 2015
Malaysia	31.3	12.2	4.3	Bande *et al.* 2012
Iran	19.2	14.2	0.03	Akhtardanesh *et al.* 2012
Thailand	20.1	24.5	5.5	Sukhumavasi *et al.* 2012

1.5.2. In Estonia

Prevalence of the virus has not been officially studied and no research on the matter can be found. Veterinarians have however formed opinions based on rough prevalences from other European countries. Veterinarian Riina Jõgila has stated in an interview for a newspaper in 2013 that approximately 10-20% of our stray intact male cat population could be infected with FIV (Postimees 2013).

2. FELINE LEUKEMIA VIRUS

FeLV is a retrovirus belonging to subfamily oncornavirus. Like other retroviruses it is an RNA virus with the enzyme reverse transcriptase (RT) to synthesize viral DNA. It is a serious pathogen and can increase the risk of lymphoma formation in cats 62 times. The risk of leukemia development also greatly increases and other concurring tumors may include virus-induced fibrosarcoma, neuroblastoma, osteochondroma. Other clinical syndromes associated with FeLV infection are immunosuppression, hematologic disorders, neuropathies, reproductive disorders and fading kitten syndrome. The virus is easily inactivated by common disinfectants or heating and is inactivated in a few hours outside host body (Hartmann 2015; reviewed by Levy *et al*. 2008; Levy, Crawford 2005; Macy 2007).

The virus has three subgroups: A, B and C and their recombinants. Only subgroup A can be naturally transmitted and is found in cell-free fluids, other subgroups are associated with cells. Therefore all naturally infected cats are infected with subgroup A, 50% of cats have also combination AB and 1% have a combination with subgroup C (AC or ABC). Subgroup A has strains varying greatly in pathogenicity, but pathogenicity will always increase in combination with other subgroups. Subgroup B is created when subgroup A uniquely recombines with endogenous FeLV envelopes at sequences already present in the feline genome creating a great number of FeLV-B strains. Subgroup AB combination most often causes thymic lymphoma and myeloproliferative disease. Subgroup C is less common and is created with subgroup A mutation. It is antigenically similar to feline oncornavirus-associated cell membrane antigen (FOCMA) which is a protein present only on FeLV-induced neoplastic cells. Infected cats usually develop severe erythroid hypoplasia and non-regenerative anemia and usually die in 1 to 2 months. Subgroups A and B are also capable of recombining with proto-oncogens and the resultant recombinants are much more potent at producing tumors (Hartmann 2015; Macy 2007).

2.1. Feline leukemia virus infection

FeLV can be considered one of the most significant infections causing morbidity and mortality in cats. In coinfection with FIV or feline coronavirus, FeLV has the most significant impact on survival. Survival times tend to be several years shorter for FeLV

positive cats compared to FeLV negative ones. Younger cats are more at risk of clinical illness (Gleich *et al*. 2009).

The virus is excreted in body fluids- mostly saliva and milk but also urine, feces, tears and respiratory secretions, and is spread vertically from queen to kittens or horizontally between coinhabiting cats or through mucous membranes or percutaneously between cats that fight. Infection typically occurs via oronasal route while grooming or biting. Transmission via saliva needs prolonged contact with infected cats, so it is possible when cats share food bowls, litter pans, bite or lick each other. Transmission can also occur *in utero*. In this case fetal resorption, abortion or neonatal death is common. 20% of kittens will survive and become persistently infected adults.

The virus can be found in local lymphoid tissue after infection, it then spreads via lymphocytes and monocytes to the periphery (thymus, spleen, lymph nodes). At this point fever, diarrhea, lymphopenia and lymphadenopathy are common. Then salivary glands and mucosal glandular epithelium are infected and begin to secrete the virus. At the same time bone marrow gets involved producing infected leucocytes and platelets. Clinical signs associated with the infection are also weight loss, fever, dehydration, rhinitis, diarrhea, conjunctivitis, oral infections, lymphadenopathy, abscesses, upper respiratory tract infections, lymphoma, myeloproliferative disease (such as erythemic myelosis), leukopenia, anemia, thrombocytopenia, hemotropic mycoplasmosis, uveitis and myelopathy leading to paralysis. FeLV infected cats can have suppressed immune function and associated secondary infections such as treatment-resistant dermatophytosis and acute generalized toxoplasmosis. Bone marrow suppression resulting from infection of hematopoietic stem cells usually leads to pure red cell aplasia and rarely to red blood cell macrocytosis. The risk of immune mediated hemolytic anemia (IMHA) is also increased. In coinfection with feline parvovirus FeLV increases the susceptibility to parvoviral diarrhea (Levy, Crawford 2005).

There are 4 possible outcomes for FeLV infection depending on the animals' immune function and the amount and subgroup of infecting virus: progressive, regressive, abortive or focal infection. Differentiating between these outcomes requires blood testing for antigen, antibodies and proviral DNA. Since the virus integrates into host genome, the infection most probably remains lifelong (Arjona *et al*. 2000; Hartmann 2015; Levy *et al*. 2008).

Progressive infection occurs when the virus replicates rapidly post-infection due to insufficient FeLV-specific immunity. This takes place firstly in the lymphoid tissues and then simultaneously in bone marrow, mucosal and glandular epithelial tissues and therefore the virus is shed in excretions. FeLV-related diseases develop in progressively infected cats in a few years and survival times are shortened. These cats are FeLV antigen and proviral DNA positive. 3-30% of cats develop progressive infection depending on infection pressure. This is greater for cats being in close contact with a shedding cat for a long period of time and lower for cats in a one-time contact with a shedding cat (Hartmann 2015; Levy *et al*. 2008).

Regressive infection develops when an effective immune response decreases viral replication before or during bone marrow infection. This occurs in approximately 2-10 % of infected cats. Proviral DNA and high amounts of antibodies can be detected in the blood, but a reversion to an aviremic state has taken place rendering the identification of FeLV antigen and culturable virus impossible. This may take two weeks to several months. FeLV-associated diseases may develop after reactivation of the infection (Hartmann 2015). Regressively infected cats do not shed the virus in saliva, but since viral DNA integrates into host DNA, proviral DNA transmission via blood transfusion is possible (Levy *et al*. 2008).

Differentiating between progressive and regressive infection can be done by repeated testing for viral antigen (usually p27) in peripheral blood (see table 2). In both cases the infected cats become antigen positive in 2-3 weeks after viral exposure and remain persistently proviral DNA positive. In case of regressive infection, the test becomes negative after 2-8 weeks (to several months) and in some cases, detectable antigenemia never develops (Levy *et al*. 2008). Provirus loads also differ between progressors and regressors. Early in the infection the load is similar, but in a few weeks the provirus load in regressively infected cats decreases while remaining high in progressively infected individuals (Hartmann 2015).

Table 2. Outcomes of FeLV infection. + represents occurrence and – non-occurrence

	Antigen (p27)	**Proviral DNA**	**Viral RNA**	**Antibodies**	**Shedding**	**FeLV-associated disease development**
Progressive	+	+	+	-	+	common
Regressive	-	+	-	+	-	uncommon

Abortive infection manifests in inability to detect proviral DNA, viral RNA or antigen in antibody- positive animals. FeLV- associated diseases are not a risk in this infection outcome (Hartmann 2015). This infection has been documented in experimental inoculations and even then, rarely (Levy *et al*. 2008). Focal infections are rare and occur when the infection is restricted to certain tissues, e.g. spleen, lymph nodes, mammary glands, small intestine. This outcome might be irrelevant in natural infection (Hartmann 2015).

25% of FeLV positive cats are expected to develop lymphoma in 2 years of diagnosis (Levy, Crawford 2005). FeLV induces tumors indirectly by immunosuppression and more importantly directly by activating proto-oncogenes and disrupting tumor suppressor genes as a consequence of FeLV genome integration into host cell genome. This can be a result of both progressive and regressive infection. Infection with FeLV can bring about virtually any hematopoietic neoplasm. 70-90% of non-lymphoid hematopoietic neoplasia (myeloproliferative disease) cases are FeLV positive. FeLV positive lymphomas are most commonly multicentric and mediastinal, but may also be spinal, ocular or renal and arise from T-lymphocytes rather than B-lymphocytes. Time from infection to tumor development varies greatly depending on age of infection, subgroup, strain and anatomic region. Experimental inoculation has shown a mean period of 5.3 months between infection and tumor development. Lymphoma develops in 25% of progressively infected cats and usually in 2 years post-infection. Age during infection is an important factor in symptom development. Younger cats develop tumors more rapidly and death due to immunosuppression before neoplastic disease is also possible.

Fading kitten syndrome due to thymic atrophy leading to severe immunosuppression in neonatal kittens is the result of early infection with FeLV. Older cats more likely have an abortive or regressive infection and milder clinical signs (Hartmann 2015; Macy 2007).

2.2. Diagnostics

Most common marker for enzyme-linked immunosorbent assay (ELISA) is protein p27, a core protein which can be isolated abundantly from body fluids (Macy 2007). Most preferred in clinic tests detect soluble antigen from peripheral blood via chromatography such as ELISA. These tests usually give a positive result in 28 days post infection and have a high sensitivity. If infection is strongly suspected but the test gives negative results retesting in

30 days or confirmation with a second soluble antigen test from another manufacturer or PCR to find provirus particles is advisable (Westman 2016).

Kittens and FeLV-vaccinated cats may be tested at any time since (maternal) antibodies do not interfere with antigen detection. Indirect immunofluorescent antibody assay (IFA) that detects cell-associated antigen is also possible, but is less sensitive. It acquires a qualified reference laboratory and gives a positive result a few weeks later post infection than ELISA since it takes time for the infection to affect bone marrow (Levy, Crawford 2005). Proviral DNA and viral RNA can be detected using PCR in 1 week post infection making earlier diagnosis than with ELISA possible. It is also the most sensitive method (Griessmayr *et al*. 2003; Levy *et al.* 2008).

2.3. Treatment

The first vaccine for FeLV was introduced in 1985. A vaccine needs to include only subgroup A since it is the only one transmitted naturally. FeLV vaccine is considered a non-core vaccine by American Association of Feline Practitioners (AAFP) and is recommended only for cats with a high risk of infection e.g. living with FeLV positive cats or roaming outdoors (Macy 2007). However, when vaccinating a FIV positive cat the vaccine might not provide protective immunity against FeLV (Bandecchi *et al*. 2006).

Vaccines appear to prevent progressive illness and thus FeLV-associated fatal disease but not infection (Levy *et al*. 2008). According to register of medicinal products authorised in Estonia, we have a FeLV vaccine Leucogen containing envelope antigen p45 and Leucofeligen FeLV/RCP in combination with other infectious disease vaccines intended to prevent clinical illness. Duration of immunity is at least 1 year.

Clinically healthy FeLV positive cats can be vaccinated against other diseases as usually. FeLV vaccine should be avoided as it gives no benefit. Sterilization-castration are beneficial in reducing roaming and sexual behavior and related stress and interaction with other cats (Levy, Crawford 2005).

Medical treatment of FeLV is generally similar to FIV treatment. Interferon therapy as antiviral and immunomodulating therapy has shown conflicting results with evidence of

clinical improvement (Leal *et al*. 2014; Macy 2007) but according to some clinicians is the best treatment given there is no direct cure for retroviruses so far (personal communication with dr. med. vet Matko Perharić, Zagreb University). Human interferon alfa or feline interferon omega can be used just as in FIV treatment. Zidovudine as an antiviral agent can also be used (Levy, Crawford 2005).

Identification and segregation of retrovirus positive cats is the best preventive measure for FeLV and FIV infection. In case of suspected infection (e.g. bite from retrovirus positive cat) the cat should be tested immediately and when negative retested a minimum of 30 days for FeLV and 60 days for FIV later. Cats living together with retrovirus positive cats should be tested annually and in those cases vaccination should also be considered (Levy *et al.* 2008).

Since the virus is easily inactivated, following of basic hygiene rules and isolation of infected cats in hospitals and catteries eliminates infection. Cats living together with FeLV positive cats in conditions not enabling isolation should be vaccinated. It should also be kept in mind that vaccination does not provide 100% protection against infection. Good nutrition and husbandry are important to maintaining good health. Raw meat and dairy products should be avoided due to risk of food-borne infections in immunosuppressed animals (Levy, Crawford 2005).

2.4. Risk factors

Risk factors for FeLV infection are similar to those of FIV infection since both are retroviruses and transmitted in a similar way. These include male gender, adulthood and outdoor access (Levy *et al*. 2008). Mixed breeds in contrast to purebreds have a higher risk and cats living in multi-cat households. Young kittens are also more susceptible (Levy, Crawford 2005).

2.5. Distribution

FeLV has a worldwide distribution (Arjona *et al.* 2000). FeLV prevalence has showed a decline in the last 20 years which might be due to screening and vaccination against the disease (Levy *et al.* 2006). Nondomestic felids can also be infected with FeLV but infection

is considered enzootic only in European wild cat populations in France and Scotland (Macy 2007). Prevalence is similar throughout the world (see table 1) being about 1-8 % in healthy cats and considerably higher in clinically ill cats or in countries with free roaming felines (Levy, Crawford 2005; Litster, Nilkumhang 2003; Malik *et al*. 1997).

No prevalence studies have been conducted in Estonia so far, nor have any interviews with respected veterinarians estimating prevalence in Estonia been published.

3. ACUTE PHASE PROTEINS

Acute phase proteins (APPs) are a group of blood proteins, named so due to their serum concentration changes of at least 25% during an acute phase response. The acute phase response is the innate immune systems' reaction to inflammation, infection, neoplasia or trauma (Stiller *et al.* 2016). These changes occur in answer to inflammatory cytokines (Eckersall, Bell 2010) in the early stages of disease processes before acquired immune response takes effect (Leal *et al.* 2014). It is therefore critical for survival during the early stage of injury (Ceron *et al*. 2005). In response to proinflammatory cytokines acute phase proteins are synthesized mainly by hepatocytes in the liver but to some extent also extrahepatically by lymphocytes, in the mammary glands, lungs, spleen, kidney, adipose tissue or gastrointestinal epithelium (Ceron *et al*. 2005; Eckersall 2004).

The function of APPs during inflammation is to modulate the efficiency of the immune system by transporting molecules to decrease their loss and protecting tissues from inflammatory mediator damage (Paltrinieri 2008). Increases in circulating APP concentrations are correlated with disease severity (Murata *et al*. 2004).

In human medicine, APP concentration measurements are used in prognosis assessment for example in HIV positive people (Kann *et al*. 2014). APPs may be used in diagnosis of disease, in giving a prognosis or monitoring response to therapy. APP increase is very rapid during inflammation making measuring APP concentrations an appropriate method for early diagnosis of subclinical disease. APP concentration decrease is rapid when the pathogen has been eliminated making it the first sign of recovery.

However, there are interspecies and individual differences in APP response. APPs are highly sensitive but unfortunately not very specific biomarkers for disease only showing the presence of inflammation or infection (Ceron *et al*. 2005; Kann *et al*. 2012). APP concentration increase can be associated with any number of diseases and also depends on the age of the animal to some extent, since adult feline monocytes produce less cytokines than those of young or old individuals (Paltrinieri 2008). The difference might also be attributable to older cats having more subclinical disease conditions (Kann *et al*. 2012). APP concentrations also differ between individuals and might have breed differences (Ceron *et al.* 2005; Paltrinieri *et al*. 2014). To avoid these problems, each animal could be used as its own reference to observe changes in APP concentrations (Leal *et al*. 2014).

Coinfection with FIV or possibly other immunocompromising infections might also effect acute phase reaction to a pathogen. For example, FIV infection decreases haptoglobin response associated with *Haemoplasma* spp. infection (Korman *et al.* 2012). Whether APP concentrations increase or decrease during FIV infection has been conflictingly reported but it is likely that FIV positive cats are able to produce an acute phase response leading to an APP increase (Leal *et al.* 2014). APP concentration increases in ill cats are persistent in contrast to healthy cats exposed to pathogens (for example corona virus) that have a transient APP increase (Giordano *et al.* 2004).

APPs can be divided into major, moderate, minor and negative responders according to the magnitude and duration of their response to stimuli (Kann *et al*. 2012). The list of APPs is ever growing owing to new discoveries, but the most well-known positive APPs are C-reactive protein, complement fractions C3 and C4, alfa-1-acid glycoprotein, lipopolysaccharide binding protein, haptoglobin, ceruloplasmin, serum amyloid A and α-globulins.

Concentration of a major APP rises 10 – 100 (even 1000-fold in humans) times when stimulated, peaking at 24-48 hours post lesion and remains high as long as the inflammation persists before declining rapidly during recovery (Ceron *et al*. 2005). Moderate APP concentrations increase 2-10 -fold, peak at 2-3 days and decline more slowly than major ones. A minor APP concentration increases 50-100%.

Negative APP responses decline during disease (Eckersall, Bell 2010; Stiller *et al*. 2016). Of these, albumin is most researched. Other negative APPs include transferrin, apolipoprotein A1, retinol binding protein, cortisol binding protein and transthyretin. Negative APPs are not widely researched in feline acute phase response, but they take part in bioactive molecule transport and decrease in them allows an increase in free bioactive molecules (Paltrinieri 2008).

Serum amyloid A (SAA) is a major APP in dogs and cats (Christensen *et al*. 2012). SAA is the most rapidly acting (concentration increase within a few hours of inflammatory stimulus) and sensitive APP in feline acute phase response and also correlates with disease severity in feline pancreatitis (Korman *et al*. 2012; Silvestre-Ferreira *et al.* 2016; Tamamoto 2009). SAA has been found to be a significant prognostic marker in cats with various neoplastic, inflammatory and other diseases (Tamamoto *et al*. 2013). There is also evidence that SAA

could be a suitable marker for assessing FAIDS prognosis (Kann *et al*. 2014).

Alfa-1-acid glycoprotein (AGP) can be classified as a major APP in feline acute phase reaction. Although feline acute phase response has been less researched than canine, AGP has been found to be a marker for feline infectious peritonitis, lymphoma, FIV and FeLV infection (Eckersall, Bell 2010; Paltrinieri 2008) albeit less sensitive than SAA (Korman *et al*. 2012).

Measuring specific APPs is for the time being a bit too time-consuming and expensive to make it a routine practice especially considering the low specificity of the result. Studies of sensitivity and specificity of available measuring methods are also lacking (Kann *et al.* 2012). There are however several methods available: colorimetry, radioimmunoassay, ELISA, radial immunodiffusion, immunoturbidimetric tests (Paltrinieri 2008).

3.1. Haptoglobin as a marker of chronic inflammation

Haptoglobin is a positive moderate APP responder in dogs and cats and a major one in ruminants. Concentration increase in response to inflammatory stimulus is less marked and slower and decline to normal levels is more gradual than in major APPs such as SAA (Silvestre-Ferreira *et al.* 2016).

Haptoglobin plays a role in hemolysis binding free hemoglobin (Hgb) since free hemoglobin is toxic and proinflammatory (Paltrinieri 2008). Haptoglobin-Hgb complexes are phagocytosed (Murata *et al*. 2004). Binding of hemoglobin also reduces the amount of free iron which invading bacteria could use giving it a bacteriostatic effect (Petersen *et al.* 2004). Kajikawa *et al.* 1999 found that feline haptoglobin concentration starts to increase 24 h post injury and reaches maximum in 48 hours. In cattle haptoglobin concentrations can increase from 0.02 g/l to 2 g/l within 2 days in case of mastitis, enteritis, peritonitis, pneumonia, endocarditis or endometritis. In dogs a moderate haptoglobin response is triggered also by corticosteroid treatment or hyperadrenocorticism (Eckersall, Bell 2010). Various conditions in cats have been associated with haptoglobin increase (see table 3) but no generally accepted reference values have been validated so far.

Table 3. Examples of conditions where an increase in haptoglobin concentration has been described in cats

Condition	Reference
Abscess	Ottenjann *et al*. 2006
Anemia of inflammatory disease	Ottenjann *et al*. 2006
***Dirofilaria immitis* infection**	Silvestre-Ferreira *et al.* 2016
Feline infectious peritonitis	Giordano *et al*. 2004
Feline immunodeficiency virus	Duthie *et al*. 1997
Hospitalisation	Kajikawa *et al*. 1999
Inflammation	Kajikawa *et al.* 1999
Surgical trauma	Ceron *et al*. 2005
Upper respiratory tract infection	Reviewed by Ceron *et al*. 2005

Although research on normal reference values of haptoglobin in felines is scarce and variable (Kann *et al.* 2012), physiological reference values are considered to be less than about 4 g/l with several conditions activating the acute phase response and increasing haptoglobin levels. This is summarized in table 4 (Duthie *et al*. 1997; Giordano *et al*. 2004; Kajikawa *et al*. 1999; Stiller *et al*. 2016).

Table 4. Healthy feline haptoglobin reference values from different studies and conditions related to concentration increase

Hp reference value	Hp increase	Reference
1-2.6 g/dl (10-26 g/l)	-	Paltrinieri 2008
0.04-3.84 g/l	-	Duthie *et al*. 1997
0.38 (0-2.57) g/l	Systemic inflammation 5.67 g/l	Stiller *et al*. 2016
0.42 g/l	Hospitalization 4.83 g/l	Kajikawa *et al*. 1999
1.30 g/l	Feline infectious peritonitis 2.13 g/l Feline coronavirus infection 1.80 g/l	Giordano *et al*. 2004

Haptoglobin levels in coinfected cats has seldom been studied. Korman *et al*. 2012 measured haptoglobin concentration in *Mycoplasma haemofelis* infected FIV positive and FIV negative cats and got results of 2.43 g/l (1.05-3.65 g/l) and 0.41 g/l (0.15-2.45 g/l), respectively. Haptoglobin concentration might also be associated with age of the animal in addition to health status. The reason for this might however be that older cats have more subclinical illnesses (Kann *et al*. 2012).

Methods for measuring haptoglobin concentration can be divided into spectrophotometry

based on haptoglobins' ability to bind hemoglobin, and immunoassays such as ELISA. No gold standard exists at this point however (Ceron *et al.* 2005; Stiller *et al.* 2016). Stiller *et al.* 2016 have found both methods appropriate for measurement and a strong positive correlation between the assays. ELISA might produce lower results and is more time consuming, but has a wider working range making it advantageous for use in critically ill patients. A commercial kit by Tridelta Development based on hemoglobin binding is used in many studies (Giordano *et al*. 2004).

4. AIMS OF THE STUDY

The aims of this study were:

- to estimate the prevalence and risk factors of FIV and FeLV infection in cats brought to the Tartu animal shelter;
- to determine whether there is an association between FIV and FeLV infection and serum haptoglobin concentration in cats;
- to assess the relation between serum haptoglobin concentration and the cats' age, gender and clinical signs of rhinitis.

5. MATERIAL AND METHODS

5.1. Study population

Blood samples from Tartu animal shelter cats were collected at Janne Orro animal clinic in 2014 and 2015. Blood sampling and FeLV/FIV testing is a part of routine check-up for shelter animals brought to the clinic. FeLV/FIV test was performed at once and serum refrigerated for subsequent laboratory analysis. Our study included 173 samples of cats from the shelter. Most cats were intact adult males, collected from Tartu area. Most of the cats displayed no clinical signs of illness (see table 5). The most common symptom was rhinitis which was used in the statistical analysis as an example of illness. Other symptoms of illness which were rare and not used in further analysis included diarrhea, conjunctivitis, gingivitis, pneumonia, abscess, eye problems and existence of wounds or parasites. Results were calculated using OpenEpi 3.01.

Table 5. Descriptive statistics of study population (n = 173)

		n	**%**	**95% CI**
Gender	male	100	57.8	50.3; 65.0
	female	73	42.2	35.0; 49.7
Reproductive Status	intact	162	93.63	89.2; 96.6
	castrated	6	3.5	1.4; 7.1
	sterilized	2	1.2	0.2; 3.8
	pregnant	3	1.7	0.4; 4.7
Age	⩾1 year	158	91.3	86.4; 94.9
	<1 year	15	8.7	5.1; 13.6
Location	Tartu	117	67.6	60.4; 74.3
	elsewhere	49	28.3	22.0; 35.4
	unknown	7	4	1.8; 7.8
Rhinitis	yes	15	8.7	5.1; 13.6
	no	158	91.3	86.4; 94.9

5.2. FeLV / FIV test

For FIV and FeLV infection assessment Virbac Speed Duo FeLV/FIV test was used for all cats in the study group. It is a method of membrane immunochromatography detecting FeLV

antigen p27 and anti-gp40 FIV antibodies from feline whole blood. The method is based on respective proteins binding with chromogen to produce a visible color change in infected animals. Speed Duo test has a sensitivity of 96.3% for FIV and 94.7% for FeLV and a specificity of 98.9% for FIV and 99.2% for FeLV (Virbac BVT).

5.3. Haptoglobin assay

Haptoglobin concentration in feline sera was measured with the standard assay for haptoglobin measurement developed by Makimura and Susuki (1982) for bovines. The method is based on haptoglobin binding with hemoglobin and the quantity of the complexes measured spectrophotometrically by using tetramethylbenzidine (TMB) as a chromogen (Alsemgeest *et al*. 1994).

5 standard solutions were prepared by serial 1:2 dilution using aliquots of acute phase pig serum with known haptoglobin concentration (2.9 g/l). Last standard was pure 0.9% NaCl. Positive and negative controls were porcine serum with high and low concentration (mean 2.34 g/l and 1.37 g/l, respectively). 5 µl standard solutions, controls and serum samples were pipetted into 10 ml tubes. 100 µl of cyanomethemoglobin solution (1 ml of concentrate (30%) + 9 ml of 0.9% NaCl) was added to each tube and incubated at room temperature for 10 minutes. The hemoglobin-haptoglobin binding reaction was stopped by adding 2.5 ml of 0.9% NaCl. 20 µl of each standard, controls and sample were pipetted in triplets to the test plate and 200 µl of the chromogen solution (6 mg of TMB + 1 ml of distilled water and diluted with chromogen buffer 1:100) added. The plate was covered and incubated at 37 degrees for 1 hour. After that 50 µl of substrate solution (10 ml of distilled water + 12 µl of 30% H_2O_2) was added and the plate incubated at room temperature for 15 minutes. During this time the samples turned blue in accordance with the haptoglobin concentration. The process was stopped by adding 50 µl of 20% H_2SO_4 which changed the indicative color from blue to yellow. The microwell plate was shaked and optic density (OD) of each sample measured with ELISA reader (Magellan Sunrise©) at 420 nm wavelength. The samples with higher haptoglobin concentrations than calibrator range were diluted 1:4 with 0.9% NaCl and the process repeated.

5.4. Statistical analysis

EpiTools online epidemiological calculator (Sergeant 2017) was used for calculating prevalences and confidence intervals of virus antibodies presence. Associations between FIV positivity with gender (male or female), age (<1 year or >1 year old), rhinitis (yes/no) and other clinical disease than rhinitis (yes/no) was analyzed with logistic regression model. As there were only 3 FeLV positive cats associations between FeLV positivity with gender and age was not studied. Associations between cats' serum haptoglobin concentrations with FIV positivity, FeLV positivity, rhinitis, gender and age was analyzed using linear regression model. Backward elimination process was used for both models for final models.

Statistical program STATA 14.2 (StataCorp LP, Texas, USA) was used for both regression models.

6. RESULTS

There were 3 FeLV positive cats and 23 FIV positive cats. Apparent prevalence for FIV is 13.3% (Wilson 95% CI 0.09; 0.192), and for FeLV 0.6% (Wilson 95% CI 0.017; 0.05).

We found a positive association between the gender of the cat and FIV positivity with male cats having a higher infection risk (OR = 5.8, 95% CI 1.7; 20.5; p = 0.006). Risk factors for FeLV infection could not be identified due to a small number of FeLV positive cats in the study group.

No associations between haptoglobin concentration and FIV or FeLV infection was found, but cats with rhinitis and male cats had higher haptoglobin concentrations (p = 0.022 and p = 0.001, respectively; Figure 2.)

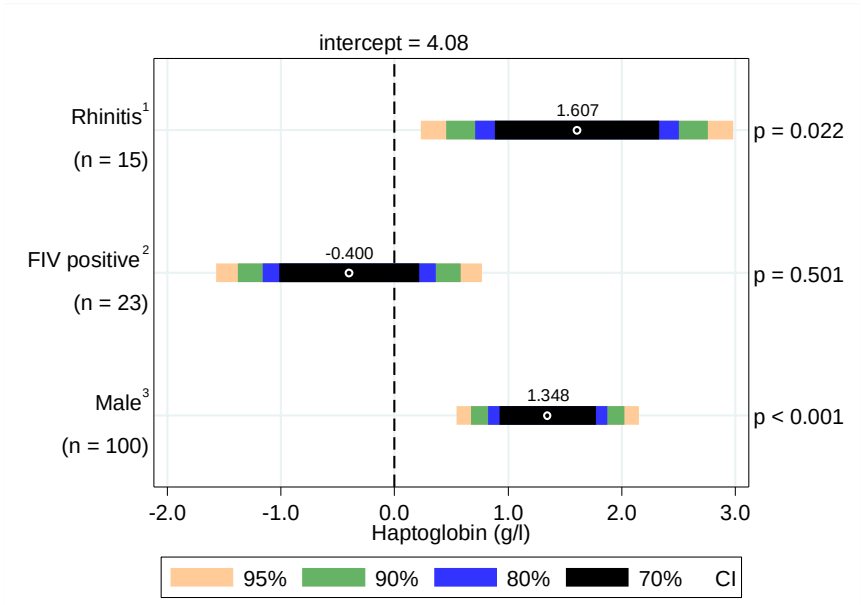

Figure 2. Cats' (n = 173) serum haptoglobin concentration associations according to the regression model coefficient plot. Model confidence intervals (CI) are presented as horizontal bars. Point estimates for variables are shown on top of the bars. Feline immunodeficiency virus (FIV) was retained into the model as confounder variable.
[1] Compared to cats without rhinitis (n = 158)
[2] Compared to FIV negative cats (n = 150)
[3] Compared to female cats (n = 73)

In our study group male cats had an average haptoglobin concentration of 5.51 g/l compared to females having an average of 4.18 g/l. Cats with clinical signs of rhinitis had an average haptoglobin concentration of 6.51 g/l in contrast to cats with no signs of rhinitis with an average of 4.18 g/l (Figure 3.).

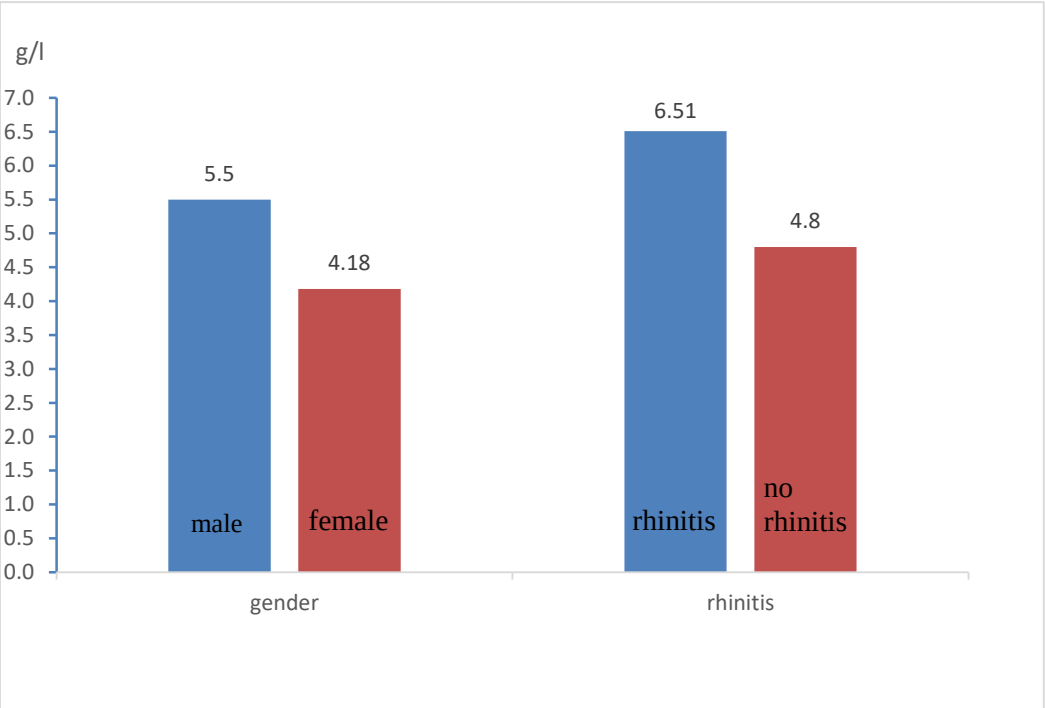

Figure 3. Average haptoglobin concentration values (g/l) for male and female cats and cats with and without rhinitis

The average haptoglobin concentration in our study group was 4.95 g/l.

7. DISCUSSION

Seroprevalence in shelter cats cannot be directly extrapolated to the overall cat population of Estonia since stray animals invariably belong to a high risk group. We can make an educated guess about prevalence based on studies from neighboring countries and our own study of shelter cats and we found the apparent prevalence of FIV to be 13.3% which correlates well with the suggestion of veterinarian Riina Jõgila (10-20%; Postimees 2013). Few prevalence studies have been published in our directly neighboring countries, but our results seem likely in the light of prevalence in Finland being 6.6% (Sukura *et al.* 1992) and in Russia 16% (Pavlova *et al*. 2015). FIV prevalence is largely dependent on the proportion of stray and free roaming cats and Russia could be considered to have more of such animals than Estonia and Finland to have less. So it would make sense if prevalence in Estonia would be somewhere in between those of Russia and Finland. Overall true prevalence might be estimated to be somewhat lower since our study population consisted mostly of cats belonging to a high risk group for FIV infection and does not represent client owned cats. A wider scale study comprising a larger number of samples from stray and client owned cats, sick and healthy cats and cats from different regions of Estonia should be conducted in order to give a more precise estimation of prevalence. In this study, only rhinitis was included as a sign of illness. Subsequent studies should take into account other disease states as well in order to distinguish sick and healthy groups.

Male sex was found to be a risk factor for FIV infection and this is in accordance with most studies published. No statistically significant correlation between infection and other factors (castration/sterilisation, age, rhinitis) was found. This could be explained by a rather small sample size. Unfortunately we had quite few samples of cats that were castrated or sterilised, young or sick with rhinitis. Additional risk factors that could be investigated are for example breed and aggressive behavior and the hypothesis is that infection risk should be higher among mixed breed and aggressive cats.

Apparent prevalence for FeLV infection was 0.6%, but this needs to be interpreted carefully since we had a very small positive sample size and no statistically sound conclusions could be drawn. It might however be plausible given the estimated prevalence worldwide to be about 1-8 % (Levy, Crawford 2005). No risk factors for FeLV infection could be found in this study due to very few positive samples.

There is always the possibility of false positive or false negative Speed Duo test results. Fortunately the test has quite high sensitivity and specificity, but samples were deemed positive according to only one test result and were not confirmed in any way. A similar approach is used in most prevalence studies worldwide and similar immunochromatographic tests used. In this respect, our study should be comparable. Future studies could however confirm positive samples with a second method (second ELISA test or PCR for example) to reduce margin for error.

Severely ill stray cats are less likely to reach an animal clinic than pet cats and are likely to be euthanized without FIV/FeLV status determination if their prognosis is poor. Therefore our apparent prevalence might be underestimated in sick cats. On the other hand, all healthy shelter cats are tested and this gives a better representation compared with for example studies conducted with pet cats visiting animal clinics since pets are mostly brought to hospitals and blood samples taken with a medical problem.

Our study shows that FIV and FeLV are a concern in the Estonian cat population and there is room for improvement in reducing the prevalence. Stray cat colonies should be eradicated and cats with owners should not be allowed to roam freely. Although the problem is probably less severe than a few decades ago, pet owning culture of Estonian people has to change in order to diminish the risk of feline infectious diseases.

We found no correlation between haptoglobin concentration and FIV or FeLV infection, but this might be due to a small number of infected and clinically ill animals in this study. Haptoglobin concentration increase was found to be associated with male gender and rhinitis. Rhinitis being an inflammatory condition was expected to cause haptoglobin increase compared to clinically healthy animals (Ceron *et al*. 2005; Kajikawa *et al.* 1999). Having proven the association exists, haptoglobin should be considered for use in clinical medicine for investigating inflammatory processes. Gender has not previously been proven to be significantly associated with haptoglobin concentration (Kann *et al*. 2012). The relation could be true in the sense that male cats might inherently have higher haptoglobin levels. It could also be due to our study population being mostly intact shelter cats who have been roaming freely and living stressful lives with males having more conflicts with other cats and possibly having more subclinical health disorders than their less aggressive female counterparts. This result is valuable in validating haptoglobin reference ranges as a possible factor effecting base levels of healthy animals.

The average haptoglobin concentration of the cats in our study was 4.95 g/l and the average of healthy cats without rhinitis was 4.80 g/l which is somewhat higher than should be expected from healthy felines on the basis of other studies (Duthie *et al*. 1997; Giordano *et al*. 2004; Kajikawa *et al*. 1999; Stiller *et al*. 2016). The most probable reason for this is the use of porcine serum as a standard solution which has a high haptoglobin concentration. For that reason we cannot rule out seemingly higher haptoglobin concentrations. Fortunately this does not affect our results since in the purposes of this study the relative differences of haptoglobin concentration between the 173 studied cats were important, not the exact values themselves. Nonetheless, porcine serum is an important factor using this method of haptoglobin assay and in future research for validating reference values a different method or standard solution should be used. Another explanation for higher haptoglobin levels would be the study group being shelter cats that might have unnoticed health issues. Both of these reasons may play a part simultaneously.

We do not yet have a certain haptoglobin concentration reference value for sick or healthy felines and this is grounds for further research. This is largely due to lack of standardized methodology. We can get extremely different reference values using different measurement methods and different laboratories. Tridelta kit is used in many studies, but there are other methods and a validated laboratory method needs to be agreed on. Every laboratory also needs their own references since analytical conditions play a major role. Species specific reagents should also be used, at the moment many laboratories use porcine reagents for example.

Human medicine dealing with HIV positive people uses viral RNA load and acute phase protein concentration measurements for estimation of disease progress and prognosis (Kann *et al*. 2014). Implementation of the same methods in veterinary medicine is something to consider since current methods are based on patient history and clinical signs which makes the outcome subjective.

A single haptoglobin measurement does not say much in the way of diagnosing a disease or checking disease progression in a patient. The patient can be used as their own reference when there is a haptoglobin result from a time when the patient was healthy and changes in concentration during disease and recovery monitored. It is also better to measure several acute phase proteins concurrently. The most exact way would be monitoring a major, moderate, minor and negative acute phase protein concurrently and their changes in time to

estimate disease process and response to treatment or differentiate between acute and chronic processes.

I believe acute phase protein measurement is a prospective method in clinical medicine and will become more widely used in the future. It is a good way of finding subclinical diseases for example in donor animals or in dairy herds for discovering subclinical mastitis. The method can be compared to taking the animals' temperature: it is a nonspecific indicator, but when irregular hints at a medical problem.

CONCLUSION

In conclusion, our study found the apparent prevalence of FIV to be 13.3% and of FeLV to be 0.6% in shelter cats of Tartu. FIV prevalence could be overestimated owing to our study population consisting of high risk animals. FeLV positive samples were too scarce to make assumptions about FeLV prevalence. The risk factor for FIV infection was found to be male gender. No risk factors for FeLV infection could be assessed due to a small sample size. Future studies comprising larger and more variable study populations should be undertaken in order to estimate FeLV prevalence more correctly and to study the group of client owned cats. Haptoglobin serum levels were significantly higher in cats with rhinitis which was to be expected and also in male cats which was an association not been proven so far. Perhaps male cats have inherently higher haptoglobin or it is an effect of our study population comprising of male intact strays with subclinical health issues. The average haptoglobin concentration in our study group was somewhat higher than was expected based on other studies, but this might also be the effect of our study population being stray cats.

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KASSIDE IMMUUNPUUDULIKKUSE VIIRUSE JA KASSIDE LEUKEEMIAVIIRUSE LEVIMUS TARTU VARJUPAIGA KASSIDEL: SEOSED VANUSE, SOO, RINIIDI JA HAPTOGLOBIINI KONTSENTRATSIOONIGA SEERUMIS

Üldkokkuvõte

Selle uurimuse eesmärkideks olid leida FIV-i ja FeLV-i levimus Eestis, hinnata võimalikke riskitegureid nakatumisele ning uurida seerumi haptoglobiinisisalduse seost immuunpuudulikkuse viiruse ja leukeemiaviiruse nakkusega, kasside vanuse ja soo ning kaasneva riniiti haigestumisega. Selleks analüüsisime 173 Tartu varjupaiga kassi vereproove. Uurimuse valimisse kuulusid peamiselt kastreerimata täiskasvanud isased kassid. Vereproovid koguti ja FIV/FeLV Speed Duo test teostati Janne Orro loomakliinikus. Haptoglobiini kontsentratsioon mõõdeti laboris sügavkülmutatud seerumist kasutades sigadele välja töötatud meetodit. Statistiline analüüs viidi läbi kasutades programme EpiTools 3.01, OpenEpi ja STATA 14.2.

Uuringu tulemusena leidsime, et FIV-i ilmnev levimus on 13.3%, kuid see võib olla kerge ülehinnang, kuna valimi kassid kuulusid kõrge nakatumisriskiga gruppi. Siiski on tulemus usutav arvestades teistes Euroopa riikides läbi viidud uuringute tulemusi. FeLV-i levimust ei saa paraku hinnata liiga väheste positiivsete tulemuste tõttu. Riskitegurid mõlemale nakkusele eeldati olevat isassugu, kõrgem vanus ja kaasnev haigus riniidi näitel. Ainus leitud riskifaktor FIV infektsioonile oli sugu ning isastel kassidel oli suurem risk haigestuda. FeLV-i riskitegureid ei olnud väikese valimi tõttu võimalik hinnata. Seose puudumine nakatumisriski ning vanuse ja riniidi esinemise vahel võib olla tingitud liiga vähesest hulgast noortest ja riniiti haigestunud kassidest valimis. Statistiliselt oluliste järelduste tegemiseks peaks läbi viima suuremaskaalalise uuringu. Vajalik oleks suurem valim ning prove tuleks koguda erinevatest Eesti regioonidest, samuti tuleb kaasata kodukasse ning teisi haigusseisundeid peale riniidi. Võiks ka uurida lisa riskitegureid nagu agressiivne käitumine.

Haptoglobiini kontsentratsiooni tõusul leiti seos isassugupoole ja riniidiga. Riniit põletikulise seisundina tõstis haptoglobiini taste ootuspäraselt. Haptoglobiini mõõtmist võiks tulevikus kasutada kliinilises meditsiinis kasside põletiku uurimises. Haptoglobiini

taseme suurenemist pole varasemas kirjanduses isase sugupoolega oluliselt seostatud. Põhjuseks võib olla isaste kasside loomulikult kõrgem haptoglobiini tase. See võib olla tingitud ka meie valimist, mis koosnes suuremalt osalt kastreerimat isastest hulkuvatest kassidest, kes on tõenäoliselt agressiivsemad ja kel on ka agressiivsest käitumisest tingituna rohkem terviseprobleeme kui emastel kassidel.

Meie uuringus leitud keskmine haptoglobiini kontsentratsioon oli mõnevõrra kõrgem kui enamuste uuringute poolt tervetel kassidel normaalseks peetakse. See oli nii ka riniiti mite põdevate kasside puhul. Erinevus võib olla tingitud meie haptoglobiini määramise meetodist, kuna kasutasime sea seerumit, millel on kõrge haptoglobiini kontsentratsioon ning seega võib tegu olla metoodika veaga. Teine põhjus võib seisneda andmekasutuses. Uuringust jäeti välja riniidist erinevad haigusseisundid, kuna neid esines vähesel hulgal. Samas oli valimi hulgas ka teisi haigeid kasse, kelle haptoglobiini tase võis olla tõusnud teistel põhjustel peale riniidi. Võimalik põhjus on ka hulkuvatel kassidel sagedasti märkamatuks jäävad terviseprobleemid ning neil loomadel võib esineda subkliinilisi põletikulisi või infektsioonilisi protsesse. Kokkuvõttes peaksime tulevikus kinnitama haptoglobiini võrdlusväärtused, kuna haptoglobiini mõõtmine vereseerumist on perspektiivikas diagnostikameetod.

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