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Case Report

A CASE-CONTROLLED STUDY OF FELV INFECTED CATS IN TEHRAN, IRAN, CONFIRMED BY IMMUNOCHROMATOGRAPHY AND RT-PCR AND CORRELATION WITH CLINICAL AND HEMATOLOGICAL FINDINGS

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Summary: Immunochromatography (ICGA), reverse transcriptase polymerase chain reaction (RT-PCR) and hematological assays were performed on blood samples of 90 cats (45 healthy or control ones and 45 sick or case ones) in Tehran, Iran, as a case-control study from June 2009 through February 2010. Prevalence of FeLV in this population was 1.1% and 2.2% as determined by immunochromatography and RT-PCR assays, respectively. Factors that were significantly associated with positive results in RT-PCR method were pale mucous membrane (P=0.026) and rhinitis (P= 0.002), which were more prevalent in FeLV-positive cats. The size of population of the household was found to be a predictor for FeLV infection, and the relative risk of FeLV infection in cats kept in multicat households is 6.6 times higher in comparison with single housed cats. The most common clinical findings in control group were gingivitis and/or stomatitis (37.8%), skin lesions (8.9%), lymphadenopathy and pale mucous membrane (6.7%), and the most frequent hematological findings were decreased PCV (24.4%), lymphopenia and decreased hemoglobin level (20%), leukocytosis and neutrophilia (13.3%). In the case group, the most common clinical findings were gingivitis and/or stomatitis (77.8%), pale mucous membrane (53.3%) and skin lesions (37.8%), and the most frequent hematological findings were lymphopenia (37.8%), anemia (26.7%), decreased Hb (24.4%) and leukopenia (15.6%).

Key words: cat; feline leukemia virus (FeLV); prevalence; immunochromatography; reverse transcriptase polymerase chain Reaction (RT-PCR)

Introduction

Feline leukemia virus (FeLV) is an oncogenic, myelosuppressive and immunosuppressive γ-retrovirus that occurs throughout the world and represents one of the most important pathogens of domestic cats. FeLV is generally transmitted horizontally in felines by close contact through saliva, blood and other body fluids (1, 2, 3). Risk factors for FeLV infection include gender (more common in males), age, illness and access to outdoor environment, whereas indoor lifestyle and sterilization are associated with reduced infection rates. FeLV infection is the infection of "social cats" because it is mostly spread through social contacts (3, 4). The role of the cat flea

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(*Ctenocephalides felis*) has also been suggested as a possible factor in transmission (5). Reported prevalence of this virus differs considerably depending on the geographical region, the cat population evaluated and especially on the method used in different studies due to differences in sensitivity and specificity of these diagnostic methods. The infection rate of free roaming cats is similar throughout the world, ranging from 1- 8% in healthy cats and up to 21% in sick cats (3). The most recent studies report a prevalence of 2.3-3.3% in North America, 0-14.2% in Asia and 3.5- 40.5% in Europe (6-30). Two previous serologic studies carried out in Iran have shown infection rates between 4.8% to 14.2% in two different regions and different populations of household and stray cats (6, 13). Routine diagnosis screening for FeLV relies on detection of the core viral antigen p27 by ICGA or ELISA, which is produced abundantly in majority of infected cats. In-clinic test kits detect soluble circulating antigen in peripheral blood. Molecular diagnostic methods like polymerase chain reaction (PCR) are becoming more popular due to their advantages over serological methods. The PCR technique is extremely sensitive and the method allows identification of the virus independently of the presence of viremia (31).

According to Iranian society for prevention of cruelty against animals (IRAN, SPCA), more than 90% of cats (*Felis catus*) in Iran are stray and most of owned cats are kept outdoors (6). The aims of this study were to determine the prevalence of FeLV infection by serological and molecular methods among client-owned cats referred to Small Animal Polyclinic, Faculty of Specialized Veterinary Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran (45 healthy or control and 45 sick or case cats). This was the first molecular assay on FeLV in Iran. Seropositivity and PCR positive results were also correlated with clinical and hematological findings such as health status, gender, age and lifestyle.

Materials and methods

Clinical examination

The study group comprised of 90 cats presented to Small Animal Polyclinic, Faculty of Specialized Veterinary Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran from June 2009 through February 2010. These client-owned cats were randomly selected with no limitation for age, gender and lifestyle. These cats were divided into two different groups according to their clinical signs (45 healthy and 45 sick cats). Regarding the most common clinical signs reported in FeLV infected cats, the diagnostic criteria was delineated. These clinical signs included weight loss, fever, dehydration, rhinitis, diarrhea, oral inflammation (gingivitis and/or stomatitis), lymphadenopathy and cutaneous lesions and abscesses (3). Each cat that had two or more of these clinical signs simultaneously was considered as a case cat and those that were presented for vaccination or a routine checkup were considered as control cats. Informed consent was obtained from each cat owner prior to the study. A detailed questionnaire was completed for each animal. Investigated parameters included putative risk factors such as age, gender, breed, health status, sexual intactness and housing conditions (only indoors or outdoors; single or multicat household; to be in contact with other cats or not).

Laboratory examination

Blood samples (2-3 ml) were drawn from jugular or cephalic vein of adult cats (in kittens, only 1 ml of blood was drawn). The collected blood was divided and poured into plain tubes and anticoagulant containing tubes (ethylenediamine tetraacetic acid). Serum samples were separated after centrifugation (for 10 minutes at the speed of 3000 rpm) for serological testing. The separated serum was kept at -20 C° before performing rapid immunochromatography assay. Complete blood counts were performed by automatic cell counter (Nihon Kohden, Tokyo, Japan) for all cats, and parameters such as hematocrit, hemoglobin and platelet counts were rechecked manually according to the guidelines of International Committee for Standardization of Hematology (ICSH). The presence of hematological disorders such as anemia (Hematocrit < 20), leukopenia or leukocytosis (less than 5500 to more than 19,500 leukocyte/µl of blood) and changes in differential leukocyte counts was recorded.

Immunochromatography assay (ICGA.

ICGA was carried out with a commercial kit (Speed Duo® FeLV/FIV, BVT Company, La Seyne sur Mer, France) for detecting p27 antigen of FeLV according to the manufacturer's instructions. Sam-

ples with positive results were retested after a minimum of 30 days according to the guidelines of the American Association of Feline Practitioners' for feline retrovirus management and only considered truly positive if they tested positive for the second time (31). The sensitivity and specificity of the kit in comparison to viral isolation was recorded as 89.1% and 97.7%, respectively.

Reverse transcription polymerase chain reaction

Reverse Transcriptase PCR assay was performed with a commercial kit (VeTeK™ FeLV Detection Kit, iNtRON Biotechnology Inc, Gyeonggi-Do, Korea) for direct detection of feline leukemia virus on the basis of a genetic database.

A commercial kit was used for RNA extraction (VeTeKTM Viral Gene-spin™ Viral DNA/RNA Extraction kit, iNtRON Biotechnology) from 150 µl whole blood according to manufacturer's instructions. Extracted RNA was collected in sterile microcentrifuge tube and stored at -40 C°.

Reverse Transcriptase PCR reaction was carried out according to instructions of the manufacturer. PCR assay were done by using PCR machine (Corbett Research Company, Mortlake, Australia). The amplified products were analyzed in 1.5% agarose gel electrophoresis, using 100bp DNA ladder (Fermentas, Vilnius, Lithuania) as a reference marker. Control RNA of the kit was used as positive control and distilled water as negative control. Primers used in this kit were able to amplify a 239bp segment of FeLV genome. Therefore, samples that had a 239bp segment of FeLV genome were considered as positive (Figure 1).

Statistical analysis

Cases positive with immunochromatography and positive with RT-PCR were set as outcome variables, while the independent variables were sex, age, health status (including most prevalent clinical signs stated above), population of the household, sexual status and abnormal hematological findings (e.g. anemia, leukopenia, leukocytosis, etc.). Prevalence was calculated as the percentage of cats with positive ICGA and PCR results. Asymptotic χ^2, Mann-Whitney U and independent sample t-test were used to test the bivariate associations between each of the putative risk factors and infection. Risk factors found to be significantly associated with risk of infection in bivariate analyses were included in logistic regression analyses. For these analyses, categorical variables were recorded as indicator variables. Regression models were built by analyzing the main effects of covariates using a forward selection procedure, with a P-value for the likelihood ratio test of <0.05 used for selection. All statistical analyses were performed with standard software (SPSS 15.0 for Windows, SPSS, Chicago, Illinois, USA). Values of $P<0.05$ were considered to indicate a statistically significant difference.

Results

In the present study, only 1 cat out of 90 cats (1.1%) tested positive for the presence of FeLV antigen, and 2 cats from this population (2.2%) were positive in RT-PCR assay, and no cat was positive by both methods simultaneously. Whereas all positive cats belonged to the case group, the overall prevalence of FeLV in control group with ICGA and PCR was 0%. Overall prevalence of FeLV infection in case group was 2.2% and 4.4% by ICGA and PCR methods, respectively. Source, health status and abnormal clinical and hematological findings of FeLV positive cats were summarized (Table 1). According to results of this study, all positive cats belonged to DSH breed and were younger than 3 years old. Two cats out of 3 positive cats (66.7%) had free access to outdoor. The same percentages of these positive cats were sexually intact and kept in multicat households. Most common abnormal clinical findings in FeLV positive cats were pale mucous membranes (100%) and gingivitis/Stomatitis (66.7%). No cat was positive in two tests simultaneously. Regression analysis confirmed these factors as significant risk factors for FeLV infection. The full logistic regression model containing selected predictors, without interactions, was statistically significant (LR $\chi^2=150.87$, $P<0.001$, likelihood=17.50).

Factors that significantly associated with positive cats in PCR assay by Asymptotic χ^2 test, were pale mucous membrane ($P=0.026$) and rhinitis ($P=0.002$), which were more prevalent in FeLV positive cats (Table 2). The comparison of quantitative variables between positive and negative cats in PCR assay by Mann-Whitney-U test showed that only the difference of eosinophil count was statistically significant between two groups and it was higher in infected cats ($P=0.043$).

Table 1: Source, signalment, health status, and abnormal clinical and hematological findings of FeLV positive cats (3 positive results out of 90 samples).

Age	Gender	Breed	Lifestyle	Type of household	Sexual status	Abnormal clinical findings	Abnormal hematological findings	FeLV ICGA	FeLV PCR
1Y	F	DSH	Indoor/ Outdoor	Multicat	Intact	Fever, Diarrhea, Pale mucous membranes	Leukocytosis, Neutrophilia	+	-
10 month	M	DSH	Indoor	Single	Neutered	Gingivitis/Stomatitis, Lymphadenopathy, Pale mucous membranes	Anemia, Thrombocytopenia	-	+
3Y	F	DSH	Indoor/ Outdoor	Multicat	Intact	Gingivitis/stomatitis, Rhinitis, Pale mucous membranes	Thrombocytopenia	-	+

Y= year, M= male, F= female, DSH= domestic shorthair

Table 2: A comparison of the qualitative variables between positive and negative cats in PCR by asymptotic χ^2 test

Variable	χ^2	df	P Calculated
Gender	0.006	1	0.936
Population of household	0.06	1	0.807
Sexual status	0.486	1	0.486
Diarrhea	0.122	1	0.727
Weight loss	0.289	1	0.591
Gingivitis/ Stomatitis	1.524	1	0.217
Lympha_ denopathy	2.055	1	0.152
Pale mucous membrane	4.958	1	0.026 *
Cutaneous abscesses and lesion	0.427	1	0.094
Rhinitis	9.87	1	0.002 *
ICGA positive result	0.023	1	0.879

*Statistically significant difference

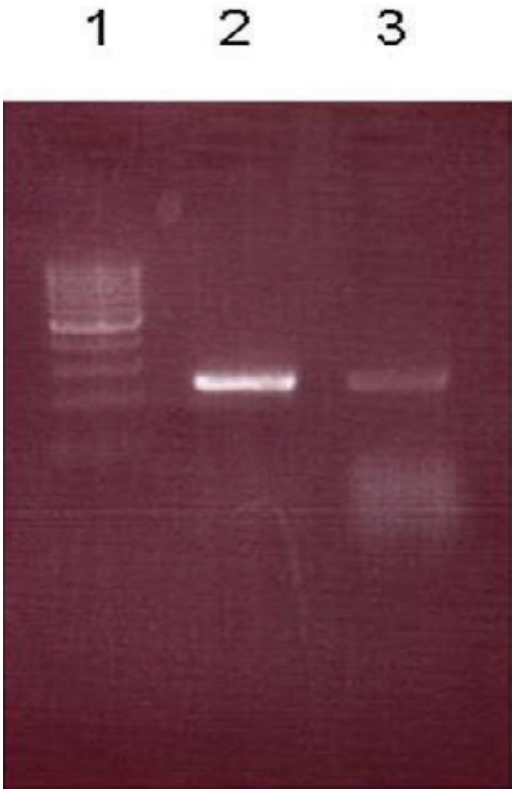

Figure 1: The positive band (column 3) beside positive control of the RT-PCR kit (column 2) and 100 bp ladder (column 1) in gel-electrophoresis

Discussion

The current study revealed an overall prevalence of 1.1% and 2.2% for infection with FeLV in Tehran, Iran, by serologic and molecular methods, respectively. Previous studies performed in Iran revealed infection rates between 1.6% to 14.2% in two different regions and different populations of household and stray cats. In the first study in Iran (Tehran), among 103 healthy domestic and stray cats, 4.8% showed positive serologic results for FeLV through ELISA method but there was no molecular analysis performed in this study (13). In another study conducted in southern Iran (Kerman) on household and stray cats, serum infection was reported to be about 14.2% (6). Our estimated prevalence is in accord-

ance with the study performed in Tehran in 2008; however, it is obviously different from the results of the study conducted in Kerman, though the same commercial ICGA kit was used for serologic evaluation in the latter. It seems that the prevalence of retroviral infection represents obvious regional patterns in some countries (8). This pattern may also be present in different parts of Iran. On the other hand, the population that was studied in Kerman comprised 70 urban stray cats. Our study was a case-controlled study which was performed on 90 client-owned cats, 42.2% of which were kept individually and 35.5% of which had no access to outdoor. This low prevalence rate may have different reasons. Firstly, it could be due to the low prevalence of FeLV in Tehran. Another probable reason may be "latent" cats, that is, cats which are permanently infected with FeLV but have no detectable antigen in their peripheral blood. Evaluating bone marrow for existence of FeLV genome is required to detect such cats, but this was not done in the present study. Prevalence of FeLV infection in cat populations differs throughout the world. The reported seroprevalence for FeLV in healthy and sick client-owned and free-roaming cats that are in accordance with our estimated prevalence were 0% in Vietnam (8, 18), 2% in Australia (15, 16), 1.3% in Taiwan (17), 2.9% in Japan (11) 3% in Switzerland (19) 1% in Finland (20) and 2.3% in USA (21). The 35.7% prevalence rate was reported in the study performed by nested PCR on 179 blood samples in Spain (22). In another study, conducted in England, 21.72% of the blood samples were positive by quantitative PCR method (30) Tozon et al. reported 17 positive cats out of 42 cats by PCR method (28).

Interestingly, no cat was positive in both tests. Although the RT-PCR test is very sensitive, retroviruses, including FeLV, are usually genetically variable, and therefore it is possible that the primer used by our RT-PCR kit were not able to detect all strains of this virus. Another reason for observed discrepancy might be relatively low sensitivity of immunochromatography test that may lead to some false negative results.

In regard to low seroprevalence of FeLV by ICGA (only 1 positive sample), it was inapplicable for statistically comparing the characteristics and abnormal clinical and hematological findings of FeLV positive and FeLV negative cats in this study, and there was no statistically significant difference between positive and negative cats in regard to the serological method. As a descriptive statistic, all three positive cats in our study were younger than 3 years old (from 10 months to 3 years old). It was believed that the susceptibility of the cats to FeLV was age-dependent, and that younger cats were more susceptible to FeLV infection (3, 4, 14). Pale mucous membranes (100%) and gingivitis or stomatitis (66.7%) were the most common clinical findings in FeLV positive cats in our survey, what is consistent with other reports, but these differences were not statistically significant (24).

Factors that were significantly associated with positive RT-PCR results were pale mucous membrane (P=0.026) and rhinitis (P=0.002), which were more prevalent in FeLV positive cats. In a similar comparison based on Mann-Whitney-U Test made with the cats that were positive in RT-PCR test, it was shown that the presence of eosinophilia can increase the probability of positive results in the RT-PCR test. This factor has not been mentioned in previous studies, and since the number of PCR positive cases was very low in our study, this might be due to a chance. By using the full logistic regression model, the population of the household was found to be a predictor of FeLV infection, and the relative risk of FeLV infection in cats kept in multicat households was 6.6 times higher in comparison with single cats. Keeping cats in permanent contact with other cats increases the chance for contact with other possibly infected cats; as a result, it increases the overall prevalence of FeLV infection (1, 4, 23). The most prevalent hematological abnormalities in FeLV-infected cats were thrombocytopenia (66.7%), anemia and leukocytosis (33.3%), which were reported by other authors (23), but in some other studies, leukopenia and lymphopenia were reported to be more prevalent (9). According to the results of this study, all positive cats belonged to DSH cross-breed. The same results were described in some other studies (24, 26). This may be related to the higher population of cross-breed DSH cats in many countries such as Iran. Two cats out of three positive cats (66.7%) had free access to open space, but the difference was not significant, even though there was a statistically significant association between the lifestyle and risk of FeLV infection in some previous studies (8, 20, 27, 28, 29).

Finally, according to the low prevalence of FeLV in both case and control cats, it seems that a proportion of this population under study, especially cats with abnormal clinical signs, may be infected with FeLV, but they are in the latent stage of the disease. Evaluating bone marrow samples for existence of

FeLV would be required to confirm this type of infection. Another possible explanation is that these cats may have been infected with other pathogens that are able to display similar clinical signs as FeLV (e.g. Feline immunodeficiency virus, Feline panleukopenia virus). Interestingly, since we used combo immunochromatography kits which are also able to detect the presence of antibodies against FI, we found a relatively high prevalence of FIV in these samples. Sixteen out 90 blood samples (17.8%) were positive for anti FIV antibodies while no cat was positive for FeLV and FIV, simultaneously, and this could perhaps explain some clinical signs. In conclusion, results of ICGA and PCR methods suggest that the overall prevalence of FeLV is very low in Tehran, in contrast to its relatively high prevalence in southern Iran (Kerman). According to the full logistic regression model, the whole model was statistically significant, and if all these putative factors can be mentioned simultaneously, it is possible to predict correctly the FeLV infection status in 94.4% of the cats.

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PREISKAVA OKUŽENOSTI MAČK V TEHERANU Z VIRUSOM FELV Z IMUNOKROMATOGRAFIJO IN RT-PCR TER KLINIČNI IN HEMATOLOŠKI REZULTATI

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Povzetek: V raziskavi v Teheranu (Iran) so bili med junijem 2009 in februarjem 2010 odvzeti krvni vzorci 90 mačkam (45 zdravim oz. kontrolnim in 45 bolnim) in pregledani z imunokromatografijo (ICGA), obratno transkripcijo in verižno reakcijo s polimerazo (RT-PCR) ter hematološkimi preiskavami. S pomočjo imunokromatografije in RT-PCR so ugotovili, da je prevalenca okuženosti s FeLV v preiskovani populaciji 1,1- in 2,2-odstotna. Dejavniki, ki so bili statistično značilno povezani s pozitivnimi rezultati, dobljenimi z metodo RT-PCR, so bili blede mukozne membrane (p = 0,026) in vnetje nosne sluznice (p = 0,002), ki je bilo bolj pogosto pri mačkah, pozitivnih na FeLV. Okuženost z FeLV se je pojavljala pogosteje v gospodinjstvih z večjim številom mačk. Relativno tveganje za okužbo z virusom FeLV pri mačkah, ki so živele v gospodinjstvih z več mačkami, je bilo 6,6-krat višje, kot pri mačkah, ki so bile v gospodinjstvu same.
V kontrolni skupini so bili najpogosteje ugotovljeni naslednji klinični znaki: vnetje dlesni in/ali želodca (37,8 %), poškodbe kože (8,9 %), limfadenopatija in bledost mukoznih membran (6,7 %). Najpogostejše hematološke ugotovitve so bile: zmanjšan PCV (24,4 %), limfopenija, zmanjšana raven hemoglobina (20 %) ter levkocitoza in nevtrofilija (13,3 %). V skupini okuženih živali so bili najpogostejši klinični znaki vnetje dlesni in/ali želodca (77,8 %), bledost mukoznih membran (53,3 %) in poškodbe kože (37,8 %), najpogostejše hematološke ugotovitve pa limfopenija (37,8 %), anemija (26,7 %), znižana raven hemoglobina (24,4 %) in levkopenija (15,6 %).

Ključne besede: mačka; virus mačje levkoze (FeLV); prevalenca; imunokromatografija; obratna transkripcija in verižna reakcija s polimerazo (RT-PCR)