

Case-control Study of Infection by Feline Immunodeficiency Virus in Cats by Immunochromatography and PCR methods in Tehran, Iran

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Abstract: Immunochromatography, PCR and hematological assays were performed on blood samples of 90 cats (45 healthy and 45 sick) in Tehran as a case-control study. Prevalance was 17.77% and 4.44% on Immunochromatography and PCR assays respectively. The results showed no significant difference was between healthy and sick groups but, Gingivitis, stomatitis, abscesses, dermal wounds and band cells were more in seropositive cats significantly. Castration, age, sex, access to outside (lifestyle) and overcrowding were not significantly effective on tests results.

Key words: FIV, Immunochromatography, PCR, Clinical and Hematological Findings.

INTRODUCTION

Feline immunodeficiency virus (FIV) is a member of the lentivirus subfamily of retroviruses. It is an RNA virus with outer envelope and nucleocapsid. FIV originally was isolated in 1986 from a cattery in north California, however, retrospective assays of stored cat sera have shown that FIV has been widely distributed worldwide since at least the 1960s (Sherding, 2006). FIV causes a lifelong infection and gradually progressive decline in immune function that leads to an acquired immunodeficiency syndrome. Manifestations include chronic weight loss, opportunistic infections, stomatitis, chronic inflammatory conditions and increased risk for malignant neoplasia (Gabor *et al.*, 2001; Sellon *et al.*, 2006). Direct inoculation of saliva through biting is the principal mode of FIV transmission however, infection can occur through mating, blood transfusion, intimate contact and during lactation (Allison *et al.*, 2003; Sherding, 2006). Adult, male outdoor cats living in large multicat household are at increased risk for territorial fighting that can lead to bite-wound transmission (Knotek *et al.*, 1999; Yuksek *et al.*, 2005; Gleich *et al.*, 2009). The prevalence in indoor pet cats in single-cat households is less than 1%. The prevalence in well-controlled purebred catteries is less than 2% (Sherding, 2006; Luria *et al.*, 2004). The prevalence in some countries in areas with a high density of free-roaming cats, such as Japan and Italy reach 25% to 30% (Maruyama *et al.*, 2003; Bandecchi *et al.*, 2006).

The routine diagnosis of FIV depends on the detection of anti-FIV serum antibodies using assays such as enzyme-linked immunosorbent assay (ELISA), Immunochromatography and Western blot (Sellon *et al.*, 2006; Sherding, 2006). Virus isolation and polymerase chain reaction (PCR) are used in specialized research labs for detecting virus or viral antigen. Immunochromatography is one of the sensitive, accurate and considered the preferred screening tests for FIV however, false negative and false positive may occur, thus, all positive immunochromatography results should be confirmed by other methods such as PCR (Sellon *et al.*, 2006; Arjona *et al.* 2007). However, because of PCR is not yet adequately standardized or validated, it is uncertain whether PCR assays detect all field strains of FIV found in cats (Sherding, 2006). With regard to these, we used Immunochromatography and PCR assays accompany with hematological test and clinical findings in this study.

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MATERIALS AND METHODS

With regard to the report of other researchers about the infection of domestic cats with feline immunodeficiency virus, and by taking into consideration a minimum incidence of 2% in healthy cats and a maximum incidence of 30% in sick cats, and with a reliability of 95% and a test power of 80%, two 45-member groups of sick and healthy cats out of a 90-member population were sampled in this study. A number of cats that were brought to the domestic animals specialty polyclinic of Tehran were selected. At first, the background and particulars of the cats were registered in a special form. Each cat underwent a thorough clinical examination and the results were registered in the form. Since the most common findings among cats infected with feline immunodeficiency virus include weight loss, fever, dehydration, diarrhea, rhinitis, gingivitis and/or stomatitis, lymphadenopathy, pale mucous membranes, and dermal abscesses, each cat which simultaneously showed two or more of these symptoms was reckoned as "sick". Those cats which were apparently healthy and were brought to the clinic for the annual clinical examination, vaccination, or anything other than treatment were considered as "healthy". After informing the cats' owners and obtaining their permission, samples of the cats' blood were drawn, 2 cc from each one. In order to separate the serum, part of the blood was centrifuged for 10 minutes with the speed of 3000 rpm. The separated serum was kept in temperature of -20 °C before rapid immunochromatographic assay was done. Checking the presence of GP40 antibody of the feline immunodeficiency virus in the samples was carried out by means of rapid immunochromatography kit made by French BVT Company. The sensitivity and specificity of the kit were 97.3% and 98.6% respectively.

On the other hand, for PCR technique, DNA extraction was carried out using the commercial Accuprep Genomic DNA extraction kit manufactured by Bioneer Company of South Korea. In order to do so, 20 microliter of the proteinase solution K available in the kit was transferred to a 1.5 milliliter sterile microtube, and then 200 microliter of the complete blood sample was added to that. After blood addition, 200 microliter of the Binding buffer, available in the kit, was also added to the complex, and the sample was put in the bain-marie of 60°C temperature for 10 minutes. After that, 100 microliter of isopropanol was added to the sample and well mixed by a pipette.

At the next stage, the samples was transferred into a column containing a DNA-absorbing filter, which was itself inside a 1.5 millimeter sterile microtube; the column was centrifuged with a speed of 8,000 rpm for one minute. After the centrifuge finished, the column containing the filter was transferred to a new sterile microtube and was washed with 500 microliter of washing buffer number 1 and 1 minute of centrifuging with the speed of 8,000 rpm. The second stage washing was carried out with the same procedure, the only difference being the use of buffer number 2. In order for the washing buffer to completely exit from the column, a one-minute centrifuge with the speed of 12,000 rpm was also carried out.

Finally, in order to separate the DNA that had stuck to the column, 200 microliter of the elution buffer was added, and then after keeping the column in the room temperature for one minute, it was centrifuged with the speed of 8,000 rpm for one minute.

The liquid resulted from centrifuge containing pure DNA was gathered in a 1.5 milliliter sterile microtube and kept in a freezer in the temperature of -40°C. After purification, A two-step PCR amplification was used with previously described (Arjona *et al.*, 2007) primer sets specific for the envelop gene of FIV. In the first reaction of PCR, 5 to 10 µl of extracted DNA (about 1 µg) was added to a PCR master mix containing 2.5 U of Taq DNA Polymerase (Cinagene, Iran), 5 µl of 10× PCR buffer (200 mM Tris-HCl, 500 mM KCl, PH 8.4), 1% DMSO, 1.7 mM MgCl₂, 0.2 mM dNTPs, and 0.4 µM of each primer [VE-1S (5'-GAGTAGATAC(A/T)TGGTT(G/A)CAAG-3') and VE-1R (5'-CATCCTAATCCTTGCATAGC-3')]. Total volume of the reaction was adjusted to 50 µl with sterile distilled water. PCR program was 35 cycles of 94°C, 1 min, 50°C, 1 min and 72°C for 2 min. There were also two steps of 94°C for 5 min, before and 72°C for 10 min, after the 35 cycles. The primers VE-1S and VE-1R expected to amplify a DNA fragment of 1230 bp. PCR product of the first reaction was subsequently used, as template, in second round of amplification (nested PCR) with primers VE-2S (5'-CAAAATGTGGATGGTGGAA (T/C)-3' and VE-2R (5'-ACCATTC(C/A)ATAGCAGT (G/A)GC-3'). Therefore, 1 µl of the first round PCR product was added to 49 µl of PCR master mix containing the reagents, at concentrations similar to the first PCR. Nested PCR program was also similar to the first PCR. Finally, the amplicons (858 bp products) of nested PCR were analysed by electrophoresis on 1% agarose gel and ethidium bromide staining.

In addition, total red blood cell count, total and differential white blood cell count, as well as hematocrit measurement were carried out according to the common laboratory methods. Finally, the statistical relationship of the positive results of the test in both healthy and sick groups with potential effective factors such as age,

gender, lifestyle, sterilization, and density of the living place was examined. Moreover, the most common hematological findings and clinical symptoms were found in cats which were positive in terms of virus infection.

RESULTS AND DISCUSSION

Of 90 animals examined, 16 (17.77%) and 4 (4.44%) were positive in immunochromatography and PCR assay respectively. In “healthy” group, the most common clinical findings are gingivitis and/or stomatitis (37.8%), skin lesions (8.9%), lymphadenopathy and pale mucus membrane (6.7%), and hematological findings are decreased PCV (24.4%), lymphopenia and decreased Hb (20%), leukocytosis and neutrophilia (13.3%). In “sick” group, the most common clinical findings are gingivitis and/or stomatitis (77.8%), pale mucus membrane (53.3%) and skin lesions (37.8%), and hematological findings are lymphopenia (37.8%), anemia (26.7%), decreased Hb (24.4%) and leucopenia (15.6%).

Discussion:

The diagnosis of FIV infection has received more attention since about one decade ago. For the first time in Tehran, through ELISA method, this infection was diagnosed to be 3.4% (Rad *et al.*, 1998). In another study applying immunochromatography method, conducted in Kerman on domestic and wild cats, this serum infection was reported to be about 19.2% (Akhtardanesh *et al.*, 2010). In the current study, which has applied immunochromatography, this infection has been diagnosed to be 17.8%; of course, with regard to the type of the study (case-control study), mere serum incidence was not the goal of the researchers. All the samples were examined by PCR method as well, through which 4.4% of the samples proved positive. All the positive cases in PCR were those positive in immunochromatography as well.

Regarding 97.3% sensitivity and 98.6% specificity of the immunochromatographic kits of the French BVT company, it seems that in diagnosing FIV, the PCR test has a high specificity but a low sensitivity, the latter being probably due to the vast genetic variety of FIV; however, other factors, such as the small quantity of the viruses in the sample could contribute to the low sensitivity of the PCR in FIV diagnosis.

In our study, the comparison of the qualitative variables in seronegative and seropositive cats, with the use of logistic regression analysis, showed that mere existence of gingivitis, stomatitis, abscesses, and dermal wounds can be useful in predicting seropositivity. Nevertheless, in another study, conducted on the West Australian cats, access to open space, masculinity, and old age were reported as FIV risk factors (Norris *et al.*, 2007). In another study conducted in Italy on cats, a serum incidence of 11.3% was reported, and logistic regression analysis showed that access to open space and old age were risk factors of this infection (Bandeccchi *et al.*, 2006). Also, a study applying PCR was carried out in Brazil, which reported the overall infection to be 4%; the incidence in apparently healthy and apparently ill cats was 1.4% and 13.95% respectively.

In this study, the presence of all the clinical symptoms was meaning fully effective on the prediction of FIV infection. Also, masculinity and old age were positively relevant in prediction FIV infection (Caxito *et al.*, 2006).

In two separate studies carried out in Turkey, old age was reported as an effective factor in predicting the FIV (Yilmaz *et al.*, 2000; yuksek *et al.*, 2005). In another study, done in the Czech Republic, castration was found to have a reverse relation with FIV infection. In this study, the most common clinical findings in ill cats were chronic urinary infections and general lymphadenopathy; this clinical relation is similar to that found in most of the studies conducted in Europe (Knotek *et al.*, 1999). In Kerman (a city in Iran), the results of logistic regression analysis showed that masculinity and old age were positively influential in predicting seropositivity (Akhtardanesh *et al.*, 2010). In a study done in Germany, the only predicting factor was reported to be old age (Gleich *et al.*, 2009).

With regard to all the cases mentioned in different studies, old age, not being castrated, access to outside, high concentration of cats' population at their keeping place, and masculinity can all be considered as risk factors and predicting factors of FIV infection, while in our study, with regard to the logistic regression analysis, none of these factors had a significant predicting effect on FIV infection. This can be due to various reasons: for example, animals' castration age in Iran is not in accordance with the international standards, and most of the cats are castrated after puberty age, while, due to territory conflicts and pair bonding, it is highly probable that the animal is infected with FIV during this period. Of course, it is also possible that the animal is infected in older ages. As for free access to outside, according to the common custom in Iran, most of the cat owners, at least in Tehran, allow the animal to have access to outside; therefore, this factor, which was the same for most of the cats in our study, might not be specifically predictive of FIV infection.

As the study continued, the comparison of the quantitative hematologic variables among seropositive and seronegative cats based on logistic regression test showed that the presence of Band cells only could increase the probability of seropositivity, while in most studies, even in authentic references, anemia, low Hb level, and leukopenia were introduced as relatively common symptoms of FIV infection (Sellon *et al.*, 2006; sherding, 2006; Gleich *et al.*, 2009).

In a similar comparison based on U-Mann-Whitney Test made on the cats which roved positive through PCR test, it was shown that the presence of basophilia can increase the probability that the PCR test is positive; this factor has not been mentioned in other researches, and since the number of PCR positive cases were very few in our study, it may not be considerable in here as well.

Finally, the point requiring attention is the noticeable difference between positive cases in the immunochromatographic test and those in PCR test. In addition to the probable low sensitivity of PCR in diagnosing FIV (e.g. because of the vast genetic variety of the virus, low number of virus particles in the samples, etc), this problem could be due to various, sometimes unknown, factors which lead to false positive cases in the immunochromatographic test.

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