EVALUATION OF ANTIBODIES ELICITED BY A NEW RECOMBINANT ANTIGEN FOR FELINE LEUKEMIA VIRUS (FeLV) INFECTION DIAGNOSTIC*

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ABSTRACT. Almeida N.R., Danelli M.G.M., Hagiwara M.K., Góes A.C.M.A., Medeiros M.A. & Mazur C. **Evaluation of antibodies elicited by a new recombinant antigen for Feline Leukemia Virus (FeLV) infection diagnostic.** [Avaliação de anticorpos extraídos de um novo antígeno recombinante para o diagnóstico da infecção pelo Vírus da Leucemia Felina (FeLV).] *Revista Brasileira de Medicina Veterinária, 35(Supl.2):95-99, 2013.* Programa de Pós-graduação em Ciências Veterinárias, Instituto de Veterinária, Anexo 1, Universidade Federal Rural do Rio de Janeiro, BR 465 Km 7, *Campus* Universitário, Seropédica, RJ 23897-970, Brasil. E-mail: nadia.ufrrj@gmail.com

A novel recombinant Feline Leukemia Virus (FeLV) capsid antigen was developed and inoculated in rabbits to elicit antibodies against it. These antibodies were evaluated by indirect immunoflourescence assay (IFA) and proved to react with the native FeLV antigen in agreement with the previous tests performed in animal samples. Considering its advantages to Brazilian scenario, the established IFA using recombinant antigen may be a practical and low cost diagnostic test to be incorpored as diagnosis of Feline Leukemia Virus.

KEY WORDS. FeLV, cats, IFA, leukemia.

RESUMO. Um novo antígeno recombinante de capsídeo do Vírus da Leucemia Felina (FeLV) foi desenvolvido e inoculado em coelhos com o intuito de obter-se anticorpos. Estes anticorpos foram avaliados pela técnica de imunofluorescência indireta (IFA) e reagidos contra o antígeno nativo do FeLV, de acordo com os testes anteriores realizados em amostras de animais. Considerando suas vantagens no cenário brasileiro, o estabelecimento da IFA utilizando um antígeno recombinante pode ser um teste prático e de baixo custo, podendo ser incorporado como diagnóstico da Leucemia Viral Felina.

PALAVRAS-CHAVE. FeLV, gatos, IFA, leucemia.

INTRODUCTION

Feline Leukemia Virus (FeLV) is a horizontally transmitted gammaretrovirus that causes diseases and death in persistently infected cats worldwide. FeLV infection is of high veterinary importance and serves as an animal model for human oncogenic di-

Rev. Bras. Med. Vet., 35(Supl.2):95-99, dezembro 2013

95

seases, anemia, and immunodeficiencies (Rohn et al. 1996).

After virus exposure, different courses of FeLV infection may be observed which are associated with cellular and humoral immune responses and proviral load in peripheral blood. Interactions between FeLV and cells of the hemolymphatic system determine whether the cat immune response or the virus infection will predominate in the host/virus relationship, thus resulting in a persistent viremia and progressive infection or self limiting, regressive infection (Rojko & Kociba 1991). It is assumed that mutual grooming and sharing of eating and drinking bowls represent the major means of FeLV transmission, due to the fact that saliva is the primary source of virus (Francis et al. 1977).

In the field, the disease control is based on the diagnosis of FeLV infections and vaccination of susceptible cats. Most available FeLV diagnostic tests detect antigens in serum, plasma or whole blood. The American Association of Feline Practitioners recommends the FeLV status of all cats, regardless of age or health status (Levy et al. 2008).

Routine diagnosis for FeLV includes ELISA and indirect immunofluorescence antibody assay (IFA) for the capsid protein p27 detection (Lutz et al. 2009). Screening tests (conventional and lateral flow ELISA tests) detect the presence of free antigen in the circulating blood, while IFA test relies on the detection of the antigen presence within infected leucocytes and platelets, confirming bone marrow infection. Most of the positive cats to IFA test have persistent antigenemia with continuous virus elimination in saliva (Hardy & Zurckerman 1991, Herring et al. 2001).

As other viral infections, FeLV immunodiagnostic tests depend on virus antigen production which can be a neck bottle, especially for retrovirus. Commonly, these viruses are hard and expensive to cultivate, and afterwards, the antigens should be obtained by cumbersome procedures of fractionation and purification. Hence, we chose to develop a recombinant antigen produced by bacteria. Such recombinant bacteria may represent an easy to deal and economic source of antigen. Anyhow, due to possible differences among native and recombinant proteins, even small ones, the reactivity of the latter need to be tested and compared to the former antigen.

In Brazil, only a few FeLV epidemiologic studies have been carried out with small numbers of samples from domestic cats tested by lateral flow ELISA, probably due to its high cost (Souza et al. 2002, Teixeira et al. 2007). A single similar study was conducted in São Paulo city, using a large sample and IFA as diagnostic procedure. (Jorge-Junqueira et al. 2011). Because of the cost and difficulty to obtain commercial antigens for routine diagnosis of retroviral infections, FeLV infection still remain underdiagnosed in domestic cats

Aiming to overcome these difficulties and contribute to expand FeLV diagnosis in Brazil, a recombinant antigen produced by *Escherichia coli* was developed and specific antibodies produced in rabbits. The elicited antibodies reactivity was tested by IFA test on cat blood smears, previously identified as positive or negative for FeLV infection by commercial IFA sets.

MATERIAL AND METHODS

Cloning procedures

A DNA sample obtained from peripheral blood mononuclear cells (PBMC) of a naturally FeLV infected cat was used successfully in order to amplify the p27 coding region by PCR. The primer design was based on the complete FeLV-A genomic sequence (Genbank/nucleotide database accession number NC001940) and the characteristics of the chosen expression vector (pET100/D-TOPO®, Invitrogen) (5'- CACCATGCCCTTGAGGGAGGG-3' and 5'-CAGA-ACTTTAGTCATCTCCTTGTGG-3'). The obtained amplicon was inserted in the expression vector in accordance to the manufacturer's recommendations. The correct p27 coding region insertion in the recombinant plasmid was analyzed by nucleotide sequencing.

Recombinant protein (r-p27) production

To produce antigen (r-p27) for the inoculums, competent *E. coli* (BL21 Star, Invitrogen™) were transformed with the recombinant plasmid by electroporation. Hence, the r-p27 was overexpressed by growing the selected recombinant bacterial clone induced with 1M IPTG (isopropyl-D-thiogalactopyranoside) in 500 mL Lurya Broth cultures. The bacterial crude lysates obtained from these cultures were analysed by SDS-PAGE to confirm the production of a recombinant protein.

Purification and reactivity evaluation

The r-p27 purification from crude extracts (poly-histidine tail based) was performed by immobilized metal affinity (IMAC) in nickel-charged resin columns (HisTrap FF crude, GE Healthcare®). The purified samples were characterized by SDS-PAGE and desalinated with PD-10 desalting columns (GE Healthcare®), according to the manufacturer's recommendations. The concentration of purified recombinant p27 was performed using the colorimetric enzymatic assay BCA (Bicinchoninic acid assay, Sigma®).

In order to evaluate the r-p27 reactivity against anti-p27 (native antigen) antibodies, a test diagnostic SNAP *combo*

96

Rev. Bras. Med. Vet., 35(Supl.2):95-99, dezembro 2013

FIV/FeLV® (*IDEXX Laboratories*) was used according to the manufacturer's recommendations.

Antibodies production

Two 7 months old New Zealand rabbits were immunized with intramuscular inoculations of 80 mg rp-27 in aluminum hydroxide each, for 4 consecutive weeks. A fifth inoculation was performed with the same protein doses, but without adjuvant as a booster, in the femoral vein. The blood collection was performed in previously anesthetized animals through intracardiac puncture.

Western blotting assay

After sera separation, the antibody titer against the r-p27 was evaluated by Western blotting (WB). WB was conducted on a nitrocellulose membrane with approximately 2,5 µg r-p27 protein/cm. Basically, this assay was carried out using the collected rabbit serum dilutions in milk TBS-T (4% non-fat milk in Tris-tween buffered saline) and horseradish peroxidase-conjugated mouse anti-rabbit immunoglobulin (Sigma-Aldrich®).

IFA evaluation of the antibodies elicited against r-p27

Peripheral blood smears in microscope conventional slides were obtained by puncturing ear tips of 30 positive (naturally infected) and 30 negative cats previously tested for at least two FeLV diagnostic techniques (commercial IFA - VRMD®, Elisa and/ or PCR). The slides were fixed using an acetone and methanol solution (3:1) at room temperature for 20 minutes. Afterwards, 20 µL of anti r-p27 antibody 10x diluted in PBS buffer were dropped in previously selected rich in leukocytes and platelets area and incubated 30 minutes at 37°C in a moisten chamber. Then, the slides were immersed in washing buffer (27 mM Na_2CO_3, 0.1M $NaHCO_3$, 0.15 M NaCL, pH 9.0-9.5) for 10 minutes. After removal of the washing buffer, the slides were dipped quickly in distilled water and allowed to dry at room temperature. Subsequently, 20 µL of the 100x diluted FITC-conjugate anti-rabbit (Molecular Probes®) were deposited in the same area, the incubation step was repeated (30 min., 37° C) and slide was washed and dried, this time in a dark room. After drying, 5 µL of washing buffer glycerin (1:9) was added and covered with a coverslip. A fluorescence microscope (Olympus BX41) with epi-illumination and a 40x lens was used to observe for the observation of the reaction.

RESULTS AND DISCUSSION
Cloning procedures

A recombinant plasmid, able to express the desired recombinant protein (p27) was obtained and its correct position and its nucleotide sequence were confirmed.

r-p27 and antibody production

The SDS-PAGE analysis of the selected recombinant bacterial culture lysates indicated a protein with approximately 30 kDa. The reactivity of this recombinant protein against an antibody for a nati-

ve p27 was proven by the colorimetric reaction of the SNAP® (IDEXX).

The antibody against r-p27 was successfully produced by rabbit inoculation. The serum of both rabbits showed a titer of 500 by WB (Figure 1).

Evaluation of the antibodies elicited against the r-p27 by IFA test

The elicited antibodies reacted against the p-27 in leukocytes and platelets of naturally infected cats blood smears, as observed in Figure 2. There was an agreement between the positive and negative results obtained here by IFA test using anti r-p27 antibodies with those obtained previously with commercial IFA reagents. Only in one case, it was obtained

Figure 1. Nitrocellulose stripe showing the reaction of the inoculated rabbit serum with the protein r-p27 by Western blotting.
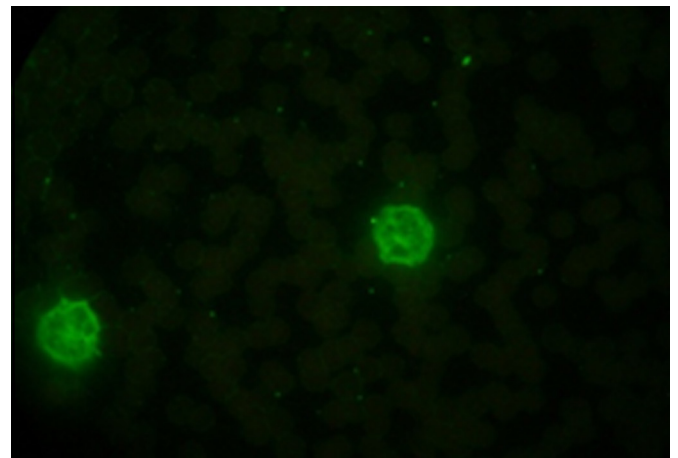

Figure 2. Immunofluorescence of a naturally infected cat blood smear obtained with the elicited antibodies against the r-p27. 100X.

Rev. Bras. Med. Vet., 35(Supl.2):95-99, dezembro 2013

97

an undefined result. No significant difference was found between the observed and expected frequencies (p=0,03; confidence interval 95% - SPSS software Version 15.0®, IBM).

From 2007 to 2011, the Brazilian cat population increased by about 50% from 12 to 18 million of animals whilst dog population was increased by 10% in the same time interval. This may reflect a change in the preference of pets, probably due to Brazil's favorable economy and practical aspects of cats care compared to dogs. This change was also observed in other countries (Pet Food Brasil 2011).

The increasing population and concentration of cats in small groups has stimulated the dissemination and persistence of viral infections. FeLV was first described by Jarrett et al. in 1964 and is one of the most common fatal pathogens for cats worldwide. The infection results mostly from oral exposure to saliva and nasal secretions containing high levels of the virus, especially through mutual grooming and shared food dishes and water bowls. Vertical transmission occurs occasionally but it is of secondary importance (Levy et al. 2006). Favored by the evolution of the world's cat population, FeLV has become a major feline pathogen, which is responsible for important diseases that can cause prolonged suffering and death.

In our recent survey, in Rio de Janeiro state, we reported a FeLV infection prevalence of 11.52% (Almeida et al. 2012). This finding is consistent with several epidemiologic studies conducted in different regions of the world (Arjona et al. 2000, Levy et al. 2006, Gleich et al. 2009).

Prophylactic measures for FeLV infections rely on identification and isolation of infected cats, as well as on vaccination of known non infected cats under high risk of exposure (Dunham & Graham 2008). In Brazil, an inactivated virus vaccine is available, but the high cost limits its usage, mainly in shelters or large group of cats. (Almeida et al. 2012).

Tests for FeLV infection were mostly developed in the ELISA format as microwell plates and lateral flow systems (rapid tests), and they are commercially available. Rapid tests are the most frequently used at veterinary clinics and animal shelters around the world, due to its simplicity and low time consumption (Crawford & Levy 2007).

The FeLV rapid test is an ELISA, with immobilized labeled antibodies that bind soluble antigen found in serum, blood or saliva of the infected cats, and is the only available test for commercial or in-practice use in North America and other countries, including Brazil. These tests are prepared in a kit format for in-practice use, or in a microwell format for laboratory use, and allow the detection of the viral nucleocapsid protein p27. The resulted chromogenic reaction is compared to a positive or negative control (O'Connor et al. 1989, Mermer et al. 1992). The Elisa test is a sensitive one, but it has to be repeated regularly to confirm non infected status and still needs to be associated to IFA test to confirm a persistent FeLV infection.

The IFA is the least expensive FeLV diagnostic assay in many countries (Almeida et al. 2012). Cats with IFA positive results are considered persistently infected because the test detects viral antigen associated with leukocytes and platelets during the secondary viremia, when progenitor cells from bone marrow are compromised (Herring et al. 2001).

Nevertheless, routine and research diagnosis for FeLV infections still remain problematic in some parts of the world such as Brazil. Despite its large worldwide diffusion and application (Hartmann et al. 2007), the ELISA based rapid test usage still depends on imported kits with highly restrictive costs in Brazil. Moreover, any alternative immunodiagnostic technique depends on the antigen source and on viral cultivation in cell cultures, which is expensive and time-consuming. Perhaps, these factors are the greatest obstacles for the availability of FeLV diagnosis in our country.

The production of viral proteins by recombinant bacteria or other biological systems has been used for different purposes as an alternative technology to classic methods of protein purification from cultivated virus. Such alternatives are of special interest for viruses. Basically, the desired coding region is ligated in a DNA vehicle as a bacterial plasmid and this construct is inserted in an appropriate biological system such as bacteria to express the recombinant protein. Cultures of recombinant bacteria represent a practical and economic source of the heterologous recombinant protein.

There is a great veterinary interest in a large scale FeLV epidemiologic survey in Brazil. Nevertheless, the rapid test costs and the difficulty to obtain the viral antigen by classical ways to establish immunodiagnostic techniques may explain the availability of just a few epidemiologic studies found in Brazil and its low numbers of surveyed animals (Souza et al. 2002, Teixeira et al. 2007). On the other hand, IFA test for FeLV is less expensive and of practical

use, because of the possibilities of preparing blood smears and send them by post (Jorge-Junqueira et al. 2011). The recombinant bacteria obtained in our work represents a good source for viral antigen production, which might be used for establishing alternative diagnostic methods other than ELISA, such as IFA. An inexpensive diagnostic method for FeLV infection allows us for large epidemiological and clinical studies in Brazil. Even more, with this novel recombinant antigen, other tests may be developed for the evaluation of FeLV antibodies in vaccinated and non-vaccinated cats.

CONCLUSION

The IFA results showed that the elicited antibodies against the r-p27 reacted efficiently against the native antigen in naturally infected cat leukocytes. Furthermore, it was proven to be a reliable diagnostic assay for FeLV infections. The establishment of this method may contribute significantly to FeLV diagnostic and epidemiological studies in Brazil and other countries.

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Rev. Bras. Med. Vet., 35(Supl.2):95-99, dezembro 2013

99