DEVELOPMENT OF AN INDIRECT ELISA FOR THE DIAGNOSIS OF FELINE IMMUNODEFICIENCY VIRUS*

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The objective of this study was to develop an indirect ELISA (r-p24 ELISA) for the diagnosis of Feline Immunodeficiency virus (FIV-B). Serum samples of 150 asymptomatic cats, of both sexes and mixed breeds and ages were used. The SNAP Combo Plus commercial kit was used as the gold standard. The r-p24 ELISA was standardized using 0.25 µg of recombinant p24 protein (r-p24) per well, serum was diluted at 1:320 and the conjugate was diluted at 1:7500. The r-p24 ELISA showed a sensitivity of 97% and specificity of 93% when compared to the gold standard with a concordance of 83%. When compared to the Western blot confirmatory test, the r-p24 ELISA showed a sensitivity of 94% and specificity of 94% with a concordance of 84%. Therefore, the standardized ELISA is an important tool for the FIV related research and for the FIV diagnosis at low cost.

KEY WORDS. Feline immunodeficiency virus, p-24 protein, Western blot, ELISA.

RESUMO. O objetivo deste estudo foi desenvolver um ELISA indireto (r-p24 ELISA) para o diagnóstico do Vírus da Imunodeficiência Felina (FIV). Foram utilizadas 150 amostras de soro de animais assintomáticos, sem distinção de sexo e idade. O kit comercial SNAP Combo Plus foi utilizado como padrão ouro. O r-p24 ELISA foi padronizado utilizando 0,25 µg da proteína recombinante p24 (r-p24) por poço,
INTRODUCTION

Feline immunodeficiency virus (FIV) is a lentivirus from Retroviridae family, distributed worldwide, that causes one of the most common infectious diseases of cats, the feline immunodeficiency, because a progressive depletion of CD4+ cells. The prevalence varies by location and the most effective way to guard against infection is to prevent exposure to FIV-infected cats (Pedersen et al. 1987, Hosie et al. 2009, Alves et al. 2011, Hartmann 2011). FIV can be transmitted through the saliva inflicted by of bites, via mucosal exposure, blood transfer, during mating, and vertically during prenatal and postnatal exposure (Sellon & Hartmann 2006, Medeiros et al. 2012).

FIV infected cats may remain asymptomatic for years, however, eventually succumb due to direct viral effects or, more commonly, to secondary infections resulting from virus-induced immunosuppression (Hosie et al. 2009, Magden et al. 2011, Korman et al. 2012, Sobrinho et al. 2012).

Domestic cat population is growing in the world and the search for preventive methods against infectious diseases is increasing. Infections with these viruses can be difficult to diagnose by clinical signs alone, becouse, laboratory diagnosis associated with clinical signs is becoming more necessary for FIV prevention (Mortola et al. 2004). Testing to identify infected cats is the mainstay of preventing transmission of the viruses (Hosie et al. 2009, Alves et al. 2011).

FIV infection in cats can be diagnosed by serologic and molecular methods (Hohdatsu et al. 1992, Hosie et al. 2009, Alves et al. 2011). Antibodies to FIV can be detected by immunofluorescence, ELISA, Western blot and radioimmuneprecipitation assays (Calzolari 1995, Calandrella 2001, O’Connor et al. 1989, Alves 2010). Of these assays, ELISAs are the most widely used in the field because they are simple to perform and available to the veterinary practitioner (Hosie & Jarret 1990). Antigens used in ELISA are cell culture derived, gradient purified FIV particles, and recombinant protein. Of the FIV proteins, the capsid protein (CA) or p24, the transmembrane (TM) and surface (SU) glycoproteins of the envelope are the most widely used for the ELISA (Reid et al. 1991, Furuya et al. 1992, Mermer et al. 1992, Calzolari 1995).

The objective of this work was to develop an indirect ELISA technique for FIV diagnosis.

MATERIAL AND METHODS

Animals, sample collection, and controls

One hundred and fifty serum samples were analyzed, 78 collected from sheltered and private cats, of both sexes and mixed breeds and ages; and 72 samples kindly donated by the Departamento de Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo. All procedures and protocols used in this work were approved by the Universidade Federal de Minas Gerais Animal Experimentation Committee (101/09). Samples positive and negative by the SNAP Combo Plus (Idexx Laboratories Inc One Idexx Dr, Westbrook, USA) and by the Polymerase Chain Reaction (PCR) were used as positive and negative controls according to Alves (2010).

Cloning, r-p24 protein expression, and protein purification

The cloning process, selection and expression of clones were performed by Mazur et al. (2010). Cultures in 500 mL of Luria Blot (LB) medium with recombinant plasmids (CA) were induced with 1mM of IPTG for 4 hours, under agitation at 37ºC. The induction of protein expression was detected by SDS-PAGE. The expressed r-p24 protein was purified using the His Trafte FF, 1 ml, (Amershams Biosciences Cor, Pisca-taway, USA). The total amount of protein in the crude extract was quantified using the NanoDrop (Thermo Fisher Scientific, Wilmington, Delaware, USA).

SDS-PAGE and Western blotting assays

Protein extracts were mixed with an equal volume of reduced sample buffer 2x, containing 2-mercaptoetanol (Merck, Darmstadt, Germany), and separated by SDS-PAGE. The gels were stained by standard methods using Coomassie Brilliant Blue. Nitrocellulose (NC) membranes were post-coated with 5% skimmed milk (with?) plus cat polyclonal antibodies (diluted 1:10 and 1:100) in TBS (pH 7.4) for 1 h at room temperature. The NC membranes were then probed with peroxidase-labelled HRP Conjugated Goat anti-Cat IgG Fc (Immunology Consultants Lab, Newberg, OR, USA) (1:1000 dilution) for 1 h at room temperature. In this study, Western blot with expressed r-p24 protein was compared with the r-p24 ELISA.

Standardization of the r-p24 ELISA method

The positive or uninfected sera (as a negative control) were used to optimize the detection system. To determine the optimal peroxidase-labeled HRP Conjugated Goat anti-Cat IgG Fc conjugate dilution, a serial 2-fold dilution of the conjugates from 5000x to 7500 was used. To optimize the r-p24
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Concentration, 1 µg, 0.5 µg, and 0.25 µg per well were tested. Sera were tested in the dilution of 1:20 to 1:1280.

**r-p24 ELISA**

To sensitize the ELISA plates (Maxisorb, Nunc, Hampton, NH, USA), 0.25 µg of r-p24 were used in 100 µL of 0.05 M carbonate buffer (pH 9.6), and the plate was incubated at -4 °C for 18 h. The antigen-coated plates were washed two times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST), and were blocked with PBS containing 5% skimmed milk for 1 h at room temperature. After washing three times, anti-cat sera diluted in blocking solution (1:320) were added and incubated for 1 h at room temperature. After washing, 100 µL/well of the diluted HRP Conjugated Goat anti-Cat IgG Fc (1:7500 dilutions) in blocking solution was added, and incubated for 1 h at room temperature. The plates were washed three times and then incubated with 0.1150 peroxidase substrate (citric acid 0.1 M, sodium phosphate 0.2 M, 30 volumes H₂O₂ and 0.3 mg/ml of Ortho-phenil-diamina (OPD) (Sigma Aldrich, VA, USA) for 15 minutes at room temperature. After this period, the reaction was stopped with 40 µL of H₂SO₄ (4 M) at 490 nm.

**Gold standard**

The commercial kit SNAP Combo Plus (Idexx Laboratories Inc One Idexx Dr, Westbrook, USA) was used as the gold standard.

**RESULTS**

**Standardization of the r-p24 ELISA method**

The optimal dilutions of antigen and test sera for the r-p24 ELISA were determined. Using the cat antibody-positive sera, the optimal dilution of the test sera was found to be 1:320. The optimal protein concentration of r-p24 as the coating antigen was 0.25 µg/well, and the optimal dilution for the HRP Conjugated Goat anti-Cat IgG Fc was 1:7500 dilutions in blocking solution was added, and incubated for 1 h at room temperature. After washing, 100 µL/well of the diluted HRP Conjugated Goat anti-Cat IgG Fc (1:7500 dilutions) in blocking solution was added, and incubated for 1 h at room temperature. The plates were washed three times and then incubated with 0.1150 peroxidase substrate (citric acid 0.1 M, sodium phosphate 0.2 M, 30 volumes H₂O₂ and 0.3 mg/ml of Ortho-phenil-diamina (OPD) (Sigma Aldrich, VA, USA) for 15 minutes at room temperature. After this period, the reaction was stopped with 40 µL of H₂SO₄ (4 M) at 490 nm.

**Sensitivity and specificity of the r-p24 ELISA**

Evaluating the intrinsic attributes of the test and using the cutoff value determined the r-p24 ELISA showed a sensitivity of 97% and specificity of 93%, when compared to the gold standard (Table 1 and 3). The positive predictive value (PPV) was 79% and the negative predictive value (NPV) was 99%. Both tests had an agreement of 0.83 in the Kappa index.

**Comparison of Western blot with golden standard and r-p24 ELISA**

The sensitivities of the r-p24 ELISA and Western blot were compared for 150 serum samples. The results of the r-p24 ELISA and Western blot were in agreement for 31 of the 150 sera (Table 2). In contrast, two serum samples that were negative by r-p24 ELISA were found to be positive by Western blot. When comparing r-p24 ELISA with the Western blot, a sensitivity of 94% and specificity of 94% was found, with a Kappa index of 0.84; and golden standard with Western blot, a sensitivity of 97% and specificity of 98% was found, with a Kappa index of 0.94 (Table 3).

**DISCUSSION**

Diagnosis methods for FIV are based on the detection of antibodies that recognize viral structural proteins, such as p24, p15 and one of the envelope glycoproteins, gp40 (Rosati et al. 2004, Teixeira et al. 2010). Some authors have described the use of recombinant antigens in highly sensitive and specific immunoenzymatic tests for FIV diagnosis (O’Connor et al. 1989, Avrameas et al. 1993, Calzolari et al. 1995, Rosati et al. 2004, Alves 2010).

### Table 1. Analysis of the cutoff point by ROC seeking a balance between specificity and sensitivity. SNAP Combo Plus x ELISA, 0.528 cut off.

<table>
<thead>
<tr>
<th>SNAP Combo Plus</th>
<th>ELISA</th>
<th>Negative</th>
<th>Positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>110</td>
<td>8</td>
<td>118</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>1</td>
<td>31</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>111</td>
<td>39</td>
<td>150</td>
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</table>

### Table 2. Analysis of the cutoff point by ROC seeking a balance between specificity and sensitivity. Western blot x ELISA, 0.528 cut off.

<table>
<thead>
<tr>
<th>SNAP Combo Plus</th>
<th>Western blot</th>
<th>Negative</th>
<th>Positive</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td>Negative</td>
<td>109</td>
<td>8</td>
<td>117</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>2</td>
<td>31</td>
<td>33</td>
<td></td>
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<tr>
<td>Total</td>
<td>111</td>
<td>39</td>
<td>150</td>
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### Table 3. Comparative analysis of ELISA, Western blot compared to the SNAP Combo Plus.

<table>
<thead>
<tr>
<th>SNAP Combo Plus</th>
<th>Western blot</th>
<th>ELISA</th>
<th>Sens.</th>
<th>Esp.</th>
<th>Kappa</th>
<th>PPV</th>
<th>NPV</th>
</tr>
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<tbody>
<tr>
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<td>118</td>
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<td>93</td>
<td>0.8344</td>
<td>79</td>
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<tr>
<td>Positive</td>
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<td>31</td>
<td>32</td>
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<td>Total</td>
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Sens. = sensitivity; Esp. = specificity; PPV = positive predictive value; NPV = negative predictive value.
In this study, the indirect ELISA using the recombinant r-p24 antigen showed a sensitivity of 97%, specificity of 93%, and Kappa index of 0.83 when compared to the gold standard SNAP Combo Plus. The SNAP Combo Plus is an immunocromatographic commercial kit sensitized with recombinant p24 and gp40 FIV antigens and monoclonal antibodies anti-FeLV p27, with 100% sensitivity and 99.6% specificity (Hartmann et al. 2007). Kashiwase et al. (1997) also demonstrated the high reactivity of recombinant p24 in serologic tests, such as ELISA and Western blot, when standardizing a capture ELISA with monoclonal antibodies. Calzolari et al. (1995), using recombinant antigens of surface (SU), transmembrane (TM) and nucleocapsid (CA), described indirect ELISA for FIV diagnosis. Using recombinant r-p24 (CA) antigen, Calzolari et al. (1995) found a sensitivity of 63% and specificity of 100%, which was different from our test, that showed higher sensitivity for FIV diagnosis. However, our standardized r-p24 ELISA showed a similar sensitivity to the ELISA standardized by Calzolari et al. (1995) with the recombinant TM protein, with sensitivity of 98% and specificity of 97%. It has been demonstrated that retroviral transmembrane proteins have more antigenicity than nucleocapsid proteins (Chong et al. 1991, Fontenot et al. 1992). However, CA is the major viral core protein, highly conserved and glycosilated, which makes it the protein of choice for the ELISA assay (Reid et al. 1991, Mermer et al. 1992, Calzolari et al. 1995). Rosati et al. (2004) demonstrated that the use of both CA and TM recombinant antigens in a single test enhances its sensitivity. Hartmann et al. (2007) compared various commercial kits for FIV diagnosis and showed that tests that use recombinant CA and TM antigens have higher sensitivity and specificity. Alvarenga et al. (1993) detected 100% of the infected animals using SU and TM (P237) synthetic peptides in the ELISA, showing that the use of synthetic antigens is an important diagnosis tool for FIV.

According to Hosie & Jarret (1990), serologic tests can create false positive results due to their low specificity. Therefore, diagnosis must be associated to clinical findings and the animal life style. Positive animals with low infection risk must have the test results confirmed by more specific tests (Teixeira et al. 2010). False negative results can be detected at the terminal stage of the disease, as a result of the immunosuppression (Teixeira et al. 2010). Mortola et al. (2004) affirm that is necessary to use highly sensitive tests to reduce the risk of infection to uninfected animals. However, when there is doubt about a test’s result a more specific test should be used, such as the Western blot (Hosie et al. 2009).

In this study, the Western blot showed a sensitivity of 97% and specificity of 98% when compared to the SNAP Combo Plus. It had a higher specificity than the r-p24 ELISA and the PCR, which showed a sensitivity of 91% and specificity of 96% (data not shown), and these results confirm that the Western blot is the Best confirmatory test for FIV diagnosis. Alvarez et al. (2007) and Thorn et al. (1987) also demonstrated that the Western blot is a good confirmatory test for equine infectious anemia virus (EIAV) and HIV. However, the r-p24 ELISA showed a Kappa index of 0.84 when compared to the Western blot.

The results found in serologic and molecular methods differ greatly (Bienzle et al., 2004, Mortola et al. 2004, Teixeira et al. 2010, Alves et al. 2011). The PCR can have doubtful results due to the great genetic variability of FIV, use of specific primers only for one subtype, low viral load during a long period of infection, and inadequate preparation of PCR components (Pedersen et al. 1989, Bachmann et al. 1997, Bienzle et al. 2004, Alves et al. 2011).

Some countries, like the United States, Japan, Australia, and New Zealand use an inactivated vaccine for subtypes A (Petaluma) and D (Shizuoka) (Uhl et al. 2002, Yamamoto et al. 2007, Ravazzollo & Costa 2007). In the US the vaccine is used since 2002, however the fast mutation rate of lentivirus interferes with the vaccination efficacy (Yamamoto et al. 2007, Teixeira et al. 2010). Vaccination induces a strong humoral response in which the antibodies produced are undistinguishable from antibodies produced by natural infection, making diagnosis difficult (Uhl et al. 2002). Diagnosis methods capable of differentiating vaccination and non vaccination antibodies are currently being studied (Kusuhara et al. 2007, Levy et al. 2008). Some countries do not have FIV vaccines commercially available, which makes diagnosis more reliable.

CONCLUSION

The rp-24 ELISA standardized in this study showed good sensitivity and specificity and is an important tool for research related to FIV and diagnosis at low cost.

Acknowledgment. This study was supported financially by CNPq, FAPEMIG and INCT de Infor-

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