ELISA r-p24: optimization and validation of an enzyme immunoassay for the diagnosis of Feline Immunodeficiency Virus infections

ELISA r-p24: desenvolvimento e validação de um ensaio imunoenzimático para o diagnóstico de infecções causadas pelo Vírus da Imunodeficiência Felina

Marli Sidoni[1]*, Hilton Jorge Nascimento[2], Mariana Miguez[3], José Godinho da Silva Junior[4], Carlos Mazur[5] & Maria das Graças Miranda Danelli[6]

[1]Bióloga, DSc. Programa de Pós-graduação em Ciência, Tecnologia e Inovação – UFRRJ, Instituto de Tecnologia em Imunobiológicos - Bio-Manguinhos, Fundação Oswaldo Cruz – FIOCRUZ, Rio de Janeiro, RJ, Brasil
[2]Químico, DSc. Instituto de Tecnologia em Imunobiológicos - Bio-Manguinhos, Fundação Oswaldo Cruz – FIOCRUZ, Rio de Janeiro, RJ, Brasil
[3]Engenheira-química, DSc. Instituto de Tecnologia em Imunobiológicos – Bio-Manguinhos, Fundação Oswaldo Cruz – FIOCRUZ, Rio de Janeiro, RJ, Brasil
[4]Químico, PhD. Instituto de Tecnologia em Imunobiológicos – Bio-Manguinhos, Fundação Oswaldo Cruz – FIOCRUZ, Rio de Janeiro, RJ, Brasil
[5]Médico veterinário, DSc. Departamento de Microbiologia e Imunologia Veterinária, Instituto de Veterinária, Universidade Federal Rural de Janeiro – UFRRJ, Seropédica, RJ, Brasil
[6]Biomédica, DSc. Departamento de Microbiologia e Imunologia Veterinária, Instituto de Veterinária, Universidade Federal Rural de Janeiro – UFRRJ, Seropédica, RJ, Brasil

Abstract

The Feline Immunodeficiency Virus (FIV) is a lentivirus that affects cats worldwide. The detection of the virus' 24 Kda capsid protein (p24) antibody is the most common way to diagnose FIV infections. In Brazil, the most used commercial assay for FIV diagnosis is a rapid ELISA test based on the lateral flow principle, with high sensitivity and specificity (SNAP® test). However, the high cost of this imported assay undermines not only the diagnosis of this infection but also its epidemiology. The objective of this study was to optimize and validate an indirect ELISA using the protein p24 recombinant antigen (r-p24) with national technology and maintaining sensitivity and specificity comparable to those of available commercial rapid ELISA tests. Twenty-six reference cats' sera samples (13 negative and 13 positive for FIV), previously analyzed by PCR and the SNAP® test, were used as reference to optimize the ELISA. Serum samples of 226 cats from private owners and from shelters of the metropolitan area of Rio de Janeiro were used to validate the assay. The sensitivity and specificity of the r-p24 ELISA were 95.4% and 99.4%, respectively. When compared to the SNAP Combo Test, the accuracy of r-p24 ELISA was 99%, with a *Kappa* index of 0.96. Our results indicate that the national technology-based ELISA r-p24 has sensibility and specificity values that are comparable to rapid ELISA kits. This test is therefore recommendable, using national technology for FIV routine diagnosis, research and epidemiological studies.

Keywords: FIV, ELISA r-p24, felines, optimization, validation.

Resumo

O Vírus da Imunodeficiência Felina (FIV) é um lentivírus que afeta gatos em todo o mundo. A detecção do anticorpo para proteína do capsídeo viral de 24 kDa (p24) é a forma mais comum para diagnosticar infecções pelo FIV. No Brasil, o ensaio comercial mais utilizado no diagnóstico FIV é um teste rápido de ELISA (SNAP® test), baseado no princípio de fluxo lateral, com alta sensibilidade e especificidade. No entanto, o alto custo deste ensaio importado inviabiliza não só o diagnóstico da infecção, como também sua epidemiologia. O objetivo deste trabalho foi aperfeiçoar e validar um teste de ELISA indireto, com tecnologia nacional, utilizando o antígeno recombinante da proteína p24 (r-p24), com sensibilidade e especificidade comparáveis às dos testes rápidos, comercialmente disponíveis. Vinte e seis amostras de soro gatos (13 negativos e 13 positivos para FIV), previamente analisados por PCR e Snap®, foram utilizadas como amostras de referência para aperfeiçoar o ELISA e 226 amostras de soro de gatos, de proprietários privados ou de abrigos da área metropolitana Rio de Janeiro foram utilizadas para validar o ensaio. O ELISA r-p24 apresentou uma sensibilidade de 95,4% e 99,4% de especificidade quando comparadas às do teste

SNAP Combo, uma precisão de 99% e um índice *Kappa* de 0.96. Nossos resultados indicam que o ELISA r-p24 apresentou sensibilidade e especificidade comparáveis às dos testes rápidos, utilizando tecnologia nacional para diagnóstico, pesquisa e estudos epidemiológicos do FIV.

Palavras-chave: FIV, ELISA r-p24, felinos, aperfeiçoamento, validação.

Introduction

The Feline Immunodeficiency Virus (FIV) is one of the main pathogens of domestic cats (*Felis catus*) and worldwide spread (Lutz, 1990). Brazil has about 22 million house cats, according to a survey of the National Health Research (PNS) (Instituto Brasileiro de Geografia e Estatística, 2013). The Brazilian Association of Pet Products (Abinpet) reported that the country has the second largest concentration of domestic cats of the world. The Abinpet estimated an annual growth rate of the Brazilian feline population by 8%. Therefore, it will reach about 40 million animals in 2020 (Associação Brasileira de Produtos para Animais de Estimação, 2014).

Once the clinical signs of FIV infected animals are nonspecific and similar to other diseases, laboratory diagnosis is critical for the identification and subsequent treatment (Hosie et al., 2009; European Advisory Board on Cat Diseases, 2012). The American Association of Feline Practitioners (AAFP) recommends the evaluation of specific antibodies against FIV in all cats to determine their immunological status, regardless of age or health condition (Levy et al., 2003). The European Advisory Board on Cat Diseases (ABCD) proposes the identification and segregation of infected cats as effective measures to minimize the risk of virus transmission to other cats, to prevent opportunistic fatal infections or lymphomas (Hosie et al., 2009).

FIV-infected cats are immunocompromised due to a progressive depletion of CD4+ T cells. After a long asymptomatic period, chronic and progressive clinical features characterize an acute immunodeficiency phase known as Feline Acquired Immunodeficiency Syndrome or FAIDS (Bendinelli et al., 1995). The biological and pathogenic similarities with HIV make FIV infected cats the most important experimental model to study the life cycle, pathogenesis, prevention and treatment of AIDS (Bendinelli et al., 1995; Burkhard & Dean, 2003).

For FIV infections, classical diagnostic techniques (virus isolation or detection of proviral DNA) are problematic, as the viral load of the infected animals is low and the lentivirus displays high genetic variability (Dunham & Graham, 2008; Hosie et al., 2009). Antibodies against FIV proteins persist throughout lifetime and can be used for reliable diagnosis of the infection. The viral capsid p24 protein is the first to be detected by immune recognition of FIV; subsequently antibodies against viral structural proteins Pol and Env can be detected (Reid et al., 1991). Therefore, the detection of the p24 antibody is so far the most common way to diagnose FIV infections.

Available commercial assays are based on different methods and viral antigens for antibody detection. The SNAP® FIV/FeLV Combo (IDEXX Laboratories Inc., Westbrook, Maine, USA) is a rapid ELISA test based on the lateral flow principle that detects antibodies against p24 and p15 (viral matrix protein). The SNAP® Combo Plus detects specific antibodies against p24, p15 and the transmembrane glycoprotein GP40. The SNAP® Combo and SNAP® Combo Plus performances are 94% sensitivity and 100% specificity, and 100% sensitivity and 100% specificity, respectively. SNAP® Combo is available in Australia, New Zealand and North America, whilst SNAP® Combo Plus is available only in Europe (Westman et al., 2015). The Witness® FeLV/FIV (AGEN Biomedical Limited, Brisbane, Australia) detects antibodies against the GP40 protein whilst Antigen Rapid FIV/FeLV (Bionote, Korea) detects antibodies against GP40 and p24 proteins. Both are immunochromatographic lateral flow tests and show a performance of 95% and 99% sensitivity and 89% and 100% specificity, respectively.

In Brazil, the detection of antibodies by rapid enzyme immunoassays is currently the most used in FIV diagnostic, due to its high sensitivity, specificity, and speed. However, because of the high cost of these commercial kits, their application is often impractical particularly when a high number of sera samples should to be tested (cats from shelters, many animals of a single owner, epidemiological studies, etc.). This undermines not only the diagnosis of FIV infections, but also its epidemiology (prevalence and incidence) in Brazil. An expansion of FIV diagnosis methods in Brazil is therefore needed.

To surpass these obstacles, our objective was to develop an indirect enzyme immunoassay with national technology, maintaining sensitivity and specificity comparable to those of the available commercial immunochromatographic tests.

Material and methods

Cats' sera samples

The experiments were performed according to protocol 057/2014 approved by the Ethics Committee on Animal Use of the Universidade Federal Rural do Rio de Janeiro (UFRRJ). Twenty-six reference sera samples (13 negative and 13 positive for FIV), previously analyzed by PCR and the SNAP® test, were provided by the Veterinary Immunology and Virology Laboratory, Veterinary Institute of the UFRRJ and used as references sera samples (negative and positive controls samples). To validate the assay, a total of 226 sera samples were used. The samples were collected from adult animals, random and independent of gender, from non-governmental organizations of the state of Rio de Janeiro from November 2014 to September, 2015.

Production and purification of r-p24 recombinant antigen

The recombinant plasmid containing the coding region for FIV p24 protein was provided by the Veterinary Immunology and Virology Laboratory, Instituto de Veterinária, UFRRJ. The expression and purification of r-p24 protein from bacterial biomass followed the protocol described by Mazur et al. (2010). Briefly, the recombinant plasmid DNA was transformed by electroporation in One Shot® BL21 Star™ cells (Invitrogen) and the DNA/bacteria mixture was spread and incubated overnight at 37°C in solid Luria Broth (LB) with ampicillin at 100mg/mL. After that, one of the obtained recombinant bacteria clones of this culture was inoculated in 0.5 mL of liquid LB with ampicillin at 100mg/mL (LB amp) and incubated at 37°C, under shaking at 200 rpm, overnight. Then, a 100 µL of this 0.5 mL culture was inoculated in a 500 mL of LB amp, incubated at 37°C under shaking at 200 rpm for 2 to 3 hours, when the OD at 600nm of 0.6 to 0.8 was reached. In this moment, 1mM IPTG (Isopropyl-β-D-thiogalactopyranoside) was added and the culture was kept incubating and shaking for more 4 hours, under the same conditions. Subsequently, it was centrifuged at 3220 x G for 15 min, the supernatant was discarded and the pellet was kept at -20°C, until the protein purification step. To purify the recombinant bacterial biomass a HisTrap™ FF column (GE Healthcare) coupled to a high performance liquid chromatography system (HPLC) Äkta Purifier 10 (GE Healthcare) was used. The quantification of r-p24 protein was estimated by the Folin-Lowry method using the Bio-Rad DC Protein Assay™ *kit* (Bio-Rad Laboratories, Inc.), according to the manufacturer´s instructions.

ELISA r-p24 optimization

Aiming to optimize the ELISA r-p24, different concentrations of the r-p24 antigen, positive and negative controlled sera samples, and anti-cat IgG rabbit immunoglobulin conjugated with peroxidase (Sigma® Co, USA) were used. To assess the optimal antigen concentration on the ELISA microplate, the coating with r-p24 protein was analyzed at concentrations of 25, 50, 100 and 200 ng/well. The sera samples were diluted at 1:50 and 1:100 and the conjugate at 1:2000, 1:4000, 1:8000, 1:16.000 and 1: 32.000. The ideal antigen dilution, reference sera samples, and conjugate were determined by cross-titration. The biggest ratio between the optical densities at 450nm (OD_{450}) of the positive and negative reference sera samples was selected to determine the optimal dilutions to be used in the assay. To perform the ELISA r-p24 assays, polystyrene microplates with 96 wells (Nunc-Immuno Plate, MaxiSorp, Denmark) were coated with the recombinant protein, r-p24, (100 ng/100 µL/well) diluted in carbonate/bicarbonate buffer, pH 9.6, (15 mM Na_2CO_3, 35 mM $NaHCO_3$). After 2 hours at 37°C, the plates were washed in 0.05% PBST (4x) and 100µL/well of each sera sample to be tested was added. The sera samples were used at 1:100 in sample buffer [0.05% PBST pH 7.6 (9 mM Na_2HPO_4, 1 mM NaH_2PO_4) at 10% (w/v) skimmed milk powder (Molico®, Nestlé USA) and 0.05% Tween 20]. After 30 minutes incubation at 37°C, the plates were washed four times in 0.05% PBST solution and 100 µL of anti-mouse IgG immunoglobulin peroxidase conjugated rabbit cat diluted at 1:8000 in PBST 0.05% plus 1% BSA (InLab®, BR) was added. After 30 minutes at 37°C, the plates were washed (4x) in 0.05% PBST and 100 µL/well of TMB Plus 2 (Kem-En-Tec Diagnostics, DK) was added and incubated at room temperature for 15 minutes. The reactions were stopped by adding 100 µL/well

of a solution containing 2M H_2SO_4. The absorbance of each reaction was measured in a microplate photometer (RChisto Sunrise, Tecan, Switzerland), using a 450 nm filter. The cutoff value was calculated as the average of the 13 negative reference sera samples plus three times the standard deviation (Jacobson, 1998; Madruga et al., 2001).

Elisa r-p24 validation

The ELISA r-p24 was developed according to the validation and diagnostic certification for infectious animal diseases criteria of the Test Manual for Diagnosis and Vaccines for Terrestrial Animals (World Organisation for Animal Health, 2013). The statistical validation of the ELISA was performed with the panel of 226 cats sera samples analyzed by SNAP® FIV/FeLV Combo test (IDEXX Laboratories, Inc. USA), performed according to the manufacturer's specifications for the presence of antibodies against FIV and FeLV. Pools of negative and positive sera samples were prepared and used as control references in the optimization of the ELISA r-p24.

Statistical analysis

The sensitivity, specificity and *kappa* index (κ) were determined through a (2x2) Contingency Table. The agreement degree of the obtained results with the ELISA r-p24 and the SNAP® Combo test was measured by the ROC curve using the program Sigma Plot version 10.0. The following compliance criteria were used: excellent (> 0.9); good (0.8 to 0.9); regular (0.7 to 0.8); bad (0.6 to 0.7); failed (0.5 to 0.6).

Results

The ELISA optimization experiments pointed that the best difference between the OD_{450} of the positive and negative sera reference samples were obtained with assays using microplates coated with r-p24 antigen at 100 ng/100 μL, sera dilutions at 1:100 in 0.05% PBST and 10% of skimmed milk powder; and rabbit anti-cat IgG conjugate diluted at 1:8000 in 0.05% PBST and 1% BSA (Figure 1).

Reference negative sera samples resulted in an average OD_{450} of 0.144 (standard deviation, sd=0.041), whilst positive samples resulted in an average OD_{450} of 1.385 (sd=0.375). The boundary between positivity and negativity (cutoff) was set at OD_{450} of 0.268 and sera samples with higher values were considered positive for the ELISA r-p24 (Figure 2).

The contingency table (Table 1) shows the results of 226 samples produced by cats' sera analysis in the ELISA r-p24 and SNAP® FIV/FeLV Combo test. Of the 226 samples that were used in the validation, 44 (19.5%) were positive and 182 (80.5%) were negative using the SNAP FIV/FeLV Combo test. The ELISA r-p24 identified 42 (95.5%) positive samples and 181 (98.9%) negative samples. Therefore, only three samples resulted in discordant results. The ELISA r-p24 had a sensitivity of 95.4%, a specificity of 99.4%, an accuracy of 0.99 and a kappa index of 0.96 (Table 2), in comparison to the reference test. The area under the ROC curve (AUC) was 0.99 (99% confidence interval: 0.969-1.019) (Figure 3).
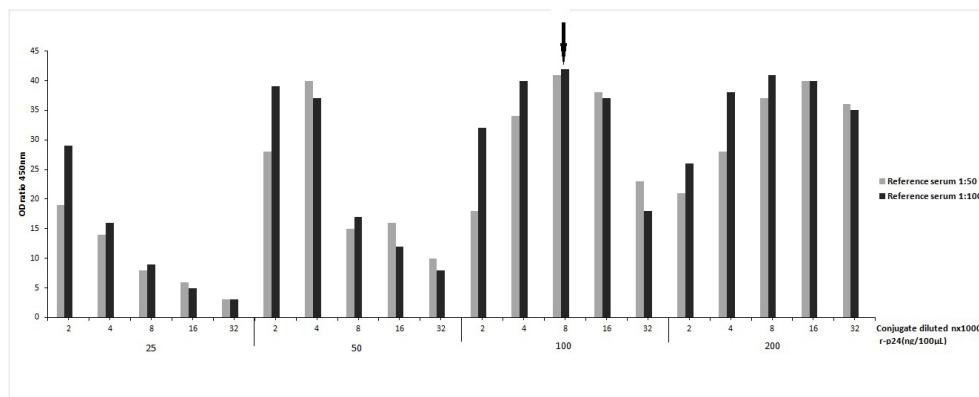
Figure 1. ELISA r-p24 optimization. Relationship of the mean OD_{450} of the positive and negative reference samples at dilutions 1:50 and 1:100; r-p24 antigen at a concentration of 25, 50, 100 and 200ng/100μL; conjugated anti-cat IgG diluted at 1:2000, 1:4000, 1:8000, 1:16000, 1:32000. The arrow indicates the best condition for testing.
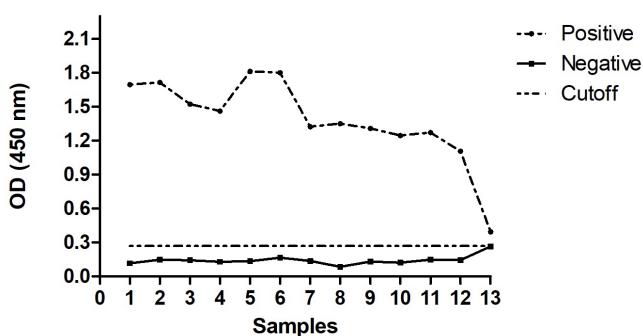
Figure 2. Graphical representation of the OD_{450} obtained in the ELISA r-p24. Cats' sera samples at 1:100 dilution, r-p24 antigen at 100ng/100µL and conjugated anti-cat IgG diluted at 1:8000. *Cutoff*= 0.268.

Table 1. Contingency table for comparing the results of the cats' sera samples tested by SNAP® FIV/FeLV Combo test and by ELISA r-p24.

	SNAP Combo FIV/FeLV		**Total**
Indirect ELISA	**FIV +**	**FIV -**	_
FIV +	42 (TP)	1 (FP)	43 (TP +FP)
FIV -	2 (FN)	181(TN)	183 (FN+ TP)
Total	44 (TP +FN)	182 (FP+TP)	226 (n)

TP(true positive); TN (true negative); FP (false positive); FN (false negative); n= TP+TN+FP+FN. Cutoff= 0.268.

Table 2. ELISA r-p24 performance.

Parameter	Formula	Value (%)
Sensitivity	TP/(TP+FN)	95.5
Specificity	TP/(FP+TN)	99.4
Accuracy	(TP+TN)/n	0.99
kappa index *(κ)*	(OP- EP)/(1-EP)	0.96

TP(true positive); TN (true negative); FP (false positive); FN (false negative); OP (observed proportion) = (TP+TN)/n; EP (expected proportion) = [(TP+FP)(TP+FN)]+[(FN+TN)(FP+TN)]/n².
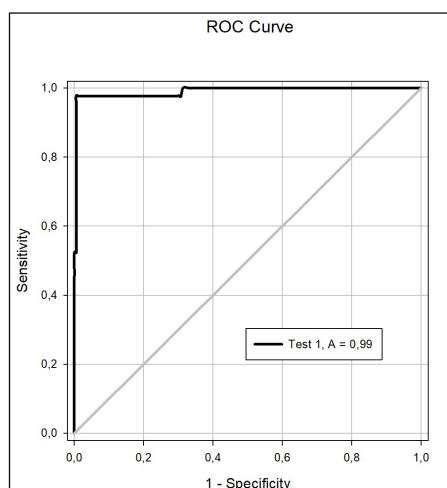
Figure 3. Receiver operator curve (ROC) demonstrating sensitivity and specific of Elisa r-p24.

Discussion

This study describes the successful optimization and validation of an ELISA assay to diagnose Feline Immunodeficiency Virus (FIV) infections in susceptible animals, using a recombinant viral capsid protein, the r-p24, as antigen.

The optimization experiments pointed the adsorption of the recombinant p24 protein of FIV occurs in just 2 hours, dispensing refrigeration during the coating process. Regardless of the used antigen concentration, the most appropriate reference sera samples dilution was 1:100 and this value was adopted for sera samples testing. As this requires only 1μL of sample per well we considerably reduced the 25μL of serum that are generally required in commercial assays. The SNAP tests that were used as reference, needed three drops of blood or serum, approximately 150μL and thus 150 times as much amount of serum than the developed r-p24 ELISA.

The negative and positive reference sera OD_{450} values (0,144 and 1,385, respectively) agreed with values that were suggested by Reid et al. (1991). According to them, the OD_{450} values of the negative control serum should not exceed 0.15 and the OD_{450} of the positive control serum should be close to 1.0.

The accuracy of 99% reflects the high precision of the ELISA r-p24 for the diagnosis of FIV infection, when compared to the SNAP® test, which is considered the gold standard. This result was reproduced when the accuracy was quantified by the area under the ROC curve and the ELISA r-p24 showed a discrimination ability of 99% between truly infected and uninfected cats. The kappa index analysis evaluated the reproducibility by the degree of agreement between the results of the two observations, taking into account the proportion of observed in relation to the expected agreement. The identified kappa index of 0.96 is regarded as almost perfect agreement between both tests (McGinn et al., 2004).

The ELISA r-p24 performance was similar to several other serological tests for FIV diagnosis. Hartmann et al. (2007) compared the quality of seven commercially available serological tests (three immunoenzymatic and four immunochromatographic) for this diagnosis. These assays detect antibodies against different FIV viral proteins (GP40, p24 and/or p15) and showed high sensitivity (92 to 100%) and specificity (99 to 100%). Based on the work Sand & Hartman (2010) , the ELISA r-p24 showed higher sensitivity (95.5%) when compared to commercial immunoassay Antigen Rapid (88.9%) and for the SNAP® FIV/FeLV Combo test. According to Camargo et al. (1987), an immunoassay is satisfactory when sensitivity and specificity are above 90%.

The development and use of laboratory tests improve the diagnosis, epidemiological studies, control and prevention of diseases, and help to understand their pathogenesis. Imported commercial tests are of high cost which limits their use in research and clinical routine disease diagnosis in Brazil. A national test with high sensitivity and specificity would increase usage, both in routine serologic diagnosis and epidemiological studies. The development of this assay attends national policies that stimulate the production of reagents for diagnostics in Veterinary Medicine diseases, reducing the technological dependency of imported products.

Conclusions

The ELISA r-p24 revealed excellent sensitivity, specificity, and accuracy, and further advantageous aspects like speed, simplicity, low consumption of control sera and test samples, and low cost compared to imported kits. The technique was developed to enable routine FIV diagnosis, research and epidemiological studies, with national technology in Brazil and other countries. The new technique represents a promising tool for the diagnosis and control of FIV, an infection that threatens the growing population of cats in the country. This might have serious implications for the economy and well-being of cat owners and the feline associated industry.

Acknowledgements

References

Associação Brasileira de Produtos para Animais de Estimação – ABINPET. (2014). *População de gatos cresce em média 8% ao ano no Brasil e está perto de se igualar a de cães.* Retrieved in 2015, March 15, from http://abinpet.org.br/site.faq

Bendinelli, M., Pistello, M., Lombard, S., Poli, A., Garzelli, C., Matteucci, D., Ceccherini-Nelli, L., Malvaldi, G., & Tozzini, F. (1995). Feline immunodeficiency virus: an interesting model for AIDS studies and an important cat phatogen. *Clinical Microbiology Reviews*, *8*(1), 87-112. PMid:7704896.

Burkhard, M. J., & Dean, G. A. (2003). Transmission and immunopathogenesis of FIV in cats as a model for HIV. *Current HIV Research*, *1*(1), 15-29. http://dx.doi.org/10.2174/1570162033352101. PMid:15043209.

Camargo, I. F., Gaspar, A. M. C., & Yoshida, C. F. T. (1987). Comparative ELISA reagents for detection of hepatitis B surface antigen (HBsAg). *Memorias do Instituto Oswaldo Cruz*, *82*(2), 181-187. http://dx.doi.org/10.1590/S0074-02761987000200005. PMid:3506632.

Dunham, S. P., & Graham, E. (2008). Retroviral infections of small animals. *The Veterinary Clinics of North America. Small Animal Practice*, *38*(4), 879-901, ix. http://dx.doi.org/10.1016/j.cvsm.2008.03.005. PMid:18501285.

European Advisory Board on Cat Diseases – ABCD. (2012). *Guidelines: feline immunodeficiency.* Retrieved in 2014, November 14, from http://www.abcd-vets.org/Pages/Home.aspx

Hartmann, K., Griessmayr, P., Schulz, B., Greene, C. E., Vidyashankar, A. N., Jarrett, O., & Egberink, H. F. (2007). Quality of different inclinic test systems for feline immunodeficiency virus and feline leukemia virus infection. *Journal of Feline Medicine and Surgery*, *9*(6), 439-445. http://dx.doi.org/10.1016/j.jfms.2007.04.003. PMid:17604205.

Hosie, M. J., Addie, D., Belák, S., Boucraut-Baralon, C., Egberink, H., Frymus, T., Gruffydd-Jones, T., Hartmann, K., Lloret, A., Lutz, H., Marsilio, F., Pennisi, M. G., Radford, A. D., Thiry, E., Truyen, U., & Horzinek, M. C. (2009). Feline immunodeficiency. ABCD guidelines on prevention and management. *Journal of Feline Medicine and Surgery*, *11*(7), 575-584. http://dx.doi.org/10.1016/j.jfms.2009.05.006. PMid:19481037.

Instituto Brasileiro de Geografia e Estatística – IBGE. (2013). *Pesquisa Nacional de Saúde 2013*. Rio de Janeiro: IBGE. Retrieved in 2015, January 3, from http://biblioteca.ibge. gov.br/ visualizacao/ livros/liv94074.pdf

Jacobson, R. H. (1998). Validation of serological assays for diagnosis of infectious diseases. *Revue Scientifique et Technique*, *17*(2), 469-526. http://dx.doi.org/10.20506/rst.17.2.1119. PMid:9713892.

Levy, J., Richards, J., Edwards, D., Elston, T., Hartmann, K., Rodan, I., Thayer, V., Tompkins, M., & Wolf, A. (2003). Report of the American Association of Feline Practitioners and Academy of Feline Medicine Advisory Panel on feline retrovirus testing and management. *Journal of Feline Medicine and Surgery*, *5*(1), 3-10. http://dx.doi.org/10.1053/jfms.2002.0202. PMid:12613492.

Lutz, H. (1990). Feline retroviruses: a brief review. *Veterinary Microbiology*, *23*(1-4), 131-146. http://dx.doi.org/10.1016/0378-1135(90)90143-J. PMid:2169669.

Madruga, C. R., Araujo, F. R., & Soares, C. O. (Eds.). (2001). *Imunodiagnóstico em medicina veterinária* (360 p.). Campo Grande: Embrapa Gado de Corte.

Mazur, C., Reis, J. K. P., Leite, R. C., Danelli, M. G. M., Hagiwara, M. K., Góes, A. C. M. A., & Medeiros, M. A. (2010). Evaluation of a recombinant p24 antigen for the detection of feline immunodeficiency virus-specific antibodies. *Pesquisa Veterinária Brasileira*, *30*(10), 877-880. http://dx.doi.org/10.1590/S0100-736X2010001000011.

McGinn, T., Wyer, P. C., Newman, T. B., Keitz, S., Leipzig, R., & For, G. G. (2004). Tips for learners of evidence-based medicine: 3. Measures of observer variability (kappa statistic). *Canadian Medical Association Journal*, *171*(11), 1369-1373. http://dx.doi.org/10.1503/cmaj.1031981. PMid:15557592.

Reid, G., Rigby, M. A., McDonald, M., Hosie, M. J., Neil, J. C., & Jarrett, O. (1991). *Immunodiagnosis of feline immunodeficiency vírus infection using recombinant viral p17 and p24*. AIDS, *5*(12), 1477-1483. http://dx.doi.org/10.1097/00002030-199112000-00010. PMid:1667575.

Sand, C., Englert, T., Egberink, H., Lutz, H., & Hartmann, K. (2010). Evaluation of a new in-clinic test system to detect feline immunodeficiency virus and feline leukemia virus infection. *Veterinary Clinical Pathology*, *39*(2), 210-214. http://dx.doi.org/10.1111/j.1939-165X.2009.00196.x. PMid:19930142.

Westman, M. E., Malik, R., Hall, E., Sheehy, P. A., & Norris, J. M. (2015). Determining the feline immunodeficiency virus (FIV) status of FIV-vaccinated cats using point-of-care antibody kits. *Comparative Immunology, Microbiology and Infectious Diseases*, *42*, 43-52. http://dx.doi.org/10.1016/j.cimid.2015.07.004. PMid:26459979.

World Organisation for Animal Health – OIE. (2013). Principles and methods of validation of diagnostic assays for infectious diseases. In World Organisation for Animal Health – OIE. *OIE Manual of diagnostic tests and vaccines for terrestrial animals*. Retrieved in 2014, January 3, from http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.05_VALIDATION.pdf