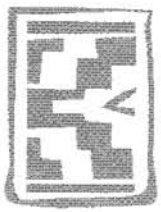

Evaluation of an indirect enzyme-linked immunosorbent assay for the detection of feline lentivirus-reactive antibodies in wild felids, employing a puma lentivirus-derived synthetic peptide antigen

M. VAN VUUREN[1], E. STYLIANIDES (nèe DE KLERK)[1], S.A. KANIA[2], E.E. ZUCKERMAN[3] and W.D. HARDY Jr[3]

ABSTRACT

VAN VUUREN, M., STYLIANIDES (nèe DE KLERK), E., KANIA, S.A., ZUCKERMAN, E.E. & HARDY, W.D. Jr. 2003. Evaluation of an indirect enzyme-linked immunosorbent assay for the detection of feline lentivirus-reactive antibodies in wild felids, employing a puma lentivirus-derived synthetic peptide antigen. *Onderstepoort Journal of Veterinary Research*, 70:1–6

An enzyme-linked immunosorbent assay (ELISA) using a puma lentivirus-derived synthetic peptide as coating antigen was evaluated as a diagnostic test for infection with feline immunodeficiency virus (FIV) or related lentiviruses in free-ranging lions. The sensitivity and specificity of the ELISA was determined using two approaches. In the first approach, the results were standardized according to certain statistical criteria, and in the second, the puma lentivirus western blot was used as the gold standard. The sensitivity of the test when compared with the standardized results was 85.4 % and the specificity 100 %. The sensitivity of the test when using the western blot as the gold standard was 78.6 % and the specificity 100 %. The test would therefore be well-suited to the screening of populations of wild felids in which FIV or related lentiviruses are endemic. The results also indicate that in spite of genetic divergence between lentiviruses isolated from *Panthera* and *Felis* spp., puma lentivirus-derived antigens can be used in immunoassays for the detection of antibodies in *Panthera* spp. reactive to FIV or related lentiviruses. The results also indicate that the lion population in the Hluhluwe-Umfolozi Game Reserve, South Africa is lentivirus negative.

Keywords: ELISA, feline immunodeficiency virus, lions, puma lentivirus, western blot

INTRODUCTION

Feline immunodeficiency virus (FIV) is a member of the genus *Lentivirus* of the family *Retroviridae*. It is distributed worldwide in domestic and feral cats. Molecular analysis has shown some of the lentiviruses from wild felids to be genetically different from FIV.

Lentivirus infections in free-ranging felids in southern Africa have received considerable attention during the last decade (Spencer 1991; Spencer, Van Dijk, Horzinek, Egberink, Bengis, Keet, Morikawa & Bishop 1992; Spencer 1993; Osofsky, Hirsch, Zuckerman & Hardy 1996; Van Vuuren, Stylianides & Durand 1997). A sizeable proportion of the lion populations in the Kruger National Park (Spencer 1991; Spencer *et al.* 1992; Van Vuuren *et al.* 1997)

[1] Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110, South Africa.

[2] Department of Comparative Medicine, College of Veterinary Medicine, University of Tennessee, PO Box 1071, Knoxville, TN 37901-1071, USA.

[3] National Veterinary Laboratory, 1 Tice Road, Franklin Lakes, NJ 07417, USA

Accepted for publication 7 October 2002—Editor

and Botswana (Osofsky *et al.* 1996; Van Vuuren *et al.* 1997) was reported to be infected with FIV or a related lentivirus, whereas lions in Namibia's Etosha National Park appear to be lentivirus negative (Spencer *et al.* 1992; Spencer 1993). Two recent small surveys confirmed the presence of FIV-related antibodies in leopards in South Africa and Botswana (Osofsky *et al.* 1996; Van Vuuren *et al.* 1997).

Laboratory tests used for the detection of antibodies against FIV in domestic cats may not have the same sensitivity and specificity for retroviruses of wild felids (Spencer *et al.* 1992; Osofsky *et al.* 1996). Similarly, different assays for the detection of antibodies against FIV or related viruses applied to free-ranging felids sometimes yield significantly different prevalence figures (Osofsky *et al.* 1996).

The discordant results that can be obtained with different tests used on the same population have been pointed out by Osofsky *et al.* (1996). They described the results obtained with five separate methods for the detection of lentivirus antibodies in free-ranging felids and found the western blot (WB) using a puma lentivirus (PLV) cell lysate as capture antigen to be the most sensitive. Where the latter method yielded 12/53 (22.6%) positive results, only 8/53 (15%) were positive with a commercial, whole virus, domestic cat FIV ELISA, 4/53 (7.5%) positive with a domestic cat FIV cell lysate WB, 3/53 (5.7%) positive with a PLV capture antigen in an indirect fluorescent antibody (IFA) test and 1/53 (1.9 %) positive with an IFA test employing a domestic cat FIV capture antigen.

Spencer *et al.* (1992) similarly found 38/74 (51 %) of their study population of lions positive when using a commercial, whole virus, domestic cat FIV ELISA, whereas 81/98 (83 %) of the same population tested positive with an FIV recombinant p24 group-specific antigen ELISA. These results can be ascribed to the sequence divergence between lentiviruses infecting domestic cats and wild felids (Kania, Kennedy & Potgieter 1997). Because of these antigenic differences, Osofsky *et al.* (1996) emphasized the fact that the choice of assay can significantly influence the results.

This paper compares the results obtained by testing 84 lion sera from four different geographic regions with an indirect ELISA employing a PLV-derived synthetic peptide antigen and two WB assays employing cell lysates from a PLV and a domestic cat FIV, respectively.

MATERIALS AND METHODS

Serum specimens

Serum specimens (n = 84) obtained during routine immobilization and investigation of free-ranging lions were provided by researchers from the Kruger National Park (n = 22) and Hluhluwe-Umfolozi Game Reserve (n = 18) in South Africa, the Department of Veterinary Services, Zimbabwe (n = 22) and the Botswana Department of Wildlife and National Parks (n = 22).

Reagents

The PLV envelope peptide used as coating antigen in the ELISA has previously been described (Kania *et al.* 1997). It corresponds to a conserved motif common to envelope glycoproteins present on HIV-1, HIV-2 and FIV and is represented in FIV by the p237 peptide.

The source of viral antigens, used on the nitrocellulose strips for the WBs, has been described by Osofsky *et al.* (1996). It essentially consists of feline lymphosarcoma cell line 3201B (VandeWoude, O'Brien, Langlier, Hardy, Slattery, Zuckerman & Hoover 1997) infected with FIV and PLV to a high multiplicity of infection and subjected to cell lysis in a lysing buffer (W.D. Hardy Jr, E.E. Zuckerman & R. Cooper, personal communication 2001).

Indirect ELISA

The test was perfomed as described by Kania *et al.* (1997). Briefly, flat-bottomed microtitre plates (Immulon 2, Dynatec) were coated with 100 mℓ PLV peptide antigen. The antigen was diluted in phosphate buffered saline (PBS) to an optimal concentration of 10 mg/mℓ The plates were held at 4 °C overnight and washed three times with PBS containing 0.05 % Tween 20 (PBS-T) (ICN Biochemicals). The test sera were diluted 1:25 in PBS-T, added to the antigen-coated wells and incubated at 37 °C for 1 h. All sera were tested in duplicate and a positive and negative control was included on each plate. Following incubation, the plates were washed as described and goat anti-cat peroxidase-conjugated IgG (Kirkegaard and Perry Laboratories) diluted 1:8000 was added. This was followed by another incubation period of 37 °C for 1 h and a final washing step. The substrate {2,2'-azinobis (3-ethylbenz-thiazoline-6-sulfonic acid)} (ABTS) was added and the plates placed on a rocker at room temperature for 20 min. The optical densities (OD) were determined at a wavelength of 405 nm.

Sera that gave an OD of double that of the negative control were recorded as positive.

Feline immunodeficiency virus and PLV WB assays

The tests were performed as previously described (Osofsky *et al.* 1996). In brief the test sera were diluted 1:50 in the test buffer (PBS containing 5 % non-fat milk powder and 5 % normal goat serum) and added to the antigen-containing nitrocellulose strips. After an incubation period of 18 h on a rocker at room temperature, the strips were washed twice with PBS-T. The biotinylated anti-cat IgG (Vector Laboratories) was diluted 1:1000 in the test buffer, added to the strips and incubated for 45 min. Two washing steps in PBS-T followed. Horseradish peroxidase Avidin D (Vector Laboratories) was diluted 1:1000 and added to the strips and again incubated at room temperature on a rocker for 45 min. After a final wash the strips were developed in the developing solution (4-chloro-1-napthol) for 7 min. The reaction was stopped by rinsing the strips several times in water. The presence of a p24 band and at least one other viral band was taken as positive, while the presence of a single band or bands not corresponding to viral proteins was recorded as inconclusive.

Calculation of results

Sensitivity, specificity and predictive values were calculated with standard formulae (Galen & Gambino 1975). Two approaches were used to obtain the numbers required to determine these values. In the first the results were standardized according to the criteria described in Table 1 (De Klerk, Anderson & Geffen 1983; De Klerk & Anderson 1985).

If the standardized result was inconclusive it was excluded from the analysis. The number of true positive and the number of false negative remaining results were used to calculate the sensitivity using the following formula:

$$\% \text{ sensitivity} = [\text{true positives}/(\text{true positives} + \text{false negatives})] \times 100$$

The specificity was calculated from the number of true negative and the number of false positive results using the following formula:

$$\% \text{ specificity} = [\text{true negatives}/(\text{true negatives} + \text{false positives})] \times 100$$

The positive and negative predictive values were calculated using the following formulae:

$$\text{Positive predictive value} = [\text{No. of positive tests in infected animals/total no. of positive tests}] \times 100$$

$$\text{Negative predictive value} = [\text{No. of negative tests in uninfected animals/total no. of negative tests}] \times 100$$

In the second approach the PLV ELISA was measured against the PLV WB that represented the gold standard. The sensitivity, specificity, positive and negative predictive values were calculated using the same formulae as described for the standardized results.

RESULTS

The comparative test results for the three procedures, namely FIV WB, PLV WB and PLV ELISA are summarized in Table 2.

Individual animals with discrepant test results and showing concordance in any two tests are listed in Table 3.

Data in Table 2 were analyzed to assess the sensitivity, specificity, positive predictive value and negative predictive value of the PLV ELISA. The results are presented in Table 4.

TABLE 1 Criteria used to standardize results obtained with three serological tests

Combination of results	Standardized result
All three tests positive	Positive
Any two tests positive and one inconclusive	Positive
Any two tests positive and one negative	Positive
One test positive and two negative	Negative
One test inconclusive and two negative	Negative
Two tests inconclusive and one negative or positive	Inconclusive
One test positive, one test negative and one test inconclusive	Inconclusive

TABLE 2 Comparative test results for antibodies against a PLV and a domestic cat FIV in the sera of 84 free-ranging lions

Location	No. of sera	FIV WB[a]			PLV WB[b]			PLV ELISA[c]	
		Pos[d]	Neg	Inc	Pos	Neg	Inc	Pos	Neg
Kruger Park	22	16	2	4	15	4	3	18	4
Botswana	22	19	2	1	20	2	0	14	8
Zimbabwe	22	3	15	4	7	10	5	5	17
Hluhluwe	18	0	18	0	0	18	0	0	18
Total	84	38	37	9	42	34	8	37	47

[a] FIV WB = Western blot using a domestic cat cell lysate as capture antigen
[b] PLV WB = Western blot using a puma lentivirus cell lysate as capture antigen
[c] PLV ELISA = Indirect enzyme-linked immunosorbent assay with a puma lentivirus peptide as the coating antigen
[d] Pos = Positive
Neg = Negative
Inc = Inconclusive

TABLE 3 Discrepant results between the FIV WB, PLV WB and PLV ELISA. Lions with inconclusive results are indicated in bold and were excluded from the calculations to determine the operating characteristics of the different tests

Location	No. of sera	Lion ID	FIV WB[a]			PLV WB[b]			PLV Elisa[c]	
			Pos[d]	Neg	Inc	Pos	Neg	Inc	Pos	Neg
Kruger Park	6	Lion 2			x		x			x
		Lion 5			x		x			x
		Lion 9			**x**			**x**	**x**	
		Lion 10			x		x			x
		Lion 13	x					x	x	
		Lion 19		**x**				**x**	**x**	
Botswana	7	Lion 26	x			x				x
		Lion 27	x			x				x
		Lion 32			x	x			x	
		Lion 36	x			x				x
		Lion 37	x			x				x
		Lion 41	x			x				x
		Lion 42	x			x				x
Zimbabwe	10	Lion 46		x		x				x
		Lion 48			**x**			**x**		**x**
		Lion 49		x				x		x
		Lion 50			**x**			**x**		**x**
		Lion 51			x	x			x	
		Lion 53		x		x				x
		Lion 54		x		x			x	
		Lion 57		x		x				x
		Lion 59			**x**			**x**		**x**
		Lion 66	x					x	x	

[a] FIV WB = Western blot using a domestic cat cell lysate as capture antigen
[b] PLV WB = Western blot using a puma lentivirus cell lysate as capture antigen
[c] PLV ELISA = Indirect enzyme-linked immunosorbent assay with a puma lentivirus peptide as the coating antigen
[d] Pos = Positive
Neg = Negative
Inc = Inconclusive

TABLE 4 Sensitivity, specificity and positive and negative predictive values of the PLV ELISA[a] by comparison with the standardized results, and with the PLV WB[b] results as the gold standard

Test	Sensitivity	PPV[c]	Specificity	NPV[d]
Standardized results	85.4 %	94.6 %	100.0 %	80.6 %
PLV WB results	78.6 %	100.0 %	100.0 %	79.1 %

[a] PLV ELISA = Indirect enzyme-linked immunosorbent assay with a puma lentivirus peptide as the coating antigen
[b] PLV WB = Western blot using a puma lentivirus cell lysate as capture antigen
[c] PPV = Positive predictive value
[d] NPV = Negative predictive value

DISCUSSION

The recognized gold standard for all lentivirus serological tests is the WB for each virus in the genus. In contrast to other serological tests such as the ELISA and IFA that allow detection of the full spectrum of antibodies, the WB allows visualization of the antigen specificity of antibodies. However, as with any serological assay, false negative and positive results can be obtained with the WB. Consequently an alternative approach to determine the operating characteristics of the PLV ELISA by standardizing the results was included (De Klerk *et al.* 1984; De Klerk & Anderson 1985). According to the criteria described in Table 1, five specimens were excluded from the calculations. The results obtained from the remaining 79 specimens were used to calculate the sensitivity, specificity and predictive values.

The PLV WB produced eight inconclusive results. Four of these were positive and four negative with the PLV ELISA. Of those that were positive with the PLV ELISA, two were positive, one inconclusive and one negative with the FIV WB. The two positive results are likely to be true positives on the basis of the high positive predictive value (PPV) of the PLV ELISA. The negative FIV WB result was not unexpected as it has been reported that the domestic cat FIV WB may not detect all feral cat lentiviruses (Osofsky *et al.* 1996). Of those that were negative with the PLV ELISA, three were inconclusive and one negative with the FIV WB.

Among the Botswana lions, several animals that were positive in the PLV WB were negative in the PLV ELISA. The positive animals did show reactivity against envelope proteins in the WB. It may be concluded that antigenic differences in the envelope proteins may be responsible for the discordant results. This is also supported by the fact that sera from three lions that were inconclusive in the WB (on the basis of no reactivity to p24) but positive in the ELISA did show reactions against envelope proteins in the PLV WB.

The sensitivity of the PLV peptide ELISA obtained in this lion study, albeit lower than the WB, confirms that a PLV-derived antigen as the capture antigen in an ELISA can be used for the detection of antibody in *Panthera* spp. even though the lentiviruses infecting pumas and lions are divergent. A likely explanation is that some regions of the envelope protein are conserved between the lentiviruses infecting wild felids. When comparing the sensitivities of the PLV-based and FIV-based WBs the perception is confirmed that wild felids should preferably be tested with immunoassays employing "feral" virus-derived antigens. More so, the ideal serological test would employ a lion lentivirus as the capture antigen to test lions.

This study has shown the PLV peptide ELISA to be a highly specific test for the detection of antibody to lentivirus infections in lions. No false positive results were detected. The PLV ELISA would therefore be a good test to screen populations in which the virus is endemic. When screening populations with a high incidence of infection, negative specimens can be re-tested with the PLV WB.

When using the standardized results for the PLV ELISA, the PPV indicated that 95 % of the positive results could be regarded as true rather than false positives. Similarly the negative predictive value (NPV) of 80.6 % for the PLV ELISA indicated that 81 % of the negative results could be considered as true rather than false negatives.

Antibodies reactive to FIV have been detected in many non-domestic felids including the leopard, lion, jaguar, tiger, panther, bobcat and pallas cat (Egberink & Horzinek 1992; Kania *et al.* 1997). Genetic analysis has shown that the lentiviruses

infecting non-domestic felids are distinct from FIV. It is therefore likely that reactivity of antibodies to lentiviruses in non-domestic felids would be stronger if antigens derived from wild felids are used as target antigens in serological assays. This observation has been confirmed by Osofsky *et al.* (1996). Although there is sequence divergence between lion and puma lentiviruses (Kania *et al.* 1997), the relatedness of these non-domestic felid lentiviruses is probably closer than to FIV.

The results also indicate that the lion population in the Hluhluwe/Umfolozi Game Reserve more than likely represents another lentivirus negative population in southern Africa.

Acknowledgements

The authors would like to thank Drs. Dewald Keet, Kathy Alexander, Dave Cooper and Norman Mukarati from the Kruger National Park, Botswana Department of Wildlife and National Parks, KwaZulu-Natal Parks Board and Department of Veterinary Services, Zimbabwe, respectively, for providing the serum specimens.

REFERENCES

DE KLERK, E., ANDERSEN, R. & GEFFEN, C. 1983. The enzyme-linked immunosorbent assay (ELISA) in the laboratory diagnosis of gonorrhoea. *South African Medical Journal*, 64:451–454.

DE KLERK, E. & ANDERSEN, R. 1985. Comparative evaluation of the enzyme linked immunosorbent assay in the laboratory diagnosis of brucellosis. *Journal of Clinical Microbiology*, 21:381–386.

EGBERINK, H. & HORZINEK, M.C. 1992. Animal immunodeficiency viruses. *Veterinary Microbiology*, 33:311–331.

GALEN, R.S. & GAMBINO, S.R. 1975. *Beyond normality; the predictive value and efficiency of medical diagnoses.* New York:John Wiley & Sons Inc.

KANIA, S.A., KENNEDY, M.A. & POTGIETER, L.N.D. 1997. Serologic reactivity using conserved envelope epitopes in feline lentivirus-infected felids. *Journal of Veterinary Diagnostic Investigation*, 9:125–129.

OSOFSKY, S.A., HIRSCH, K. J., ZUCKERMAN, E.E. & HARDY, W.D. 1996. Feline lentivirus and feline oncovirus status of free-ranging lions (*Panthera leo*), leopards (*Panthera pardus*), and cheetahs (*Acinonyx jubatus*) in Botswana: A regional perspective. *Journal of Zoo and Wildlife Medicine*, 27:453–467.

SPENCER, J.A. 1991. Survey of antibodies to feline viruses in free-ranging lions. *South African Journal of Wildlife Research*, 21:59–61.

SPENCER, J.A. 1993. Serological survey of sera from lions in Etosha National Park. *South African Journal of Wildlife Research*, 23:60–61.

SPENCER, J.A., VAN DIJK, A.A., HORZINEK, M.C., EGBERINK, H.F., BENGIS R.G., KEET, D.F., MORIKAWA, S. & BISHOP, D.H.L. 1992. Incidence of feline immunodeficiency virus reactive antibodies in free-ranging lions of the Kruger National Park and the Etosha National Park in southern Africa detected by recombinant FIV p24 antigen. *Onderstepoort Journal of Veterinary Research*, 59:315–322.

VANDEWOUDE, S.O., O' BRIEN, S.J., LANGLIER, K., HARDY, W.D., SLATTERY, J.P., ZUCKERMAN, E.E. & HOOVER, E.A. 1997. Growth of lion and puma lentiviruses in domestic cat cells and comparisons with FIV. *Virology*, 233:185–192.

VAN VUUREN, M., STYLIANIDES, E. & DURAND, A. 1997. The prevalence of viral infections in lions and leopards in southern Africa. *Proceedings of a symposium on lions and leopards as game ranch animals, Onderstepoort, South Africa, 1997*: 168–173.