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A preliminary investigation of exposure to rabies virus in selected wildlife in the Kruger National Park, South Africa

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Rabies is a zoonotic disease caused by members of the genus *Lyssavirus* and causes fatal encephalitis in warm-blooded vertebrates. Rabies has been previously confirmed in domestic dog populations in close proximity to the Kruger National Park (KNP) and can potentially threaten conservation efforts. Domestic dogs infected with rabies virus occasionally enter the KNP and may be a source of rabies exposure to wildlife. Therefore, the aim of this study was to determine if wild carnivores in the KNP have been exposed to rabies virus, based on the presence of antibodies. Serum samples from the African wild dog (*Lycaon pictus*), spotted hyena (*Crocuta crocuta*), lion (*Panthera leo*), leopard (*Panthera pardus*) and banded mongoose (*Mungos mungo*) were tested for the presence of rabies-specific antibodies using the BioPro enzyme-linked immunoassay kit (BioPro ELISA kit). Selected sera were tested in parallel with the fluorescent antibody virus neutralisation test (FAVNT). Of the 168 carnivore serum samples screened, eight (4.8%) had a percentage blocking (PB) ≥ 40, indicating the presence of rabies-binding antibodies and confirmed with the FAVNT to be very low levels of rabies virus neutralising antibodies (range 0.00 IU/mL–0.22 IU/mL). Rabies-binding antibodies detected by the BioPro ELISA kit and rabies virus neutralising antibodies shown by the FAVNT should however be interpreted with caution because of the lack of validation and species-specific cut-off values for wild carnivores.

Conservation implications: The results of this study will assist in understanding the epidemiology of rabies in the KNP carnivores, especially exposure risk. The use of rabies diagnostic tools developed for domestic animals for disease surveillance in the KNP carnivores was also evaluated and the outcomes will further support research on rabies in free-ranging wildlife populations.

Keywords: rabies; BioPro ELISA; antibody detection; conservation; virology.

Introduction

Rabies is a disease caused by members of the genus *Lyssavirus* in the family *Rhabdoviridae*. The disease is fatal to humans and other warm-blooded vertebrates, following infection, once clinical signs are apparent. Late in infection rabies virus is found in the saliva and is usually transmitted by the bite of an infected animal (Swanepoel 2004). In South Africa, two genetically distinct and epizootiological groups of rabies virus are found, namely the canid rabies biotype and mongoose rabies biotype (Mansvelt 1962; Nel et al. 1997).

The first outbreak of rabies in South Africa occurred at Port Elizabeth (in the Eastern Cape Province) in 1893 involving domestic dogs (*Canis familiaris*), domestic cats (*Felis catus*) and a few cattle (*Bos taurus*) (Swanepoel et al. 1993). In 1928, rabies was confirmed in two human cases who had incidentally been bitten by a yellow mongoose (*Cynictis penicillata*) (Herzenberg 1928). In 1950, rabies was identified in black-backed jackals (*Canis mesomelas*) and cattle (*Bos Taurus*), the disease spread from the northern border district of the Limpopo province, and by 1952, was confirmed in Mozambique, Swaziland and Mpumalanga province (Swanepoel et al. 1993). In 2008, an outbreak in dogs occurred in the Mpumalanga province and subsequently spread to areas surrounding the Kruger National Park (KNP) (Mkhize et al. 2010).

Densely populated towns and villages around the KNP are associated with a high number of domestic dogs that enter and interact with wildlife within the boundaries of the KNP (Grover 2015). Cases of domestic dogs with rabies have been reported entering the KNP (L. De Klerk-Lorist [State Veterinarian Skukuza, DAFF] pers. comm., 2014). All of these positive cases were confirmed

with the fluorescent antibody test (FAT) on brain tissue samples (Agriculture Council-Onderstepoort Veterinary Research [ARC-OVR] reference: 2014-D-8316; 2014-D12860; 2014-D-17045; 2014-D-8312; 2014-D-15228). In 2013, an African wild dog (*Lycaon pictus*) (AWD) from a pack that was previously seen interacting with a domestic dog (*Canis familiaris*) was tested for exposure to rabies using a fluorescent antibody virus neutralisation test (FAVNT). This animal had a rabies virus neutralising antibody titre of 0.5 international units per millilitre (IU/mL), which is considered to be adequate seroconversion against immunisation with a rabies vaccine. As AWDs have not yet been routinely vaccinated against rabies virus in the KNP, this result suggested exposure of the carnivore to a *Lyssavirus*.

The extent and nature of rabies exposure in the KNP wildlife is currently unknown, and the most recent research conducted was in 1995 and 2012. Van Heerden et al. (1995) investigated the health and disease status of AWD; 31 serum samples were tested with a blocking enzyme-linked immunoassay (ELISA) method and no rabies-binding antibodies were detected. Prager et al. (2012) tested another 26 AWD using a rapid fluorescent focus inhibition test (RFFIT), and no rabies neutralising antibodies were detected.

Many densely populated towns and villages exist adjacent to the borders of the KNP, which host numerous owned and free-roaming domestic dogs (Conan et al. 2015). In 2008, a rabies outbreak occurred in the Nkomazi local municipality (southern boundary of the KNP), spreading westwards to the City of Mbombela and northwards to Bushbuckridge (western boundary of the KNP) and other areas surrounding the KNP (Mkhize et al. 2010). From 2006 until 2016, a total of 586 domestic dog positive rabies cases were laboratory-confirmed, for both Limpopo- and Mpumalanga-province municipality bordering KNP (DAFF n.d.). Between 2009 and 2014, Grover (2015) investigated rabies occurrence in both wildlife and domestic animals in private game reserves bordering the KNP and the Bushbuckridge municipality and found that 42.4% of animals sampled tested positive for rabies (344 animals of 28 different species were sampled, of which 236 were domestic dogs). As the KNP is surrounded by a high population of domestic dogs with known rabies status, research investigating the exposure to the KNP wildlife is important.

Exposure can be detected by the presence of serum antibodies in unvaccinated animals. Several serological methods are available; one such method detects rabies-binding antibodies, such as the ELISA test, and another method detects rabies neutralising antibodies, such as the FAVNT. Test performance is expressed by sensitivity and specificity values, using a recognised reference or gold standard method (Altman & Bland 1994). The FAVNT is the gold standard method for detection of rabies neutralising antibodies, with high levels of sensitivity and specificity (Cliquet, Aubert & Sagné 1998). Although the FAVNT is considered the gold standard, it requires specialised reagents, equipment and containment facilities to handle virus; therefore, laboratories prefer an ELISA platform for serological testing. The BioPro enzyme-linked immunoassay kit (BioPro ELISA kit) is a blocking ELISA method that has been used to detect rabies antibodies in domestic dogs and cats (Wasniewski & Cliquet 2012) and wild carnivores, including red foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procyonoides*) (Wasniewski et al. 2013).

Therefore, the aim of this preliminary study was to detect potential exposure of certain wildlife carnivore species to rabies virus in the KNP using serological test methods.

Research methods and design

Sample collection

A total of 168 serum samples were obtained from the Veterinary Wildlife Services (VWS) biobank in the KNP. Samples were selected on the basis of species, age and sample collection date. Preference was given to lion, spotted hyena, leopard, AWD and banded mongoose samples. Sera from the older individuals within each species were chosen with the assumption that the opportunity for rabies exposure would increase with age. Samples with collection dates after 2009 were used because a rabies outbreak in domestic dogs occurred in 2009 in the surrounding areas of the KNP (Mkhize et al. 2010). Geographical location of where the animals were sampled was taken into consideration in order to concentrate on animals sampled closer to villages and towns bordering the KNP. However, because of the limited samples available in the biobank, this cannot be considered a preference. As a positive control, seven serum samples from previously vaccinated AWD, lion and leopard were received from the biobank of the National Zoological Gardens of South Africa (NZG) (Pretoria).

Enzyme-linked immunoassay

The BioPro ELISA kit is a blocking ELISA test for detection of rabies glycoprotein G antibodies in fox sera (Mojžiš, Korytár Jerg 2008). Serum samples, stored at –80 °C, were thawed and heat inactivated for 30 min at 56 °C to reduce possible background that may interfere with product formation thereby ensuring more consistent results (Namekar et al. 2012). A 96-well microplate layout template was designed to include positive, negative and test kit controls according to manufacturer's instruction. Test sera were diluted 1:1 as prescribed in the manufacturer manual using the diluent provided.

Test sera (100 μL/well) were added to the microtitre plates pre-coated with rabies antigen, the plates were incubated at 2 °C to 8 °C with gentle shaking overnight (18 h to 24 h). Positive and negative controls and 100 μL of control sera provided in the kit were added to designated wells. Plates were washed six times using the washing solution provided (1:10 dilution), afterwards 100 μL biotinylated anti-rabies antibody diluted 1:100 was added to each well and plates were incubated for 30 min at 37 °C with gentle shaking. Plates were washed four times with diluted washing solution and

100 µL Streptavidin peroxidase conjugate, diluted 1:100, was added to each well and incubated for 30 min at 37 °C with gentle shaking. Tetramethylbenzidine (TMB) substrate (100 µL/well) was added to the test plates, and the plates were placed in a box, to avoid direct light and incubated for 15 min to 30 min at room temperature with gentle shaking. After incubation, 50 µL of the supplied stop solution was added to each well and the optical density (OD) was measured at a wavelength of 450 nm using an ELISA microplate reader (Original Multiskan EX; Labsystems Inc.).

The ELISA results were calculated by using the OD measurement obtained from the ELISA microplate reader and presented as a percentage of blocking (PB) value. According to the manufacturer's instructions, samples with a PB < 40 are considered negative for rabies-binding antibodies and samples with PB ≥ 40 are considered to have rabies-specific antibodies present. Samples with a PB ≥ 70 are considered positive with an equivalent antibody level of ≥ 0.5 IU/mL (O.K. Servis Biopro S.R.O 2014).

Fluorescent antibody virus neutralisation test

Rabies virus neutralising antibody titres were determined at the OIE Rabies Reference Laboratory (ARC-OVR) using the FAVNT as described previously (Cliquet et al. 1998). The serum samples for FAVNT were selected based on the results obtained from the BioPro ELISA kit. As the samples used in this study were primarily from unvaccinated animals, very low levels of antibodies, if any were expected to be present and therefore any samples with a PB of ≥ 35 were tested using the FAVNT. Serum samples were thawed and heat-inactivated for 30 min at 56 °C and thereafter kept at 4 °C to 6 °C. All the works were carried out with sterile techniques in a biosafety cabinet (BSC, Baker, United States of America), and appropriate personal protective equipment was worn at all times.

The FAVNT was conducted in 96-well microtitre tissue culture plates, using challenge rabies virus (CVS), ATCC® VR-959™CVS-11 strain (Anses, France) and Baby Hamster Kidney cells. The CVS stock was diluted to a working concentration of 100$TCID_{50}$/50 µL. Each test included an OIE positive serum sample prepared in dogs and a negative control reference serum (both procured from Anses, France). The positive control serum is an OIE positive reference serum diluted to 0.5 IU/mL, the negative control serum is a pool of naïve laboratory dog sera (OIE Reference Laboratory for Rabies, Nancy, France). Dulbecco's modified Eagle's medium (DMEM)/nutrient mixture F12 supplemented with 10% heat-inactivated foetal bovine serum (FBS) and antibiotic antimycotic solution (10 000 units penicillin, 10 µg amphotericin B per mL [Sigma-Aldrich catalogue number A5955, United States of America) was used for diluting the positive and negative controls, CVS and test sera. One hundred microlitres of reconstituted DMEM/F12 was added to all the wells of the control plate and all the wells for the test sera. Fifty microlitres of each test serum sample, as well as both positive and negative control sera were added into four consecutive wells, and then serially diluted from 1:3 to 1:432. Following a virus back-titration (in the control plate), 50 µL of the diluted CVS was added to each well (with the exception of the cell control), after which the plates were incubated at 37 °C in an incubator with 5% CO_2 for 1 h. After incubation, 50 µL of BHK cell suspension (with approximately 4×10^5 cells/mL) were added to each well and the plates were incubated for 48 h at 37 °C in 5% CO_2. At the end of the incubation, the spent medium from the plates was discarded into a virucidal solution (F10, Health and Hygiene, South Africa), and the plates were lightly inverted on absorbent towel to drain off residual media. The plates were then fixed by adding 200 µL of 80% cold acetone to each well, and left to stand for 10 min. The used acetone was discarded into a virucidal solution and the plates were left to dry (maximum 5 min). After the plates had dried sufficiently, 50 µL of fluorescein isothiocyanate (FITC) anti-rabies monoclonal globulin (light diagnostics TM Rabies DFA Reagent 5100, EMD Millipore Corp., United States of America), diluted at 1:100, was added to each well. The plates were incubated for 45 min in a humidified environment at 37 °C. The contents of the plates were discarded and the plates were rinsed twice with phosphate buffer saline (PBS) pH 7.2. Excess PBS was removed by briefly inverting the plates on absorbent paper and the total area of each well was observed for the presence of fluorescence using a fluorescent microscope. If no fluorescence was observed, the well was considered to be negative, and if one or more fluorescing particles were seen, the well was considered to be positive for fluorescence. The logD_{50} titres of the CVS titration, the naïve reference serum, the positive reference serum and the test sera were calculated according to the Spearman–Kärber (1931).

Data analysis and statistical methods

Data from carnivores tested for rabies antibodies were handled in Microsoft Excel 2007 using a spreadsheet, recording the following variables: VWS laboratory number, date of sampling, species, microchip number (if applicable), age class (old adult [OAd], adult, young adult [Yad] and juvenile [Juv]), sex (male [M] and female [F]), place of capture (according to the KNP designated sections), GPS (global positioning system) of sampling point, ELISA plate, OD, ELISA PB result, FAVNT titration dilution result, FAVNT result in IU/mL and distance to closest community. Distance between sampling points and nearest towns and villages were determined through gDistance function of rgeos package (Bivand & Rundel 2017), using community point reference from Chief Surveyor General, 1:50 000 topographic layers (including farm parcel boundaries), 2008.

A distribution map was compiled using ArcGIS Desktop (Esri 2017) using the GPS position of sampling with the distinguishing between different species and PB results of the ELISA, where PB ≥ 40 is positive. Wilcoxon rank-sum test (one-sided) was run to compare vaccinated and unvaccinated PB results. Statistical analyses were carried out with R Core Team software (R Core Team 2017). Data were analysed by

logistic regressing using a generalised linear model (GLM) to determine an association between animals with a positive rabies-binding antibody result (PB ≥ 40) for any variables that might affect positive serology. The following variables were assessed: species, age, year, month, place of capture and distance to closest community (formula = status ~ distance to closest community + species + age + sex + year + month + place). A *p*-value < 0.05 was considered indicative of a statistically significant association.

Ethical consideration

Ethical approval to conduct the study was obtained from the Tshwane University of Technology, TUT Senate Committee for Research Ethics (reference no. AREC2016/03/001).

Results

All carnivores with a rabies vaccination history, the positive control group, showed an ELISA PB ≥ 70 and a FAVNT result ≥ 0.5 IU/mL (Table 1). Results of the ELISA with a PB ≥ 70 are considered positive with an equivalent antibody level ≥ 0.5 IU/mL (O.K. Servis Biopro S.R.O 2014). According to the OIE and WHO, a titre of 0.5 IU/mL is the minimum neutralising antibody titre required for a level of immunity, which allows the host to protect itself against rabies infection. The same measurement is used to confirm a satisfactory response to vaccination (OIE 2013).

Percentage blocking less than 40 is considered negative for rabies-binding antibodies and samples with PB ≥ 40 are considered to have rabies-specific antibodies present. Samples with a PB ≥ 70 are considered positive with an equivalent antibody level of ≥ 0.5 IU/mL (O.K. Servis Biopro S.R.O 2014). A titre of 0.5 IU/mL is the minimum neutralising antibody titre required for a level of immunity that allows the host to protect itself against rabies infection. The same measurement is used to confirm a satisfactory response to vaccination (OIE 2013).

Of the 168 carnivore serum samples tested, eight (4.8%) had results with PB ≥ 40. None of the samples showed a PB value of ≥ 70 or the equivalent FAVNT result of ≥ 0.5 IU/mL (Table 2).

A total of 12 AWD serum samples were tested and one (8.3%) showed PB values ≥ 40. The one sample with a PB of 42.3 had a neutralising antibody titre of 0.00 IU/mL. Two AWD samples with PB < 40 but ≥ 35 had neutralising antibody titre levels of 0.17 IU/mL and 0.22 IU/mL. None of the 10 banded mongoose samples showed PB values ≥ 40.A total of 25 leopard serum samples were tested and one (4%) showed a PB value ≥ 40, with a neutralising antibody titre of 0.02 IU/mL. Three leopard samples with PB < 40 but ≥ 35 had neutralising antibody titre levels of 0.01 IU/mL, 0.06 IU/mL and 0.07 IU/mL. A total of 84 lion serum samples were tested and four (4.8%) showed PB values ≥ 40; these four samples had neutralising antibody titres ranging from 0.00 IU/mL 0.13 IU/mL. The two lion samples with PB < 40 but ≥ 35 both had a neutralising antibody titre of 0.17 IU/mL. A total of 37 spotted hyena serum samples were tested and two (5.4%) showed PB values ≥ 40, with neutralising antibody titre levels of 0.00 IU/mL and 0.06 IU/mL. Three spotted hyena samples with PB < 40 but ≥ 35 had neutralising antibody titre levels of 0.02 IU/mL, 0.01 UI/mL and 0.06 IU/mL.

Of the 70 carnivores from Skukuza tested, only four showed a PB ≥ 40. A total of nine carnivores from Tshokwane, 10 from Crocodile Bridge, 12 from Stolsnek and 15 from Satara were tested and one carnivore in each section showed PB ≥ 40 (Figure 1).

The Wilcoxon rank-sum test (one-sided) was run to compare vaccinated and unvaccinated PB results and showed a clear distinction between vaccinated and unvaccinated carnivores ($p < 0.005$). Interpreting a result above 40 PB as positive, a logistical regression model was built to search for any variables that might affect positive serology. Variables included species, age, year, month, place of capture and distance to closest community. None of the variables evaluated had a significant impact on the outcome as evaluated by a *p*-value below 0.05.

Discussion

In this study, a panel of wild carnivore serum samples was used to determine the presence of rabies-specific antibodies using a rabies ELISA test to assess exposure of wildlife in KNP to lyssaviruses. Rabies-binding antibodies were detected in samples of AWDs, leopard, lions and spotted hyenas using a PB cut-off of ≥ 40. However, the eight positive carnivore serum samples on the ELISA test showed little correlation to the FAVNT used to detect rabies virus neutralising antibodies.

TABLE 1: Enzyme-linked immunoassay and fluorescent antibody virus neutralisation test results of carnivores with a rabies vaccination history.

Date sampled	Species	Age class	ELISA PB	FAVNT (IU/mL)	Vaccination history		
					First vaccine	Second vaccine	Third vaccine
2015-04-09	AWD	Adult	101.1	0.87	2003/06/05	-	-
2014-08-12	AWD	Adult	87.8	0.87	2003/06/05	2006/09/29	2014/02/20
2015-05-26	Lion	Adult	95.9	1.15	2014/04/23	-	-
2015-05-26	Lion	Adult	95.8	1.15	2014/12/05	-	-
2015-09-02	Lion	-	98.6	7.94	2014/12/05	-	-
2015-07-29	Lion	-	103.3	54.96	2014/12/05	-	-
2007-06-13	Leopard	Adult	70.6	0.87	2001/05/31	2003/09/10	-

Note: PB 40 is considered negative for rabies binding antibodies and samples with PB ≥ 40 are considered to have rabies-specific antibodies present. Samples with a PB ≥ 70 are considered positive with an equivalent antibody level of ≥ 0.5 IU/ml (O.K. Servis Biopro S.R.O 2014). A titre of 0.5 IU/ml is the minimum neutralising antibody titre required for a level of immunity which allows the host to protect itself against rabies infection. The same measurement is used to confirm a satisfactory response to vaccination (OIE 2013).

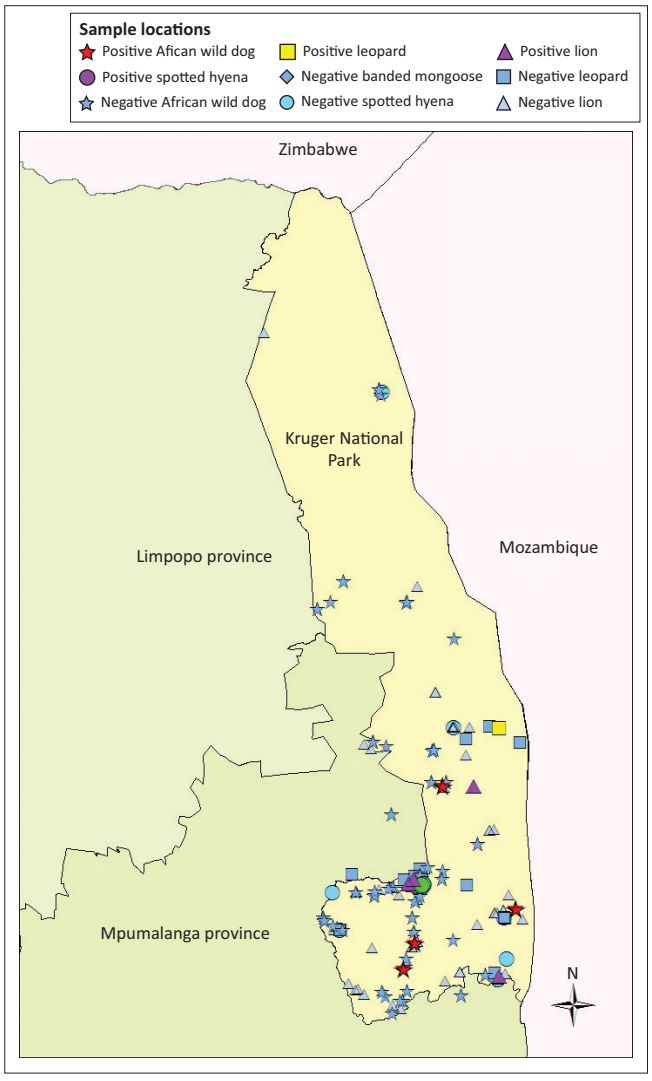
ELISA PB, enzyme-linked immunoassay percentage blocking; FAVNT, fluorescent antibody virus neutralisation test; IU/mL, international units per milliliter; AWD, African wild dog (*Lycaon pictus*).

TABLE 2: Results of carnivores with BioPro enzyme-linked immunoassay kit PB greater than or equal to 35.

Date of sampling	Species	Microchip	Age class	Place of capture	Latitude (S)	Longitude (E)	ELISA PB (%)	FAVNT IU/mL
2013-02-19	AWD	4C3D677E63	Adult	Stolsnek	-25.216609	31.564169	42.3	0.00
2015-05-12	AWD	7110352843	Adult	Pretoriuskop	-25.02338	31.33254	38.0	0.17
2015-12-17	AWD	-	Adult	Tshokwane	-24.6015	31.68886	37.0	0.22
2009-01-29	Leopard	-	Old adult	N'wanetsi	-24.452385	31.977681	36.9	0.06
2012-08-28	Leopard	4C3D34684D	Adult	Satara	-24.39525	31.8956	42.8	0.02
2012-10-12	Leopard	-	Adult	N'wanetsi	-24.452391	31.977704	39.7	0.01
2016-03-27	Leopard	-	Old adult	Pretoriuskop	-25.168919	31.268776	35.9	0.07
2010-02-15	Lion	4A593A5474	Adult	Skukuza	-24.97455	31.55965	59.1	0.13
2010-03-04	Lion	4A194A3B06	Adult	Crocodile bridge	-25.34588	31.8942	44.1	0.02
2010-06-18	Lion	-	Adult	Pretoriuskop	-25.015213	31.421688	37.0	0.17
2012-03-06	Lion	-	Adult	Skukuza	-24.991722	31.539668	40.8	0.01
2014-09-16	Lion	4C3C0F4D4C	Old adult	Satara	-24.391735	31.71723	36.5	0.17
2014-10-13	Lion	-	Adult	Tshokwane	-24.619123	31.794867	44.7	0.00
2008-09-23	Spotted hyena	-	Adult	Crocodile bridge	-25.27679	31.925391	39.6	0.02
2008-12-05	Spotted hyena	-	Adult	Skukuza	-24.9926	31.59997	35.5	0.01
2009-09-23	Spotted hyena	-	Adult	Pretoriuskop	-25.168919	31.268776	35.4	0.06
2012-07-01	Spotted hyena	4C39173F7F	Juvenile	Skukuza	-24.996444	31.591873	49.6	0.00
2013-09-10	Spotted hyena	7110250901	Adult	Skukuza	-24.992068	31.598761	49.3	0.06

Note: PB 40 is considered negative for rabies binding antibodies and samples with PB ≥ 40 are considered to have rabies-specific antibodies present. Samples with a PB ≥ 70 are considered positive with an equivalent antibody level of ≥ 0.5 IU/ml (O.K. Servis Biopro S.R.O 2014). A titre of 0.5 IU/ml is the minimum neutralising antibody titre required for a level of immunity which allows the host to protect itself against rabies infection. The same measurement is used to confirm a satisfactory response to vaccination (OIE 2013).

AWD, African wild dog (*Lycaon pictus*); GPS, global positioning system; S, South; E, East; ELISA PB, enzyme-linked immunoassay percentage blocking; FAVNT, fluorescent antibody virus neutralisation test; IU/mL, international units per milliliter.



Source: ArcGIS Desktop version 10.5.1, 2017, computer software, Esri, Redlands, CA.

FIGURE 1: Distribution of ELISA results from sera of free-ranging wild carnivores in Kruger National Park.

Sera from vaccinated carnivores were subjected to both the BioPro ELISA kit and the FAVNT analyses. The results indicated that AWD, lion and leopard develop high levels of rabies-binding antibodies and high titres of rabies virus neutralising antibodies.

The BioPro ELISA kit was developed and validated as a blocking ELISA test for detection of rabies glycoprotein G antibodies in fox sera and has not been validated for the African carnivore species tested in this study. The BioPro ELISA kit test results suggest 4.8% of the carnivores tested in this study had been exposed to a lyssavirus. One can assume that the exposure to carnivores to lyssavirus would be from rabies-positive domestic dogs from villages and towns bordering the KNP. However, there was no association found between a carnivore with rabies-binding antibodies and that carnivore's distance to the closest village or town. A carnivore closer to the community will have a higher possibility to make contact with rabies-positive domestic dogs; therefore, this could suggest that the antibodies found in the study are false positive. However, the GPS location where the carnivore was immobilised for sampling might not be in close proximity to where the animal was possibly exposed to rabies. For example, lion home ranges vary between 50 km^2 and 107 km^2, AWD between 150 km^2 and 1110 km^2, leopard between 14.8 km^2 and 76.2 km^2, spotted hyena between 25 km^2 and 130 km^2 and banded mongoose between 0.8 km^2 and 1.0 km^2 (Mills & Bester 2005). Although findings of naturally acquired antibodies in wildlife, clinically unaffected by rabies, have also been reported in Tanzania (East et al. 2001), Bolivia (Deem, Davis & Pacheco 2004), Brazil (Araujo et al. 2014; Jorge et al. 2010), Namibia (Thalwitzer et al. 2010) and Zambia (Berentsen et al. 2013). Neutralising antibody detection tests were used in these studies rather than ELISA, which

detect rabies-binding antibodies and therefore results are difficult to compare.

The disassociation between the ELISA and FAVNT results could also suggest an occurrence of false positive. On initial comparison, the ELISA may appear to be less sensitive and specific than the FAVNT (Cliquet et al. 1998). However, the FAVNT detects neutralising antibodies and the ELISA detects rabies-binding antibodies (Barton & Campbell 1988; Moore et al. 2016; Vengušt et al. 2011). As the two methods detect different humoral responses, cut-off values should be independently determined and results considered separately. The ELISA method detects all antibodies against rabies viral antigens coated on the ELISA plate, which may include non-neutralising antibodies (Vengušt et al. 2011). The ELISA measures the ability of an antibody to bind to the rabies glycoprotein and parts of the antigen (epitopes) may prompt the production of non-neutralising antibodies (Cliquet et al. 2003). The FAVNT detects biologically active serum neutralising antibodies, which may be a subset of all the binding antibodies.

Studies evaluating test performance of the BioPro ELISA kit, using FAVNT as the gold standard observed differing sensitivities, specificities, level of agreement and cut-off values. The BioPro ELISA kit has been used to detect rabies antibodies in unvaccinated domestic dogs and cats, calculating an assay specificity of 100% using 315 sera samples (Wasniewski & Cliquet 2012). A total of 701 sera samples from vaccinated dogs and cats were tested and a sensitivity of 84.7%, specificity of 97.6% and 86.2% level of agreement with FAVNT were calculated. Based on the Wasniewski and Cliquet (2012) study, a true negative result was designated as any sample with < 0.5 IU/mL on FAVNT and 70 PB on the BioPro ELISA kit. Wasniewski et al. (2013) determined the specificity of the BioPro ELISA kit in comparison to the FAVNT, using serum from 91 unvaccinated red foxes (*Vulpes vulpes*) and 117 unvaccinated raccoon dogs (*Nyctereutes procyonoides*). A specificity of 100% was calculated for the BioPro ELISA kit, using cut-off values of 40 PB and 0.24 IU/mL for the FAVNT. Using similar cut-off values, 408 red fox sera and 274 raccoon dog sera collected from areas where oral vaccination campaigns had been conducted were tested to calculate a level of agreement between the BioPro ELISA kit and FAVNT. An overall level of agreement of 95.0% was calculated, 95.1% for red fox sera and 94.9% for sera from raccoon dogs. Bedeković et al. (2016) used the BioPro ELISA kit for testing thoracic liquid and muscle extraction of orally vaccinated foxes to identify rabies antibodies in these samples. A total of 147 samples were tested, either as heat-inactivated or not-treated samples, using cut-off values of 0.1 IU/mL for the FAVNT and 40 PB for the BioPro ELISA kit. The BioPro ELISA kit had a calculated sensitivity of 93.36%, specificity of 79.1% and diagnostic viability of 94.3% using heat-inactivated sera. When non-treated sera were tested, the BioPro ELISA kit had a sensitivity of 79.2%, specificity of 92.98% and diagnostic viability of 89.16%. Cut-off values as low as 0.1 IU/mL (Jorge et al. 2010) and 0.11 IU/mL (Araujo et al. 2014) have been used for neutralising antibody tests when screening for rabies exposure in wildlife. If a cut-off value of 0.2 IU/mL (Berentsen et al. 2013) is used, the FAVNT results of this study would also suggest that there is no evidence of rabies virus neutralising antibodies in the selected carnivores from the KNP. A single case of an AWD with a FAVNT titre of 0.22 IU/mL and PB < 40 (interpreted as negative on ELISA) was observed in this study. A possible explanation could be non-specific factors such as cytotoxicity or other virus neutralising activities of the sample that resulted in overestimation of rabies virus neutralising antibodies when using the FAVNT (Barton & Campbell 1988; Cliquet et al. 2003).

The 4.8% samples considered positive for the presence of rabies-binding antibodies by the BioPro ELISA kit are all low PB values in comparison to the high PB values obtained from the rabies vaccinated carnivores. Whether or not this is true, evidence of rabies exposure will require further investigation.

Firstly, limitations of this study are the number of samples tested that represents only a very small percentage of the total population size. Secondly, neither of the two serological tests has been validated for use in wildlife carnivores as was done here. Another possible limitation of the study is the locality where the samples were taken, as these are not all in close proximity to the boundary of KNP. Therefore, in order to make future studies more meaningful, it is crucial that an extensive population-based validation of both the BioPro ELISA kit and the FAVNT be performed in order to obtain species-specific cut-off values. Future research should include proactive surveillance by collecting brain samples from carnivore carcasses in order to get a more accurate picture of the impact rabies have on the KNP wildlife. Research to improve the non-lethal diagnostic tools we do have need to focus on finding a negative control population with zero rabies exposure, including no vaccination history and no contact with domestic animals or the possibility of contact with other rabies infected wildlife.

Conclusion

Serological testing of samples opportunistically collected from the AWD, leopard, lion, spotted hyena and banded mongoose in KNP showed the presence of rabies-binding antibodies in 4.8% of the samples, with very low values of rabies virus neutralising antibodies. Rabies-binding antibodies detected by the BioPro ELISA kit should be interpreted with caution and would require further investigation. An extensive population-based validation for both the BioPro ELISA kit and the FAVNT should be performed in order to obtain species-specific cut-off values. It is recommended that continuation of surveillance be carried out to evaluate exposure to rabies virus because infection pressure may change, especially as towns and villages continue to grow along the borders of KNP. For now, the surveillance would have to rely on using the validated diagnostic tools that are available (e.g. FATs on tissue samples) and for future surveillance to develop the validation of serological methods, such as the ones used in this study.

Successful domestic dog rabies vaccination campaigns for towns and villages bordering KNP should enhance protection for wildlife from rabies. Although sample numbers were limited, the preliminary results suggest that rabies vaccination of AWD, lion and leopard can induce high levels of protective antibodies, which can be detected using currently available assays. This is valuable information that requires further research as it may provide additional management options for future control of this disease in wild carnivores.

In conclusion, this preliminary study showed that carnivores in the KNP have potentially been exposed to rabies but a validation of the tests used and continued surveillance is recommended.

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Competing interests

The authors declare that they have no financial or personal relationships that may have inappropriately influenced them in writing this article.

Authors' contributions

The publication draws substantially from the dissertation of L.R. C.B. and J.K. acted as supervisors to the dissertation. C.S. critically revised the manuscript for important intellectual content. All authors approved the final version to be published.

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Data availability

Data sharing is not applicable to this article as no new data were created or analysed in this study.

Disclaimer

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