Further progress in understanding Anthrax in the Etosha National Park

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ABSTRACT

Culture and serological results obtained during a 5-week field study on anthrax in the Etosha National Park are presented. With one exception – a water sample from a natural fountain – *Bacillus anthracis* was only isolated from animal specimens or environmental samples associated with animals known to have or suspected of having died of anthrax and no environmental "reservoir" for the bacterium could be identified. In laboratory tests, vegetative forms of *B. anthracis* inoculated into water samples declined rapidly in number and spore forms showed no inclination to germinate. Water holes would not appear to be sites of germination and multiplication of *B. anthracis* therefore. A preliminary serological survey indicated that naturally acquired anthrax specific antibody is rare in herbivores but common in carnivores in the Park with titres in lions reflecting the degree of anthrax activity in their ranges.

INTRODUCTION

The updated figures on confirmed and suspected anthrax mortalities in the Etosha National Park, South West Africa/Namibia, since the last report (Turnbull *et al.* 1986b) are shown in Table 1; their geographical distribution is shown in Figure 1. These represent just the few anthrax carcasses seen by patrolling staff and tourists out of the many that are not seen before predators and scavengers have removed all trace and it would appear that the disease is the major uncontrolled cause of mortality among certain herbivores in the Park.

TABLE 1: Mortalities in the Etosha National Park due to anthrax, 1984–87

Year	C/S*	Zebra	Wilde-beest	Spring-bok	Elephant
1984	C	87	48	0	4
	S	12	4	6	4
1985	C	11	4	1	6
	S	13	14	4	13
1986	C	31	6	3	1
	S	7	2	2	7
1987	C	8	4	3	15
	S	6	0	0	3
% of all mortalities 1984/87**		52	53	7	41

* C = confirmed by M'Fadyean stain and, on occasion, culture;
S = suspected

** Observed mortalities

Note: 2 rhino also died of anthrax during this period
1982 Game Count: Zebra 8 800; Wildebeest 2 200; Springbok 30 000; Elephant 2 400; Rhino 300

As reviewed in the previous report (Turnbull *et al.* 1986b), ecological considerations had led to the belief that gravel pits resulting from excavations of gravel for tourist roads were related in a major way to the incidence of anthrax in the Park (Ebedes 1976; Berry 1981), but subsequently no microbiological evidence for any continuing relationship of this kind could be found (Turnbull *et al.* 1986b); not a single isolation of *Bacillus anthracis* was made from 45 sets of pooled water and soil samples collected from 12 of the gravel pits, 16 natural fountains and 3 natural pans on a field study in 1983 during the anticipated annual peak incidence of anthrax. Measurements of pH and total aerobic bacterial counts revealed no marked differences between waters from artificial and natural water holes.

The problems of detecting low numbers of *B. anthracis* in environmental samples, especially those such as Etosha samples which carry a high background flora of the closely related *B. cereus*, were recognised in that study. This was compounded by the abnormally low rainfall in 1983 and the consequent reduced peak incidence of anthrax at that time. With that in mind together with confusion arising from the conflicting reports of 46% – 85% *B. anthracis* isolation rates from natural gravel pit waters in 1969 and 1971 (Ebedes 1976), re-examination was deemed necessary. In addition, the fate of *B. anthracis* inoculated into samples of these waters clearly needed to be studied.

Progress has been made in development of antibody and antigen detection systems since that time and it is now possible to examine blood and other animal specimens for the information these can reveal on the epidemiology and pathogenesis of anthrax and acquired resistance to it thereby aiding better understanding of the disease in the Park.

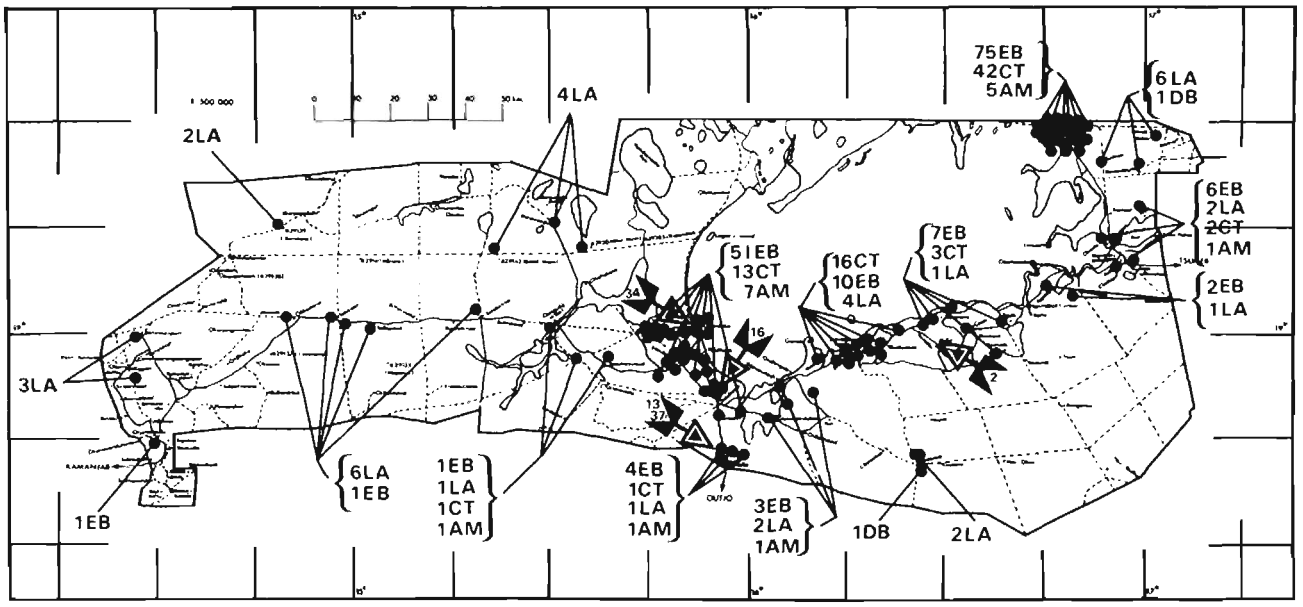

FIGURE 1: Sites of confirmed and suspected cases of anthrax in the Etosha National Park, 1984 – March 1987. AM = Springbok; CT = Wildebeest (Blue); DB = Rhino (Black); EB = Zebra (Burchell's); LA = Elephant.

A second field study was therefore organised and carried out during March 1987 with the aims of (a) the re-examination of environmental samples for the presence and persistence of *B. anthracis* and (b) the application of antibody and antigen detection systems. The results of work centred around that field study are reported here.

METHODS

Collection of environmental specimens

A total of 81 environmental samples unconnected with any known anthrax infection were examined. As summarised in Table 2, these comprised 12 waters, 29 muds and soils and two sets of 20 soil samples collected along two transects.

Water samples were collected from the perimeters of the relevant water holes in oven-sterilized 1 litre jars. Immediately following collection, the jars were placed in a portable refrigerator carried in the Park vehicle. They remained at refrigerated temperature until analysed.

Soil and mud samples were taken from several points around and across dried-out or partially dried-out water holes using sterile tongue blades or, where the caked mud was too hard, a metal spatula. Approximately 50 g of soil or mud were collected into a single sterile Whirlpak bag (Nasco – available from Astell Scientific, London SE6, UK) from any one dry water hole. In the case of transect samples, 10–20 g were collected in separate Whirlpak bags from each collection point along the transect.

Isolation media

The principal media for isolation attempts were heart infusion agar (Difco), nutrient agar (Oxoid), 5% sheep blood agar and the polymyxin-lyosozyme-EDTA-thallous acetate (PLET) agar of Knisely (1966) formulated as described by Carman *et al.* (1985) but with a lysozyme content of 300 IU.

Isolation procedure for environmental samples

Aliquots of water samples were heated at 60°C for 30 min for the dual purpose of killing background vegetative flora and heat shocking any anthrax spores present. For each aliquot, 0,25 ml was then transferred to each of 4 PLET agar plates and spread over the surface, allowed to dry and the plates incubated at 35°C for 48 h. The soils and muds (10–20 g samples) were suspended with firm agitation in 2 ml/g of sterile distilled water and the heavy debris allowed to settle. The supernatant was then subject to the same procedure as the water samples.

The PLET plates were examined at 24 and 48 h for suspect anthrax colonies. These were sub-cultured onto heart infusion or blood agar. It was sometimes helpful at this point to look for gamma-phage and penicillin sensitivity and, if blood agar was used, for haemolysis also. Alternatively, these could be checked on subsequent plating. The phage suspension was applied with a 10 μl loop spread in a ± 5 mm circle in a region of the culture where confluent growth was expected and a penicillin disc placed at some distance from the phage on another region of anticipated confluent growth.

Conclusive confirmation of the identity of an isolate as *B. anthracis* lay in demonstration of the capsule. For this, a small amount of the growth – about the size of a pinhead – was transferred with a loop from a

TABLE 2: Environmental samples examined for *B. anthracis*

Sample	Site (no. of samples examined)	Isolation of *B. anthracis*
WATERS	Adamax	
	pan	
	gravel pit	
	Aus	
	Chudop	
	Kalkheuwel	
	east	
	west	
	Kapupuhedi	
	Leeubron-Okondeka	
	Okondeka	
	source	
	middle pool	
	terminal pool	+*
	Ondongab	
MUDS	Adamax	
	pan (2)	
	gravel pit (2)	
	Chudop (3)	
	Kalkheuwel	
	east (3)	
	west (3)	
	Leeubron (2)	
	Okondeka [midway] (1)	
	White Elephant pan (3)	
SOILS	Adamax	
	pan (1)	
	gravel pit (1)	
	Grunewald gravel pit (5)	
	White Elephant pan (3)	
TRANSECT 1	Eastward from road south of Okondeka just north of the junction to Leeubron. 20 samples at 40 pace intervals	
TRANSECT 2	Eastward from Adamax gravel pit. 20 samples at 40 pace intervals.	

* all other samples were negative

colony to a tube containing 2 ml of heparinised horse blood. (Theoretically blood from any species should do, but horse seems to give the best results). After incubation in a water bath for 5–8 h at 37°C, a 10 μl loopful of the blood was spread on a microscope slide and heat fixed. The smear was covered with polychrome methylene blue (M'Fadyean's stain – BDH Chemicals Ltd, Poole, Dorset, UK) for 30 s, washed (into hypochlorite solution), dried and examined under an oil immersion lens for the presence of capsulated bacilli.

Isolation procedure for animal specimens (Table 3)

Blood taken from the jugular vein of a plains zebra *(Equus burchelli* – EBI 87:03:21 RJ) a few minutes after death was plated directly onto heart infusion agar. Samples of blood-stained soil from the region of the nose and anus and urine-soaked soil were cultured onto both PLET and heart infusion agar. Ear clippings were available from three other plains zebras, two wildebeest *(Connochaetes taurinus),* and one springbok *(Antidorcas marsupialis)* during the field study. Direct cultures on PLET and heart infusion agar were possible for all these although one particularly dry specimen required prior soaking in sterile water. Bone, skin and associated soil samples were available from one each of the zebra, wildebeest and from the springbok.

Six randomly collected bone specimens not associated with the above animals or any other known anthrax deaths were examined both by direct culture and, after being ground up, in the same manner as soil samples. Faecal specimens as listed in Table 4 were collected and examined in a manner similar to soil samples.

Parameters in water hole samples

The water holes from which waters were collected for

TABLE 3: Diagnosis of cause of death in animals dying just before or during the field study

Animal	Identity	Blood smear (M'Fadyean stain)	Culture	Comment
Zebra	EBI 87:03:09 TA	+	+	Ear held refrigerated in plastic bag 10 days before used for smear and culture
Zebra	EBI 87:03:11 PL	Not possible	+	Ear held refrigerated in specimen pot 8 days before culture. Too dry for smear to be made
Zebra	EBI 87:03:21 RJ	+	+	Observed in dying moments. Blood obtained from jugular within minutes of death.
Wildebeest	CTI 87:03:22 HB	Not possible	Ear-/soil +	Not much left of carcass when first observed
Zebra	EBI 87:03:24 TA	–	–	Subsequently found to be a capture myopathy death. Carcass had been dumped for the scavengers
Springbok	AMI 87:03:29 FJ	+	+	Swab from nares and samples of skin
Wildebeest	CTI 87:04:02 TA	+	Not done	Normal ear clipping examined

TABLE 4: Faecal culture results

Animal of origin	Collection site*	Culture	Comment
Vulture‡	Vicinity of EB1 87.03.21 RJ (anthrax positive) 5 days later	+	Heavy growth, almost pure *B. anthracis.*
Vulture‡	Site of EB 87.03.24 TA (anthrax negative)	–	This was the zebra that proved to be a capture myopathy death
Jackal	Vicinity of and seemingly associated with EB1 87.03.09 TA (anthrax positive)	+	Well desiccated faeces eaten out probably by dung beetle
Jackal	Site of AM 87.03.29 FJ (anthrax positive)	–	The faeces were fresh and probably represented previous meals
Hyena	*ca.* 500 m from AM 87.03.29 FJ (anthrax positive)	–	The faeces were old and desiccated; clearly dropped before AM 87.03.29 FJ died

* see Table 3
‡ Whitebacked Vultures *Gyps africanus*

TABLE 5: Natural parameters in the water holes

Hole	Natural/ gravel pit	pH (± 0,2)	† Protein µg/ml	Organic C (ppm)	cfu/ml	
					Total	Spore
Adamax pan	N	9,1	<2	70	1 250 000	25 000
Aus ‡	N	8,7	±35	185	1 500 000	10
Chudop	N	8,4	<2	61	4 000	1 000
Kapupuhedi	N	10,7	<2	188	3 200	1 000
Okondeka	N					
source		7,8	<2	50	45 000	50
middle*		8,9	<2	43	90 000	200
terminal**		10,4	<2	132	55 000	200
Ondongab	N	9,4	<2	121	15 000	10
Adamax	G.P.	9,3	<2	43	270 000	350
Kalkheuwel	G.P.					
west		9,2	<2	36	3 300	200
east*		9,9	<2	38	7 500	500
Leeubron-Okondeka	G.P.	10,0	<2	133	550 000	500

† Bio-Rad protein assay kit

‡ Visibly contaminated with faecal matter/urine

* Something very like *B. athracis* isolated (phage + / penicillin sensitive / non-haemolytic) but failed to produce capsule; ultimately designated *B. cereus*

** Confirmed *B. anthracis* 10 – 100/ml

study and the parameters examined are shown in Table 5. pH was measured on site at the point of collection and subsequently again in the laboratory with a portable Griffin PHJ-251-W pH meter (Gallenkamp & Co Ltd, London) calibrated with appropriate pH 7,0 and 9,2 standards (BDH Chemicals) before each reading.

Protein estimates were made with the Bio-Rad protein assay kit (Bio-Rad Laboratories, München, W. Germany) using bovine serum albumin standards supplied with the kit.

For the purposes of organic carbon level determinations, included as a guide to potential nutrient support for bacterial growth and multiplication, 2 ml aliquots of the water samples were autoclaved (115°C for 20 min) in small vials with tightly fitting screw cap lids. These were transported back to the PHLS Centre for Applied Microbiology & Research (CAMR) UK, and analysed for inorganic and total carbon with a Beckman Tocamaster 915-B using $Na_2CO_3/NaHCO_3$ standards. Since only filtered water samples (0,45 µm or 0,22 µm FP030/3 low protein absorption filters [Schleicher and Schuell, Dassel, W. Germany]) could be used for studies on the viability of *B. anthracis* in the waters, both unfiltered and filtered samples were examined to ensure that filtration had not significantly altered the chemical parameters of the samples.

Natural aerobic bacterial levels in the waters were

assessed by the drop count method described previously (Turnbull *et al.* 1986b). Spore levels of aerobic spore forming bacilli were estimated by repeating the counting procedure on the sample after being held at 60°C for 30 min.

Fate of *B. anthracis* inoculated into water hole samples

As indicated above, examination of the behaviour of *B. anthracis* in the water samples required the prior filtration of the waters through low protein bacterial filters.

Aliquots (9,9 ml) of the filtered samples were put in test tubes and inoculated with 0,1 ml of spore or vegetative cell suspensions of *B. anthracis* such as to give an initial count of $\pm 10^5$ cfu/ml in each sample. The tubes were held in a water bath at 35°C and 0,2 ml aliquots withdrawn generally at 0, 2, 4, 8 and 24 h for counting.

In the absence of a safety cabinet at the Etosha Ecological Institute, for safety reasons, only the avirulent live spore animal vaccine (Sterne) strain could be used for these studies at Etosha. The spores used were derived directly from the veterinary vaccine (Onderstepoort) by washing and resuspension three times in sterile distilled water with final suspension to a count of $\pm 10^7$ spores/ml. The vegetative cell suspension was prepared by spreading a blood agar plate with 1 ml of the veterinary vaccine. After incubation for 7 h at 35°C, the vegetative cell growth was washed off with ice cold buffered saline (PBS) followed by centrifugation and resuspension in fresh ice cold PBS three times. The vegetative cell count and confirmation of the absence of spores from the final suspension was done by viable cell counts before and after heating aliquots at 60°C for 30 min.

In an attempt to confirm that the behaviour exhibited by the Sterne vaccine strain was truly representative of the indigenous virulent *B. anthracis* in the Park, the waters were transported under refrigeration to the Central Veterinary Laboratory, Windhoek, and the study extended using strains LA3/83 isolated from the carcass of an elephant at Gobab during the 1983 field study and AGP/86 isolated from Adamax gravel pit soil collected in April 1986 and sent to the Anthrax Reference Laboratory at CAMR (Table 11). The spore suspensions were prepared at CAMR and sent to the Central Veterinary Laboratory, Windhoek, in readiness for this work. Vegetative cell inocula were prepared at Windhoek in the same manner as the Sterne strain.

ELISA for specific antibody and antigen

The competitive inhibition enzyme-linked immunosorbent assay (ELISA) detailed previously (Turnbull *et al.* 1986a) was used. In the absence of peroxidase-conjugated antibodies to each species, antisera raised in rabbits or guinea pigs to the ammonium sulphate precipitated immunoglobulins of the species studied were used at dilutions of 1:500 to 1:800 (50 µl/well) after the stage of incubation of the sera under test. This was followed by addition of commercially conjugated anti-rabbit or anti-guinea pig globulins; after this, the procedure continued as previously described (Turnbull *et al.* 1986a).

For detection of PA and LF, the wells on microtitre plates were coated with ammonium sulphate precipitated immunoglobulins from rabbit antisera to PA and LF at a concentration of 20 µg/ml. After drying, blanking was done with 4% bovine serum albumin. Specimens (blood or supernatants from ear clippings, soil, rumen contents or faeces soaked in sterile distilled water) were doubly-diluted through two lines of wells, the second line carrying an additional 20µg/ml anti-PA or anti-LF in the dilution fluid (phosphate-buffered saline with 0,5% Tween 20,50 µl/well). After 30 min incubation, the plates were washed and mouse anti-PA or anti-LF (as appropriate) was added to all wells at a delution of 1:500. Subsequent additions of peroxidase-conjugated anti-mouse serum and substrate ABTS were the same as for the antibody ELISA. Estimations of PA and LF levels were based on comparisons of end-point titres with those of concurrently run purified PA or LF preparations. End-point titres were read at the point where divergent readings between the two lines of wells (the ELISA line and the ELISA inhibition line) had re-converged to a 15% difference.

RESULTS

Overall isolations of *B. anthracis*

Successful isolations of *B. anthracis* during the period of the field study were as shown in Table 6. Only a single positive isolation was made from a sample or specimen not associated with a known case of anthrax – namely the water from the terminal pool of the Okondeka natural water hole. The result was confirmed with a second sample taken 3 days later and it was estimated that the water contained 10–100 *B. anthracis* spores/ml. In contrast, the organism was isolated from five of 11 soil samples collected from sites where the carcasses of animals known to have or suspected of having died of anthrax had lain.

Of the specimens from the four zebras, two wildebeest and one springbok that died just before or during the field study, it was possible to confirm anthrax as the cause of death in all but one of the zebras which subsequently was found to have been a capture myopathy death (Table 3). A marker was placed at the site of the nasal exudate of one of the zebras confirmed as an anthrax case (EBI 87:03:21 RJ) and *B. anthracis* was easily isolated from soil samples collected from this site 5 days, 3 weeks and 5 months later.

The results of isolation attempts from carnivore faeces in relation to herbivore deaths are summarised in Table 4. Clearly *B. anthracis* is excreted in the faeces of

TABLE 6: Overall isolations of *B. anthracis* during the 19.3.87–2.4.87 field study

Sample / Specimen	No. + / total examined	Comments
Water samples	1 / 12	10–100 cfu *B.anthracis* / ml. Confirmed with second sample 3 days later
Mud / soil from water holes	0 / 29	
Other (routine) soils	0 / 40	
Soils from sites of dead animals	5 / 11	
Faecal		
– vulture	1 / 2	Associated with + / – carcass respectively
– jackal	1 / 2	Associated with + carcasses
– hyena	0 / 1	Not associated with carcass
Bones	0 / 6	Separate sites. Not associated with known + carcass
Animals		
– zebra	3 / 4	Negative later found to be capture myopathy death
– wildebeest	2 / 2	Soil beneath one +; the other judged + by M'Fadyean stain – *B. anthracis* not successfully isolated
– springbok	1 / 1	

carnivores for a period of time after consuming meat from animals which have died of anthrax.

None of the randomly collected samples of bones yielded *B. anthracis.*

Parameters in the water samples

The pHs, protein and organic carbon content and the total viable and spore counts in the water samples that were collected are summarised in Table 5. No pattern of association between any or all of these parameters was apparent, nor was there anything obviously different about the water from the Okondeka terminal pool – the only environmental sample not associated with a known anthrax death which yielded *B. anthracis.* It did have the second highest pH (10,4) recorded among the waters examined.

Fate of *B. anthracis* inoculated into water hole samples

The results obtained with Sterne strain spores and vegetative cells are shown graphically in Figure 2 which is a composite of three repeat studies. The vegetative cells inoculated at time zero rapidly declined in numbers and within 24 h were below the level at which a count was possible. In four of the samples almost complete kill off had taken place within 2 h of inoculation.

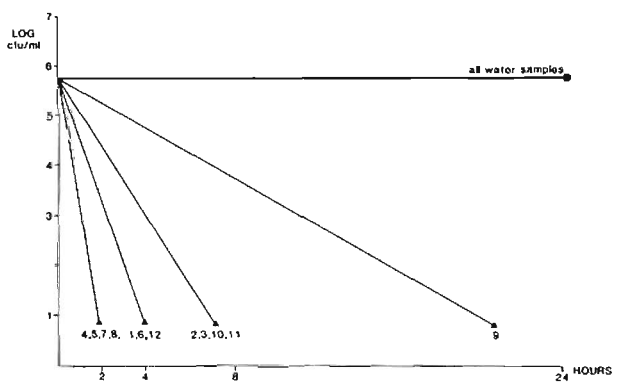

FIGURE 2: Fate of Sterne strain vegetative cells (▲) and spores (●) in the water samples*.

* 1 = Adamax gravel pit; 2 = Adamax pan; 3 = Aus; 4 = Leeubron-Okondeka gravel pit; 5 = Okondeka source; 6 = Okondeka middle pool; 7 = Okondeka terminal pool; 8 = Kapupuhedi; 9 = Kalkheuwel west gravel pit; 10 = Chudop; 11 = Kalkheuwel east gravel pit; 12 = Ondongab.

The spores, in contrast, were unaffected by any of the waters and remained at a steady level. There was no apparent inclination to germinate in any of the samples. Vegetative cells of the Sterne strain sporulate less readily than is generally the case with wild type strains and due to the problems encountered in obtaining and maintaining spore-free vegetative cell preparation of the two Etosha isolates LA3/83 and AGP/86, examination of the behaviour of these was approached in two ways.

In the first method (the waters had now been held under refrigeration for 2–3 weeks since collection), spore levels were monitored over the first 24 h at which time sufficient heart infusion broth (4% final concentration – initially determined in distilled water) was added to permit germination. Vegetative cell and spore levels were monitored up to 64 h. Before addition of the heart infusion broth (HIB) supplement, spore levels remained steady in all water samples. After supplementation, a variety of responses was seen ranging between the two extremes illustrated in Figure 3. In sample no. 8 (from Kapupuhedi with the highest pH – 10,7 [Table 5]), apparently no germination occurred while in sample no. 9 (Kalkheuwel west gravel pit), complete germination did occur and there may even have been a little (maximum 1 log) increase in vegetative cell numbers. In sample no. 1 (from Adamax gravel pit), complete germination seemingly occurred with, in a manner analogous to the Sterne strain (Figure 2), decline and death of the vegetative cells before resporulation could occur. The events apparently occurring in all the water samples are summarised in Table 7.

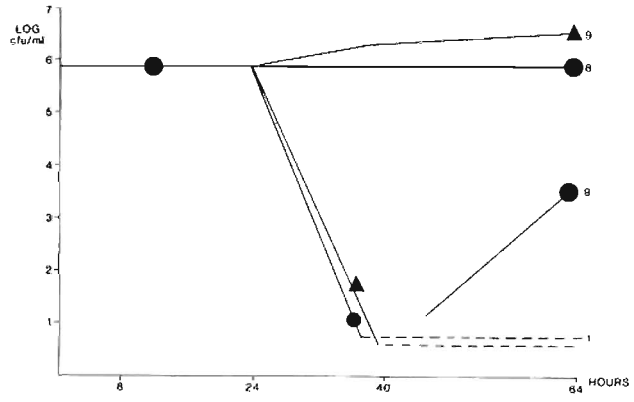

FIGURE 3: Outside events* following inoculation of spores of *B.anthracis* strains LA3/83 and AGB/86 into the water samples‡ before and after supplementation at 24 h with heart infusion broth (4% final concentration).

*Fuller details of these and intermediate events are given in Table 7.
‡1 = Adamax gravel pit; 8 = Kaupuhedi; 9 = Kalkheuwel west gravel pit.
▲ = vegetative cells; ● = spores.

The second method (the waters had now been held 3–4 weeks [at refrigerated temperature] since collection) when preparation of a spore-free vegetative cell suspension of strain AGP/86 had been achieved, was the same as that used for the Sterne strain. Direct inoculation with these vegetative forms into the HIB-supplemented (4% final concentration) water sample at a starting concentration of 6 x 10^5 cfu/ml resulted in either a slight increase (± 1 log) in total numbers (samples 1, 4, 5, 6, 10, 11) or a slight fall in total numbers (samples 3, 7, 12) but in all cases except sample 7, spore numbers had risen to 10^4 to 10^6 by 24 h; in sample 7 the appearance of spores was delayed but by 5 days, the *B. anthracis* was present exclusively in the spore form. In sample 8, sporulation was complete within the first 7 h after inoculation.

Serology

Preliminary to departing for the field study, ELISA tests for antibodies to the anthrax toxin components, Protective Antigen (PA), Lethal Factor (LF) and Edema Factor (EF), were carried out on sera obtained at the time of the previous field study in 1983. The results are shown in Table 8.

Initially it was thought that the positive sera from the black rhino *(Diceros bicornis)* represented naturally acquired infection but subsequent examination of the records showed that in November/December 1981, following the deaths of a number of rhinos from anthrax, a precautionary helicopter administered dart vaccination campaign had been carried out and it is probable that rhinos 1–4 in Table 8 had acquired their antibodies from vaccination at that time. The sera had been collected during a translocation exercise in June–July 1982.

All sera examined from lions *(Panthera leo)* in the Etosha Park were positive for anti-PA and, where tested, anti-LF and anti-EF also (Tables 8, 9). Control negative sera were obtained from two lions in the National Zoological Gardens, Pretoria (kindly supplied by Dr. H. Ebedes and Dr. R. Burroughs), and subsequently, the sera of two lions in the coastal region of Namibia – which appears to be free of anthrax – also proved to be negative (Table 9).

A similar contrast (Table 9) was seen between the sera of three Etosha spotted hyena *(Crocuta crocuta* – all positive for anti-PA) and four coastal region black-backed jackals (*Canis mesomalis* – all negative).

During the field study period, sera were examined from lions that had been bled on more than one occasion. It was readily apparent (Table 10) that titres changed with time in any one animal presumably reflecting the extent of recent exposure to *B. anthracis*

TABLE 7: Fate of two Etosha isolates of *B. anthracis* inoculated as spores into the water samples before and after supplementation at 24 h with heart infusion broth (4% final concentration)

Strain	Water samples*	Before supplement added	After supplementation with HIB at 24 h		
			Germination	24–40 h	40–60 h
AGP/86	4,5,6,10	Spores steady	Yes	Spore numbers decline. Vegetative numbers steady	Spore numbers rise again except ‡10
AGP/86	3,7,11	Spores steady	Yes	Spores decline. Vegetative numbers decline	Spores undetectable in 3,11; remain low in 7
LA3/83	3,6,9,11	Spores steady	Yes	Spores decline. Vegetative numbers decline	Spore numbers rise again
AGP/86	1	Spores steady	Yes	Spore and vegetative cells decline	Apparently have died out
LA3/83	1,4,5,7	Spores steady	Yes	Spores and vegetative cells decline	Apparently have died out
Both	8	Spores steady	No	Spores steady	Spores steady
Both	9	Spores steady	Yes	Spores decline. Vegetative cells steady or rise a little (<1 log)	Spore numbers rise again. Vegetative numbers higher than at start (max. 1 log)

* 1 = Adamax gravel pit; 3 = Aus; 4 = Leeubron-Okondeka gravel pit; 5 = Okondeka source; 6 = Okondeka middle pool; 7 = Okondeka terminal pool; 8 = Kapupuhedi; 9 = Kalkheuwel west gravel pit; 10 = Chudop; 11 = Kalkheuwel east gravel pit.

TABLE 8: Antibody titres to anthrax toxin components in some Etosha wildlife species.

Species	No.	Antibody titres to		
		PA	LF	EF
Rhino*	1	64	Neg	n
	2	64	w	n
	3	1024	128	n
	4	w	Neg	n
	6 – 10	Neg	Neg	n
Zebra	1,2	Neg	Neg	n
Elephant	1 – 3	Neg	Neg	n
Lion	1	2048	1024	Neg
	2	1024	512	2048
	3	2048	2048	2048
	4	512	256	1024
	5	2048	1024	2048
	6	2048	4096	2048
	7	512	1024	256

w = weak; evidence of antibody but below level at which titre could be assigned

n = not done

* = the rhinos with positive titres may have acquired these from an aerial vaccination campaign carried out a few months earlier

TABLE 9: Serum antibodies to the protective antigen (PA) components of anthrax toxin in four herbivore and three carnivore species

Species	Location	+ for anti-PA antibodies / total examined
Rhino	Etosha	*4/10
Elephant	Etosha	**0/16
Giraffe	Etosha	1/4
Zebra	Etosha	**0/10
Lion	Etosha	16/16
Lion	Coastal region, Namibia	‡0/2
Lion	Pretoria zoo	0/2
Hyena	Etosha	‡‡3/3
Jackal	Coastal region, Namibia	**0/4

* may have been vaccine induced in previous aerial dart vaccination operation

** no positive control available yet for the test

‡ test done at Etosha after field study

‡‡ no negative control available yet for test

or its toxin. Furthermore, higher titres were apparently associated with a greater likelihood of exposure to anthrax as defined by foci of cases in the Park and the locations of the prides (Figure 1) [sera and information on the lions kindly supplied by Dr H.H. Berry].

The only example of a positive herbivore serum sample that could not be explained by vaccination, and thus presumably represented infection with recovery, was that of a giraffe *(Giraffa camelopardalis)* with an anti-PA titre of 1:1028 (Table 9).

Other data acquired

The blood collected from EBI 87:03:21 RJ within minutes of its death was found to have 7 x 10^8 cfu of *B. anthracis* per millilitre. PA and LF levels as determined by capture ELISA were in the order of 30 and 105 μg/ml respectively. The ELISA was also found to be capable of detecting these antigens in ear clippings from two of the animals that had died of anthrax.

TABLE 10: Naturally acquired antibodies to PA in lions

Lion no.	Location of pride	Date bled	Anti-PA titre
2	Halali	81/2	4096
		81/5	16400
13	Ombika	81/8	4096
		82/5	w
		82/7	w
		84/5	w
16	Okaukuejo	82/7	16400
		85/7	8192
34	Okondeka	82/7	32800
		84/3	65600
		85/8	65600
37	Ombika	82/7	w
		84/5	2048

w = evidence of antibody but below level at which titre assignable

DISCUSSION

Isolation of *B. anthracis* from environmental samples

The low level of detectable *B. anthracis* in environmental samples not associated with known anthrax deaths noted in the previous (1983) field study (Turnbull *et al.* 1986b) was again observed in the second (1987) field study (Table 11). Of the 81 such environmental samples examined (Table 2), just one yielded *B. anthracis.* In contrast, 5 of 11 samples of soil from sites where animals had died of anthrax were positive.

As pointed out previously (Turnbull *et al.* 1986b), the procedures used for isolating and confirming the identity of *B. anthrcis* in the 1969 and 1971 surveys (Table 11) were not published, so a comparison of the methods used then and subsequently is not possible. However, there remains today considerable room for improvement in isolation methodology for *B. anthracis;* needed particularly iş the development of a selective enrichment procedure. As demonstrated by the 1986 isolation (Table 11), the present method of plating 1 ml of a suspension of 10 – 20 g in distilled water may fail to detect low numbers of *B. anthracis* spores, particularly against a high background of spores of other *Bacillus* species. As far as acquiring the disease is concerned, it only takes one spore to reach the right infection site in an animal to initiate infection and the relevance of our low isolation rates to the cycles of infection in the Park remains unclear.

Certainly it is clear that the soils and waters in even the areas of high anthrax incidence are not universally permeated with high numbers of *B. anthracis.* It may

TABLE 11: Isolation of *B. anthracis* from water holes and "routine" soil samples, 1969–87

Type of water hole	1969*	1971*	1983	1986**	1987
Gravel pit	16/23 (70%)	19/23 (85%)	0/15 (0%)	1/2‡	0/5 (0%)
Natural fountain/pan	11/24 (46%)	19/24 (80%)	0/21 (0%)	0/4	1/6 (17%)
Other "routine" soil samples	na	na	na	na	0/40 (0%)‡‡

* From Ebedes (1976)
** Sent to Anthrax Reference Laboratory, CAMR
‡ By guinea pig isolation; negative by *in vitro* methods
‡‡ Transects – see Table 2
na not applicable

be that survival of the organism either shed by the dying animal as vegetative cells or possibly even in its spore form is limited under the harsh sun. The failure on the previous field study (Turnbull *et al.* 1986b) to recover *B. anthracis* from sun-exposed stones and leaves contaminated with the blood of a zebra that had been dragged by a lion from where it had died of anthrax to a place in the shade gives support to this theory. Long term isolation studies from sites where animals have died of anthrax are now underway to clarify this.

Isolation of *B. anthracis* from animal samples

With one exception (wildebeest CT1 87.03.22 HB), *B. anthracis* was successfully cultured from all carcasses suspected of representing anthrax deaths (Tables 3, 6). There was not a great deal left of CT1 87.03.22 HB when found and the decision as to its cause of death was based on a positive soil sample from what was thought to have been blood-stained soil at the site.

The failure to isolate *B. anthracis* from any of the bone samples may reflect that the animals concerned had not died of anthrax or that numbers of the bacteria had fallen to very few for some reason. The result was interesting in view of the implication of bonemeal as one of the principal agents by which *B. anthracis* is transmitted from endemic to non-endemic regions of the world. It was also thought that examination of bones might be a way of more accurately establishing the incidence and distribution of anthrax in the Etosha National park and further culture work on bone samples, including a number from confirmed anthrax cases, may be worth doing to examine this further.

This is not the first report of isolation of *B. anthracis* from vulture faeces; Ebedes (1976), reviewing a number of reports incriminating vultures in the dissemination of anthrax, cites Bullock (1956) as having isolated *B. anthracis* from vulture faeces and, although bacteriological details are not given, it is also reported (Anon 1978) that the bacterium was isolated for up to two weeks from faeces of vultures fed anthrax spores experimentally in the Kruger National Park. Apparently the droppings of those fed with vegetative forms as opposed to spores were consistently negative.

In passing, it is to be noted that Pienaar (1967) considered that dissemination of anthrax in three major epizootics in the Kruger Park (1959, 1960, 1962) could be attributed largely to vultures but more through washing off infected gore adhering to their feathers in a water hole after feeding or regurgitating excess ingested meat into the water than via faeces. However, he states that "the excreta of vultures and all carrion feeders [even rats] have been shown to contain anthrax spores which pass untouched through their digestive tracts". However, no bacteriological evidence was actually supplied in that paper.

In the present study, it was conceivable that the vulture faeces *(B. anthracis* not isolated) collected from the vicinity of the capture myopathy zebra *(B. anthracis* not isolated) [Table 4] might have been positive from a previous meal elsewhere. However, the undigested contents of a meal probably pass through the gastrointestinal tract of a vulture within 24 h. The important indication though is that *B. anthracis* does not appear to colonize the vulture intestine and is expelled completely from the gut in a relatively short period.

Dissemination of *B. anthracis* by other carnivores is also discussed by Ebedes (1976). He cites three early reports (1920, 1941 and 1956) of isolation of *B. anthracis* from the faeces of carnivorous animals fed on infected meat, but faeces collected in his own study from 18 lions and 6 black-backed jackals after they had been seen feeding on anthrax carcasses were all negative. Again, bacteriological details are absent and it is not clear what time period elapsed between consumption of the anthrax carcasses and the collection of faeces. However, faecal swabs taken from an immobilised lion 8 h after it had fed on a plains zebra that had died of anthrax during the 1983 field study also failed to yield *B. anthracis* (P.C.B. Turnbull and J.M. Hofmeyr, unpublished results).

It is probable that there is a great deal to be learnt about the ecology of anthrax in the Etosha National Park from systematic bacteriological examinations of carnivore faeces over an extended period encompassing several seasonal cycles.

Anthrax and gravel pits versus natural water holes

As was the case with the previous study (Turnbull *et al.* 1986b), this study has failed to demonstrate bacterio-

logical support for the contention that gravel pits are associated in a major way with the incidence of anthrax. In fact, comparative analysis of locations of anthrax carcasses in the periods 1976-81 and 1982-87 has revealed a noticeable shift in the predominant anthrax foci away from the gravel pit region. Before 1981, the majority of anthrax carcasses were sited in an area of approximately 1500 km² immediately west of the Pan. Since 1982, elephant deaths have been more or less evenly spread across the Park; other sitings have been distributed all round the south, east and north-east of the Pan, i.e. largely near the roads and thus probably reflecting the greater chance near the roads of anything smaller than an elephant being observed before all traces have been removed by scavengers. The distribution of confirmed and suspected cases in the period between the last report (Turnbull *et al.* 1986b) and the present field study is shown in Figure 1.

There is, in fact, no way now of ruling out the possibility that the gravel pits, when first dug, did in some way upset the ecological balance in favour of an increased incidence in anthrax but the results from these past two field studies suggest that, although the disease continues, they no longer have a special role in its perpetuation, at least in years with less than average rainfall at the peak anthrax season.

Parameters in the water holes

As observed earlier, among the parameters studied (Table 5), nothing emerged that was distinctively different between the natural water holes and the gravel pits. The alkaline nature of the calcareous Etosha soil is well established though the readings in Table 5 are generally higher than those reported previously (Winter 1985; Turnbull *et al.* 1986b). The only sample from which *B. anthracis* was isolated (Okondeka terminal pool) had a pH of 10,4; *B. anthracis* would not normally be expected to germinate and multiply at this pH.

Several of the other water holes had as high or higher organic carbon levels than the Okondeka terminal pool. It was assumed by Turnbull *et al.* (1986b) that the organic content of water from natural water holes would be higher than that of gravel pits. In general, this seems to have proved true, the mean organic carbon in the natural water holes was 105 ppm as compared with 62 ppm in the gravel pits. However, numbers of samples examined were statistically small and complete cross-over as seen in the ranges found – natural water holes 38–188 ppm; gravel pits 36–133 ppm.

The highest bacterial counts were not strongly associated with the highest organic content. Although the two highest total viable counts were in samples from natural water holes, the mean total viable count in natural water hole (370 300) cfu/ml) and gravel pit (207 750 cfu/ml) waters did not differ significantly and there was complete cross-over in the ranges of counts – natural water holes 3 200 – 1 500 000 cfu/ml, gravel pits 3 500 – 550 000 cfu/ml.

Protein levels were almost all below the level that could be detected with the Bio-Rad Protein Assay kit. The level in the sample from Aus was clearly attributable to recent deposition of animal waste and may have been temporary.

The conclusion of this present study thus remains unchanged from those of Winter (1985) and Turnbull *et al.* (1986b) that any ecological association that may exist between anthrax and the water in natural water holes or gravel pits cannot be simply associated with readily measured parameters in these waters.

Fate of *B. anthracis* inoculated into the water samples.

Interpretation of the results presented in Table 7 and Figures 2 and 3 must be carried out in the light of three considerations. Firstly, the Sterne strain which, for safety reasons was the only one that could be utilised at Etosha in the freshly collected water samples, is not entirely typical of *B. anthracis* in other aspects than inability to produce capsule. In comparison with wild-type strains, it is, amongst other things, relatively reluctant to sporulate. Secondly, by the time the waters were examined for effect on wild type strains at the Central Veterinary Laboratory, Windhoek, two to four weeks had elapsed between collection and study. Thirdly, it was necessary to use filtered water samples for detection and counting of the inoculated *B. anthracis* which could not have been done without prior removal of the natural bacterial flora.

Within the confines of these factors, it would appear that waters in the Etosha National Park supply little opportunity for germination of anthrax spores or growth of vegetative forms needed in the water holes. Vegetative Sterne strain quickly died out in all the water samples. Even with 4% heart infusion broth supplementation (contributing an extra 400 ppm organic carbon), there was little evidence of significant growth by the wild type strains in any of the samples. The results suggest that vegetative forms entering the waters (e.g. as shed by a dying or dead animal) would quickly sporulate; the spores would then remain stable for an undertermined period.

Serology

The two virulence factors of *B. anthracis,* the capsule and the toxin, appear to be entirely specific for anthrax; there is no evidence that they are elaborated by any other organism or that any other organism produces cross-reacting antigens. The poly-D-glutamic acid capsule is only weakly antigenic, but the toxin components, particularly the one known as "Protective Antigen" (PA) lend themselves well to confirmatory diagnosis or vaccine studies (Turnbull *et al.* 1986a).

Very little is known about naturally acquired immunity to anthrax or to what extent susceptible herbivores

in enzootic regions acquire the infection and recover. Bergman (cited as a personal communication by Ebedes 1976) detected "an immunity" in Etosha zebra by an indirect fluorescent antibody test, but further details are not given. In fact, the development of sensitive antibody test systems with reliable specificities has only been possible since purified anthrax toxin components became available in the present decade. In the light of an assurance that anthrax vaccine has not been administered to giraffes in the park, the positive anti-PA titre in a giraffe (Table 9) must be assumed to represent *bona fide* naturally acquired antibody reflecting infection with recovery at some stage of this animal's life.

Of relevance here was a subsequent examination in the Anthrax Reference Laboratory, CAMR, of 20 sera collected in September 1987 from a dairy herd (approximately 100 animals altogether) which had experienced the death of one cow from anthrax in early September 1986. Apart from the cow that died, no other animals had shown signs of illness and none had been treated or vaccinated. Of the 20 sera examined, one had anti-PA/LF/EF titres of 1:1024/1:2048/negative respectively giving a firm indication that this animal had, like the giraffe, experienced sub-clinical anthrax. To our knowledge, this is the first convincing evidence on record that susceptible herbivores can, on occasion, acquired mild or subclinical anthrax and recover.

Vaccine induced and naturally acquired antibody cannot be reliably distinguished so it was not possible to determine whether any of the positive titres in the four rhino (Tables 8, 9) were the result of naturally acquired antibody. The positive lion sera, on the other hand, undoubtedly represent naturally acquired antibody assumed to result from eating meat off carcasses of animals that died of anthrax. It is not possible to say, however, whether the positive titres result from actual infection following ingestion of the meat or simply from absorption through the intestinal wall of antigenic digests of the toxin, or both. Nor is it possible to say whether the antibody contributes in any way to the lions' resistance to the disease. Lions can succumb to anthrax (Pienaar 1967) although this appears to be rare.

Two important observations emerge from the results of the assays on multiple sera from lions (Table 10). Firstly it is apparent that titres rose or fell in three of the animals; presumably this reflects the extent of recent exposure to *B. anthracis* or its toxin. Secondly, the higher titres were associated with a greater likelihood of exposure to anthrax as defined by the Park's foci of cases (Figure 1). Further to these are the comparative findings between Park and coastal region lions, hyenas and jackals (Table 9). Taken together, these observations suggest that monitoring serum anti-PA titres (anti-LF would serve equally well) in carnivores could be a sensitive method of gauging anthrax activity in an area.

An attempt to obtain positive control zebra serum by vaccination failed. The antisera and other ELISA reagents were all checked and confirmed as fully reactive. It is conceivable that serum antibody titres had not developed adequately at the time of collection of the blood three weeks after immunization. Sterne, as long ago as 1946, noted that equines develop protective immunity in response to the vaccine at a slow rate compared with, for example, bovines. An alternative explanation was the possibility that the vaccine had been neutralised by administration of a commercially prepared tube of Panalog (Squibb/Ciba-Geigy) delivering nystatin 100000 U, neomycin SO_4 2,5 mg, thiostreptone 25000 U and triamtinolone acetonide 1 mg, to the immobilisation dart wound while the animal was still anaesthetised.

Other data acquired

An extraordinarily high terminal bacteraemia has long been known to be a characteristic of anthrax and was at one time thought to be the cause of death through capillary blockage, depletion of the host's oxygen or both. It is, however, a subject that appears to have escaped systematic study and information is confined to the occasional sporadic reference. The finding of 7×10^8 cfu/ml in the blood of EB1 87:03:21 RJ can be placed among recorded figures of 10^7/ml in mice, rats and rhesus monkeys, 10^9 in chimpanzees (Lincoln *et al.* 1967 – no reference to strain of *B. anthracis* involved) and 2.5×10^8, 4×10^8 and 1.2×10^9 cfu/ml with Ames, New Hampshire and Vollum strains in guinea pigs (Turnbull *et al.* in prep.).

The capture ELISA used to measure PA and LF levels in the zebra blood is still being developed and its absolute precision has yet to be refined. With that proviso, the values of 30 μg/ml PA and 105 μg/ml LF in the blood of EB1 87:03:21 RJ compare with findings in guinea pigs generally in the order of 20 – 100 μg/ml PA and 10–150 μg/ml LF (Turnbull *et al.* in prep.).

SUMMARY AND CONCLUSIONS

This paper reports the results of studies on several aspects of anthrax in the Etosha National Park, SWA/Namibia, centred around an intensive field study carried out in March and April 1987 – the seasonal peak for anthrax activity.

Of 81 environmental samples not associated with known anthrax deaths, just one – a water sample from the terminal pool of the Okondeka natural fountain – yielded *B. anthracis*. In contrast five of 11 samples of soils from sites of anthrax carcasses were positive for *B. anthracis*.

Of specimens from four zebras, two wildebeest and a springbok that died just before or during the field study, anthrax was confirmed as the cause of death by isolation of the bacterium in all but one of the zebras which subsequently was found to have died of capture myopathy.

B. anthracis was also isolated from vulture faeces under trees surrounding the site of one of the zebras that had died of anthrax and from jackal faeces associated with a second zebra that had died of anthrax but it was not isolated from vulture faeces collected from around the site of the capture myopathy death or from samples of jackal and hyena faeces collected at a distance from any known anthrax carcass. Neither was the organism isolated from 6 samples of bones randomly collected and again not associated with known cases of anthrax.

In an attempt to establish the special role, if any, of water holes in the anthrax cycle, measurements of pH, protein, organic carbon content, total bacterial counts and spore counts were taken. There was nothing that strongly differentiated natural water hole waters from gravel pit waters; nor did the water from the Okondeka terminal pool differ in any obvious manner from waters of other water holes.

Vegetative cell preparations of the Sterne strain of *B. anthracis* fell below detectable within 2 h of inoculation into four of the (filter sterilized) water samples and, by 24 h, could not be recovered from any of the 12 samples studied. Spore levels after inoculation of Sterne strain spores remained steady; no germination occurred. Spores of two Etosha isolates also showed no inclination to germinate in the waters prior to addition of heart infusion broth to 4% final concentration. After such supplementation, a variety of responses was seen ranging from no germination in one sample, through germination with rapid decline in numbers, analogous to the behaviour of the Sterne vegetative forms, to germination with slight increase (maximum 1 log) in final numbers. In all cases in which germination occurred and the vegetative cells survived, re-sporulation had begun by 24 to 40 h. The water samples, though kept refrigerated, were 2 - 4 weeks old by the time the tests with the Etosha isolates were done. It is unlikely that natural nutrients in the waters ever reach a level equivalent to 4% heart infusion broth and the results suggest that the waters are all, albeit to varying degrees, inhibitory to germination and multiplication of *B. anthracis* spores. Vegetative cells shed by dying or dead animals into the waters probably decrease in number before a proportion are saved by sporulation.

In a serological survey on a number of herbivore and carnivore species, specific antibodies to anthrax toxin components were found in four of 10 rhino, one of four giraffe, zero of 16 elephant, none of 10 zebra but all of 16 Etosha lions and all of three Etosha hyenas were positive. Two lions from the non-enzootic coastal region and two from the National Zoological Gardens, Pretoria, and four jackals from the coastal region were all negative. The antibodies in the rhino may have resulted from a previous helicoptor vaccination campaign but the positive giraffe appears to represent naturally acquired antibody presumed to reflect mild infection and recovery in this herbivore at some point in its life. Higher titres in Etosha lions were geographically associated with the Park's foci of greater anthrax activity and it appears that monitoring serum antibodies offers a sensitive method of gauging anthrax activity in an area.

The terminal bacteraemia in a zebra that died of anthrax was found to be 7 x 10^8 cfu of *B. anthracis* per ml and its terminal blood levels of PA and LF lay in the order of 30 and 105 μg/ml.

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