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Serology and anthrax in humans, livestock and Etosha National Park wildlife

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SUMMARY

Results are presented from a number of epidemiological studies using enzyme immunoassays (EIA) based on the purified anthrax toxin antigens, protective antigen, lethal factor and oedema factor. Studies on sera from a group of 62 human anthrax patients in Turkey and from cattle in Britain following two unrelated outbreaks of anthrax show that EIA using protective antigen can be a useful diagnostic aid and will detect subclinical infections in appropriate circumstances. A serological survey on wildlife in the Etosha National Park, Namibia, where anthrax is endemic, showed that naturally acquired anthrax-specific antibodies are rare in herbivores but common in carnivores; in carnivores, titres appear to reflect the prevalence of anthrax in their ranges. Problems, as yet unresolved, were encountered in studies on sera from pigs following an outbreak of anthrax on a farm in Wales.

Clinical details, including treatment, of the human and one of the bovine outbreaks are summarized and discussed in relation to the serological findings.

INTRODUCTION

In former days, the diagnosis of anthrax was, for the most part, considered to be a clear-cut affair; either the patient or animal developed readily recognizable signs and was treated accordingly (or died) or they did not have anthrax. Consequently it was of no great concern generally that supportive serological diagnosis was unavailable.

The need for serology in relation to anthrax was first apparent in the context of the development of a vaccine for human use [1, 2]. The principal tool of those days was gel diffusion [3] using culture filtrates of the Sterne vaccine strain of *Bacillus anthracis* as the antigen, with serial dilutions of either the antigen or the serum under test.

While, for detection of antigens, this served an invaluable purpose over two

decades of vaccine development and, in fact, remains part of the quality assurance procedure in the production of the vaccine today, it was too insensitive for direct detection of antibodies in the sera of human vaccinees. For these, one early approach was back titration, again using gel diffusion, of standard antigen (a Sterne strain culture filtrate) which had been pre-incubated with the serum under test against standard antiserum from a horse hyperimmunized with repeated injections of the live spore animal vaccine. Very rarely this method would detect antibody in sera from actual anthrax cases [4]. At that time, a titre of 8 was considered very high.

An improved method of purifying the protective antigen (PA) component of anthrax toxin using Dowex I-XI [5–7] led to the application of indirect microhaemagglutination (IMHA; passive haemagglutination) for detecting antibodies to PA. The first truly informative data on the development of specific antibody in human anthrax was obtained with this [8] but there is apparently no record of it being used subsequently either for confirmation of infection or for epidemiological investigations.

The first application of the enzyme immunoassay (EIA) to anthrax serology also utilized the Dowex-I-XI-purified PA [9] and EIA was shown to be significantly more sensitive than IMHA using this PA [9] or that purified to a higher degree through hydroxylapatite [10]. Subsequently, when it became possible to obtain all three anthrax toxin components, PA, LF (lethal factor) and EF (oedema factor) in highly purified form [11], EIA was used to determine the antibody titres to all three in sera from a number of human anthrax patients in Zimbabwe [10, 12, 13]. EIA for anti-PA antibodies was also used to confirm the retrospective diagnosis of anthrax in an epidemic in Switzerland [14]. An EIA for antibodies to the capsular polypeptide was used in a serological follow-up to an outbreak of cutaneous anthrax in Paraguay in 1986 [15] and retrospective confirmation of a large outbreak in humans in Thailand in 1982 was done by means of EIAs for all three toxin components and the capsular polypeptide [16].

In the veterinary field, our earliest record of an attempt at anthrax serology is a reference [17] to the use of indirect fluorescent antibody tests on the sera of a small sample of zebra in the Etosha National Park, Namibia, in a search for evidence of naturally acquired immunity. In fact very little attempt seems to have been made, until recently, to apply serology to animal anthrax: presumably this was partly because of the lack of a perceived need and partly because of the problem of obtaining a suitable specific antigen.

More recently EIA has been applied on a small scale for the purpose of obtaining a better understanding of the epidemiology of anthrax in wildlife and livestock and the natural acquisition of antibodies (as opposed to vaccine-induced antibodies) in these [18] and in humans [19, 20].

Serological detection of antibody, particularly by gel diffusion in former days, and by EIA since about 1985, has been a fundamental tool in examining the function of existing human and animal anthrax vaccines and, thereby, in the development of putative new alternatives [10, 12, 21–25]. EIA or other serological systems such as immunofluorescence have also been applied to detection of anthrax toxin or other allegedly specific antigens in studies of pathogenesis [26] and spore structure [27], or new approaches to diagnosis [28, 29].

This paper summarizes accumulated information gained from the application of enzyme immunoassays for antibodies to anthrax toxin antigens (predominantly anti-PA) in sera from unvaccinated humans or animals in various epidemiological exercises.

METHODS

Human sera

Ninety serum samples from 62 patients diagnosed as cases of cutaneous anthrax in central Turkey were examined for the presence of antibody to PA. From 23 of these patients, 2 or 3 serum samples were collected over periods of 2–4 weeks. In a further 4 patients, collection of single serum specimens was done a specified number of days after the appearance of a lesion. In the remaining 35 cases it was not possible to specify the time period between appearance of the lesion and the time of collection of serum.

The clinical details of the patients, including a summary of their occupations, the supposed sources of infection, the sites of the lesions, the therapy given and the results of attempts at laboratory confirmation by demonstration of characteristic Gram positive bacilli in the lesion and by culture are given in the Results.

Unrelated to the 90 sera from Turkey, seven serum specimens from rangers or researchers in the Etosha National Park, Namibia, with a claim to have more than usual occupational exposure to anthrax were tested for antibodies to PA. Two of these were of European extraction; the remaining five were of Bushman heritage. None had received anthrax vaccine.

Bovine sera

Two sets of sera associated with unrelated incidents of anthrax were examined. Reference has already been made to the first incident [18] in which one cow in a dairy herd of approximately 100 animals in the West Country had died of anthrax in September 1986. No other cow had shown signs of illness and none was treated. Twenty random serum samples were collected from this herd 1 year later in September 1987 and tested for antibodies to PA.

The second set of bovine sera was collected after an outbreak of anthrax in the Midlands in July–August 1990. In this outbreak 10 cows died; 2 were confirmed as anthrax cases and the remaining 8 were suspected cases. In addition to these 10 animals were 14 others which, immediately upon observation of onset of illness (rapid respiration, elevated body temperature) of signs of submandibular or other oedema, were treated with penicillin, streptomycin, flunixin and, in one case, alphaprostol. One of these 14 cows died and was confirmed as being an anthrax case; the remaining 13 recovered without confirmation of diagnosis but were suspected of having had anthrax.

Sera were collected in September 1990 from 39 cows in this herd including the 13 treated animals. The identities were not revealed to the laboratory, however, until after the anti-PA test results had been sent to MAFF. In a follow-up to this exercise, a further 38 sera were collected, a certain proportion coming from the same herd and the rest coming from two other herds also in the Midlands but some distance away and with no histories of anthrax. The identities of the sera were again kept hidden until after the results had been sent to MAFF.

The positive controls in the first (1986/7) set of sera were sera from two vaccinated cows. The positive controls in the 1990 tests were one of these vaccinated animals and the positive serum from the 1986/7 set.

Porcine sera

Receipt of sera from pigs began after the outbreak of anthrax on an intensive pig-rearing unit in north Wales [30]. Some reference to the serology that was done has already been made [30].

Initially, ten serum samples from pigs on the affected farm were submitted for testing; two of these came from animals designated 'positive cases' which had been treated for 14 days with penicillin. The remainder came from 'contacts', four of which had also been given penicillin treatment. No positive control (vaccinated animal or previously confirmed case) or negative control sera were available at that time.

Subsequently, 200 further sera were collected from the affected farm and also, to supply negative controls, 30 sera from unrelated farms with no histories of anthrax.

Sera from African wildlife

The examinations covered here represent an extension of the previously reported serological studies on herbivores and carnivores in the Etosha National Park Namibia which is endemic for anthrax [18]. Initially begun as an attempt to determine whether naturally acquired immunity played any clear role in the cycles of infection among the different species, sera have been collected over the years whenever immobilization was being carried out and have been stored at −22 °C.

In addition to those already examined [18], the following sera were tested for anti-PA, anti-LF and anti-EF antibodies during a field study which took place in April–May 1991: 12 from elephant (*Loxodonta africana*), 15 from giraffe (*Giraffa camelopardalis*), 7 from spotted hyaena (*Crocuta crocuta*) and 1 from a brown hyaena (*Hyaena brunnea*), 8 from black-backed jackal (*Canis mesomelas*), 16 from lion (*Panthera leo*) including 1 desert region lion, 19 from black rhino (*Diceros bicornis*), 20 from springbok (*Antidorcas marsupialis*), 1 from a wildebeest (*Connochaetes taurinus*) and 24 from zebra (*Equus burchelli*).

With the exception of the lion from the anthrax-free Namib Desert region and the brown hyaena which had been caught on a farm south of western Etosha, all these sera came from animals immobilized within the Etosha National Park itself.

Included among the herbivore samples were sera from four elephants (numbers 118, 606, 609, 626) known to have received a dose of veterinary vaccine 12–18 months before collection of the serum that was tested. One of the rhinos (Suzi), which had originally been brought into the Etosha Ecological Institute in 1983 as a weanling orphaned by poachers and which had been subsequently released as a young adult into the Park, had been vaccinated at least three times, the last occasion in 1988. A proportion of the other rhinos may also have received a dose of the vaccine either during an immunization session in 1989 involving 32 rhinos in the western section of the Park or during translocation exercises; some 20 of

these animals have been translocated over the past 8 years. It was not possible, however, to identify the serum samples being tested as having come from rhinos known to have been or not to have been vaccinated.

Enzyme immunoassay (EIA)

The same inhibition EIA as described elsewhere [10, 18] was used. Concentrations of the purified toxin antigens for coating the wells of the 96-well EIA plates and for the diluent in the inhibition lines of wells were in the order of 20 μg/ml. To block unbound sites on the wells following coating, 2–4% bovine serum albumin was used for human sera, 2–5% ovalbumin for bovine and wild herbivore sera, 4% fetal calf serum or 5% ovalbumin for wild carnivores and, during the various trials with the porcine sera, 2–4% bovine serum albumin was used initially to be replaced subsequently with 5% ovalbumin. Commercial horseradish peroxidase-labelled conjugates (Sigma Chemical Co., Poole, UK) were used in the 'single sandwich' assays on human, bovine and porcine sera. For the wild animals, as described previously [18], a double sandwich assay was needed, utilizing antisera raised in rabbits or guinea-pigs against precipitated immunogobulins from the sera of each of the individual species followed by commercial anti-rabbit or anti-guinea-pig conjugates (Sigma).

RESULTS

Human case histories

The patients in this study comprised 29 housewives, 18 farmers, 4 shepherds, 1 watchman on a farm, 1 'worker', a driver, a restaurant keeper, an unemployed man, a 15-year-old student, 3 children (2 female, 1 male) and 2 male individuals with no further details.

In the majority of the adults, the disease was associated with a history of slaughtering, skinning and chopping up meat from an animal presumed to have had anthrax. The source of infection was not known in 5 of the housewives, 3 of the farmers, both unspecified male individuals, 1 of the shepherds, the student, the male child and the unemployed man. The mothers of both female children had also contracted cutaneous anthrax and one of the children was known to have handled the same implicated meat as her mother.

Fingers and thumbs were the sites of the lesions in 42 of the cases with more extensive hand involvement in 12 cases; lesions on the wrist with or without recorded hand, finger or thumb lesions were noted in 8 cases, on the arm in 2 cases, the eyelid in 3 cases and, in 1 case each, the roof of the nose, the chin and neck, the knee, the foot and the toe.

Bacteriological confirmation of diagnosis by observation of characteristic Gram-positive bacilli and/or isolation of *B. anthracis* was attempted in all but 2 housewives, 1 farmer, 1 of the female children and the 2 unspecified male individuals. A summary of the bacteriological confirmation test results as related to treatment reportedly given before presentation to the relevant authors of this paper (MD and BA) is given in Table 1.

In all but one of the cases, treatment was given from the time of first presentation including those patients who specified they had received some prior

Table 1. *Bacteriological confirmation and serological test results on sera from 62 patients clinically diagnosed as cases of anthrax*

Previous treatment	No. of cases*	Bacteriological confirmation	Antibodies to protective antigen	
			No. positive	Titres
Yes	19	9 (47%)	8 (42%)	64 (2), 128, 256, 1024, 2048, 4096, 16384
No	24	22 (92%)	9 (38%)	128 (2), 256 (3), 512 (3), 2048
NK†	13	7 (54%)	5 (39%)	16, 64, 128 (3)

* Bacteriological tests not done for 6 patients.
† Not known.

treatment. The single exception, one of the housewives, was deemed to have been adequately treated previously (with penicillin and cotrimoxazole for 10 days). In all but 5 instances, treatment consisted of administration of penicillin for 7–15 days in most cases but for 20 days in 2 cases and 35 days in 1 case. In the other 5 instances, in which the patients had histories of penicillin allergy, treatment schedules consisted of tetracycline for 10 days in 2 of the cases and, in 1 case each, chloramphenicol for 10 days, erythromycin for 7 days and streptomycin for 7 days.

All but 3 of the individuals, 2 of the housewives and the restaurant keeper, recovered without complications; one of the housewives required tracheotomy to prevent asphyxiation by laryngeal oedema. The patient supplied the information that the lesion had begun as an itching insect bite. On admission to the infectious diseases unit, initial treatment consisted of penicillin infusion, 20 million units daily for 3 days followed by intramuscular procaine penicillin for a further 17 days. In the second housewife with complications, a skin graft was required to repair the anthrax lesion on her left wrist. Similarly, surgical reconstruction was necessary to repair damage to the eyelid of the restaurant keeper following recovery from his anthrax infection at that site.

Bacteriological and serological (anti-PA) findings in the human subjects

In the 62 Turkish patients, all clinically diagnosed as having anthrax, the presence in the lesions of characteristic Gram-positive bacilli was observed in 37 (59·7%) and *B. anthracis* was isolated from 20 (32·2%). Only in one case, the farm watchman, was the bacterium isolated without Gram-positive bacilli first being seen. These 38 patients were considered as being bacteriologically-confirmed cases.

Antibodies to PA were found in the sera of 22 (35·5%) of the patients; 17 of these were among the bacteriologically confirmed cases and 5 had not been successfully confirmed bacteriologically. None of the six individuals in which attempts at bacteriological confirmation had not been made had anti-PA antibodies. Antibody titres obtained are shown in Table 1.

Table 2 summarizes the relationship between detection of antibodies and (i) bacteriological confirmation and (ii) the period between the appearance of the lesion and the collection of blood for serology. As would be expected, higher proportions of positives were found when two or more sera were collected from a

Table 2. *Antibodies to protective antigen in humans following cutaneous anthrax*

(A) *Serological results* versus *bacteriological confirmation*

No. of serum samples	Anti-PA + /bacteriol. +	Anti-PA + /bacteriol. − *
⩾ 2	10/14 (71 %)	3/7 (43 %)
1	7/24 (29 %)	2/9 (22 %)

(B) *Timing of blood collection for serology*

No of days†	No. anti-PA + all confirmed cases‡
0 7	5/21 (24 %)
⩾ 8	15/18 (83 %)

* Not including the 6 persons who were not tested bacteriologically: none of these had detectable anti-PA antibody.

† Number of days after the first appearance of the lesion.

‡ Total number of patients confirmed by bacteriology and/or serology in each (0–7 day or ⩾ 8 day) group

patient several days apart and when the sera were collected more than a week after the appearance of the lesion.

None of the seven sera from rangers and researchers in the Etosha National Park was positive for anti-PA. One of the researchers who handled lions on a regular basis had at one time been recorded as having signs of anti-PA antibodies (weak positive) but this could not be confirmed in the tests carried out on this occasion.

Positive controls in these tests were sera from vaccinated individuals.

Bovine studies

Only 1 of the 20 randomly collected serum samples collected in September 1987 from a herd in which 1 cow had died of anthrax the previous September had an anti-PA titre (1024). This compared with titres of 512 and 1024 in the vaccinated controls.

In the second more extensive survey on sera from the herd in the Midlands which lost ten cows in July and August 1990, anti-PA antibody was demonstrable in the sera of 10 of the 13 cows that had shown clinical signs of illness, been treated and recovered and also in sera from a further 6 of 22 animals that had not exhibited signs of illness. The proportion of seropositives, therefore, was 77 % in the group clinically judged to be developing the symptoms of anthrax (and thus treated) compared with 27 % in the symptomless (untreated) animals. The distribution of anti-PA titres was 256 in 1 cow, 512 in 5, 1024 in 5, 2048 in 1, 4096 in 2 and 8192 in 3 cows with ranges of 512–8192 in the symptomatic animals and 256–4096 in the asymptomatic.

In the follow-up test on 38 sera submitted blind and comprising a mixture of selected seropositives from the affected farms and controls from two other distant and unaffected farms, all eight seropositives from the affected farm were readily identified. Apart from two relatively low positive readings (64, 256), the remaining sera were negative.

Table 3. *Antibody titres to anthrax toxin components in carnivores and herbivores in the Etosha National Park**

Species	Identity numbers	No. of animals	Titres to PA	Titres to LF	Titres to EF
Lion					
Panthera leo	25	1	8192	8192	32768
	30, 35	2	32768	32768	65536
	36	1	32768	16384	16384
	43	1	16384	32768	65536
	102	1	16384	32768	32768
	203	1	Neg	w	Neg
	204	1	4096	2048	2048
	205	1	512	N.D.	w
	208	1	512	1024	N.D.
	232	1	2048	1024	2048
	233	1	2048	8192	4096
	234	1	1024	256	512
	235, 248	2	1024	1024	1024
	Desert	1	Neg	Neg	Neg
Hyaena					
Crocuta crocuta	564, 871, 833–837	7	> 1024	> 1024	> 1024
Hyaena brunnea	832	1	Neg	Neg	Neg
Jackal					
Canis mesomelas	129	1	> 1024	512	2048
	130	1	> 1024	128	w
	131	1	128	64	Neg
	132, 133, 831	3	256	Neg	Neg
	134	1	512	Neg	2048
	136	1	1024	Neg	Neg
Elephant					
Loxodonta africana	†	12	Neg	Neg	Neg
Giraffe					
Giraffa camelopardalis	†	15	Neg	Neg	Neg
Rhino					
Diceros bicornis	†	19	Neg	Neg	Neg
Springbok					
Antidorcas marsupialis	198	1	512	> 1024	512
	199	1	Neg	512	64
	511	1	256	128	256
	†	17	Neg	Neg	Neg
Wildebeest					
Connochaetes taurinus	114	1	Neg	Neg	Neg
Zebra					
Equus burchelli	†	24	Neg	Neg	Neg

* w, weak reaction; evidence of antibody but below level at which titre could be assigned. The absolute titres in the hyaenas, jackals and springbok with readings of > 1024 could not be determined due to insufficient antigens for the further tests necessary.

† Too numerous to list.

N.D., not done.

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Table 4. *Seroconversion to anthrax toxin components in six herbivore and three carnivore species in the Etosha National Park**

Species	Location	No. seropositive /total tested
Elephant	Etosha	0/28
Giraffe	Etosha	1/19
Rhino	Etosha	†4/29
Springbok	Etosha	3/20
Wildebeest	Etosha	0/1
Zebra	Etosha	0/34
Lion	Etosha	30/31
	Desert region	0/3
	Pretoria zoo	0/2
Hyaena		
C. crocuta	Etosha	10/10
H. brunnea	Adjacent farm	0/1
Jackal	Etosha	8/8
	Desert region	0/4

* Accumulated results including those recorded previously [18] together with those reported in this study.

† Believed to have been vaccine-induced [18].

Pigs

As reported previously [30], EIA on the first 10 sera (from 2 pigs showing clinical signs and 8 contacts) appeared to indicate a high proportion of seropositives; only 1, a contact, was negative. On repeated testing, the negative serum (O 53) remained consistently negative (8 repeats) and 1 of the positives (O 58) consistently exhibited a titre of 4096 (5 repeats). Repeat tests on the other sera gave variable titres from negative to 8192.

Subsequently, analysis of results obtained with a portion of the 200 further sera from the affected farm and 30 sera from unrelated farms highlighted a problem interpreted as a non-specific binding to protein on the EIA plate peculiar to pig sera. Pre-adsorption of the sera with horse erythrocytes appeared to remove this effect but all of 15 pig sera tested after adsorption were negative and, in the absence of a positive control, the merits of this approach could not be assessed.

Lack of manpower and resources precluded further work on the problem at that point.

Wildlife sera

The results of application of the EIA to the sera from wildlife during the April–May 1991 field study are given in Table 3. Among the herbivores, anti-anthrax toxin antibodies were found in the sera of just three springbok. It was found that serum from a vaccinated horse functioned satisfactorily as a positive control for the zebra, i.e. using anti-zebra immunoglobulins at the appropriate stage of the EIA on this horse's serum. None of the sera from the elephants known to have been vaccinated 12–18 months beforehand, nor any of the rhino sera, including that of 'Suzi', who had received at least three doses of vaccine prior to 1989, had demonstrable anti-PA/LF/EF antibodies. No positive controls,

therefore, were available for the elephant, giraffe, rhino or wildebeest sera but the activity of the anti-species serum for each of these was confirmed by gel diffusion.

In contrast, and in keeping with previous findings [18], almost all the carnivores were positive. In all but one of the lions and all the spotted hyaenas, positive responses to all three toxin components were found; only one of the Etosha lion sera was negative to all three components and one other gave a clear anti-PA signal but negative anti-LF/anti-EF responses. Among the jackals, all 8 gave positive anti-PA responses but with generally lower titres than were found in the lions and hyaenas; in addition, 5 were negative for anti-LF and 6 were negative for anti-EF antibodies. The negative control for the lions was the serum from the lion from the anthrax-free Namib Desert region. The activity of the anti-spotted hyaena immunoglobulins against the brown hyaena serum was confirmed by gel diffusion; the brown hyaena serum therefore appeared to supply a satisfactory negative control for the hyaenas. No negative control was available on this occasion for the jackals.

The accumulated results of all the serological tests done to date on wildlife sera are given in Table 4.

DISCUSSION

The opportunity to study sera from well documented cases of human anthrax has arisen only occasionally since the technology for applying EIA to the detection of anthrax specific antibody became available. At the time that sera were collected from 97 patients in Zimbabwe between 1982 and 1984 [11] the EIA was still in its infancy and the tests were further hampered by problems of communication between the centres involved. Of 97 clinically-diagnosed patients from whom serial blood samples were collected in that study, only 11 (11·3%) were reported to have seroconverted and neither seroconversions nor titres had shown any relation to the time of onset of the disease or to the extent or site of the lesion. It was concluded in that study that development of antibodies to PA could not be relied on to provide serological support for diagnosis of anthrax. Titres of antibodies to PA, LF and EF in a number of these sera have been given elsewhere [10, 12]. In contrast, the results of a second retrospective study on sera from 13 persons infected with anthrax between 1978 and 1981 in Switzerland led the authors [14] to conclude that EIA is a reliable and rapid method for the detection of anti-PA antibodies in infected persons. In that study, however, no information was given on the patients other than the diagnosis of anthrax and their titres.

The results presented in this paper confirm that EIA offers a good supplementary diagnostic aid for cutaneous anthrax subject to the usual rules, conditions and provisos that apply to any serological confirmatory diagnostic test, namely that (i) two or more serum samples taken 2–4 weeks apart will give greater diagnostic reliability, (ii) if only one serum sample is taken, it will be of greater diagnostic reliability if it is collected more than a week after the onset of symptoms, and (iii) negative or weak results be interpreted in the light of the early treatment the patient may have received.

Further evidence for the value of serodiagnosis in anthrax was supplied in a study on 12 sera from an outbreak in Paraguay in 1986 during which at least 21 persons had developed cutaneous anthrax [15]. Analysed by electrophoretic-

immunotransblots (EITB). 11 of the sera were found to have anti-PA antibody and 6 of these also had anti-LF antibody. An EIA was used to show that 11 (not precisely the same 11 as were positive by EITB) had anti-capsular antibody also.

In a retrospective analysis of sera from 19 of 76 human cases of anthrax that occurred in northern Thailand in 1982 [16], EITB detected antibodies to PA and LF in 13 of 18 (72%) of the patients while EIA detected anti-PA in 68% (6 of 9 with cutaneous anthrax and 7 of 10 with oral-oropharyngeal anthrax), anti-LF in 42% (8 patients) and anti-EF in 26% (5 patients).

Apart from these oral-oropharyngeal cases, experience in human anthrax serology has been confined to cutaneous cases. The events that led to villagers in Zambia apparently developing anti-anthrax antibodies [20] can only be speculated on: many were believed to have eaten meat within the preceding 2 years from hippos that had died of anthrax, but no clinical anthrax cases had been observed. Antibodies may have arisen from subclinical intestinal or cutaneous infection resulting from eating or handling these or other anthrax carcases. The authors of the report on the outbreak in Thailand [16] regarded 3 anti-PA titres found among 43 healthy unexposed Thai control villagers as 'apparent false positives'.

In contrast to the villagers in Zambia, none of the seven rangers or researchers in the Etosha National Park deemed to have had higher than average potential exposure to anthrax showed evidence of anti-PA antibodies. On the other hand, natural acquisition of EIA-detected antibodies to crude preparations of PA was reported in workers involved in the production of the live spore veterinary vaccine [19]. One worker with a particularly high titre had, in fact, reported a number of needle-stick injuries with the challenge strain. The history of a blood donor with a positive anti-PA titre in the study of Kleine-Albers and co-workers [14] was not known.

The studies in both bovine groups and in the wild herbivores supplied the first laboratory-based evidence that we are aware of that these highly susceptible animals may on occasion suffer subclinical anthrax infection. One cow in the first (1986/7) bovine study and six in the later (1990) study which had not been observed with symptoms had significant anti-PA titres.

In the second bovine study, the EIA successfully identified and confirmed the diagnosis of anthrax in 10 of 13 cows that had showed signs of illness and had been treated successfully. In the remaining three cows, the possibilities are that either these were immunogenically poor responders or they were wrongly identified as ill during the exercise of observing the herd for signs indicating that an animal should be treated on the suspicion that it was developing the acute disease.

Similarly, among the Etosha Park wild herbivores, three springbok and one giraffe have now been detected with antibodies to anthrax toxin components. None of these positive readings could be attributed to vaccination.

The high prevalence of antibodies in Etosha carnivores has already been discussed [18]. In that study, it was observed that, in lions, titres rose and fell with time and that higher titres were geographically associated with areas within the Park of higher anthrax incidence. Thus the titres appeared to represent the extent of exposure of lions to anthrax carcases and to reflect the extent of anthrax in their ranges.

It would appear that the antibody titres in lions are frequently very high. In

contrast, the anti-PA titres in the jackals in this study were relatively low with anti-LF and anti-EF titres correspondingly low or negative. In part this probably reflects the jackals' more varied diet and their lower dependence on – and therefore lower exposure to – anthrax carcases for their food. All the jackals tested had been caught within the Okaukuejo restcamp where they scavenge regularly from tourists and rubbish-bins. Also the jackal sera were collected during a period not within the peak anthrax season.

The protective action of the antibodies in carnivores against developing clinical disease is uncertain. Carnivores are normally regarded as being quite, but not exclusively, resistant to anthrax [31]. Reports emerging from the Kruger National Park in South Africa indicate that a substantial number of lions were among the casualties of a recent large epidemic of anthrax in that Park. Anthrax is regarded as endemic in only a small portion of the Kruger Park at its northern tip [32] and most lions in the Park would not be expected to have the naturally acquired antibodies found in their Etosha counterparts.

The EIA has now been used with marked success in a wide variety of animal species. The problems encountered with the pig sera were, therefore, unexpected. It is hoped that the opportunity will arise to re-investigate the matter and re-examine the sera which are in frozen storage in the meantime. In the absence of a significant bacteriological survey at the time of the incident in the pigs [30], a successful serological study could be useful in ascertaining some of the facts about the spread of anthrax on the affected farm.

Two considerations of major importance in the types of study reported in this paper are the specificity, and therefore the reliability, of the test and the persistence of the antibody titres developing in the human or animal. The toxin antigens of *B. anthracis* are genetically encoded on the totally anthrax-specific plasmid pXO1 and, in several years of study, no cross-reacting antigens have come to light. The inhibition EIA utilizing these antigens in purified form appears to be entirely specific for the corresponding antibodies and considerable confidence in the system has developed with its long term use for studying vaccine-induced antibodies [10, 12, 24]. It is hard, therefore, to know how to interpret the two positive bovine sera in the second (1990) bovine study from farms with no history of anthrax or vaccination.

The production of all three toxin antigens would appear to be necessary for virulence in *B. anthracis* infection; in *in vitro* culture they are produced simultaneously so theoretically, antibody development of all three would be expected following infection. In practice, data of previous studies [10, 12, 16] indicate that anti-PA antibodies are the most diagnostically reliable; occasional anti-PA-positive/anti-LF-negative sera were encountered but not vice versa. In this study, the single exception was the anti-PA-negative/anti-LF-positive/anti-EF-positive springbok serum. EF is more difficult to purify than PA and LF and seemingly less stable; anti-EF readings may frequently be negative in anti-PA-/anti-LF-positive sera. With considerations of time, cost and that only anti-PA is of significance in protection [10, 12, 21, 24], we now regard it as legitimate to rely just on EIA for anti-PA in routine anthrax serology.

In terms of duration of antibody, the results of studies in humans [10, 12] and guinea-pigs [24] indicated that detectable antibodies following immunization with

'chemical' (human-type) vaccines persist for an indefinite period. The rise and fall of titres in lions [18] and the absence of detectable antibodies in the sera of four elephants and one rhino known to have been vaccinated more than 12 months before suggest that antibodies acquired naturally or in response to the live vaccine may be less persistent. During the April–May 1991 Etosha field study, a brief survey of anti-PA and anti-LF titres in 8 horses held at the Ecological Institute and vaccinated with the Sterne strain live spore animal vaccine (Veterinary Research Institute, Onderstepoort, South Africa) revealed that, in 4 of the horses, antibodies could not be detected in sera taken 10 months after the first vaccination. The other 4 had, over the years, received 2 to 9 annual doses of vaccine; both anti-PA and anti-LF antibodies were detected in the sera of these 10 months after their most recent dose.

Unlike the previous finding [18] of four positive rhino sera, attributed to an earlier vaccination campaign, in this study EIA did not identify rhinos that may have received vaccine during the 1989 immunization campaign or while being translocated. Possibly none of those tested had ever been vaccinated (there are approximately 300 black rhino in the Etosha National Park) or those that had been had only received a single dose many months or years before. Another possibility discussed in that earlier report [18], is that antibiotics frequently administered to dart wounds in immobilized animals may affect the performance of vaccine administered at the same time.

Naturally-induced antibodies are probably identical to those produced in response to the live spore vaccine. Factors which affect their persistence, such as the infecting dose of *B. anthracis*, the species concerned and individual-to-individual differences, are poorly defined at this point. Possibly more positives would have been detected in the 1986/7 bovine study if the sera had been collected earlier.

With respect to diagnosis and treatment in the human cases in Turkey, it is recognized that polychrome methylene blue (M'Fadyean capsule stain) would have been preferable to Gram stain for demonstrating anti-PA in material from the lesions of the patients. Successful demonstration or culture of anti-PA from a lesion is affected by previous treatment the patient may have received and this may account for the five serologically positive patients in whom anthrax had not been confirmed bacteriologically.

Administration of penicillin appears to be rapidly effective. Examination of the fluid from the cutaneous lesion of a human case in Zimbabwe within 24 h of a single intramuscular dose of procaine penicillin (10 mg/kg body weight) showed the capsulated bacilli in an advanced state of disintegration (P. C. B. Turnbull, personal observation). A similar observation was made by Mansley [33] examining smears from infected cows following treatment and the immediate effectiveness of treatment is borne out in the present report by the effectiveness of single doses of penicillin, streptomycin and flunixin administered to cows showing symptoms. In the study on 621 cases of human anthrax in Zimbabwe [11], it was noted that the evolution of the cutaneous lesion (as distinct from the viability of the infecting *B. anthracis*) was not influenced by the duration of penicillin treatment and the initial course of treatment of 10–14 days in 1980 had been reduced to one of 2–4 days by 1984.

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