Isolation of *Bacillus anthracis*, the agent of anthrax, in the Etosha National Park

by

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CONTENTS

1 Introduction 321
2 Methods 322
2.1 Media 322
2.2 Development of the TCB medium 322
2.3 Field bacteriology at Etosha 322
3 Results 325
3.1 Laboratory trials on selective media 325
3.2 General bacteriology and use of selective media in the field 325
3.3 pH and bacterial counts 327
4 Discussion 328
5 Conclusion 330
6 Summary 330
7 Acknowledgments 331
8 Dedication 331
9 References 331

1 INTRODUCTION

Anthrax has long been enzootic in South West Africa/Namibia. While vaccination has reduced the incidence in domestic animals, the disease has remained widely prevalent in wildlife. Ebedes (1976) reported that anthrax was responsible for >50% of the total recorded deaths in the Etosha National Park and, between 1966 and 1974, 1635 animals from 10 different species (cheetah, eland, elephant, gemsbok, giraffe, kudu, ostrich, springbok, wildebeest and zebra) were recorded as dying from anthrax. Wildebeest and zebra were the most severly affected. The disease is, in fact, capable of affecting a wider spectrum of species and records show that, between the Etosha and the Kruger National Parks, it has been reported in 27 species (Ebedes, 1981).

Mortalities in the Etosha National Park continue to occur annually (Table 1) and constitute a major problem in terms of both undesired mortalities and the pressures that monitoring the disease, disposal of carcasses and other control measures place on severely limited staffing resources. In terms of undesired mortalities, the decline of the Blue Wildebeest to dangerously low numbers is of particular significance. Herds estimated at some 25,000 in 1953 numbered approximately one-tenth of that in 1978, anthrax being regarded as responsible for a major portion of this fall.

Ecological studies in the Etosha National Park — that is, studies relating the incidence of the disease in defined areas to conditions characteristic of or peculiar to those areas — have led to the belief that gravel pits

TABLE 1: Mortalities due to anthrax* in the Etosha National Park 1975–1984

Year	Zebra	Wildebeest	Springbok	Elephant	Gemsbok	Rhinoceros	Kudu	Giraffe
1975		9	1					
1976	21	9	1					
1977	12	17	9					
1978	45	10	13		2			
1979	39	18	16	2	1			
1980		13	1	2			1	
1981		2	1	70				1
1982		2	1	8		4	7	
1983	2			4				
1984	87	48		2		1		

* Confirmed by M'Fadyean-stained blood smears

resulting from excavations of gravel for roads are related in a major way to the incidence of anthrax (Ebedes, 1976; Berry, 1981). Bacteriological support for this theory was supplied in the finding that, during the rainy season of 1969, 16 of 23 gravel pits (70%) as compared with 11 of 24 (46%) natural pans were contaminated with *Bacillus anthracis* (Ebedes, 1976). In a repeat study in 1971, 85% of the gravel pits and 80% of the natural pans were recorded as positive for this agent. Berry (1981) suggests the gravel pits act as "favourable incubator areas for sporulation".

Nevertheless, the microbiological reasons for an association between gravel pits and the incidence of anthrax have not been properly elucidated and the microecology of *B. anthracis* in these artificial water holes, natural water holes and the soils of the Park is almost totally unknown. Furthermore, attempts to control anthrax empirically by approaches such as chlorination of the artificial holes have met with little success; the disease continues to be a major problem in the Etosha National Park. Because of this, bacteriological and serological studies were initiated with a view to attaining a better understanding of the cycle of the disease in the Park, thereby hopefully pointing the way to more effective control measures.

2 METHODS

2.1 Media

The selective polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar of Knisely (1966), a trimethoprim-colistin blood (TCB) agar developed specially in response to the need in the Etosha situation for a selective medium which would be easier to prepare and use than PLET (Oppenheim and Koornhof, 1980; McGetrick *et al.*, 1982) and plain 5% horse blood agar were used and compared in attempts to isolate *B. anthracis* from water hole samples and from carcasses of animals that had died or were suspected of having died of anthrax.

The formula for the PLET medium was as recommended by Knisely (1966) and contained 30 units/ml polymyxin, 40 μg/ml lysozyme (Merck 5282), 300 μg/ml disodium EDTA and 40 μg/ml thallous acetate added to molten autoclaved heart infusion agar (Difco) cooled to 50°C before the additions were made. The pH was adjusted to 7.3. The TCB medium consisted of Oxoid DST agar base containing 5% horse blood, 7.5 μg/ml colistin (polymyxin E) and 175 μg/ml trimethoprim.

The trimethoprim being somewhat insoluble was first dissolved in 2—3 ml of dimethylsulfoxide (DMSO) and added at the same time as the blood and polymyxin; the polymyxin was also pre-dissolved in a little sterile distilled water. The purpose of the polymyxin in both media is to supress growth of gram negative bacteria.

2.2 Development of the TCB medium

Preliminary tests by Oppenheim and Koornhof (1980) led to their observation that trimethoprim incorporated into blood agar resulted in a medium which allowed *B. anthracis* to grow while partially or completely suppressing *B. cereus*, *B. subtilis*, *B. megaterium* and *B. polymyxa*. McGetrick *et al.* (1982) tested this medium more extensively in the laboratory using vegetative and spore preparations from 12 unrelated strains of *B. anthracis*, 46 strains of *B. cereus* and 8 strains of *B. thuringiensis* and trimethoprim levels of 100, 150, 200 and 250 μg/ml.

Starting with suspensions of 10^6 to 10^7 vegetative bacilli or spores per ml, 10-fold dilutions were prepared and inoculated onto TCB, PLET and blood agar by a modification (ICMSF, 1978) of the drop count method of Miles and Misra (1938). Percentage inhibition on the selective TCB and PLET agars as compared with the plain blood agar were calculated.

2.3 Field bacteriology at Etosha

2.3.1 Water and soil examination

During a 4-week period from mid-March to mid-April 1983, a total of 45 sets of or pooled water and soil

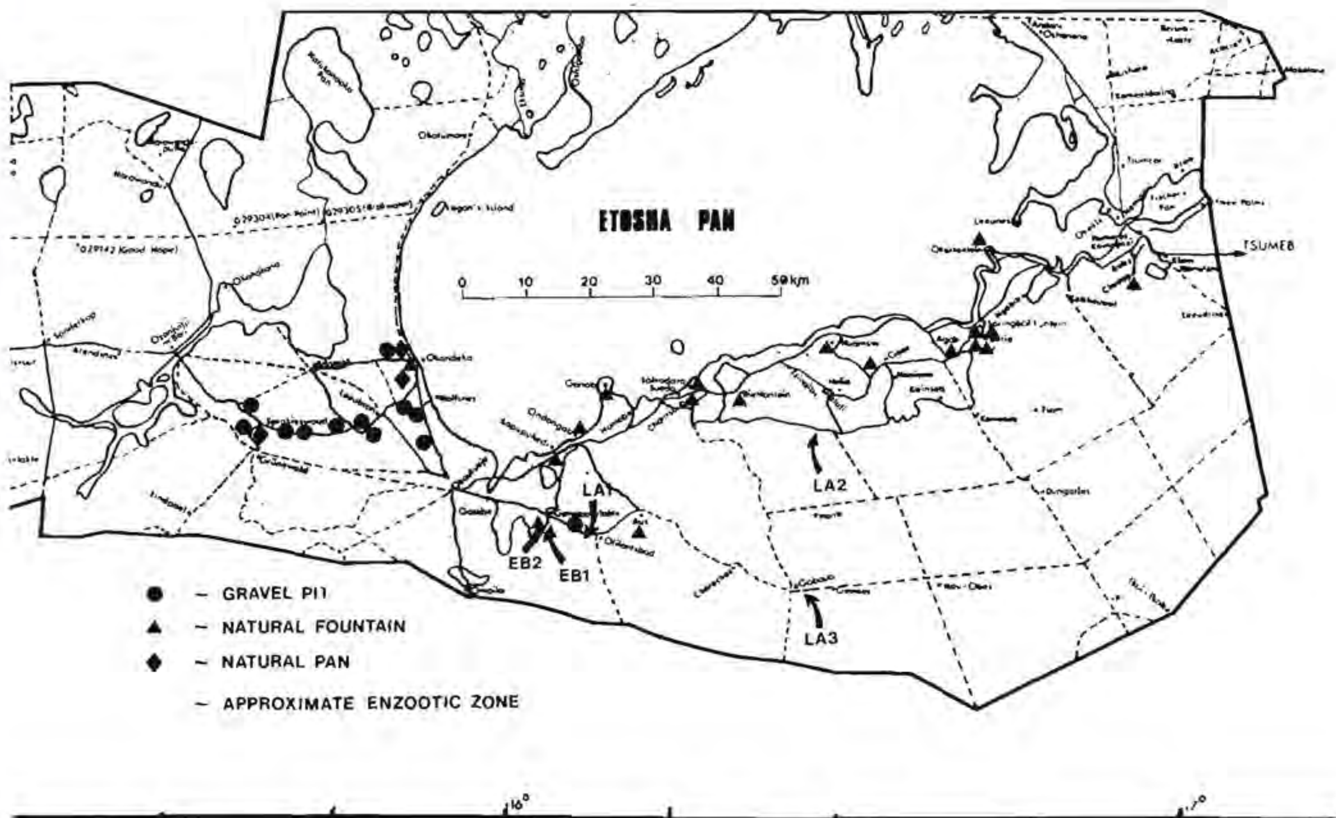

FIGURE 1: Sites of water and/or soil sampling and locations at which carcasse of animals that had died from anthrax were found.

samples were collected from 12 of the gravel pits, 16 natural fountains and 3 natural pans (Fig. 1). The gravel pits and natural pans all lay in a broadly defined "anthrax enzootic region" as based on the area of the Park where most cases of the disease occur; all but two of the natural fountains lay outside this area.

Since rainfall that season was particularly low, 9 of the 12 gravel pits and 1 of the 3 natural pans were dry. At these, surface and deep (>30 cm below surface) soil samples were collected from numerous sites around the holes; these samples were pooled in 500 ml jars as two separate entities (i.e. "surface" and "deep"). Where water was present, undisturbed samples and samples taken after the mud had been stirred up, were collected from numerous sites around the holes and were again pooled in 500 ml jars as two separate entities.

The pH's of the pooled water samples were recorded on site; those of the pooled soil samples were determined in the laboratory following suspension of 25g of sample in 20 ml distilled water.

Natural bacterial levels in the water samples were assessed by the modified (ICMSF, 1978) drop count method of Miles and Misra (1938). Duplicate 20 μl drops of sequential 10-fold dilutions of the water samples were dropped onto blood agar plates which were, in turn, incubated at room temperature (25°C to 35°C over a 24-hour period) for 48 hours. The aerobic bacterial level was determined by counting the colonies that had developed at the dilution where between 5 and 35 colonies had grown and making the appropriate calculations.

B. anthracis was looked for in the pooled samples by the following approach. The undisturbed and stirred water samples were plated directly onto blood agar, PLET and TCB both before and after being heated to 70°C for 10 minutes in a water bath. In the case of the soil samples, 10—20 ml sterile buffered saline (pH 7.4) — enough to just produce a fluid suspension — were added to 25 g soil. This suspension was shaken thoroughly and then plated onto the three agar media both before and after being heated to 70°C for 10 minutes. After the appropriate incubation times, presumptive *B. anthracis* colonies were picked off and plated onto blood agar and TCB. Penicillin discs (10 unit discs cut into quarters) and drops of γ-phage suspension were applied (Plate 3) and the plates incubated

overnight. Where appropriate, repeated subculture was carried out until pure cultures were obtained.

Attempts were made to produce an *ad hoc* enrichment broth to retard the growth of *B. cereus* and other *Bacillus* species in favour of any *B. anthracis* present using brain-heart infusion broth containing trimethoprim and colistin at the same concentrations as used in TCB agar. Freshly collected zebra blood with and without added trimethoprim was also used in an attempt to selectively enrich for *B. anthracis* in soil and water samples.

In the attempted enrichments of water samples, 5 ml of the supplemented brain-heart infusion broth, zebra blood or supplemented zebra blood were added to both heated (70°C for 10 minutes) and unheated pellets obtained by centrifuging 10 ml volumes of the water samples at approximately 2500 x g for 30 minutes. In the case of the soil samples, duplicate suspensions of 25 g of sample in 25 ml of brain-heart infusion broth were prepared and one of the suspensions was heated to 70°C for 10 minutes; trimethoprim and colistin were added to final concentrations of 175 μg/ml and 7.5 μg/ml respectively. For zebra blood enrichment attempts, 5 ml of freshly collected blood were added to approximately 5 g of heated and unheated soil sample pre-wet with buffered saline. The enrichment cultures were held 8—10 hours at 37°C and then subcultured onto the three plating agars.

2.3.2 Specimens from carcasses

During the 5-week period of the field study, five animals — three elephant and two zebra — were found to have died of anthrax. The locations of the carcasses when found are shown on the map (Fig. 1). In all cases, the carcasses had been opened in two to several places by lions and one of the elephants (LA3) was estimated to have been dead for five days at the time of discovery.

Swabs applied to the opened sites were plated on site (Plate 1) onto the three agar media in the case of the two zebra and one of the elephants. Swabs of ear clippings from all the animals taken for standard M'Fadyean smears were also cultured. Soil specimens which had been clearly wet by urine and an oozing lesion behind the tail of LA1 were taken for culture (Plate 2).

The *ad hoc* enrichment broths were also assessed with the specimens obtained from and around these dead animals. Swabs of ear clippings were transferred to test-tubes containing 5 ml of enrichment broth or fresh zebra blood with and without trimethoprim; these were incubated for 8 hours at 37°C followed by plating onto blood agar and TCB. The contaminated soils from under LA1 and EB1 were suspended in the trimethoprim-colistin enrichment broth and fresh zebra blood and incubated for 8—10 hours at 37°C

PLATE 1: Taking cultures from LA1 on site.

PLATE 2: Taking samples of the urine-contaminated soil under LA1. Note also the inguinal tear by lion before the carcass was sighted.

followed by subculture onto the three plating agars as before (2.3.1).

3 RESULTS

3.1 Laboratory trials on selective media

Fig. 2, from the work of McGetrick *et al.* (1982), shows that, under laboratory conditions, both PLET and TCB, when compared with conventional blood agar, effected significant degrees of inhibition on the growth of the large proportion of the 46 *B. cereus* and 8 *B. thuringiensis* strains while inhibiting the 12 *B. anthracis* strains to a lesser degree or not at all (Fig. 2).

On PLET, there was some difference between the results obtained with spore suspensions of *B. anthracis* and those found with vegetative cell suspensions; five of the 12 spore preparations exhibited <50% recovery rates while only three of the vegetative equivalents showed any inhibition at all. Apart from that, inhibition of growth, or lack of such inhibition, for all three *Bacillus* species was essentially the same regardless of whether they were inoculated as spores or vegetative cells.

For the purposes of illustration, Fig. 2 shows the inhibition patterns obtained with just two of the four trimethoprim levels tried in the TCB agar. The greater selectivity found at a trimethoprim level of 200 μg/ml as compared with 100 μg/ml can be seen. In the event, a concentration of 175 μg/ml was chosen as probably optimal for field use.

At the laboratory bench level, the inhibition of growth on PLET of some of the *B. anthracis* spore cultures marginally favoured TCB as a selective medium. The need in the case of PLET for the plates to be incubated for 48 hours (as compared with overnight for TCB) and what was felt to be the more ready recognition of the *B. anthracis* colony on the blood-based TCB agar added further weight to the subjective preference for TCB in anthrax isolation work.

3.2 General bacteriology and use of selective media in the field

B. anthracis was not found in any of the 45 pooled soil or water samples collected from the gravel pits, natural fountains or natural pans. *B. cereus,* on the other hand, abounded; in only three of the 45 samples as isolation of presumptive *B. cereus* not recorded (Table 2). Mixed growth was encountered in about a quarter of the samples (Table 2); in the soil samples, this largely comprised other *Bacillus* species while in the water samples, coliforms (undoubtedly originating from faecal contamination by the animals) were often fairly

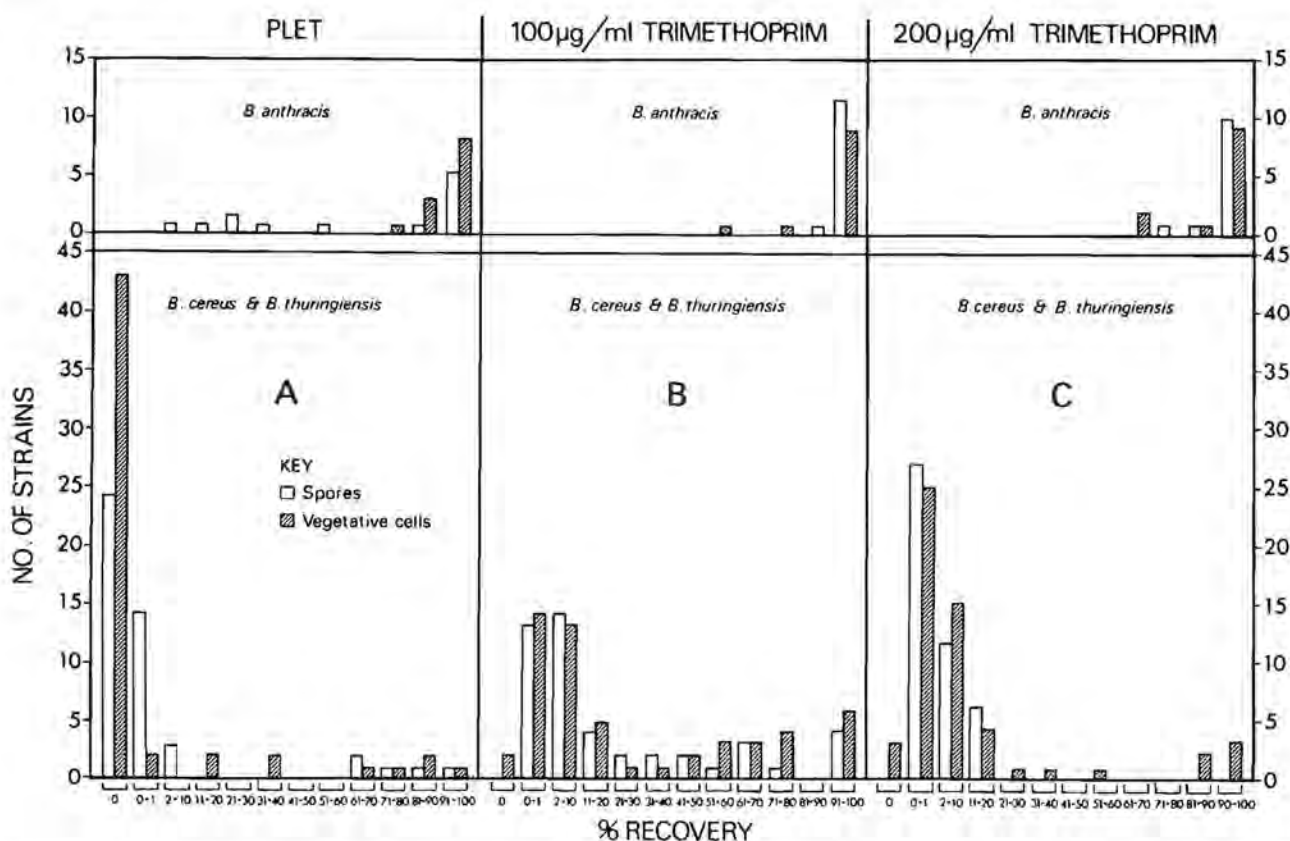

FIGURE 2: Selective inhibition (expressed in terms of percentage recoveries) of *B. cereus* and *B. thuringiensis* in favour of *B. anthracis* by PLET (A) and TCB (B, C) media in laboratory tests. The results with two of four levels of trimethoprim actually tested in TCB are shown (B, C). Both media were equally effective at the laboratory level in allowing growth of vegetative cells of *B. anthracis*, but recoveries of the organism, when in the spore form, were generally higher and more consistent with TCB. For field use, a level of 175 µg/ml of trimethoprim was chosen as optimal for TCB.

abundant also. Total viable aerobic bacterial counts in the water samples were summarised in Table 4.

As can be seen in Table 2, under the challenge applied by the Etosha field specimens, *B. cereus* succeeded in overcoming the inhibitory functions of both PLET and TCB in approximately half the samples. Often the extent of growth of the *B. cereus* was reduced as compared with plain blood agar but generally it still remained significant on the inhibitory media.

B. anthracis was successfully isolated on direct plate culture from the three elephants and two zebra found dead of anthrax (Table 3). Soil contaminated with the urine of LA1 and soil from under EB1 were also positive. As seen in Table 3, however, only in one instance was a specimen positive on the selective media while being negative on non-selective blood agar and in this instance, it appeared to be a chance event anyhow (only a single colony was found on TCB). The absolute merits of the inhibitory media were thus not proved with the positive samples.

As illustrated in Plate 3, the selective media did have a noteworthy value in that they "cleaned up" the cultures. Both PLET and TCB were effective in this respect but the advantages of TCB over PLET appeared to be that (1) TCB could be read after overnight incubation as compared with a 48 hour period necessary for PLET and (2) haemolysis could be looked for on TCB as an additional differential guide to colonies which were or were not likely to be *B. anthracis*.

TABLE 2: Comparison of the three isolation media used. 1. Primary isolation results following direct plate culture of 45 soil and water samples.

Condition	5% Horse blood agar			TCB			PLET		
	No growth	*B. cereus**	Other growth	No growth	*B. cereus**	Other growth	No growth	*B. cereus**	Other growth
Unheated samples	0	42	3	15	20	10	17	25	13
Heated samples	3	36	6	27	17	1	20	14	11

* with or without other growth

TABLE 3: Comparison of the three isolation media used. 2. Primary isolation results following direct plate culture of specimens from five animals found dead with anthrax and two related soil specimens.

Source	5% horse blood agar	TCB	PLET
LA 1			
Trunk lesion	+	+	+
Ear	+	+	+
Urin	+	+	?
Inguinal tear	+	+	?
EB 1			
Thorax	+	+	+
Hind quarter	+	+	+
Flesh from 3 regions	+	+	+
EB 2			
Lung	+	+	n
Spleen	+	+	n
Abdomen	+	+	n
LA 2 – ear	+	+	+
LA 3 – ear	–	+*	n
Soil contaminated with LA 1 urine	+	+	+
Soil under EB 1	+	+	n

* 1 colony only n = not done

The *ad hoc* selective enrichment attempts in brain-heart infusion broth with added trimethoprim and colistin and freshly collected zebra blood with and without trimethoprim and colistin also failed to yield higher *B. anthracis* isolation rates than the direct plate cultures on blood agar. All the water hole samples yielded essentially pure *B. cereus* when the enrichment cultures were plated out on blood agar, TCB and PLET and *B. anthracis* was only isolated with difficulty from enrichment cultures of the contaminated soil under LA1 and from less than half the animal specimens that had been positive by direct plate culture.

Of interest in the isolation work done during this study was that *B. anthracis* was not isolated from (1) mud and water samples collected from the vicinity of LA3 which had lain for an estimated five days at the time of discovery in a shallow water pan: (2) stones and leaves contaminated with blood from EB2 which had been dragged by lions for some distance from where it was presumed to have died to where it was found.

Since these field trials, isolation of *B. anthracis* from soil samples from Gruinard Island (see below) has been attempted on TCB and compared with PLET medium prepared in the Vaccine Research and Production Laboratory, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, UK. While counts equivalent to 100 *B. anthracis* colony forming units per gram were found on the PLET medium, the organism was not detected amongst numerous colonies of *B. cereus* and other *Bacillus* species on the TCB.

3.3 pH and bacterial counts

The soil and water pH levels in the three types of water hole and the total viable bacterial count in water where it was present are summarised in Table 4. In general, the expected alkalinity was confirmed although the outside range, 6.9 to 9.8, was quite extensive. However, the mean pH values in the three types of water holes were essentially identical — all in the vicinity of 8.

No marked differences between the three types of water hole were apparent from the total aerobic bacterial counts. The overall levels in the natural fountain waters were generally rather higher than the gravel pits and natural pans. These figures presumably reflect the relative levels of organic matter and the corresponding available bacterial nutrients in the different types of water.

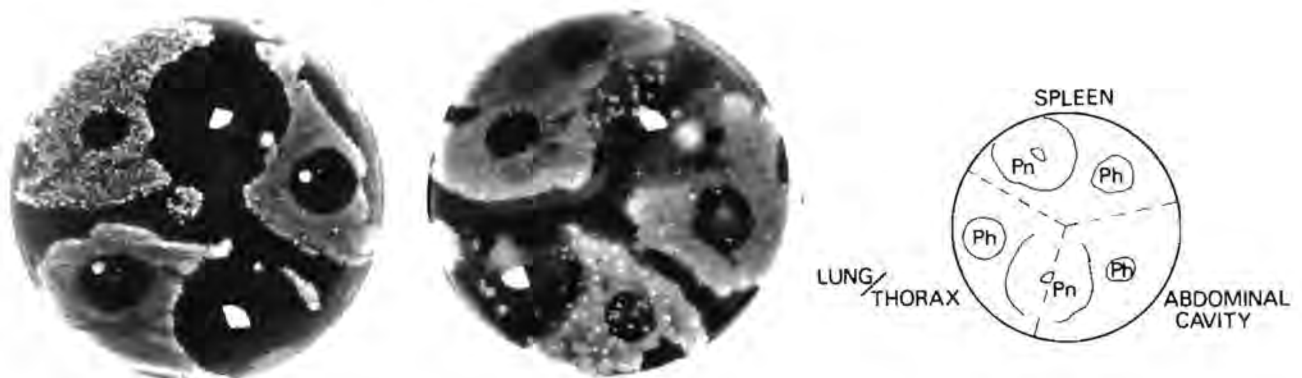

PLATE 3: The "clean-up" effect of TCB illustrated with the primary isolation cultures from EB2. Swabs from the regions shown were plated on site onto the three sections of 5% blood agar (left) and TCB (centre) plates. Drops of phage (Ph) and penicillin quarter-discs (Pn) were placed as illustrated (right) and the plates incubated overnight at 37°C. Note that *B. anthracis* is readily identified on the non-selective blood agar but is more readily purified from the TCB. These cultures could, in fact, be read after 8 hours at 37°C.

TABLE 4: pH and aerobic bacterial levels in the water holes

Type of water hole	Number examined	Containing water	pH		Aerobic colony count/ml*			
			mean	range	Number examined	Unstirred	Stirred	
							Mean	range
Natural fountains	16	yes	8.0	6.9–9.8	2	6000 & 0.5m	na	5m & 10m
					14	nd	5m	1m–10m
Gravel pits	3	yes	8.2	7.6–9.0	3	6000–1.5m	1.6m	1.2m – 2m
	9	no**	7.7	7.5–8.7	na	—	—	—
Natural pans	2	yes	8.0	7.6–8.2	2	55000 & 90000	na	1.8m & 2.1m
	1	no**	8.2	na	na	—	—	—

*m = million
** soil pHs measured using 25 g soil suspended in 20 ml distilled water
na = not applicable; nd = not done.

DISCUSSION

The failure to isolate *B. anthracis* from the water hole water and soil samples contrasts with two previous reports (Table 5) but is in agreement with the findings of Winter (1985) as discussed in greater detail below. The reasons for the discrepancies apparent in Table 5 can only be speculated upon. Unfortunately, the bacteriological procedures used for isolation and identification of *B. anthracis* in those earlier studies were not published and a comparision of methods used is not possible.

Positive isolations of *B. anthracis* from routine soil samples (i.e. samples not taken from the immediate vicinity of the carcass of an animal that has died of anthrax) are, in the experience of one anthrax reference laboratory, exceedingly rare (Carman *et al.*, 1984). This appears to be borne out by the paucity of published reports of successful isolation of *B. anthracis* from field samples of soils. Probably representative of the occasions when this has been done successfully is the report of Shapovalova (1966) in which the isolation methodology relied on infection of mice and guinea pigs with soil suspensions and cultures.

In the somewhat unusual case of Gruinard Island off the northwest coast of Scotland, now, >40 years after artificial contamination by the detonation of an experimental biological warfare bomb containing spores of the Vollum strain of *B. anthracis*, this organism can still be isolated readily (Manchee *et al.*, 1983).

TABLE 5: Isolation of *Bacillus anthracis* from water holes in the Etosha National Park.

Type of water hole	1969*	1971*	1983
Gravel pits	16/23 (70 %)	19/23 (85 %)	0/15 (0 %)
Natural pans/ fountains	11/24 (46 %)	19/24 (80 %)	0/21 (0 %)

* from Ebedes, 1976

However, the Gruinard and Etosha situation vary in a number of ways:

(i) the Gruinard soil is an acid (pH 4.2—4.7 — Manchee *et al.*, 1981) organic peat and carries relatively low numbers of competing spore formers (<10^4/g — M. Broster, personal communication) as compared with levels in the alkaline calcareous Etosha soil as high as 10^6/g noted in this study. As well as having a possible bearing on the microecologies of *B. anthracis* in the two soils, this may indicate that the chance of detecting low numbers of *B. anthracis* in the Etosha soil using conventional laboratory media is substantially less than is the case with Gruinard soil.

(ii) Much of the soil in the semi-desert Etosha lacks any vegetation and is exposed for a very large proportion of the year to intense sunlight and relatively high day-time temperatures; the ground surface can become extremely hot during the day. The climate on Gruinard Island is mostly cool to cold and wet throughout the year and even on clear summer days, the surface soil, supporting a luxuriant growth of sphagnum moss, grass, heather and bracken, is unlikely to exhibit temperature rises significantly above the air temperature.

(iii) The level of contamination of the Gruinard soil with *B. anthracis* spores was exceedingly high in the vicinity of the detonated bomb and, even after 40 years, soil samples containing several hundred spores per gram were found (Manchee *et al.*, 1983). This is unlikely to be matched in the harsh, dry and hot conditions of Etosha under which probably only a small proportion of the vegetative forms of *B. anthracis* shed by the dying or freshly dead animal will succeed in sporulating. Decomposition bacteria and conditions are unlikely to allow extensive survival and sporulation of the *B. anthracis* in the rotting carcass either.

(iv) The nature of the Gruinard microflora is such that PLET appears to be more effective in inhibiting species present other than *B. anthracis* than was the case in Etosha.

Although positive isolation of *B. anthracis* was made after a stage of heating at 70°C for 10 minutes from the contaminated soils under LA1 and EB1 and from a number of the animal specimens when several days old, it is possible that 70°C may be too high a temperature to use when looking for small numbers of *B. anthracis* spores in soil samples in general; Manchee *et al.* (1981) heated their soil samples at 60°C for 1 hour.

The importance of field trials on products showing promise in the laboratory was well illustrated with the selective media. Numerical assessments of rises or falls in bacterial counts necessarily involves artificially standardised starting suspensions of bacteria or their spores which may differ greatly from field conditions. Thus the effective inhibition of most of the *B. cereus* and *B. thuringiensis* by PLET and TCB noted in the laboratory (Fig. 2) was found to be less effective in the field. One shortcoming of the initial laboratory trials, in fact, was that they did not include tests on mixed cultures; the results shown in Fig. 2 were obtained with just pure cultures of the various strains.

The two media did inhibit non-anthrax bacteria sufficiently to "clean up" the *B. anthracis* isolates from the animal specimens and contaminated soils under LA1 and EB1. They thus proved useful in purification of the isolates; in this respect, TCB, with its shorter incubation requirement and added advantage of distinguishing haemolytic colonies, was of greater value than PLET. The disadvantage with TCB lies in batch-to-batch variation resulting from the poor solubility of trimethoprim.

In preliminary trials, the addition of trimethoprim to PLET did not appear to improve its inhibitory effect against problem non-anthrax bacilli (J.A. Carman, personal communication). It is of interest in considering the role of trimethoprim as the active agent in TCB against *B. cereus* and other non-anthrax bacilli that, as judged by routine antibiotic sensitivity assays, *B. cereus* is resistant to trimethoprim (Turnbull and Kramer, 1983).

The failure to isolate *B. anthracis* from Gruinard Island soil on TCB in later preliminary trials gives some cause for anxiety over the negative isolation results with Etosha soils. In retrospect, the apparent effectiveness of this medium when used for isolation of *B. anthracis* from the carcasses may have been the result of a combination of relatively high numbers of *B. anthracis* and a predominantly gram negative background flora in these sources and it may not have been suited to looking for relatively low numbers of *B. anthracis* in soil with an extensive background flora of gram positive bacilli. Failure to isolate on the PLET conceivably could have been due to lack of field experience with this medium. Follow-up studies with further isolation attempts to confirm the negative soil findings reported here appear to be called for.

As implied throughout this paper, the chief problem bacterium from the standpoint of differentiation from *B. anthracis* is *B. cereus*. These are closely related and some authorities regard the anthrax bacterium as a variety of *B. cereus* referring to it as *B. cereus* var *anthracis* (Gordon *et al.*, 1973). The two can be hard to distinguish; the topic of their differentiation is covered in detail by Parry *et al.* (1983). *B. thuringiensis* and *B. mycoides* are other close relatives of *B. cereus* and these and certain other *Bacillus* species, such as *B. megaterium*, *B. subtilis*, *B. licheniformis* and *B. polymyxa*, which at times also resemble the *B. cereus* group, can make it difficult when they are present in large numbers on primary isolation plates to pick out the occasional colony of *B. anthracis* lying in their midst.

The urgent need in relation to improvement of detection and isolation of *B. anthracis* from soils and other specimens with low levels of contamination is for an effective enrichment broth. The *ad hoc* use in this study of brain-heart infusion broth containing trimethoprim and colistin and freshly collected zebra blood with and without the antibiotics did not result in improved isolation rates. Blood is used by other workers (Carman *et al.*, 1984) as a selective enrichment medium for *B. anthracis*. They recommend a 5-hour incubation period; it is possible that the zebra blood would have given better results if held for this shorter period.

As reviewed by Ebedes (1981) and Hambleton *et al.* (1984), anthrax is a disease of great antiquity and one that has drawn the attention of microbiologists since the science of microbiology began. Despite the extensive work done on the disease, particularly prior to 1960, little is known about how *B. anthracis* maintains its cycle of infection. Animal reservoirs of infection have not been identified and, as already discussed as well as demonstrated in this study, detecting foci of environmental contamination is extremely difficult.

As reviewed by Shapovalova (1966) and Van Ness (1971), it has been shown in laboratory experiments that, under appropriate conditions, spore germination, vegetative cell multiplication and re-sporulation can occur in soils. Van Ness postulated that suitably wet depressions containing rotting vegetation can provide "incubator areas" in which this cycle can occur. The association of peak anthrax mortality rates in the Etosha with the rainy season is certainly in line with such a theory. The wet and overcast conditions during this season possibly also allow a greater proportion of vegetative *B. anthracis* shed by dying animals to sporulate. Together these factors may result in greatly enhanced environmental spore levels. The association of the disease with water holes may, in turn, result from the spores being washed down to the holes by the rain or from increased thirst of the sick animal drawing it to the water hole, or both these factors.

The failure to isolate *B. anthracis* from the water hole soils during this study may be attributable, at least in

part, to the abnormally low rainfall in that particularly dry year (March/April, 1983).

Biting insects are believed to transmit anthrax (Van Ness, 1971; Davies, 1983; Parry *et al.*, 1983); increased insect densities associated with the wet season may contribute further to the peak incidence of the disease at this time of the year.

It is more difficult to offer a plausible explanation for the differential association of anthrax in the Etosha National Park (Ebedes, 1976) with artificial water holes. The simple parameters of pH and aerobic bacterial counts (Table 4) did not reveal marked differences that could readily account for any such differential association although the generally higher aerobic counts in natural fountain water reflected, as expected, the higher organic content of this water type than gravel pit water.

In a water quality survey undertaken in the Etosha National Park between 1974 and 1979, Winter (1985) analysed water samples from fountains and boreholes (but not gravel pits apparently) for 17 chemical parameters, bacterial levels and quantitative isolation of *B. anthracis* bacteriophage. No significant differences were found between concentrations of the 17 parameters in water holes in anthrax and non-anthrax areas during comparable seasons. Althoguh his total viable counts were somewhat lower than those recorded here, he confirmed the absence of a significant difference in the bacterial levels in water samples from the two areas.

In agreement with the findings presented here, Winter concluded that selective isolation of *B. anthracis* by available procedures, including the use of PLET, was not possible and that the isolation plates were always overgrown by related organisms. He did note, however, an inverse relationship between presence of *B. anthracis* bacteriophage and the occurrence of anthrax; anthraphage was isolated mainly in non-anthrax areas and also in greater frequency during the dry season than during the wet season. It is conceivable that higher phage levels may result in lower *B. anthracis* numbers and thereby contribute to the lower incidence of the disease in the non-enzootic areas.

5 CONCLUSIONS

The failure in this study to isolate *B. anthracis* from water and soil samples collected from natural fountains, natural pans and gravel pits may be attributable to a number of factors: (i) the abnormally low rainfall and the consequent failure of a postulated ecological anthrax cycle; (ii) insufficiently sensitive methods and media for detection of low numbers of *B. anthracis*; (iii) insufficiently selective media for inhibiting the large numbers of non-anthrax *Bacillus* species, particularly *B. cereus,* present in all samples.

As judged by positive isolations from the five animals found dead of anthrax and positive soils under two of these, the inhibitory media used were helpful for purifying the isolates but not adequate for primary isolation purposes despite promising results in laboratory tests. Apparent ecological associations between anthrax and artificial water holes are not readily attributable to pH or other chemical differences or differences in concentrations of other aerobic bacteria.

It is hoped that the opportunity will arise in the near future to re-attempt to isolate *B. anthracis* from these different sites using improved methods and media and at a time when rainfall is relatively high. Studies on the growth or decline of the organism in samples of water from the different sources following inoculation with appropriate spore suspensions are envisaged and may throw light on differences with respect to anthrax between artificial and natural water holes.

In addition, work is underway to develop sensitive methods of detecting immunity to anthrax in man and animals and it is intended that these should be used as soon as is feasible to determine whether natural antibody can be detected in any of the suspectible species. A plan of appropriate core vaccination programmes and a suitable vaccination schedule can then be drawn up for species in danger.

6 SUMMARY

During a four week period from mid-March to mid-April 1983, 45 sets of pooled water and soil samples were collected from the circumferences of 12 gravel pits, 16 natural fountains and 3 natural pans in the Etosha National Park. The gravel pits and natural pans all lay in a broadly defined "anthrax enzootic" region. *B. anthracis* was not isolated from any of these samples. It was, however, isolated from five animals found dead of anthrax and from contaminated soil beneath two of these. The positive animal and soil samples were used to assess under field conditions a newly developed selective agar (TCB agar) containing 175 μg/ml trimethoprim and 7.5 μg/ml colistin and to compare it with an existing selective polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar. Despite promising results in forerunning laboratory trials, neither media were sufficiently inhibitory to effectively prevent growth on primary culture of non-anthrax bacilli, particularly *B. cereus,* under the challenge posed by the Etosha samples. On the other hand, they were of value in secondary purification of positive isolates. *Ad hoc* selective enrichment attempts using brain-heart infusion broth with trimethoprim at the same level as in TCB and freshly collected zebra blood did not result in better isolations. The aetiological association between anthrax and artificial water holes could not be readily attributed to pH differences or related to marked differences in aerobic bacterial counts in the two types of water. Negative isolations

from environmental specimens may be attributable in part to the abnormally low rainfall during the particular season in which the study took place. The relationship between the reported findings and the aetiology of anthrax in the Etosha National Park is discussed.

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8 DEDICATION

This paper is dedicated to the memory of Dr. Ian M. Hofmeyr, killed in a vehicle accident in the Etosha National Park on 6 November, 1984. His wildlife expertise held worldwide acclaim and his untimely death came as an inestimable loss to the Etosha National Park and to the cause of nature conservation both in southern Africa and the world as a whole.

9 REFERENCES

BERRY, H.H.
1981 Abnormal levels of disease and predation as limiting factors for wildebeest in the Etosha National Park. *Madoqua* 12: 242—253.

BROSTER, M.G.
1984 Personal communication. Defence Microbiology Division, Chemical Defence Establishment, Porton Down, Salisbury, Wilts, UK.

CARMAN, J.A.
1984 Personal communication. Vaccine Research and Production Laboratory, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts, UK.

CARMAN, J.A., HAMBLETON, P. and MELLING, J.
1984 The isolation and identification of *Bacillus anthracis*. *In:* Collins, C.H. and Grange, J.M. (Editors). *Isolation and identification of microorganisms of medical and veterinary importance*. London: Academic Press.

DAVIES, J.C.A.
1983 A major epidemic of anthrax in Zimbabwe. II. Distribution of cutaneous lesions. *Central African J. Med.* 29: 8—12.

EBEDES, H.
1976 Anthrax epizootics in Etosha National Park. *Madoqua* 10: 99—118.

EBEDES, H.
1981 A new look at anthrax. *In:* Fowler, M.E. (Editor). *Wildlife diseases of the Pacific basin and other countries*. Proceedings of the 4th International Conference of the Wildlife Disease Association, 25—28 August, 1981, Sydney, Australia.

GORDON, R.E., HAYNES, W.C. and PANG, C.H-N.
1973 The genus *Bacillus*. United States Department of Agriculture, Agricultural Research Service, Agriculture Handbook No. 427, Washington, D.C.: U.S. Government Printing Office.

HAMBLETON, P., CARMAN, J.A. and MELLING, J.
1984 Anthrax: the disease in relation to vaccines. *Vaccine* 2: 125—132.

ICMSF
1978 *Microorganisms in foods. 1. Their significance and methods of enumeration*, 2nd edition, pp 119—120. International Commission on Microbiological Specifications for Foods (ICMSF). Toronto, Canada: University of Toronto Press.

KNISELY, R.F.
1966 Selective medium for *Bacillus anthracis*. *J. Bacteriol.* 92: 784—786.

MANCHEE, R.J., BROSTER, M.G., MELLING, J., HENSTRIDGE, R.M. and STAGG, A.J.
1981 *Bacillus anthracis* on Gruinard Island. *Nature* 294: 254—255.

MANCHEE, R.J., BROSTER, M.G., ANDERSON, I.S. and HENSTRIDGE, R.M.
1983 Decontamination of *Bacillus anthracis* on Gruinard Island? *Nature* 303: 239—240.

McGETRICK, A.M.T., TURNBULL, P.C.B., OPPENHEIM, B.A. and KOORNHOF, H.J.
1982 Selective media for *Bacillus anthracis*. Proceedings of the second annual congress of the South African Society for Microbiology, 15—17 September, 1982, Pretoria.

MILES, A.A. and MISRA, S.S.
1938 The estimation of the bactericidal power of the blood. *J. Hyg. (Camb).* 38: 732—748.

OPPENHEIM, B.A. and KOORNHOF, H.J.
1980 A selective medium for anthrax. Proceedings of the congress of the South African Society of Pathologists, 7—9 July, 1980, Johannesburg.

PARRY, J.M., TURNBULL, P.C.B. and GIBSON, J.R.
1983 A colour atlas of *Bacillus* species. Wolfe Medical Atlas series No. 19. London: Wolfe Medical Publications Ltd.

SHAPOVALOVA, M.F.
1966 A method of isolating anthrax bacilli from the soil. *Zh. Mikrobiol., Epidemiol. i Immunobiol.* 43: 144—146 (In Russian).

TURNBULL, P.C.B. and KRAMER, J.M.
1983 Non-gastrointestinal *Bacillus cereus* infections: an analysis of exotoxin production by strains isolated over a two-year period. *J. Clin. Pathol.* 36: 1091—1096.

WINTER, C.T.
1985 The water quality of water-holes utilised by game in the Etosha National Park. *Madoqua* 14: 145—153.

VAN NESS, G.B.
1971 Ecology of anthrax. *Science* 172: 1303—1307.