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THE BIOCHEMICAL, MORPHOLOGICAL AND VIRULENCE PROFILES OF *BACILLUS ANTHRACIS* ISOLATED IN THE KRUGER NATIONAL PARK

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ABSTRACT

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The biochemical, morphological and virulence profiles of 44 *Bacillus anthracis* isolates, obtained from various localities and carcass remains of wild animals in the Kruger National Park, South Africa, were examined. The morphological characteristics tested for included: the formation of capsules on bicarbonate agar, the motility of the vegetative organism, the presence of haemolysis on blood tryptose agar, the sensitivity of the vegetative organism to bacteriophage, the production of lecithinase on egg yolk agar, the liquefaction (hydrolysis) of gelatine and the capability of each isolate to produce mucoid colonies when grown on bicarbonate agar with horse serum in an atmoshere containing CO_2. The API 50CHB and 20E systems were used to evaluate the biochemical activity of each isolate. The virulence of each isolate was determined by its LD50, using an inbred line of Balb/C mice.

A clear pattern in the biochemical reactions emerged that appeared to be specific for each isolate. On the API 50CHB test strip, only 2 of the 44 isolates gave a 100 % positive reaction to all 10 of the biochemical substances to which it was supposed to react, 9 gave positive results to 90 %, 19 were positive to 80 %, and 14 were positive to 70 %. The reactions on the API 20E were completely different from what was expected, with only 1 of the biochemical activities (gelatinase production) showing a positive reaction to all the isolates. The virulence test indicated that 27/44 isolates could be regarded as highly virulent with a LD50 of <1 000 organisms, and the rest of the isolates as virulent with a LD 50 of 1 001—10 000 organisms. The other morphological characteristics demonstrated the typical nature of *Bacillus anthracis.* Three control isolates, one being the non-capsular, avirulent toxigenic Sterne strain were included in this study.

INTRODUCTION

The Kruger National Park (KNP) in South Africa covers a vast expanse of subtropical savanna woodland area, where large numbers of game animals still occur in their natural state. Apart from being a wildlife sanctuary, it is also an important endogenous anthrax area, a number of animals playing an important role in the epidemiology of the disease. Although a number of animal species, including cheetah, eland, elephant, gemsbok, giraffe, ostrich, buffalo, springbok, wildebeest and zebra are susceptible to *Bacillus anthracis* infection, it is also known that species like the kudu and roan antelope are more vulnerable. It has been reported that this disease can infect and cause mortalities in 27 species of animals occurring in the Kruger and Etosha National Parks (McConnell, Tustin & De Vos, 1972; Turnbull, Hofmeyr, McGetrick & Oppenheim, 1986; De Vos, 1989).

Various methods and procedures, including the use of differential and selective media, have been described to isolate and identify *Bacillus anthracis* (Knisely, 1965; Knisely, 1966) and to distinguish it from other *Bacillus* species. The most important of these being *Bacillus cereus* (Leise, Carter, Friedlander & Freed, 1959; Cottral, 1978; Norris, Berkeley, Logan & O'Donnell, 1981; Parry, Turnbull & Gibson, 1983; Doyle, Keller & Ezzell, 1985; Carman, Hambleton & Melling, 1985; Logan & Berkeley, 1984; Logan, Carman, Melling & Berkeley, 1985; Turnbull *et al.*, 1986; Bisping & Amtsberg, 1988). The biochemical and morphological characteristics of *Bacillus anthracis* have been described in great detail by most of these authors and play an important role in the identification of this organism. The study by Logan *et al.* (1985) utilized the API 50CHB and 20E systems and demonstrated that even the avirulent and slightly virulent isolates of *Bacillus anthracis* gave the typical biochemical reactions of this organism. The *Bacillus anthracis* isolates used in their study originated from different sources in the United States of America and the United Kingdom, and were isolated from cattle, soil and human infections.

The *Bacillus anthracis* isolates used in this study were isolated from specimens from an active, epidemiological region that included contaminated soil and animal carcasses in the Kruger National Park. The paucity of descriptive information on typical organisms regarding their morphological and biochemical characteristics, isolated from an African environment, prompted this investigation. This study was therefore initiated to confirm and document some of the biochemical, morphological and virulence characteristics of each of the 44 isolates of *Bacillus anthracis* isolated in the Kruger National Park. The biochemical activities of the isolates were tested with the API 50CHB and 20E test systems, while the morphological characteristics were evaluated regarding the ability of each isolate to form capsules, the absence of haemolysis on blood agar, the presence of mucoid colonies on bicarbonate agar, susceptibility to a bacteriophage, motility, lecithinase production and hydrolysis of gelatine. The virulence was determined by means of the LD50 test. The avirulent Sterne isolate of *Bacillus anthracis,* along with 2 other isolates of this organism, were included as positive controls.

MATERIAL AND METHODS

Bacillus anthracis *isolates*

The 44 different isolates of *Bacillus anthracis* were isolated from different areas in the KNP during routine epidemiological surveys. For comparison, 3 isolates already identified as *Bacillus anthracis* from the Veterinary Research Institute, Onderstepoort, were included as positive controls. One of these was the avirulent, non-capsular and toxigenic Sterne

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vaccine isolate (Control 3). Isolate numbers 444, 448, 450, 479, 481, 482, 484, 486, 487, 488, 489, 493, 494, 495 and 497 were isolated from the remains of animal carcasses that could not be identified. The rest of the isolates were obtained from soil specimens.

The initial isolation was done on PLET medium (Knisely, 1965; Knisely, 1966), followed by subculture on sheep blood tryptose agar from which single colonies were freeze dried. The absence of haemolysis and pathogenicity in mice were the parameters for tentative identification.

Biochemical tests

The isolates were first inoculated on 5 % sheep blood tryptose agar[1] (BTA) and incubated aerobically overnight at 37 °C. Suspensions of the vegetative organisms were made in sterile physiological saline and compared to a McFarland standard opacity tube No. 3 before the test strips were inoculated with vegetative organisms. The API 50CHB test strips[2], containing 49 different carbohydrates and 20E[2] with 20 different biochemical tests, were inoculated according to the instructions of the manufacturer. The reactions were noted after 24 and 48 h, but the biochemical reagents for the 50CHB and 20E galleries were added after the 48 h viewing. The reactions were scored from 1–5, and scores of 3, 4 and 5 were regarded as positive.

The results presented in Table 1 indicate the number of isolates with positive reactions for each of the biochemical tests.

Preparation of spore suspension for virulence tests

The isolates were activated on 5 % sheep blood tryptose agar and incubated overnight at 37 °C. Subcultures were made on the sporulation agar medium of Norris *et al.* (1981), containing manganese sulphate[3], and incubated for 48 h at 37 °C. The spores were harvested in sterile distilled water and diluted to contain 1×10^4, 1×10^5 and 1×10^6 cells per mℓ. The dilutions were placed in boiling water for 10 min in order to kill vegetative organisms, to confirm spore formation and stimulate germination. Colony counts were performed on BTA from the same spore dilutions immediately after intraperitoneal injection into mice.

LD50 tests

A local, inbred line of Balb/C mice, males and females, mass-measuring between 18–22 g, were used. They were housed under disease-free conditions and fed *ad lib.* a commercial pelleted ration and sterilized water. Groups of 5 mice were used for each dilution. An injection of 0,1 mℓ containing the required spore concentration, was given intraperitoneally to each animal. After this, each group was accommodated in a Type 2 cage on a wire mesh platform, without bedding, and placed in a Class 3 stainless steel cabinet with a Hepa filtered air outlet.

The animals were inspected daily for 7 days, during which time all the dead animals were removed and autoclaved for 60 min at 121 °C. After 7 days, all the remaining animals were euthanized with carbon dioxide. The LD50 calculations were made, as described by Reed & Muench (1938) and Aulisio & Stanfield (1984). An LD50 with <1 000 organisms was regarded as highly virulent (+++), between 1 001–10 000 as virulent (++), >10 000 as low virulent (+) and those with no mortalities as avirulent (−).

Motility

A stab culture with a sterile glass Pasteur pipette was made into motility medium (Cowan, 1974) in 15 × 90 mm stoppered glass test tubes, and incubated aerobically for 48 h at 37 °C. The lack of motility was recorded as the absence of growth away from the line of stab inoculation. A 2nd method to determine motility was used by mixing a loop of an overnight culture from tryptone soy broth[1], containing the vegetative organisms, in a drop of sterile saline and observing the organism in a hanging drop below a cover slip with phase contrast at 1 000 × magnification.

Haemolysis

The isolates were streaked on 5 % sheep blood tryptose agar and incubated aerobically for 48 h at 37 °C. Haemolysis was observed visually after 24 and 48 h and recorded. The plates were then left at room temperature for 14 days before a final examination for haemolysis was made and finally discarded.

Growth on bicarbonate agar

Each isolate was inoculated onto nutrient agar[1] containing 0,5 % sodium bicarbonate and 20 % horse plasma, and incubated in a jar with carbon dioxide generated from an Anaerocult C[3] bag, for 24 h at 37 °C. The isolates were then examined for the presence of mucoid colonies (Parry *et al.*, 1983).

Capsule staining procedure

After overnight incubation on the bicarbonate agar, mucoid colonies from each isolate were selected on macroscopical appearance. A wet preparation was prepared from each isolate by suspending some of these organisms in ± 50 µℓ of 40 % formalin on a microscope glass slide and mixing it with the same volume of Winsor & Newton, black Indian ink (951)[4]. This was covered with a cover slide, compressed lightly to allow the viewing of single organisms and examined at 400 × magnification under phase contrast (Parry *et al.*, 1983).

Susceptibility to bacteriophage

A bacteriophage, isolated from a watering hole in the Etosha National Park, was kindly made available to us by Dr H. Jaeger from the Central Veterinary Laboratory in Windhoek. It was assumed that it had similar properties to the original gamma phage, as it only lyses *Bacillus anthracis* and not *Bacillus cereus*. Sheep BTA was inoculated with a 0,2 mℓ suspension of vegetative cells to produce confluent growth on the agar surface. This was inoculated with 0,1 mℓ of a dilution containing approximately 10^7 bacteriophage plaque-forming units per mℓ. After allowing the phage inoculum to dry for 30 min, it was incubated for 24 h at 37 °C (Logan *et al.*, 1985). An area devoid of growth indicated susceptibility to the bacteriophage.

Lecithinase production

Egg yolk agar plates were prepared and inoculated with each isolate and incubated for 48 h at 37 °C (Kautter, Lynt & Solomon, 1984).

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[3] Merck Chemicals, P.O. Box 1998, Midrand 1685
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Hydrolysis of gelatine

Gelatine media were prepared in 17 × 120 mm glass stoppered test tubes and inoculated with a stab culture of each isolate. Incubation took place for 4 weeks at 22 °C, after which it was placed in the refrigerator for 4 h to enhance solidification (Parry *et al.*, 1983).

RESULTS

Biochemical tests with API 50CHB and 20E

With the API 50CHB test strip positive reactions were recorded for all the isolates (44/44) with ribose, glucose, N-acetyl-glucosamine, sucrose and trehalose (Table 1). The majority of all the isolates (43/44) tested positive for maltose and esculine. Some isolates had low positive rates for certain of the biochemical reactions, such as d-xylos (1/44), galactose (2/44), sorbitol (1/44), arbutine (5/44), lactose (2/44), melezitose (2/44), and d-fucose (1/44). All 3 of the control isolates, including the avirulent Sterne isolate, gave positive reactions to ribose, glucose, N-acetyl-glucosamine, fructose, esculine, sucrose, trehalose, maltose, starch and glycogen. Negative reactions were recorded with all 44 isolates for glycerol, arabinose, L-arabinose, L-xylose, xyloside, sorbose, rhamnose, alpha-methyl-D glucocide, alpha-methyl-D mannoside, dulcitol, inositol, mannitol, amygdalin, salicin, melibiose, inulin, raffinose, xylitol, gentiobiose, D-turanose, D-tagatose, D-xylose, L-fucose, D-arabitol, L-arabitol, 2-keto gluconate and 5-keto gluconate.

TABLE 1 The percentage of *Bacillus anthracis* isolates positive for each of the biochemical characteristics (excluding the control isolates)

Biochemical test	Isolates pos. (total)	Percentage of isolates with positive reaction %
Amidon/starch	13/44	30
Arbutine	5/44	11
Cellobiose	10/44	22
D-fucose	1/44	2
D-xylose	1/44	2
Esculine	43/44	98
Fructose	17/44	39
Galactose	2/44	4
Gluconate	11/44	25
Glucose	44/44	100
Glycogen	14/44	32
Lactose	2/44	4
Maltose	43/44	98
Melezitose	2/44	4
N-acetyl-glucosamine	44/44	100
Ribose	44/44	100
Sorbitol	1/44	2
Sucrose	44/44	100
Trehalose	44/44	100

The information leaflet accompanying the API 50CHB test kit illustrates quite clearly the 10 substrates to which *Bacillus anthracis* could react positive. Out of the total of 44 isolates, 14 gave positive reactions with 70 % of the substrates, 19 reacted with 80 %, 9 with 90 % and only 2 isolates reacted with 100 % of the substrates. The results with the API 20E test strip indicated that only with the gelatine (41/44), Voges Proskauer (6/44) and glucose (1/44) did isolates give positive reactions. All the other tests were negative.

Virulence tests (LD50)

Of the 44 isolates of *Bacillus anthracis* tested for virulence with the LD50 test, 27 were regarded as highly virulent (+++) with a calculated LD50 value of <1 000 organisms, and 17 isolates as virulent (++) with a LD50 value of 1 001–10 000 organisms (Table 2). No avirulent isolates were encountered, apart from the Sterne non-pathogenic/avirulent, non-capsulated vaccine isolate (control isolate 3). Control isolates 1 and 2 were of high (+++) and low (+) virulence, respectively.

TABLE 2 The LD50 values for each of the *Bacillus anthracis* isolates calculated after 7 days

Isolate No	LD50 × (10^x)	No. of orgs	Virulence
369	<3	<1 000	+++
437	3,80	6 310	++
438	<3	<1 000	+++
439	<3	<1 000	+++
440	3,49	3 090	++
441	<3	<1 000	+++
442	<3	<1 000	+++
443	<3	<1 000	+++
444	<3	<1 000	+++
446	3,37	2 344	++
447	<3	<1 000	+++
448	<3	<1 000	+++
449	<3	<1 000	+++
450	<3	<1 000	+++
451	3,21	1 622	++
466	3,37	2 344	++
467	3	1 000	+++
468	3,15	1 413	++
469	3,53	3 388	++
470	3	1 000	+++
471	4	10 000	++
474	<3	<1 000	+++
475	3,37	2 344	++
476	<3	<1 000	+++
477	3,50	3 162	++
478	<3	<1 000	+++
479	<3	<1 000	+++
480	3,83	6 761	++
481	<3	<1 000	+++
482	<3	<1 000	+++
483	3,50	3 162	++
484	<3	<1 000	+++
485	3,83	6 761	++
486	<3	<1 000	+++
487	<3	<1 000	+++
488	<3	<1 000	+++
489	<3	<1 000	+++
490	<3	<1 000	+++
491	3,83	6 761	++
492	3,88	7 586	++
493	3,88	7 586	++
494	<3	<1 000	+++
495	<3	<1 000	+++
497	3,88	7 586	++
672 (cont 1)	<3	<1 000	+++
673 (cont 2)	4,30	19 953	+
OP (cont 3)	0	0	−

+++ = high virulent; ++= virulent; += low virulent; −= avirulent

Morphological characteristics

Motility

All the isolates tested, including the 3 controls, were non-motile (Table 3), with the motility medium and the direct microscopic method.

Haemolysis

Haemolysis was absent in all the isolates after 48 h, but in 13 isolates slight haemolysis was present after 14 days.

TABLE 3 The morphological characteristics of the *Bacillus anthracis* isolates

Isolate No.	Haemolysis on BTA		Mucoid colonies	Gelatine hydrolysis	Susceptible to bacteriophage
	After 24 h	14 days			
369	−	−	+	−	+
437	−	−	+	−	+
438	−	−	+	+	+
439	−	−	+	+	+
440	−	−/+	+	+	+
441	−	−	+	−	+
442	−	−/+	+	+	+
443	−	−	+	−	+
444	−	−	+	+	+
446	−	−/+	+	−	+
447	−	−/+	+	+	+
448	−	−/+	−	−	+
449	−	−/+	+	−	+
450	−	−/+	+	−	+
451	−	−/+	+	−	+
466	−	−/+	+	−	+
467	−	−/+	+	−	+
468	−	−/+	+	+	+
469	−	−	+	−	+
470	−	−	−	−	+
471	−	−	+	−	+
474	−	−	+	−	+
475	−	−	+	−	+
476	−	−	+	+	+
477	−	−	+	+	+
478	−	−	+	−	+
479	−	−	+	−	+
480	−	−	+	+	+
481	−	−	+	+	+
482	−	−	−	+	+
483	−	−	+	+	+
484	−	−	+	−	+
485	−	−	+	−	+
486	−	−	+	−	+
487	−	−/+	+	+	+
488	−	−	+	+	+
489	−	−	+	+	+
490	−	−	+	+	+
491	−	−	+	+	+
492	−	−	−	+	+
493	−	−	+	+	+
494	−	−	−	+	+
495	−	−/+	+	−	+
497	−	−	+	+	+
672 (cont 1)	−	−	−	−	+
673 (cont 2)	−	−	−	+	+
OP (cont 3)	−	−	−	−	+

Growth on bicarbonate agar

Five of the 44 isolates which had a dry and butyrous appearance failed to develop mucoid colonies on the bicarbonate agar. The mucoid appearance of the positive colonies was easily distinguished and very characteristic. When touched with a plastic disposable microbiological loop the mucoid colonies had a stringy and tenacious consistency.

Presence of capsule

Capsules were demonstrated in all but 1 of the isolates examined under phase contrast with the wet Indian ink preparation. The capsules were distinctly visible as a white, well-demarcated area surrounding the vegetative organism. The Sterne avirulent isolate did not have a capsule.

Susceptibility to bacteriophage

All the *Bacillus anthracis* isolates were susceptible to the activity of the gamma-like bacteriophage, including the avirulent non-capsular Sterne strain and the 2 positive controls.

Lecithinase production

All the isolates produced lecithinase, visible as a distinct zone of opacity on egg yolk agar.

Hydrolysis of gelatine

Gelatine hydrolysis occurred in 50 % of the isolates.

DISCUSSION

The information provided by the API 50CHB test on *Bacillus anthracis* was useful to confirm its identification. The reactions for *Bacillus cereus* and *Bacillus anthracis* were very similar with the API 50CHB system (Logan *et al.*, 1984; Logan *et al.*, 1985), and it is very difficult, if not impossible, to distinguish between these 2 species if this is the only parameter measured. There was no difference between the biochemical and morphological characteristics of virulent, low virulent and avirulent *Bacillus anthracis* isolates (Logan *et al.*, 1985). In this study, the only avirulent isolate, the Sterne strain, utilized the same biochemical substrates and also had the same morphological characteristics, apart from its virulence, than the other isolates. All the isolates (100 %) tested in this study utilized ribose, d-glucose, N-acetyl-glucosamine, sucrose and trehalose, whereas only 98 % utilized maltose and esculin. This corresponds with the findings of Logan *et al.*, (1985). Differences were noted for starch and glycogen, our

findings indicating 31 % and 33 % of isolates positive as compared to 97 % and 92 % of isolates obtained by Logan *et al.*, (1985), respectively. The results with the API 20E test system were very disappointing, with only the liquefaction of gelatine giving consistent, positive results with most of the isolates; the rest of the substrates were not utilized at all. Results similar to ours were obtained elsewhere (Dr H. Jaeger, personal communication, 1990). This is in contrast to the results obtained by Logan *et al.*, (1984; 1985). No obvious reason for these differences is apparent.

One of the most important differences between *Bacillus cereus* and *Bacillus anthracis* is the mortality caused by spores of the latter organism and is indicative of its virulence when administered parenterally to mice. The determination of the LD50 values provides this information. The use of an inbred strain of Balb/C mice, injected with dilutions of *Bacillus anthracis* spore suspension, provided highly reproducible data. Of the KNP isolates examined, 61 % (27/44) were highly virulent and 39 % (17/44) virulent. An interesting fact emerging from these results was that 87 % (13/15) of the carcass isolates and only 48 % (14/29) of the soil isolates were highly virulent (+++) and the rest virulent (++). The reason for this phenomenon is not known.

Of the 3 controls only the Sterne strain was avirulent, the other 2 being highly virulent and virulent. Another important difference between *Bacillus cereus* and *Bacillus anthracis* is the distinct motility of *Bacillus cereus*. Although the motility medium was included here as a means of detecting motility, we found the direct microscopic method with the hanging drop more convenient, reliable and faster to perform. A definite advantage of the microscopic method is the direct detection of bacterial contamination, which could mar the interpretation of the motility reaction with inoculation of the motility medium. With the direct microscopic observation, the complete lack of motility on the part of *Bacillus anthracis* was very obvious and the typical chain-like growth pattern was observed.

None of the *Bacillus anthracis* isolates, including the 3 controls, showed any signs of inducing haemolysis when observed after 24 and 48 h of incubation. However, after being left at room temperature for 14 days signs of slight haemolysis were evident in 13 isolates. A similar phenomenon has been observed elsewhere (Dr H. Jaeger, personal communication, 1990). The reason for this is not known. With 5 exceptions, all the isolates had a distinct mucoid appearance after incubation on bicarbonate agar. These isolates had a drier and more butyrous texture, and only 1 of them did not have a capsule. But with the LD50 test, 4 of them were highly virulent (+++) and one virulent (++). This could be the result of a mutation, affecting the presence of the capsule but not its virulence. The selection of mucoid colonies is essential when demonstrating capsules, and making wet preparations with Indian ink negativestain, is a very convenient and effective method. The bacteriophage was active on all of the *Bacillus anthracis* isolates and clear zones of bacteriolysis were seen on BTA. Contaminated cultures are easily demonstrated by this method, as witnessed with the growth of the contaminating organism present in the lysed area.

The biochemical utilization of substrates to identify bacterial organisms is commonly used, *Bacillus anthracis* being no exception. From the results obtained in this study it is deduced that for an organism to be classified as *Bacillus anthracis* it is not necessary for it to utilize all 10 substrates, as indicated by the API 50CHB identification table. The evaluation of other characteristics play an important role in making a final decision on the identification of *Bacillus anthracis*. These include the absence of motility, absence of haemolysis on BTA, the presence of a capsule, susceptibility to a specific bacteriophage (gamma phage) and its virulence. The production of lecithinase and hydrolysis of gelatine provides additional important information on a specific isolate, but is not crucial when separating it from other species like *Bacillus cereus*. All the isolates examined in this study could be regarded as organisms representing some of the possible variations of organisms identified as *Bacillus anthracis*.

It is clear that *Bacillus anthracis* isolates from the Kruger National Park share many properties with isolates from other localities (Logan *et al.*, 1985), but that variations in the biochemical and morphological profiles can and does occur.

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