

ANTHRAX

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SUMMARY

Anthrax is primarily a disease of herbivorous animals, although all mammals including humans, and at least some avian species can contract it. Anthrax is caused by *Bacillus anthracis*. Animals usually acquire the bacterium by the ingestion of contaminated substances (feed, grass, water or infected carcass) and hence suffer most commonly from the gastrointestinal form of the disease. Cutaneous anthrax probably does occur on occasion through the bite of bloodsucking flies or via a wound or abrasion.

There are three primary clinical forms of anthrax in animals: the peracute form in which death occurs within a few hours of the onset of clinical signs; the acute form in which death occurs from 24 h to a few days after onset; and the subacute or localised form which lasts for several days and may end in recovery. Fever, muscle tremor, dyspnoea, and congestion of the mucosae may be seen in the peracute form and the animal soon collapses and dies after terminal convulsions. Severe depression and listlessness are usually seen first in the acute form, although they are sometimes preceded by a period of excitement. In the subacute form, the animal may at first appear depressed or excited and then show inappetence, weakness, prostration and death. The body temperature is high and may reach 42°C, and respiration rapid and deep. The mucosae are congested and haemorrhagic, and the heart rate much increased.

Identification of *B anthracis*

The detection of capsulated bacilli in large numbers in a smear prepared from blood or tissue fluid and stained with polychrome methylene blue (M'Fadyean's stain) is sufficient for the diagnosis of anthrax. It is also usually easy to identify isolates of *B anthracis* cultured on nutrient or blood agar because of their characteristic appearance. This appearance is better defined on blood agar where colonies have a matt appearance and are fairly flat, similar to *Bacillus cereus* but generally rather smaller, tackier, white or grey-white, and are non-haemolytic or only weakly haemolytic.

Other useful characteristics to confirm the identity of *B anthracis* are sensitivity to penicillin and the diagnostic gamma bacteriophage although some genotypes are not sensitive to these agents. Polymerase chain reaction (PCR) assays are available for detecting the toxin and capsule genes. Such PCRs can confirm the virulence as well as the identification of *B anthracis*.

The isolation of *B anthracis* from old, decomposing carcasses, processed specimens (bone meal, hides), or environmental samples such as contaminated soil requires procedures that involve the thermal destruction of saprophytic contaminants and culture on PLET (polymyxin, lysozyme, EDTA [ethylene, diamine tetraacetic acid], thallos acetate) agar.

Serological tests

There are no commonly used or commercially available serological tests for the diagnosis of anthrax in animals.

Status of anthrax in Australia and New Zealand

In Australia, anthrax usually occurs as sporadic incidents in herbivores and in areas that have neutral to alkaline subsoil and on floodplains along waterways. New Zealand has been free from anthrax since 1954.

Introduction

Aetiology

Anthrax is caused by the bacterium *Bacillus anthracis*, which is a Gram positive rod 3.0-5.0 µm long and 1.0-1.2 µm wide. *B anthracis* produces two pathogenicity factors: a capsule which is carried on the pX02 plasmid, and a tripartite toxin consisting of a protective antigen, an oedema factor and a lethal factor. The tripartite components are carried on the pX01 plasmid. Both factors are necessary for normal virulence. The capsule is thought to inhibit phagocytosis by leucocytes during infection and the toxin components are thought to be responsible for oedema and death.¹

There is a lack of molecular polymorphisms within *B anthracis*.² The biochemical, serological or phage typing methods available in the case of other pathogens have proved to be of no value for identifying different strains of *B anthracis*.

However, use of a novel molecular typing system based on rapidly evolving variable-number tandem repeat (VNTR) loci has differentiated strains.³ In this system, fluorescently labelled PCR primers were used to produce PCR amplification products from 8 VNTR regions in the *B anthracis* genome. Six major groups were identified: the A subdivision which consisted of groups A1, A2, A3 and A4, and the B subdivision which consisted of groups B2 and B1. All Australian isolates that have been typed were classified in the A3 group.

More recently, use of a quantitative PCR and inoculation of guinea pigs with a range of *B anthracis* strains showed that *B anthracis* virulence is associated with the clonality and virulence of plasmids pX01 and pX02.⁴ An increasing gradation of virulence of *B anthracis* strains was demonstrated to be associated with increased plasmid copy numbers.

Epidemiology

Anthrax is a disease of most animal species, caused by *B anthracis*. Until the development of effective veterinary vaccines in the 1930s, anthrax was a major cause of heavy losses of cattle, sheep and goats throughout the world. This is still the case in affected regions of developing countries.⁵ During the past 30 years, a progressive, worldwide reduction in anthrax in animals has followed national programmes in which vaccination was a key element.⁶

In domestic animals and wild animals, an outbreak of anthrax usually tends to affect one species more than others and only a proportion of those exposed are usually affected. In New South Wales there is a northern zone in which bovine and ovine anthrax occur with equal frequency and a southern zone where bovine anthrax is 4 times as common as ovine, with death rates in cattle 13 times greater than in sheep.⁷

Mortality can be very high, especially in herbivores which play a central role in anthrax epizootics. As death approaches, haemorrhagic effusions from the nostrils, mouth and anus further contaminate the soil. The blood at death carries from 10^8 to $> 10^9$ bacilli per mL. These bacteria sporulate on exposure to oxygen in the air.

The usual cycle of infection is:

1. Uptake of spores by the animal feeding or drinking.
2. Entry of spores through a lesion along the gastrointestinal tract and carriage to the regional lymph nodes and beyond.
3. Multiplication in the lymph nodes and spleen.
4. Endothelial breakdown of vessels and sudden release of bacilli and toxin leading rapidly to death.
5. Shedding of vegetative bacilli by the dying or dead animal.
6. Sporulation on exposure to oxygen in the air.
7. Infection of a further animal by the spores, which may in the meantime have been widely spread by fomites or by the wind.⁵

The vegetative form of *B. anthracis* is easily inactivated by exposure to moderate temperatures, disinfectants and the putrefactive action of bacteria in an unopened carcase. However, spores survive in the environment for a long time. Spores prepared by Pasteur in 1888 were still viable 68 years later.⁸ Spores in soil can also survive for many decades.^{5,9} Subsoils that are pH 9 and calcareous in nature are conducive for spore survival.¹⁰

Clinical signs

Death of sheep or cattle without prior signs should lead to suspicion of anthrax, particularly when bloody fluid exudes from an orifice of a carcase in an 'anthrax area'. This is most common at the beginning of an outbreak. The course of such peracute infections is probably about 1-2 h. However, fever, muscle tremor, dyspnoea, and congestion of the mucosae may be observed. The animal soon collapses and dies after terminal convulsions. Acute infections run a course of about 48 h. Severe depression and listlessness are usually observed first, although they are sometimes preceded by a period of excitement. The body temperature is high, up to 42°C, respiration is rapid and deep, mucosae are congested and haemorrhagic, and the heart rate is much increased. Pregnant cows may abort. Dairy cattle may show a much decreased milk production and the milk may be blood stained or deep yellow in colour.¹¹

Subcutaneous oedema and sometimes colic is seen in horses before death. Pigs are usually feverish, dull and anorexic with swelling of the neck and face and sometimes with blood-stained froth at the mouth. Swelling around the pharynx restricts respiration, causing laboured breathing and cyanosis of mucous membranes. Dysentery and/or constipation may also be seen. Pigs die 1-7 days after these signs develop and piglets die of septicaemia. Anthrax bacilli localise in the lungs, causing respiratory signs and blood-stained froth from the mouth. Dogs and cats are generally highly resistant to anthrax.¹⁰

Gross pathology

If anthrax is suspected, the carcase should not be opened as exposure of vegetative *B. anthracis* in spilled blood to atmospheric oxygen leads to sporulation, contamination of the environment and risk of infection of the pathologist. However, on necropsy, tarry unclotted blood, absence of rigor mortis and an enlarged, haemorrhagic spleen are indicators of anthrax.¹²

An enlarged spleen is a characteristic feature of anthrax in cattle but is uncommon in sheep, pigs, cattle and horses. The mesentery may be thickened and oedematous, with excess peritoneal, pleural and pericardial fluid. Petechial haemorrhages may be visible in many organs, and the intestinal mucosa may be dark red and oedematous, with some areas of necrosis. Not all the signs are uniformly present in all cases of anthrax.^{10,12}

Diagnostic Tests

Identification of *Bacillus anthracis*

Demonstration of encapsulated *B. anthracis* in smears of blood or tissues from fresh anthrax-infected carcasses and growth of the organism on blood plates are straightforward. There may be difficulty with samples from pigs and carnivores in which the terminal bacteraemia is frequently not marked, or in animals that were treated with antimicrobial substances.¹⁴ If blood smears from pigs and carnivores are negative, then, to eliminate the possibility of anthrax, smears should be prepared from localised lesions with care to minimise exposure of blood and exudates to air.

Fresh specimens

Demonstration of capsule

Virulent encapsulated *B. anthracis* is present in tissues and other body fluids from animals that have died from anthrax. These capsules can be demonstrated in a thin smear of blood or tissue fluid and stained with the preferred stain, polychrome methylene blue (M'Fadyean's reaction) (Appendices 1 and

2).⁶ However, the capsule may not be readily detected if the animal has been dead for 6 hours or more. The capsule stains pink, whereas the bacillus cells stain dark blue. The cells are found in pairs or short chains and are often square-ended. The Gram stain does not reveal the capsule. Capsules stain reddish-mauve in Giemsa-stained smears although reports suggest that Giemsa stains give variable results.⁶ The capsule is not present on *B anthracis* grown aerobically on nutrient agar or in nutrient broths, but can be seen when the organism is cultured for at least 5 h in a few millilitres of blood (defibrinated horse blood is preferred).¹⁰

Care should be taken when handling stained smears as viable spores may be present in the material.

Culture and identification of B anthracis

B anthracis should be cultured under PC3 conditions. It is usually easy to identify *B anthracis* and to distinguish it from other *Bacillus* spp, including *Bacillus cereus*, which is an almost ubiquitous component of the environmental microflora. An isolate with the following characteristics is identified as *B anthracis*. On nutrient or blood agar after 24 h incubation, colonies have a matt appearance, fairly flat, similar to *B cereus* but generally rather smaller, tackier, white or grey-white on blood agar, and often having curly tailing ('Medusa head' appearance) at the edges. Colonies have high tenacity, that is when the colony is pushed and part of the colony pulled up with a loop, the colony will form a peak like beaten egg whites. They are also non-haemolytic or only weakly haemolytic. The vegetative cells of *B anthracis* are large measuring 3-5 µm in length and 1.0-1.2 µm wide and most isolates are non-motile. Ellipsoidal central spores, which do not swell the sporangium, are formed at the exponential cell growth phase. *B anthracis* isolates are able to produce the capsule in blood or on anaerobic culture on nutrient agar containing 0.7% sodium bicarbonate when incubated at 37°C in the presence of CO₂. Most isolates are sensitive to the gamma-phage¹⁵ and penicillin.⁶ A method for the propagation of the diagnostic gamma phage is described in Appendix 3.

Identification of B anthracis from old, decomposed specimens and environmental specimens including soil

The isolation of *B anthracis* from old, decomposing carcasses, processed specimens (bone meal, hides), or environmental samples such as contaminated soil requires specialised procedures. These types of specimens are likely to contain saprophytic contaminants that may outgrow and obscure *B anthracis* on non-selective agars. A procedure for the isolation of *B anthracis* from such specimens is described in Appendix 4. Reports of procedures for direct detection of *B anthracis* in soils and other environmental specimens using PCR are emerging. However, none has become routinely applicable at present.

PLET agar (Appendix 5)¹⁵ is the best selective agar for the isolation of *B anthracis* from animal or environmental specimens contaminated with other organisms including other *Bacillus* species.⁶ It is recommended for the culture of *B anthracis* after preparation of samples as described in Appendix 4.

If all other methods fail, then animal inoculation methods may be required. Failure of culture procedures may be because specimens are from animals that received antibiotic therapy before death or environmental samples contain sporostatic chemicals. Adult mice or guinea pigs are the animals of choice (Appendix 6).

Identification of virulence factors using the polymerase chain reaction (PCR)

PCRs have been developed for the detection of pX01 and pX02 plasmids. A method has been outlined in Appendix 7.⁶ Suitable primers for confirming the presence of the pX01 and pX02 plasmids and methodology are also listed in Appendix 7 (Table 1).^{17,18} These primers are effective for confirming the presence or absence of pX01 and/or pX02 in pure cultures of isolates from animal (including human) or environmental specimens or samples. They are unsuitable for the direct detection of *B anthracis* in such specimens or samples.¹³

For the rare possibility of strains of *B anthracis* lacking both pX01 and pX02 a chromosomal marker should also be run (Appendix 7) (Table 2).⁶ It is advisable to run these primers alone rather than in a multiplex PCR with protective antigen (PA) and capsule primers.

A multiplex PCR for the characterisation of *B anthracis* isolates has been developed.¹⁹ The assay amplifies *lef*, *cya*, *pag*, (pX01) and *cap* (pX02) genes, and a *B anthracis* specific marker.

Serology

B anthracis is antigenically very closely related to *B cereus*. The only unshared antigens that lend themselves to differentiating these two species by immunological approaches are the anthrax toxin genes, produced during the exponential growth phase, and the capsule of *B anthracis*. There has also been little need for serological tests for the diagnosis of anthrax in animals. Most of the interest in developing serological testing has been for research on humoral responses in humans, and to a lesser extent in animals, for evaluating vaccines and for epidemiological studies involving naturally acquired seroconversions in humans, livestock and wild animals.¹³

Currently the best serological procedure is the enzyme-linked immunosorbent assay (ELISA) in microtitre plates coated with the PA component of the anthrax toxin. The toxin antigens appear to be truly specific for *B anthracis*, although there is at present no commercial source for these tests.¹³

An immunochromatographic field test, based on the detection of PA, produced by the US Navy Research Medical Centre is currently being evaluated in a number of countries including Australia. This test, which can produce a result within 5–15 min, may provide an effective means for the field diagnosis of anthrax (Hugh-Jones M. Personal Communication).

Status of anthrax in Australia and New Zealand

In Australia, anthrax usually occurs as sporadic incidents in herbivores and in areas that have neutral to alkaline subsoil and on floodplains along waterways. Sporadic cases occur throughout central New South Wales and into northern and north-eastern Victoria. Western Australia has recorded cases in a very localised and isolated area northwest of Albany. Two cases of anthrax were reported in Queensland in 2002. The remainder of Western Australia and Queensland, the Northern Territory, South Australia and Tasmania are considered to be free from anthrax. New Zealand is also considered to be free from anthrax. New Zealand has been free from anthrax since 1954.

In 1997, there was an unusual outbreak in northern Victoria in which 202 cattle and 4 sheep died on 83 farms. On average each year in Australia cases occur on only about 6–12 farms. Usually few cases (1–3 cattle or 5–20 sheep on average) occur on each affected farm.^{10,20}

In New South Wales, there is a northern zone in which bovine and ovine anthrax occur with equal frequency and a southern zone where bovine anthrax is 4 times as common as ovine, with death rates in cattle 13 times greater than in sheep.⁷ The reason for this difference may be that the persistence of *B anthracis* in the soil may be different in the two zones, different predisposing factors directly affect the relative incidence of anthrax in sheep and/or that the modes of transmission of *B anthracis* may be different.

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Appendices

Laboratory workers should use good laboratory practice as outlined in the Australian/New Zealand Standard for Safety in laboratories when working with specimens from suspected anthrax cases and when culturing *B anthracis*. Use of a safety cabinet when handling materials that may be infected, and the safe disposal of discarded materials and disinfection of contaminated equipment should be mandatory.¹³

Appendix 1

Preparation of blood and body fluid for staining with polychrome methylene blue (M'Fadyean's stain)

1. Spread a small (1-5 μ L) drop of blood or body fluid on a microscope slide.
2. Air-dry and fix by dipping in absolute or 95% methanol or ethanol for 30-60 s.
3. Re-dry smear.
4. Place a large drop of polychrome methylene blue (M'Fadyean's stain) (Appendix 2) on the smear.
5. Spread with an inoculating loop to cover all parts of the smear.
6. Leave for 30-60 s.
7. Wash stain off with water.
8. Blot, dry and examine.
9. The capsule stains pink, whereas the bacillus cells stain dark blue.⁶

Appendix 2

Preparation of polychrome methylene blue (M'Fadyean's stain)

1. Dissolve 0.3 g of methylene blue in 30 ml 95% ethanol.
2. Add 100 ml of a 0.01% potassium hydroxide (KOH) (w/v) and mix.
3. Allow the solution to stand exposed to air, with occasional shaking, for at least 1 year to oxidise and mature before use.

Addition of K_2CO_3 to a concentration of 1% hastens the 'ripening' of the stain. The efficacy of the stain should be established by testing in parallel with an earlier, functional batch of stain on clinical specimens as it has been demonstrated that stains that give positive reactions with cultures of *B anthracis* cultured artificially in horse blood sometimes do not give positive results in the field.¹⁴

M'Fadyean's stain may be bought commercially but needs to conform with the quality control standards mentioned previously.

Appendix 3

Propagation and use of the diagnostic gamma bacteriophage

(Contact the Anthrax Reference Laboratory – Victorian Institute of Animal Science, 475-485 Mickleham Road, Attwood, Victoria, Australia, 3049 – Phone 0392174200 for phage sources)

1. Prepare a “lawn” of growth of *B anthracis* on Mueller Hinton Agar plates containing 5% sheep’s blood.
2. Incubate 4-6 h at 37°C. If there is sufficient vegetative growth (heavy and visible to the naked eye) then seed with phage. If the growth is thin, then incubate overnight before seeding.
3. Approximately 2 mL of the stock gamma bacteriophage is dropped over the surface of the vegetative growth. The plate is tilted to allow the phage to cover the whole area of growth. This may be repeated until the whole surface of the vegetative growth has been covered with phage.
4. Incubate the plates at 37°C overnight.
5. Store the incubated plates at -20°C overnight.
6. Remove the plates from the freezer and allow to thaw at room temperature for 2 h.
7. Pour off the brownish-red liquid and pre-filter through a Whatman 3 filter paper.
8. Filter through a 0.22 µm filter into a storage vessel.
9. To confirm the potency of the phage, prepare serial dilutions from 1:1 to 1:10,000 in saline and test with *B anthracis* for susceptibility as described in steps 3 and 4 of this Appendix.
10. Store the phage at 2-8°C. Do not freeze.¹⁴
11. Inoculate the suspect organism onto a blood or nutrient agar plate by streaking a lawn over the whole plate or part of the plate. Several isolates can be inoculated onto one plate.
12. Place a 10-15 µL drop of the phage suspension on the streaked area.
13. Allow the drop of phage suspension to soak in and incubate at 37°C overnight.
14. If the culture is *B anthracis* the area under the phage will be devoid of bacterial growth due to lysis of bacterial cells by the phage.¹³

Appendix 4

Identification of *B anthracis* from old, decomposed specimens and environmental specimens including soil.

1. Blend the sample in two volumes of sterile distilled or deionised water.
2. Heat in a water bath at 62.5°C for 30-60 min.
3. Prepare tenfold dilutions to 10⁻³.
4. Inoculate 10-100 µL from each dilution onto 3 blood agar plates and 250-300 µL onto 3 PLET agar plates (see Appendix 5).
5. Incubate all plates at 37°C.
6. Blood agar plates are examined after overnight incubation for typical colonies as described previously. PLET plates are examined after 40-48 hours.¹⁴

Appendix 5

Preparation of PLET agar

1. Heart infusion agar is made up according to the manufacturer's instruction. Difco heart infusion agar (or Difco heart infusion broth with other agar base) (Difco Laboratories, Detroit, MI 48232-7085, USA) is preferred. Heart infusion agars of other manufacturers or other nutrient bases, such as brain-heart infusion, can be made to work, but the optimal concentrations need to be determined; the concentrations recommended for normal use are unlikely to be optimal for use in PLET. A starting point for determining the optimal concentration is 25g/L of dehydrated broth plus agar at the manufacturer's recommended concentration.
2. EDTA (0.3 g/L) and thallos acetate (0.04 g/L) are added before autoclaving (*Note: thallos acetate is poisonous and should be handled with care; avoid skin contact or inhalation of the powder while weighing out*).
3. After autoclaving, the agar is cooled to 50°C and polymixin (30,000 units/L) and lysozyme (300,000 units/L) are added.
4. After swirling to ensure an even suspension of ingredients, the agar is poured into petri dishes.⁶

Appendix 6

Inoculation of animals for the isolation of *B anthracis*

1. If soil samples are to be used, the animals should be pretreated on the day before testing with both tetanus and gas gangrene antiserum. The samples should be prepared as described in Appendix 4 including heat-shock at 62.5°C for 15 min.
2. Inject mice subcutaneously with 0.05-0.1 mL; guinea pigs with 0.4 mL (0.2 mL in each thigh muscle).
3. Any *B anthracis* present will result in death after 48–72 h and the organism can be cultured as described previously.¹⁴

Appendix 7

PCR for the detection of pX01 and pX02

Template DNA for PCR from *B anthracis* colonies can be prepared as follows:

1. Transfer a loopful of growth from a young *B anthracis* culture on nutrient agar to 25 µL of sterile deionised (or distilled) water.
2. Heat at 95°C for 10 min.
3. Following cooling to approximately 4°C, 5 µL is taken for the PCR reaction.⁶
4. PCR can be carried out in 50 µL volumes using 200 µM of each of the primers dATP, dCTP, dTTP and dGTP, 1.5 mM MgCl₂ and 2.5 units of amplitaq polymerase, all in NH₄ buffer, followed by addition of 5 µL of the template DNA.

5. The following PCR cycle can be used: 1 x 95°C for 5 min; 30 x 95°C for 0.5 min followed by 55°C for 0.5 min followed by 72°C for 0.5 min; 1 x 72°C for 5 min; cool to 4°C
6. After the PCR cycle, 10% of the reaction volume of tracking dye (0.02% xylene cyanol, 0.02% bromophenol blue and 50% glycerol) is added to each PCR tube.
7. 10 µL is loaded into wells on a 1% agarose gel in TBE (45 mM Tris-base, 45 mM boric acid, 1 mM EDTA pH 8.0, diluted 1 in 10). A 1-kilobase ladder for size markers should be included in the outer wells of the gel.
8. The gel is electrophoresed at 80 V for 1 h followed by staining in ethidium bromide solution for visualisation under UV light.

Table 1. Primers for pX01 (Protective antigen) and pX02 (capsule antigen)

Target	Primer ID	Sequence 5'-3'	Product size	Concentration
Protective antigen (PA)	PA 5 3048-3029	TCC-TAA- CAC- TAA-CGA-AGT- CG	597	1 mM
	PA 8 2452-2471	GAG-GTA-GAA- GGA- TAT- ACG- GT		
Capsule	1234 1411-1430	CTG-AGC-CAT- TAA TCG-ATA-TG	847 bp	0.2 mM
	1301 2257-2238	TCC- CAC-TTA CGT- AAT- CTG- AG		

Table 2. Primers for chromosomal antigen

Target	Primer ID	Sequence 5'-3'	Product size	Concentration.
S-layer	Upper 391-413	CGC-GTT-TCT ATG- GCA- TCT CTT- CT	639 bp	0.2 mM
	Lower 1029-1008	TTC- TGA- AGC TGG- CGT- TAC AAA-T		