ANTHRAX

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Part 1. Diagnostic Overview

Summary

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

There are three primary clinical forms of anthrax in animals: peracute, acute and subacute. The peracute disease progresses rapidly and death occurs within a few hours after the onset of clinical signs which include fever, muscle tremor, dyspnoea, and congestion of the mucosae. Shortly afterward, the animal will often have terminal convulsions, collapse and die. Development of the acute form of the disease will result in death from 24 hours to a few days after onset of clinical signs, which may include a period of excitement followed by severe depression and listlessness. Subacute or localised disease is more prolonged, lasting for several days, and is not always fatal.

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.

An enlarged spleen is a characteristic feature of anthrax in cattle but it is uncommon in sheep, pigs and horses. The mesentery may be thickened and oedematous, with excess peritoneal, pleural and pericardial fluid. Failure of blood to clot is also a useful indicator.

Demonstration of encapsulated B. anthracis *in stained smears of blood from fresh anthrax-infected carcases is diagnostic for anthrax. Culture of* B. anthracis *or detection of virulence factors and chromosomal genes of* B. anthracis *are also definitive for the diagnosis of anthrax. The immunochromatographic test (ICT) is a rapid, in-field, test for anthrax available from the National Anthrax Reference*

Laboratory. The ICT hand-held kit is very useful in that obtaining a result in the field may preclude the need for on-site necropsies, thereby avoiding unnecessary occupational risks and environmental contamination.

Aetiology

Anthrax is caused by the spore forming bacterium *Bacillus anthracis*, a Gram positive rod 3.0-5.0 µm long and 1.0-1.2 µm wide. *B. anthracis* produces two pathogenicity factors: a poly-D-glutamic acid capsule carried on the pX02 plasmid and a tripartite toxin consisting of protective antigen (PA), oedema factor and lethal factor carried on the pX01 plasmid. Both factors are necessary for normal virulence. The capsule is thought to inhibit phagocytosis by leucocytes during infection which allows *B. anthracis* to grow unimpeded in the infected host. The tripartite toxin components are responsible for the characteristic signs and symptoms of disease and death.[1]

More recently, use of a quantitative PCR and inoculation of guinea pigs with a range of genetically diverse *B. anthracis* strains showed that virulence varied according to clonality and pX01/pX02 plasmid copy number. Increased plasmid numbers contributed to an increased level of virulence of the isolate. [2]

The genome of *B. anthracis* is highly monomorphic, which makes differentiation of the different strains difficult.[3] Biochemical, serological and phage typing methods available in the case of other pathogens have failed to discriminate the different isolates of *B. anthracis.* As a consequence, new approaches have been developed which have focused on the use of rapidly evolving variable-number tandem repeat (VNTR) loci to determine phylogenetic relationships among *B. anthracis* strains.[4] Multiple-locus VNTR analysis (MLVA) has been successfully applied to *B. anthracis* isolates worldwide. Analysis of diverse strains has separated *B. anthracis* into two major clonal groups: group A containing 4 or more minor branch clusters, and group B containing two minor branch clusters. Group A is the most common group worldwide, with group B restricted in number and geographical range. All of the Australian isolates typed to date fit within group A branch cluster 3, as confirmed by recent genotyping analysis of an expanded set of Australian *B. anthracis* strains by the National Anthrax Reference Laboratory.[4,5]

Clinical Signs

In an area where anthrax is known to occur, the sudden death of one or two animals within a flock or herd with no prior signs of disease should lead to the suspicion of anthrax, particularly when bloody fluid is seen to exude from orifices. This is most common at the beginning of an outbreak. The course of such peracute infections is probably about 1-2 hours. However, fever, muscle tremor, dyspnoea, and congestion of the mucosae may be observed. Initial cases are frequently found dead near water. The animal soon collapses and dies with terminal convulsions. Acute infections run a course of about 48 hours. Severe depression and listlessness are usually observed first, although they are sometimes preceded by a period of excitement. The animal may exhibit a high body temperature (up to 42^{o}C), an increased heart rate with respiration rapid and deep, and the mucosae will be congested and haemorrhagic. Pregnant cows may abort. Terminally ill dairy cattle may show decreased milk production and the milk may be blood-stained or deep yellow in colour.[6]

The clinical manifestations of anthrax may vary considerably from species to species. Subcutaneous oedema, and sometimes colic and diarrhoea, are seen in horses before death. Pigs are regarded as being more resistant to anthrax than cattle, sheep, goats and horses, but outbreaks resulting in significant mortalities can occur.[7] The disease in pigs may manifest in either the pharyngeal or intestinal form. Clinical signs may include fever, depression and anorexia with swelling of the neck and face. Swelling around the pharynx can restrict respiration, causing laboured breathing and cyanosis of mucous membranes. Dysentery and/or constipation may also be seen. Pigs may die 1–7 days after these signs develop, and piglets die of septicaemia. Anthrax bacilli can localise in the lungs, causing respiratory signs and blood-stained froth from the mouth. Dogs and cats are highly resistant to anthrax.[8]

Epidemiology

Anthrax is a disease of most animal species. 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.[9] During the past 30 years, a progressive, worldwide reduction in anthrax in animals has followed national programmes in which vaccination is a key element.[10]

In domestic livestock and wild animal populations, an outbreak of anthrax usually involves one species over other equally susceptible species and only a proportion of those exposed are usually affected. This phenomenon is seen worldwide. The difference in disease incidence may be influenced by the way in which different species feed. For instance, cattle pull plants out of the ground when grazing and ingest more potentially contaminated soil than sheep, which tend to bite plants off at ground level.[7] 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 four times more common than ovine anthrax. In the southern zone, death rates in cattle are 13 times greater than in sheep.[11] In Victoria, anthrax incidents are primarily restricted to cattle.

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 into the bloodstream leading to rapid death.
5. Shedding of vegetative bacilli from bloody discharge into the environment by the dead or dying animal.
6. Sporulation of released vegetative bacilli on exposure to oxygen in the air.

7. Infection of further animals by the uptake of spores, and potential spread of spores by fomites, scavenging animals, or by the wind.[9]

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 have been shown to survive for many decades; Wilson and Russell in 1964 demonstrated the survival and virulence of anthrax spores in dry soil after 60 years[12] and Jacotot and Virat[14] in 1954 showed spores prepared by Pasteur in 1888 were still viable 68 years later.[8] However, the longest survival claim is likely to be the recovery of anthrax spores from bones estimated on carbon-dating to be 200±50 years.[13] Subsoils that are pH 9 and calcareous in nature are considered conducive for spore survival.[8]

Occurrence and Distribution

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 along an anthrax 'belt' which runs through central New South Wales and into northern and north-eastern Victoria. Western Australia recorded outbreaks on three cattle farms in a very localised and isolated area west of Albany in 1994. 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 has been free from anthrax since 1954.[8]

In 1997, there was an unusual outbreak in an intensive dairy farming area in northern Victoria in which 202 cattle and four sheep died on 83 farms.[16] In 2007, the largest outbreak in 20 years in New South Wales occurred in the Hunter Valley. This outbreak was well outside the anthrax 'belt'. A total of 53 cattle and one horse died on 11 properties.[16] On average each year in Australia, cases occur on about 6–12 farms. Usually few animals (1–3 cattle or 5–20 sheep, on average) are infected on each affected farm.[8,16]

Gross Pathology

If anthrax is suspected, the carcase should not be opened as exposure to atmospheric oxygen of vegetative *B. anthracis* in spilled blood leads to sporulation of the vegetative bacilli, contamination of the environment, and increased risk of infection of the attending veterinarian and field personnel. However, on necropsy, tarry unclotted blood, absence of rigor mortis and an enlarged, haemorrhagic spleen are indicators of anthrax.[18]

An enlarged spleen is a characteristic feature of anthrax in cattle but it is uncommon in sheep, pigs 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 lesions are uniformly present in all cases of anthrax.[8,18]

Diagnostic Test Methods

Demonstration of encapsulated *B. anthracis* in stained smears of blood from fresh anthrax-infected animals is diagnostic for anthrax. However, if the animal has been dead for >24 hours the capsule may not be readily detected in a blood smear. Culture

of *B. anthracis*, or detection of virulence factors and chromosomal genes of *B. anthracis*, are also definitive for the diagnosis of anthrax. Detection of virulence factors and chromosomal DNA is a more reliable option than culture, which may be subject to overgrowth with contaminants, especially when the animal has been dead for some time.

In Australia, *B. anthracis* isolates and/or DNA should be sent to the National Anthrax Reference Laboratory, Victoria for confirmatory testing.

A rapid, in-field, test for anthrax has been developed by the US Naval Medical Research Center and evaluated at the Australian National Anthrax Reference Laboratory. This test is now readily available in Australia for use within 48 hours of death.

Currently, there are no commercially available serological tests for the diagnosis of anthrax in animals.

Part 2. Test Methods

Collection of Specimens for Laboratory Diagnosis

The preferred sample for diagnosis of anthrax is blood. Due to the zoonotic potential associated with the handling of suspected anthrax carcases, blood that can be accessed via a needle without the need to open the carcase, e.g. from jugular, mammary, limb, ear or other peripheral veins, is recommended. If venous blood cannot be obtained without opening the carcase, then a sample can be collected from blood pooled in the nasal cavity. Blood can be submitted for culture (in approved laboratories), ICT test, smear preparation and PCR.

As an alternative to a blood sample, thin blood smears can be prepared from peripheral blood vessels, e.g. those in the ear. Smears should be sent from more than one affected, or suspected, animal where possible. The blood smears should be air-dried and not fixed by heat or other agents. One end of the slide must be left clean. Where blood or other body fluids cannot be collected because of carcase decomposition a 2 cm square portion of the ear should be submitted for PCR. From animals other than sheep and cattle, where the number of circulating bacilli in the blood is low, smears from any affected organs, e.g. swellings in throat and lymph nodes of pigs, should be collected.

Demonstration of Capsule Using Optical Microscopy

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 of aged polychrome methylene blue (M'Fadyean's reaction).[10] Smears are considered positive if blue square-ended rods, usually in short chains, surrounded by a pinkish-red capsule (Figure 1),[18] or shadons (capsular material)[10] are observed. A single ellipsoidal spore may be seen in the centre of the vegetative bacilli but they do not swell the sporangium. In Giemsa-stained blood smears, the capsule appears reddish-mauve although reports suggest that Giemsa stain gives variable results.[10] The Gram stain does not reveal the capsule.

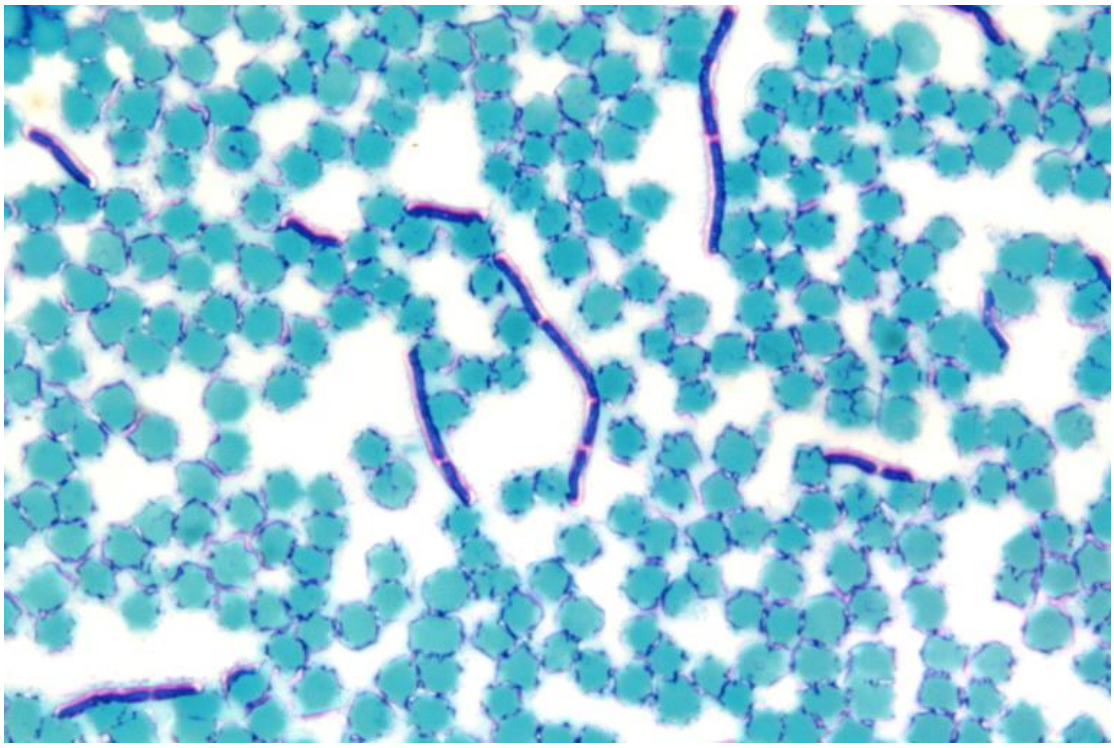


Figure 1: Blood smear containing *Bacillus anthracis* stained with polychrome methylene blue, demonstrating pinkish-red capsules.

Care should be taken when handling stained smears as viable spores may be present in the material, even after heat fixation.

It is important to note that if the animal has been dead for ≥24 hours, the capsule may not be readily detected in a blood smear. The capsule is also not present on *B. anthracis* grown aerobically;[8] hence, culture and/or PCR should be undertaken on an appropriate sample for the exclusion of anthrax.

Culture and Identification of *B. anthracis*

In Australia, only those laboratories accredited under the Security Sensitive Biological Agents (SSBA) Regulatory Scheme, such as the National Anthrax Reference laboratory, may undertake culture for *B. anthracis*. These laboratories may wish to culture blood, or swabs of the blood exuding from any of the orifices. In New Zealand, only the Animal Health Laboratory at the Investigation and Diagnostic Centres, Ministry of Agriculture & Forestry may undertake testing for *B. anthracis*.

It is usually easy to identify *B. anthracis* and to distinguish it from other *Bacillus* spp, including *B. 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 hours incubation, colonies are 3-5 mm in diameter, have a matt appearance and are fairly flat. They are similar to *B. cereus* but generally smaller, tackier, white or grey-white on blood agar, and often have curly tailing ('Medusa head' appearance) at the edges. Colonies have high tenacity; i.e. 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, and most isolates are non-motile and sensitive to gamma-phage[19] and penicillin.[10] A method for the propagation of the diagnostic gamma phage is described in Part 3. Alternatively, isolates can be confirmed as *B. anthracis* using PCR.

The capsule is not present on *B. anthracis* grown aerobically on nutrient agar or in nutrient broths. To induce the production of capsules the organism is cultured for at least 5 hours in a few mL of blood (defibrinated horse blood is preferred)[8] or grown on nutrient agar containing 0.7% sodium bicarbonate at 37^{o}C and incubated in the presence of CO_2 (20% is optimal but a candle jar will also work). The encapsulated *B. anthracis* look different to that described above as they are mucoid. The capsule can be visualised by making smears, fixing and staining as described above.

Identification of *B. anthracis* from Old, Decomposed Specimens and Environmental Specimens including Soil

The isolation of *B. anthracis* from old, decomposing carcases, 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 Part 3. Reports of procedures for direct detection of *B. anthracis* in soils and other environmental specimens using PCR are emerging; however, none is routinely applicable at present.

Polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar[19] is the best selective agar for the isolation of *B. anthracis* from animal or environmental specimens contaminated with other organisms, including other *Bacillus* species.[10] PLET agar is recommended for the culture of *B. anthracis* after preparation of samples as described in Part 3.

If all other methods fail, then animal inoculation may be required. Adult mice or guinea pigs are the animals of choice (Part 3). Failure to culture *B. anthracis* may be because specimens are from animals that have received antibiotic therapy or where environmental samples contain sporostatic chemicals.

PCR Assays

A number of PCR assays, including real-time PCR, have been employed around the world to demonstrate and confirm the presence of the virulence genes, and a chromosomal marker specific for *B. anthracis*.[7] A multiplex PCR assay, approved by SCAHLS, has been developed for the detection of pX01 and pX02 plasmids, and a chromosomal gene in blood smear scrapings, blood, bacterial cultures and tissues.[20]

Blood smears

Following microscopic examination of blood smears, blood material for PCR is obtained by scraping the area of the smear coated with immersion oil, with a scalpel blade. Material is transferred to a 1.5 mL microfuge tube containing 0.2 mL sterile dH_20 and a pellet made by centrifugation at 16,000 ***g*** for 3 minutes at room temperature (RT). The supernatant is discarded and the pellet subjected to DNA extraction.

Ear tissue

Excise a small section of ear tissue (approximately 50 mg), transfer the material into a 1.5 mL microfuge tube containing 0.2 mL sterile dH_20 and homogenise with a 1.5 mL pestle (Eppendorf). Centrifuge at 16,000 ***g*** for 30 seconds. Transfer supernatant into another 1.5 mL tube and centrifuge at 16,000 ***g*** for 3 minutes at RT. The supernatant is discarded and the pellet subjected to DNA extraction. This procedure can also be used for other tissues.

Extraction of DNA

DNA can be extracted from blood, stained blood smears, ear tissues and bacterial colonies. The pellet harvested from stained blood smears is resuspended in 0.2 mL InstaGene matrix (Bio-Rad) and bacterial colonies for PCR analysis are suspended in 0.1 mL InstaGene matrix. Tubes are mixed briefly by vortexing, incubated at 56 °C for 20 minutes, briefly vortexed, then incubated at 100°C for 20 minutes (the manufacturer's recommended incubation of 100°C for 8 minutes was found to be inadequate for the inactivation of *B. anthracis* spores in some samples and thus extended to 20 minutes). A pellet is made of particulate matter by centrifugation at 16,000 ***g*** for 1 minute at RT and the aqueous phase is transferred to a fresh tube. DNA extracts are stored at -20°C.

Multiplex PCR analysis

Detection of *B. anthracis* by multiplex PCR is performed to amplify targets located on the chromosome, pXO1 and pXO2 as in the following table.

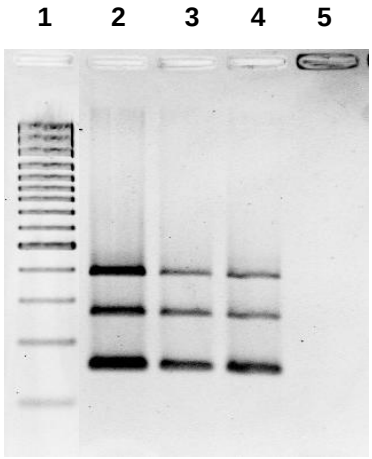
Primer sets to identify *B. anthracis* targets [21]

Target	Primer	Sequence	Size
Chromosomal	Ba813R1	TTAATTCACTTGCAACTGATGGG	152 bp
	Ba813R2	CGATAGCTCCTACATTTGGAG	
pXO1	*lef* 3	CTTTTGCATATTATATCGAGC	385 bp
	lef 4	GAATCACGAATATCAATTTGTAGC	
pXO2	*cap* 57	ACTCGTTTTTAATCAGCCCG	264 bp
	cap 58	GGTAACCCTTGTCTTTGAAT	

To minimise the effects of amplification inhibitors, DNA templates are treated with GeneReleaser (Bio Ventures) immediately prior to PCR. In a 0.2 mL PCR tube, 2 µL template DNA is added to 6 µL GeneReleaser slurry, and heated in a microwave at 770 W for 6 minutes. Tubes are immediately cooled on ice and a PCR cocktail is added to achieve a final volume of 20 µL, containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM each of primers Ba813 R1, Ba813 R2, *cap* 57 and *cap* 58, 1 µM each of *lef* 3 and *lef* 4, and 0.7 U Taq DNA polymerase. The *lef* primers are used at twice the concentration of the *cap* and Ba813 primers to favour amplification of this larger product.

PCR is performed in a thermal cycler. Cycling conditions are: 4 minutes at 94°C, 35 cycles of 40 seconds at 94°C, 40 seconds at 57°C, 40 seconds at 72°C, followed by 5 minutes at 72°C. Amplification products are separated on a 2% (w/v) agarose gel in TBE, stained with ethidium bromide or equivalent (e.g. SYBR®Safe) and bands visualised by UV or blue light transillumination. A multiplex PCR result is considered to be positive if two or three of the targets are amplified (Figure 2).

Figure 2: Multiplex PCR result



Rapid Immunochromatographic Test (ICT)

The Anthrax ICT is a SCAHLS-approved, in-field, diagnostic test, which detects the PA component of the circulating anthrax toxin. The test was developed by the US-Naval Medical Research Center[23] and is available in Australia from the National Anthrax Reference Laboratory. The ICT has been undergoing continual evaluation in Australia for the past 8 years[24, 25] and has gained widespread acceptance amongst field veterinarians in Victoria. The test is also being used and evaluated in Canada, South America and the USA and has been used with success in South Africa.[26] The test kit is now being produced by the Department of Primary Industries, Victoria and can be either supplied on demand, or stocks held at 4^{o}C at veterinary offices and laboratories. It is a rapid and simple to perform assay for the on-site diagnosis of anthrax that offers a result within 15 minutes.

The ICT is a hand-held kit which contains capture anti-PA antibodies and an in-built control. Basic training in the ICT must be undertaken before kits are used by field or laboratory personnel.

ICT test method

The ICT and sample buffer must be stored at 4°C, or kept cool on ice if taken into the field. The ICT is held within a foil package and this should be examined to ensure that the packaging is intact and that the kit is within the expiry date. If the expiry date has been surpassed, check with the supplier as to the procedures to follow. Before use, ensure that the kit and buffer are at room temperature.

1. To prepare the sample for the ICT, place 1 drop of blood into the supplied 3mL of sample dilution buffer, replace lid and gently mix.
2. Remove the ICT from the foil package and position on a flat surface, place approximately 6 drops of the sample preparation into the sample delivery well, or until the sample delivery well is full.
3. Allow a maximum of 15 minutes for the sample to move through the ICT. A pink line should appear in the control window within 15 minutes and if PA is present in the specimen a pink line will also appear in the test window (Figure

3). If the control line is not visible at 15 minutes, the ICT is not valid and a further test should be conducted.

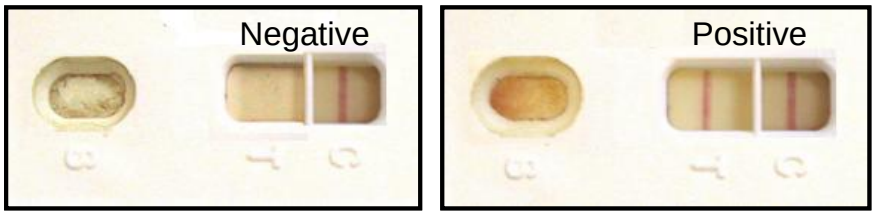


Figure 3: Negative and Positive ICT; (S) sample delivery well, (T) test window, (C) control window.

In samples taken from animals and processed within 48 hours post mortem, the sensitivity of the test approaches 100% (95% CI 93.98-100%). On this basis, it is recommended that the ICT only be performed on samples from animals within 48 hours post-mortem. Where the test is used beyond 48 hours post-mortem, the sensitivity will decrease; however, any positive ICT can still be regarded as a true result. If a positive result is obtained from testing in the field, the result should be confirmed using an approved laboratory method of diagnosis. In the case of a negative result, appropriate samples for investigation of sudden or unexpected death should be submitted to a diagnostic laboratory in attempt to make a definitive diagnosis.

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 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.[22]

Serological enzyme immunoassays for the confirmation of the diagnosis of anthrax and for epidemiological and research aids, have been used. Available immunoassays are based on the toxin antigens, primarily the PA component of the anthrax toxin as the toxin antigens appear to be truly specific for *B. anthracis*. These assays are not widely used and are confined to a few specialist laboratories that are capable of preparing or purchasing the reagents required.[7]

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Part 3. Reagents and Test Kits

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, the safe disposal of discarded materials and disinfection of contaminated equipment should be mandatory.[27]

Preparation of a polychrome methylene blue (M'Fadyean's stain) stained smear for visualisation of capsule

1. Spread a small (1-5 µL) 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 seconds.
3. Re-dry smear.
4. Place a large drop of polychrome methylene blue (M'Fadyean's stain) on the smear ensuring that all areas are covered.
5. Leave for 30-60 seconds.
6. Wash stain off with water.
7. Blot, dry and examine.
8. The capsule stains pink, whereas the bacillus cells stain dark blue.[10]

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

1. Dissolve 0.3 g of methylene blue in 30 mL 95% ethanol.
2. Mix 100 mL of 0.01% potassium hydroxide (KOH).
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 a functional stain using clinical specimens rather than laboratory cultured *B. anthracis*. It has been shown that stains that give positive reactions with artificially cultured *B. anthracis* do not always give positive results in the field.[22]

Commercially purchased M'Fadyean's stain must also conform to the quality control standards mentioned above.

Propagation of the diagnostic gamma bacteriophage

1. Prepare a "lawn" of growth of *B. anthracis* on Mueller Hinton Agar plates containing 5% sheep's blood.

2. Incubate for 4-6 hours 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 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 them to thaw at room temperature for 2 hours.
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 phage potency, 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 Section.
10. Store the phage at 2-8°C. Do not freeze.[22]

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 15-20 minutes.[28,7]
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.
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.[22]

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 25 g/L of dehydrated broth plus agar at the manufacturer's recommended concentration.
2. EDTA (0.3 g/L) and thallous acetate (0.04 g/L) are added before autoclaving.
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.[10]

Inoculation of animals for the isolation of *B. anthracis*

1. If soil samples are to be used, the animals should be pre-treated on the day before testing with both tetanus and gas gangrene antiserum. The samples should be prepared as described above, including heat-shock at 62.5^{o}C for 15 minutes.
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 hours and the organism can be cultured as described previously.[22]