

# **Anthrax (*Bacillus anthracis*)**

## **Laboratory case definition**

The Public Health Laboratory Network (PHLN) has developed standard case definitions for the diagnosis of key diseases in Australia. This document contains the laboratory case definition for *Bacillus anthracis*.

**Authorisation:** PHLN

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## **1 PHLN Summary Laboratory Definition**

### **1.1 Condition:**

Anthrax

#### **1.1.1 Definitive Criteria**

- Isolation of *Bacillus anthracis* from blood, sterile sites, sputum, nasal swabs, wounds, intestinal contents or other clinical specimens.

#### **1.1.2 Suggestive Criteria**

- Detection of *B. anthracis* by nucleic acid test (NAT) covering the genes coding for capsule and virulence factors; OR
- Gram positive square-ended bacilli, non-motile and encapsulated (well shown with polychrome methylene blue stain), from blood or other normally sterile site.

## **2 Introduction**

Anthrax is one of the oldest recorded diseases of animals. Anthrax was the first disease of humans and other animals in which the causative agent was definitely demonstrated as a specific microorganism by the French biologist Casimir-Joseph Davaine in 1863 and in 1876 by the German bacteriologist Robert Koch, who isolated the organism in pure culture. It was also the first infectious disease against which a bacterial vaccine was found to be effective, by Louis Pasteur in 1881. These discoveries led to the origin and development of the modern sciences of bacteriology and immunology.

Anthrax is a zoonotic disease that is transmissible to humans through handling or consumption of contaminated animal products. The aetiologic agent of anthrax, *Bacillus anthracis*, is a spore forming gram-positive bacillus. *B. anthracis* produces central or paracentral spores without significant swelling of the bacilli. The bacilli stain Gram positive and are typically large and square-ended. They are 1.0 to 1.3 µm by 3.0 to 10 µm in size, and occur singly or in chains of two or three bacilli. *B. anthracis* spores can remain viable in soil for many years<sup>3</sup>.

Humans can become infected with *B. anthracis* by handling products or consuming undercooked meat from infected animals. Infection may also result from inhalation of *B. anthracis* spores from contaminated animal products such as wool or the intentional release of spores during a bioterrorist attack. Human-to-human transmission has not been reported. Three main forms of anthrax occur in humans: cutaneous, gastro-intestinal, and inhalational<sup>3</sup>.

Virulent *B. anthracis* are typically distinguished from closely-related soil *Bacillus* sp. by the presence of two virulence plasmids, designated pXO1 and pXO2. However in the last decade, *B. cereus* isolates carrying pXO1 and pXO2-like plasmids containing *B. anthracis* toxin and /or capsule synthesis genes have been isolated from severe cases of human and primate disease (references <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4892579/>, <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1594744/>) (refer below). The pXO1 plasmid contains the *lef*, *cya* and *pag* genes, which encode for lethal factor, oedema factor and the protective antigen, respectively. The pXO2 plasmid contains the *cap* gene, which encodes for the capsule<sup>6</sup>. Molecular tests aimed at rapidly confirming the identity of an isolate and determining whether the isolate is virulent or not target these genes in particular<sup>6</sup>. Following infection, the protective antigen combines with, the lethal factor or the oedema factor to produce the lethal toxin and the oedema toxin, respectively. The protective antigen allows the binding of the lethal and oedema factors to the cell membrane and facilitates their passage across the cell membrane where they mediate their toxic effect<sup>10</sup>.

In September 2016, ProMed reported the isolation of a new strain of *Bacillus cereus* (*Bacillus cereus* biovar *anthracis*) from apes in Cote d'Ivoire and Cameroon, Africa. This new strain of *Bacillus cereus* possesses the two virulence plasmids associated with *Bacillus anthracis*, namely pXO1 and pXO2. The organism shares some of the phenotypic characteristics of *Bacillus cereus* but causes symptoms indistinguishable from anthrax. To date, this organism has only been detected in Africa, although anthrax-like variants have been reported elsewhere. *Bacillus cereus* biovar *anthracis* has the same potential to pose a severe public health threat as does *B. anthracis*.

## A. Cutaneous Anthrax

Cutaneous anthrax is the most common form of anthrax and accounts for about 99% of natural occurring anthrax. Cutaneous infections occurs when the bacterium or spore enters a cut or abrasion on the skin, such as when handling contaminated wool, hides, leather or hair products (especially goat hair) from infected animals. The incubation period is between 1-7 days, but may be up to 12 days. Skin infection begins as a raised itchy bump or papule that resembles an insect bite. Within 1-2 days, the bump develops into a fluid-filled vesicle, which ruptures to form a painless ulcer (called an eschar), which is usually 1-3 cm in diameter, with a characteristic black necrotic (dying) area in the centre. Pronounced oedema is often associated with the lesions because of the release of oedema toxin by *B.*

*anthracis*. Lymph glands in the adjacent area may swell. Approximately 20% of untreated cases of cutaneous anthrax result in death either because the infection becomes systemic or because of respiratory distress caused by oedema in the cervical and upper thoracic regions. Deaths are rare following appropriate antibiotic therapy, with lesions becoming sterile within 24 hours and resolving within several weeks<sup>2,3</sup>.

## B. Gastrointestinal Anthrax

The gastrointestinal form of anthrax may follow the consumption of contaminated meat from infected animals and is characterised by an acute inflammation of the intestinal tract. Gastrointestinal anthrax is more common in endemic areas where people eat the meat of animals that have died suddenly. Gastrointestinal anthrax is essentially cutaneous anthrax occurring in the intestinal tract. Initial signs of nausea, loss of appetite, vomiting, and fever are followed by abdominal pain, vomiting of blood, and severe diarrhoea. The mortality rate is difficult to determine for gastrointestinal anthrax but is estimated to be 25%-60%<sup>2,3</sup>.

## C. Inhalation Anthrax

This form of anthrax results from inhaling *B. anthracis* spores, and is most likely following an intentional aerosol release of *B. anthracis*. After an incubation period of 1-6 days (depending on the number of inhaled spores, can range from 2-43 days), disease onset is gradual and nonspecific. Fever, malaise, and fatigue may be present initially, sometimes in association with a nonproductive cough and mild chest discomfort. These initial symptoms, lasting 1-4 days, are often followed by a short period of improvement (ranging from several hours to days - note: this was not seen in the 2001 US mail inhalational cases), followed by the abrupt development of severe respiratory distress with dyspnoea (laboured breathing), diaphoresis (sweating), stridor (high-pitched whistling respiration), and cyanosis (bluish skin colour). Inhalational anthrax is often associated with gastrointestinal symptoms (see above) and confusion. Haemorrhagic meningitis may occur. Shock and death usually occur within 24-36 hours after the onset of respiratory distress, and in later stages, mortality approaches 100% despite aggressive treatment. Physical findings are usually nonspecific. The chest X-ray typically shows a widened mediastinum, due to lymphadenopathy, and pleural effusions, with or without infiltrates<sup>2,3,9</sup>.

## D. Injection Anthrax

Since 2000, a new form of anthrax has been reported in injecting drug users, linked to use of contaminated heroin. Cases do not present with typical signs of cutaneous anthrax, rather soft tissue involvement around the injection site, often progressing to severe complications. Some cases reported gastrointestinal symptoms such as nausea, vomiting and abdominal pain, while a smaller number experienced neurological symptoms, meningitis, intracranial or subarachnoid haemorrhage. In many cases, anthrax was not suspected or diagnosed with delay, likely contributing to the eight fatalities from 18 published cases.<sup>12</sup>

Most *B. anthracis* strains are sensitive to a broad range of antibiotics. Penicillin, ciprofloxacin, or doxycycline (but not cephalosporins) are usually recommended for the treatment of anthrax. A combination of antibiotics may be more effective than a single antibiotic in severe disease. To be effective, treatment should be initiated early. If left untreated, the disease is almost always fatal<sup>2</sup>.

## 3 Laboratory Diagnosis/Tests

### 3.1 Culture of clinical specimens for *Bacillus anthracis*

*B. anthracis* can be detected by Gram stain of the blood and by blood culture with routine media. Blood cultures collected at the time of presentation or early in the course of illness, before treatment is started, are usually positive<sup>9</sup>. Only vegetative encapsulated bacilli are present during infection. Spores are not found within the blood, partly because CO<sub>2</sub> levels in the body inhibit sporulation. In patients with neurological symptoms, cerebrospinal fluid (CSF) examination may reveal Gram positive bacilli. Culture must be confirmed by an appropriate Reference laboratory using PCR and phage lysis tests.

#### 3.1.1 Media

For optimal isolation of *B. anthracis*, direct plating onto sheep or horse blood agar (BA) is recommended. This may be direct from clinical specimens or from subcultures of blood culture medium.

In addition, it is recommended that clinical material is also inoculated into a suitable enriched broth, such as brain heart infusion broth or trypticase soy broth<sup>2</sup>.

For non-sterile site specimens, inoculation onto PLET (Polymixin-Lysozyme-EDTA-Thallous acetate) agar<sup>5</sup> is recommended. In addition, cultures overgrown with *Proteus* sp. can be “cleaned up” by placing some of the subculture material in ether for a minute (approximately). A few drops of the ether suspension on blood agar and PLET will subsequently yield *Proteus*-free colonies of *Bacillus anthracis*.

#### 3.1.2 Suitable specimens

##### Cutaneous Anthrax

Vesicular stage: The organism is best demonstrated in this stage. Gloves should be worn when collecting specimens. Soak two dry sterile swabs in vesicular fluid from a previously unopened vesicle.

Eschar stage: Rotate two swabs beneath the edge of the eschar without removing the eschar<sup>2</sup>. Alternatively, biopsy material may be submitted.

##### Gastrointestinal Anthrax

If the patient is able to produce a stool specimen, stool cultures should be performed.

In later stages of disease, blood cultures will yield the organism if specimens are obtained prior to antibiotic treatment. It is recommended that stool specimens are also inoculated onto PLET agar. In addition, ascitic fluid may yield positive cultures.

##### Inhalation Anthrax

If respiratory symptoms are present and sputum is being produced, obtain a specimen for culture and smear.

In later stages of disease (2-8 days post exposure) blood cultures, CSF or pleural fluid may yield the organism, if specimens are drawn before antibiotic treatment<sup>2</sup>. *B. anthracis* may be detected directly in clinical specimens by Gram's stain and/or polymerase chain reaction (PCR) if the diagnosis is strongly suspected. Specimens from internal sites, such as pleural fluid, are not recommended unless absolutely necessary for diagnosis because of the risk to operators collecting the specimen.

NB. Nasal swabs should only be used to support a confirmed exposure to *B. anthracis* spores, or during an ongoing epidemiological investigation.

### 3.1.3 Test sensitivity

No mathematical data available.

Depends on the quality and type of the specimen, the type of media chosen, and whether enrichment is used prior to direct plating. There may be enough organisms in the blood to see them on direct smears by Gram's stain. *B. anthracis* appears as short chains of 2-4 cells which are encapsulated as evidenced by clear zones around the bacilli. The presence of large encapsulated Gram-positive rods in the blood is strongly presumptive for *B. anthracis* identification. Antibiotic treatment may inhibit the growth of *B. anthracis*.

### 3.1.4 Test specificity

No mathematical data available.

The isolation of encapsulated non-motile Gram-positive rods from a normally sterile site, or from respiratory/faecal samples with a suitable history, confirmed by specific biochemical, phenotypic or molecular techniques. The isolation of *Bacillus anthracis* is notifiable in all States and Territories of Australia.

### 3.1.5 Predictive values

A negative culture does not exclude the diagnosis of anthrax.

### 3.1.6 Suitable acceptance criteria

After incubation of BA (blood agar) plates for 15-24 hours at 35-37°C, well-isolated colonies of *B. anthracis* are 2-5 mm in diameter. The flat or slightly convex waxy colonies are irregularly round, with edges that are slightly undulated (irregular, wavy border), and have a ground-glass appearance. There are often comma-shaped projections from the colony edge, producing the "Medusa head" colony. *B. anthracis* grows best aerobically, but can grow anaerobically. Colonies on BA usually have a tenacious consistency. When teased with a loop, the growth will stand up like beaten egg white<sup>1,2</sup>.

On PLET agar<sup>3</sup>, the colonies are smaller (1-2 mm) and have a very distinctive appearance, with a central raised white core and an off-white undulate periphery. After 24 hours incubation, many colonies look like miniature spiral galaxies with an arm coming off from either side, but as the colony grows, they more closely resemble fried-eggs.

NB. PLET agar typically inhibits *B. cereus* strains.

### 3.1.7 Suitable internal controls

Properly documented, relevant, quality control program for each type and batch of medium used, e.g. growth of *B. anthracis* (Sterne strain) on each batch of PLET, with no growth of *B. cereus*.

### 3.1.8 Suitable test validation criteria

Culture-based isolation of *B. anthracis*, confirmed by biochemical, other phenotypic or molecular methods, is the gold standard.

### 3.1.9 Suitable external QC program

The Royal College of Pathologists Australasia, with support from the Commonwealth Department of Health, have initiated a Biosecurity Quality Assurance Program which covers both *Bacillus anthracis* and its near neighbours.

### 3.1.10 Special considerations

In contrast to colonies of *B. cereus* and *B. thuringiensis*, colonies of *B. anthracis* are not -b-haemolytic. However, weak haemolysis may be observed under areas of confluent growth in ageing cultures and should not be confused with b-haemolysis. *B. anthracis* grows rapidly; heavily inoculated areas may show growth within 6-8 hours and individual colonies may be detected within 12-15 hours. This trait can be used to isolate *B. anthracis* from mixed cultures containing slower-growing organisms.

The Laboratory Response Network in the CDC have released the following guidelines for expected reactions of *Bacillus cereus* biovar *anthracis*:

1. Resistant to gamma phage on primary isolation, some clones positive
2. Non-haemolytic (interpretation may vary after 24 hours)
3. Motility is variable
4. Capsule production on Bicarbonate agar in the presence of CO<sub>2</sub>
5. LRN PCR = BA1 & BA2 positive, BA3 is negative.

### 3.1.11 Analysis of suspicious powders

A separate protocol has been developed to describe the methodology for the analysis of suspicious powder samples received as a result of a hoax or bioterrorist action for *B. anthracis*<sup>2</sup>.

NB. It should be noted that material received as part of a suspected bioterrorism incident will likely consist of a powdered suspension of spores. Whilst such material may be cultured overnight in broth, or directly onto blood agar plates to provide a presumptive result in 24 hours, it presents some difficulties for direct detection by PCR because of the difficulties in extracting DNA from the spore. Testing for spores cannot be validated in Australia because of the unavailability of suitable powdered spore preparations.

## 3.2 Identification of *Bacillus anthracis*

There are two levels of action in the identification of *Bacillus anthracis*:

A. **Diagnostic laboratories** - *B. anthracis* will generally be detected in routine clinical laboratories first unless all samples are directly diverted to a public health laboratory because of prior notification of a hoax or suspected biological attack. *B. anthracis* is a Risk Group 3 organism, and diagnostic laboratories should immediately refer all cultures and material to a public health (PHLN) laboratory as soon as there is any suspicion of *B. anthracis*, and institute appropriate containment and decontamination measures in the laboratory.

- There are specific protocols in place for the transfer of clinical material and cultures containing suspect Security Sensitive Biological Agents (SSBA) between diagnostic laboratories and public health reference laboratories. These are regulated under Commonwealth laws relating to SSBA.

B. **Reference laboratories** - State or Territory reference laboratories (PHLN) have the appropriate levels of containment for identification, confirmation and molecular characterisation of isolates of *B. anthracis*.

### 3.2.1 Conventional biochemical tests

#### 3.2.1.1 Suitable specimens

A pure culture on solid medium (blood agar or nutrient agar slope).

#### 3.2.1.2 Staining

Polychrome Methylene Blue (MacFaydean's stain) is the single easiest test available for presumptive confirmation of *B. anthracis* in blood films and exudates. The *B. anthracis* cells stain blue with an obvious thick capsule which stains red surrounding the cells. This stain may also be used to demonstrate capsule formation in cultures grown under 5% CO<sub>2</sub> atmosphere on bicarbonate agar.

#### 3.2.1.3 Media

Virulent strains of *B. anthracis* are the only organisms that produce rough colonies when grown aerobically and mucoid colonies when grown on a 0.8% sodium bicarbonate medium under a 5% CO<sub>2</sub> atmosphere. If the plates are re-incubated aerobically after mucoid colonies have formed, rough outgrowths from the margins will appear, showing reversion to the rough form.

#### 3.2.1.4 Test sensitivity

The test sensitivity will depend on the tests performed. The demonstration of medusa-head colonies with a tenacious consistency, Gram-positive staining bacilli, smooth colonies on 0.8% bicarbonate agar incubated in 5% CO<sub>2</sub>, and characteristic staining with polychrome methylene blue stain are traditionally considered to be reliable characteristics for confirmation of a *B. anthracis* isolate. (Table 1) *B. anthracis* is non-motile in motility medium (note that *Bacillus cereus* var. *mycoides* will also give a non-motile reaction). Motility can be tested by inoculating growth from a culture into Trypticase Soy Broth (TSB) for 2 hours at 37°C<sup>8</sup>. Characterisation of isolates by other phenotypic tests is unreliable and increases the hazard of exposure of laboratory workers.

Table 1

Test	Expected Reaction
Colony morphology	Colonies exhibit “tenacity” when teased up with a loop
Gram’s stain	Gram positive bacilli with spores - look like railway carriages end-to-end
Polychrome Methylene Blue stain	Blue bacilli and spores surrounded by thick red capsule (when taken from blood samples or Bicarbonate agar)
Motility	Negative
Growth on Blood Agar	No haemolysis in young cultures
Growth on Bicarbonate agar in CO <sub>2</sub>	Smooth mucoid colonies
Gamma phage lysis	Inhibition of growth

#### 3.2.1.5 Test specificity

No mathematical data available. The traditional phenotypic characteristics described in 3.2.1.4 may not satisfactorily identify some *Bacillus* spp resembling *B. anthracis*, nor do those tests assess the virulence capacity of *B. anthracis* strains so molecular identification methods are required for final confirmation. Laboratory personnel should be particularly aware that the Sterne strain does not produce a capsule and therefore will not give a typical reaction on Bicarbonate agar.

#### 3.2.1.6 Predictive values

A negative result in one test does not preclude a diagnosis of *B. anthracis*. Further testing as outlined above may be necessary to confirm the identification. Isolates should conform to the major criteria outlined above.

#### 3.2.1.7 Suitable test criteria

An isolate that exhibits phenotypic and biochemical characteristics consistent with documented reactions for *B. anthracis*. The major distinguishing features of *B. anthracis* are typical reaction in polychrome methylene blue stain, non-motility, colony tenacity and production of mucoid smooth colonies on bicarbonate agar incubated in CO<sub>2</sub> but rough colonies when incubated aerobically.



Identification can also be confirmed by fatty acid analysis, using the Maldi-TOF(Bioterrorism panel), (requires the security relevant reference library for proper ID. Labs should take care as the normal reference library can result in *B. anthracis* being identified as *B. cereus*. Reference <http://jcm.asm.org/content/54/3/764.full>. *B. anthracis* and *B. cereus* still need additional tests to resolve, even with the biosecurity database.) and gamma phage lysis. There is some data to suggest that the standard Bruker prep will not reliably decontaminate anthrax spores. Specific preps for PC3 level organism are available. Drevinek et al. Letters in Applied Microbiology, 55, 40-46. 2012, Weller et al. PLOS ONE, December 2015.

#### 3.2.1.8 Suitable internal controls

Test each batch of biochemical substrates with positive and negative control strains.

Results of all testing recorded and the records maintained.

#### 3.2.1.9 Suitable validation criteria

Correct biochemical and phenotypic reactions exhibited by a standard *B. anthracis* strain.

#### 3.2.1.10 Suitable external QC program

RCPA Biosecurity QAP.

#### 3.2.1.11 Kits/automated systems for biochemical identification

None available.

#### 3.2.1.12 Antibiotic Sensitivity Testing

One of the concerns in any potential bioterrorist incident is that the agent has been modified in a laboratory to increase virulence and/or antibiotic resistance. For this reason, it is of paramount importance that any isolates of *Bacillus anthracis* are tested early on for their resistance profiles. Antimicrobial susceptibility for penicillin, doxycycline, tetracycline and ciprofloxacin should be performed using Etest or similar gradient diffusion methodology. Breakpoints for *B. anthracis* are available in Table 21 of the CSLI M45-A3 2015 document. There are no corresponding published breakpoints available for EUCAST.

### 3.3 Molecular Identification and Typing

PCR testing typically relies upon the demonstration of a gene found in the *B. anthracis* chromosome (but not specific for only *B. anthracis*) and 2-4 plasmid genes that code for the virulence factors (capsule, protective antigen, lethal factor and oedema factor<sup>®</sup>) which can be multiplexed. This test can confirm that an isolate is *B. anthracis* and also distinguish between virulent and non-virulent (i.e. non-capsulated strains lacking pXO2 or non-toxin producing strains lacking pXO1) strains. It should be noted that because there can be a difference in the copy number of the two plasmids (typically pXO1 has been shown to have higher copy numbers per cell than pXO2), a PCR test which shows only one plasmid positive in a multiplex test should be followed up with a singleplex test on all targets. This is particularly important when investigating a potential bioterrorist attack. This molecular assay has only been validated on cultures. Due to the rarity of anthrax cases, this assay has not been validated for direct detection of spores in environmental samples or detection of bacilli in clinical samples.

There have now been a number of reports of *Bacillus sp* (not *B anthracis*) possessing either the pXO1-like or pXO2-like virulence plasmids. As a result, it has become even more important to use a gene target that will definitively identify a *Bacillus* isolate as *Bacillus anthracis*. Several chromosomal loci have been suggested, including ,BA813, BA5345, BA5510, *rpoB*, *gvrA*,*gyrB*, *saspB*, *plcR* and rRNA genes.

Typing of strains is best performed by whole genome sequencing given the high resolution necessary for distinguishing across the low genetic variability seen in *B. anthracis* strains. This would be particularly useful if multiple isolates were recorded from different States or Territories at the same time.

Molecular typing of *B. anthracis* has traditionally been performed by Multi-locus VNTR analysis (MLVA) which uses the combined power of multiple alleles at several marker loci<sup>4</sup>. It allows individual strains of anthrax to be assigned to particular genotypes, making geographic tracing a possibility if the strain is a unique genotype associated with one particular area of the world. In addition, although quite complex and time consuming, the methodology is reproducible at different laboratories, enabling rapid comparison of results. Typing is now best performed by whole genome sequencing given the high resolution necessary for distinguishing across the low genetic variability seen in *B. anthracis* strains. In the event of a number of anthrax cases in Australia however, cultures would be sent to the Center for Disease Control (CDC) for comparison against their large collection of *B. anthracis* strains.

The Department of Health has made RAMP (Rapid Analyte Measurement Platform) readers and kits available to a number of PHLN laboratories. These kits are specifically designed to enable rapid presumptive confirmation of anthrax spores. As a result, the usual sample for analysis is white powder preparations received as part of a threat scenario. These kits should not be used on clinical material, firstly because of the lack of spores in clinical material, and secondly because of the danger of false positives caused by some clinical samples, eg. Urine.

Some PHLN labs have access to the GeneXpert system or the Biofire Film Array which can be used with a specific bioterrorism panel to provide rapid molecular confirmation of *Bacillus anthracis* from powdered spore suspension.

### 3.4 Serodiagnosis

Although serology for *B. anthracis* is offered in a few overseas laboratories, there are no laboratories in Australia offering this testing. Where serology is considered an option, paired sera are preferred. These should be forwarded to the jurisdictional PHLN laboratory who will undertake to send them to the CDC for testing.

### 3.5 Immunofluorescence on Tissue

The Centres for Disease Control, Atlanta, Georgia criteria do not include immunofluorescence as a confirmatory test on clinical material, but Direct Fluorescent Antibody reagents are considered to provide confirmatory evidence of anthrax in cultures. However, DFA reagents for both capsule and cell wall polysaccharide are no longer available in jurisdictional PHLN laboratories

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