Standard Operating Procedures for Collection, Transportation, Preservation and Processing of samples for Diagnosis of Anthrax

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Anthrax is primarily a disease of herbivores caused by Bacilus anthracis. Anthrax is a bacterial disease caused by the spore– forming Bacillus anthracis, a Gram-positive, rodshaped bacterium, the only obligate pathogen in the large genus Bacillus. The disease was one of the main causes of uncontrolled mortality in cattle, sheep, goats, horses and pigs worldwide. Reports of the World organization for Animal health (oie) show that the disease is still enzootic in most countries of Africa and Asia, a number of european countries and countries/areas of north and South America; it still occurs sporadically in many other countries.

Anthrax is a severe problem in southern and eastern india, with a significant human incidence because the disease is poorly controlled. Outbreaks in wildlife also occur. It is absent however from the western state because of the low soil pH. *Bacillus anthracis* has always been high on the list of potential agents with respect to biological warfare and bioterrorism because of its infective spore form. The sporulated forms shed by an animal dying or dead from anthrax generally provide the source of infection of other animals, human and environment. Therefore handling of anthrax infected carcasses, collection of samples for diagnostic purpose from dead animals or from the environment requires skills and knowledge about the standard operating procedures.

Collection of samples:

Equipment and materials for specimen collection

All-purpose kit

The following list is for guidance with specimen collection:

- Leak-proof specimen containers, wide-mouth in the case of environmental samples
- Secondary containers for “double-bagging”
- Secure carrying containers (e.g. good-quality cool box, metal box, plastic mailing pots, etc.)
- Sterile swabs, forceps, scissors, syringes (1 ml) and needles (approx. 19 gauge), spatulas or spoons
- Sterile water and/or saline
- Microscope slides and slide carriers

- Culture plates and inoculating loops (if appropriate to make the primary culture at the site)
- “Sharps” disposal containers
- Labels and markers or pens
- Adhesive tape
- Autoclavable discard bags for disposables
- Autoclavable discard bags for tools, clothing, boots, etc.
- Stock hypochlorite solution and water to make up working solution (5000–10 000 ppm) and hand-washing facilities (e.g. large water container and basin)
- Paper towels.

Personal protective equipment

- Laboratory coat, gown, or overall, as appropriate to the situation, should be worn. Sleeves should be long with an elastic cuff.
- Double disposable gloves and (if appropriate, e.g. large animal dead on floor) overshoes or sterilizable boots should be used. The outer gloves should be changed as necessary to avoid spreading contamination. The skin should not be exposed between the gloves and the sleeves.
- Existing cuts or abrasions should be dressed before putting on personal protective equipment (PPE).

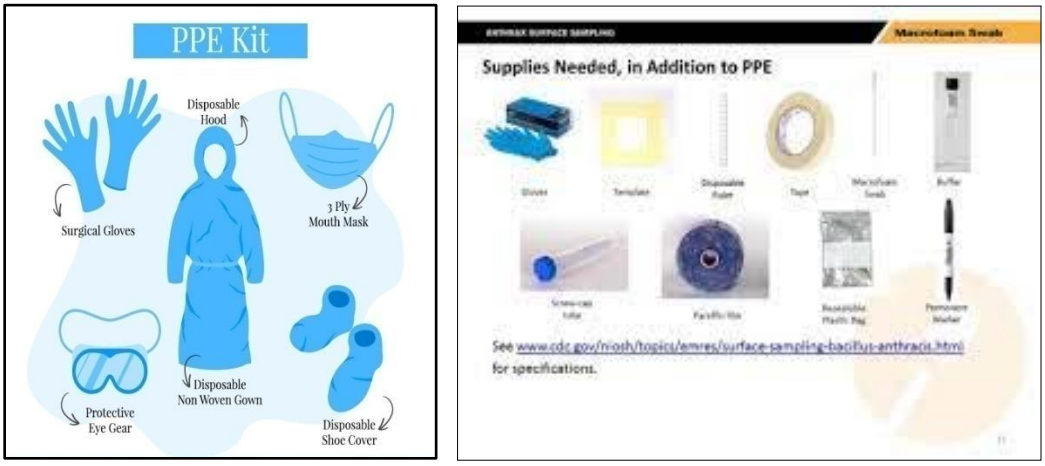


Figure 1. Personnel Protective Equipment Kit

Type of specimens

The standard procedure or approach will depend on the type of specimen being collected for examination which, will fall broadly into:

- Fresh specimens from untreated animals

- Specimens from treated animals or humans
- Specimens from old and decomposed animal carcasses or animal products or
- Environmental specimens, including those from suspected deliberate release events

Containment during collection of specimens and materials

Specimens from lesions or freshly dead animals may be handled at strict hazard levels with the following safety precautions:

- Use of adequate protective clothing (gloves, gowns with tight wrists and ties at the back)
- If the samples are not being processed in a safety cabinet, protective eye shields and good-quality face masks may be advisable to protect the operator from other (non-anthrax) infectious agents that might be present
- Keeping of high-quality, properly positioned facilities for hand-washing
- Careful dressing of skin abrasions.

Old dried-up specimens, such as old hides, that are liable to give off dust during processing, should be handled in a bio-safety cabinet, preferably class 3.

Containment during collection of Environmental and suspect deliberate release samples

- Environmental samples from sites suspected of having been contaminated naturally (e.g. carcass sites) are to be handled in a biological safety cabinet.
- Samples under suspicion of being artificially contaminated *must* be handled in a class 3 bio-safety cabinet.

Specimen collection from animals in the field

Preferably a veterinarian or microbiologist trained in handling disease-causing agents should do the sample collection. This may not always be possible, or only possible with a substantial delay, and farmers/owners/managers may have to collect the samples. The following advice aims at covering either situation:

- Need an apron or coverall if anticipate extensive handling of the carcass.
- Need disposable covers for hands and feet and strong bleach solution (10, 000 ppm).
- Dress cuts or abrasions on exposed areas, especially hands and arms.

- The professional approach is to wear apron or coverall, disposable gloves and overboots, or boots that can be disinfected. It may be appropriate to wear two pairs of disposable gloves (double gloving); the outer gloves can then be changed as and when needed without exposing the hands. Minimal alternatives are strong plastic bags as overboots and, for the hands, evert a plastic bag, insert the hand that will touch the carcass into the everted bag and grasp tissue to be sampled; insert swab, or cut off sample with other hand; reverse bag over sample or swab and seal and label the bag. In the case of cutting off a piece of tissue, insert the cutting implement into another plastic bag for transport to where it can be disinfected (strong bleach for 1 hour) or sterilized (boiled for 30 min or pressure cooked for 15–20 min).
- After specimen collection, discard disposable items into disposal bags for subsequent sterilization or incineration. Similarly, non-disposable items should be put into discard containers for subsequent sterilization or disinfection. Care should be taken to ensure that sharp objects should be in a container such that they cannot pierce easily. The containers themselves should be sterilized, incinerated or disinfected.
- Wash hands thoroughly with soap and water.

Collection of Environmental samples

- Disposable or reusable apron or coverall (as appropriate to the potential hazard of the sample) should be worn.
- Where the possibility exists of aerosolizing and inhaling dust, a respirator is advisable, preferably a quality assurance tested full-face respirator. (*Caution: the operator should be trained by a qualified person in correct wearing and use of the respirator*.)
- For samples related to known or suspected deliberate release, a quality assurance tested full-face respirator should be regarded as mandatory. (*Caution: the operator should be fitted and trained by a qualified person in correct wearing and use of the respirator*.)
- Double disposable gloves and overshoes or sterilizable boots should be worn. The outer gloves should be changed as necessary to avoid spreading contamination.
- Existing cuts or abrasions should be dressed before putting on PPE.

Figure.2. Collection of samples from dead animals and environment

Table.1 Appropriate specimens to be collected from animals suspected /died of Anthrax

CIRCUMSTANCE	SPECIMEN	CONTAINER	DIAGNOSTIC TESTS
Fresh carcass	Blood from vein (0.1 ml) or, fluid from body cavity with sterile syringe	2ml vial	Microscopy, Culturing, Molecular dignosis
	Tissues- Ear or eyelid clippings (Highly vascularized)	2ml vial	Microscopy, Culturing, Molecular diagnosis
	Swabs: From nostrils, Eye sockets, bloody material	2ml swab tubes (sealable specimen container)	Microscopy, Culturing, Molecular diagnosis

CIRCUMSTANCE	SPECIMEN	CONTAINER	DIAGNOSTIC TESTS
Putrefied carcass	Bloody soil from under the head or tail	2ml swab tubes (sealable specimen container)	Microscopy, Culturing, Molecular diagnosis
	Tissues- Ear or eyelid clippings (Highly vascularized)	2ml swab tubes (sealable specimen container)	Microscopy, Culturing, Molecular diagnosis
Very old carcass, hides, bones, soil around/under carcass, etc.	Swabs of nostrils and eye sockets. Soil from where body fluids are believed to have fallen.	2ml swab tubes (sealable specimen container)	Culture on selective agar.

Note : Smear and culture should be done within hours of collecting blood. Vegetative cells disintegrate in blood held for much more than a day. If a delay in reaching the laboratory is expected, the smear should be made on a slide immediately after collection and the blood should be collected on a dry swab.

Table.2 Appropriate specimens to be collected from Humans suspected cases of Anthrax

Specimen Type	Collection Time	Collection Frequency	Collection Procedures	Transport Media	Shipping & Handling (S&H)**
Cutaneous: or Vesicular (early) stage or Eschar (late) stage	**At illness**		• Un roof vesicle and aspirate fluid, or collect with two sterile swabs. • Collect with only synthetic tip swabs (e.g. Dacron, Nylon, Polyester) with non-wooden shaft. • Insert swab (dacron) beneath the edge of the eschar, rotate swab or obtain an aspirate.	NA	• **Transport device:** Sterile leak-proof container. • **Transport:** Ship specimens at room temperature. • **Storage:** If arriving at lab ≥72 hrs of collection, refrigerate at 2-8°C.
Gastro-intestinal			Collect stool (> 5 grams) into a leak-proof sealed container.	NA	• **Transport device:** Sterile leak-proof container. • **Transport:** Ship cold (2-8°C) on ice packs. • **Storage:** Refrigerate at 2-8°C.
Inhalational			Collect Cerebral Spinal Fluid (> 1ml); only if signs of meningitis occur.	NA	• **Transport device***:* Sterile leak-proof container. • **Transport:** Ship cold (2-8°C) on ice packs. • **Storage:** Refrigerate at 2-8°C.
Whole blood in EDTA (PCR only)	**≤4 days post symptom onset**		Collect into lavender-top EDTA collection tubes.	NA	• **Transport device:** Lavender-top EDTA tube. • **Transport:** Ship cold (2-8°C) on ice packs. If previously frozen, ship on dry ice. • **Storage:** Refrigerate at 2-8°C. If arriving at lab ≥72 hrs of collection, freeze at ≤-70°C.

Sputum	**At illness. For optimal isolation, collect within 72hrs of symptom onset.**		Collect expectorated specimen into a sterile container.	NA	• **Transport device:** Sterile leak-proof container. • **Transport:** Ship cold (2-8°C) on ice packs. • **Storage:** Refrigerate at 2-8°C.

Reference: Specimen Collection and Submission Instructions for *Bacillus anthracis*, Washington state Department of Health

Collection and Preparation of Specimens for Anthrax Diagnosis

a. Collection of blood

- Collect 10 ml of blood aseptically from a peripheral vein using the appropriate gauge needles and a Vacutainer™ serum separator tube (SST); this will yield approximately 5 ml of serum.
- Before keeping the tube for centrifugation to separate serum, allow the Vacutainer™ drawn blood to sit at room temperature for at least 30 minutes, but no longer than 60 minutes.
    - The clot should be completely formed within 30 minutes.
    - Trauma-induced haemolysis should be minimized if the blood is separated within 60 minutes of collecting the whole blood specimens.
- For field collections where serum separation is not possible within 60 minutes of collection, store the specimen at 4°C using cold packs, and ship it to a laboratory with centrifugation capabilities as soon as possible (ideally less than 24 hours).

b. Separation of the serum

- Centrifuge the specimen before storing or shipping to completely separate serum from the blood cells.
- After centrifuging the serum, use a sterile technique to transfer the serum into a plastic freezing vial with a leak-proof screw-cap (e.g. 2 ml Screw Cap Micro Tube with an O-ring in the lid or equivalent).
- Label the plastic vial(s) with all of the following:
    - Patient name and/or identification number
    - Specimen collection date and time
    - Whether the sample is an “acute” or a “convalescent” specimen
- Freeze the serum immediately after it has been transferred into the plastic freezing vial(s).
    - Store the specimen at -20° C

Collection of Samples

Collection of Blood and preparation of blood smear

- Collect tarry coloured un clotted blood oozing from the natural orifices of animals died of anthrax in a sterile blood collection vials
- Prepare blood smear by placing a drop of blood on one end of the slide, and using a spreader slide to disperse the blood over the slide’s length.
- Air dry the slide and transport immediately to the laboratory.
- During collection and transportation of blood samples strictly follow the Bio-safety measures.

Collection of Ear piece samples

Collect ear piece samples in a sterile sample container with Boric acid powder and transport immediately to laboratory on ice. During collection and transportation samples strictly follow the bio-safety measures.

Collection of Soil samples

- Collect soil samples from different carcass burial sites, livestock habitats, where the animals were died of anthrax and also from endemic areas.
- Top layer of soil covering 12x15 cm in area and 2.5 cm depth should be removed from collection site and approximately 500 gm of soil to a maximal depth of 20cm must be collected using a scoop into labelled Bio-hazard Plastic bags.
- Carefully transfer to the laboratory for processing with strict bio- safety measures.

Labeling

The following information should be recorded:

- A reference code or number marked in indelible ink on the container; and, either on the container or a sample documentation sheet
- The date and time of sampling
- The location of the sampling point
- The type of sample
- The reason for sampling
- The identity of the person taking the sample.

Figure.3.Labeling of samples

Containment for transport of clinical material ("double-bagging")

- The specimens should be collected into sterile containers using aseptic techniques.
- The containers should be wiped down with hypochlorite (10 000 ppm) and, with outer gloves changed first, put into an outer, secondary container (double-bagged). If the secondary container is a plastic bag, then this should be of good quality. It should, in turn, be sealed and, for transport, be put into a good-quality cool box or a strong plastic or metal container with a lid that can be made secure.
- The secondary and outer containers should bear the relevant hazard labels.

Figure.4. Packaging of samples

VI. Packaging the specimen for submission

- Insert absorbent material (such as absorbent sheets) into a sealable plastic bag (bag #1) and place the plastic vial(s) containing specimens into the bag.
- Line a leak proof secondary packaging, such as an appropriate plastic screw-top canister (shipping canister), with absorbent material.
    - Use enough absorbent material to absorb the entire contents of the plastic vials, in the event, there is a leak or spill.
- Place the sealed plastic bag into the shipping canister.
    - Place a biohazard label on the outer surface of the shipping container.
- Place dry ice into a polystyrene foam-lined box (the over pack).
    - Use at least 6 pounds (~3kg) of dry ice for each day of shipping time; at least 12 pounds (~6 kg) is required for overnight shipment (2 days).

- Divide the weight of the ice in pounds by 2 to get the approximate weight in kg.

- Place the shipping canister into the over pack.

Basic triple packaging system

The system consists of three layers as follows.

1. Primary receptacle.

A labeled primary water tight, leak-proof receptacle containing the specimen. The receptacle is wrapped in enough absorbent material to absorb all fluid in case of breakage.

2. Secondary receptacle

A second durable, water tight, leak-proof receptacle to enclose and protect the primary receptacle(s). Several wrapped primary receptacles may be placed in one secondary receptacle. Sufficient additional absorbent material must be used to cushion multiple primary receptacles.

3. Outer shipping package

The secondary receptacle is placed in an outer shipping package which protects it and its contents from outside influences such as physical damage and water while in transit.

Figure.5. Triple layer Package method

Specimen data forms, letters and other types of information that identify or describe the specimen and also identify the shipper and receiver should be taped to the outside of the secondary receptacle. Hand carriage of infectious substances is strictly prohibited by international air carriers, as is the use of diplomatic pouches for that purpose.

The maximum net quantity of infectious substances which can be contained in an outer shipping package is 50 ml or 50g if transport is by passenger aircraft. Otherwise, the limit per package is 4L-4Kg for transport by cargo aircraft or other carriers.

Primary receptacles exceeding 50 ml in combination packing must be oriented so the closures are upward, and labels (arrows) indicating the UP direction must be placed on two opposite sides of the package. The passenger aircraft quantify limits do not apply to blood or blood products for which there is no reason to believe they contain infectious substances, when in receptacles of not more than 500 ml each and with a total volume of not more than 4L in the outer package.

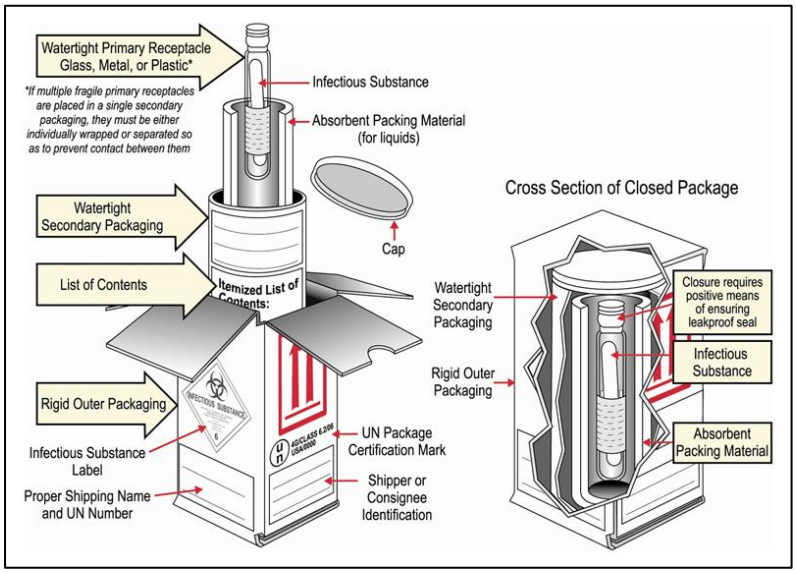


Figure.6. Triple Layer Packing cross section

Storage/ Preservation:

Cold Chain:

Specimen may need to be transported long distances before being tested. The conditions of transport can greatly affect the viability and reactivity of the specimens, resulting in lower sensitivity of testing or, in extreme cases, false negative results. Therefore, it is extremely important to ensure a collected material is stored and transported in a way that ensures its viability in the laboratory. Cold chain maintenance is important for maintaining the stability of the specimen. Ideally the cold chain is never broken.

- Generally, specimens should be stored at 2–8 °C or frozen (-20^0C).

- Preferably they should be transported in cool boxes, especially in hot weather and when the time interval between collection and delivery to the laboratory is likely to be more than 1–2 hours.
- Serum is very sensitive to heat. Reactivity to antibody detection decreases at high temperatures. These samples should be transported either with wet ice, ice packs or if the distance is long frozen ice packs.
- The outer packaging should be insulated to keep the temperature consistent. Ideally the package will have enough cold packaging to arrive at its destination and still be cool to the touch.
- If using dry ice, the outer packaging used must be breathable to release the CO_2 that will build up in the package.
- Failure to use a breathable outer box could result in the buildup of too much pressure which can damage the package, the specimen and potentially hurt personnel.

- For shipping of samples by mail or courier, the appropriate procedures with relevant paperwork must be followed.

General Safety procedures during specimen collection

- Before specimen collection, put on the chosen clothing, including double gloving. Ensure disinfectant, disposal bags and hand-washing equipment are ready.
- Existing cuts or abrasions should be dressed before putting on PPE.
- After specimen collection, rinse or wipe down gloved hands with 10% hypochlorite solution and discard outer gloves.
- Discard used PPE into disposal bags, separating auto clavable and non-auto clavable items. Inner gloves should be discarded last. Sharps should be placed in a sharps container.
- Handling person needs an apron or coverall if he anticipates extensive handling of the carcass.

- Need disposable covers for hands and feet (see below) and strong bleach solution (10 000 ppm).
- Dress cuts or abrasions on exposed areas, especially hands and arms.
- The professional approach is to wear an apron or coverall, disposable gloves and over boots, or boots that can be disinfected.
- It may be appropriate to wear two pairs of disposable gloves (double gloving); the outer gloves can then be changed as and when needed without exposing the hands.
- In the case of cutting off a piece of tissue, insert the cutting implement into another plastic bag for transport to where it can be disinfected (strong bleach for 1 hour) or sterilized (boiled for 30 min or pressure cooked for 15–20 min).
- After specimen collection, discard disposable items into disposal bags for subsequent sterilization or incineration.
- Similarly, non-disposable items should be put into discard containers for subsequent sterilization or disinfection. Care should be taken to ensure that sharp objects are in a container they cannot pierce easily. The containers themselves should be sterilized, incinerated or disinfected.
- Wash hands thoroughly with soap and water.

Disinfectants and fumigants used during collection of specimens

- Prepare hypochlorite solutions (10 000 ppm) freshly every day. Preferably handle sodium hypochlorite wearing gloves and eye protection. Avoid spilling it on clothes. Remember it corrodes ferrous metals.
- Use formalin in well-ventilated areas, wearing gloves and face shield while handling it. If handling it in an enclosed space with little ventilation, or if large volumes are involved, a full-face chemical respirator should be worn (*the operator should be fitted and trained by a qualified person in correct wearing and use of the respirator*). Care should be taken that as it is injurious to skin and mucous membranes.

Disinfection decontamination and disposal

- Basically all specimens and used disposables should be autoclaved when finished with. Whether in the laboratory or in the field, these should have been collected into autoclavable bags or other suitable containers which are then autoclaved at 121 °C for ≥ 1 hour, preferably followed by incineration.
- Contaminated autoclavable non-disposable items should also be deposited in autoclavable containers and ultimately autoclaved.
- Microscope slides, coverslips and other sharp items should be placed in autoclavable sharps containers and autoclaved, preferably followed by incineration.
- There may be circumstances where it is appropriate to immerse items in hypochlorite solution (10 000 ppm) initially and then to autoclave and incinerate them later.
- Disinfect or fumigate non-autoclavable materials.
- Laboratory clothing should be autoclaved before being sent to the laundry. Non-disposable boots should be washed down into an autoclavable basin or bucket, and the washings autoclaved. The boots then should be disinfected by immersion in hypochlorite (10 000 ppm available chlorine) or 10% formalin and allowing them to dry for about 30 min before reuse.

Sterilization (Fumigation/UV)

Equipment that cannot be autoclaved, boiled or immersed in disinfectant solutions should be fumigated. Where fumigation is not readily achieved and a safety cabinet fitted with a UV light is being used, this should be utilized applying the same principles of arranging the items to be sterilized in such a way as to ensure the UV light reaches into and around them to maximum extent . UV should not be relied on alone for decontamination, but should be used in conjunction with wiping the items to be decontaminated with toweling moistened with hypochlorite or possibly formalin.

Ideally, cabinets and rooms should be fumigated when suspected of being contaminated. Where this is not possible, they should be given a very thorough floor-to-ceiling wipe-down with hypochlorite solution (10 000 ppm)

Ref: Anthrax in Humans and Animals. 4th edition.Geneva: World Health Organization; 2008.

Processing of samples/materials for diagnosis Anthrax from Clinical and Environmental samples

Anthrax is diagnosed by examining blood (or other tissues) for the presence of the bacteria. Bacterial culture followed by confirmatory tests–including phage and penicillin sensitivity and PCR to detect genes specific to B.anthracis–is currently considered the gold standard approach for the diagnosis of anthrax. Samples must be collected carefully to avoid contamination of the environment and to prevent human exposure to the bacteria. Blood samples from relatively fresh carcasses will contain large numbers of *B. anthracis*, which can be seen under a microscope, cultured and isolated in a laboratory, or detected by rapid tests, e.g. polymerase chain reaction (PCR) or qPCR etc.

Bio-safety Level-III lab

Bio-safety level-III laboratory facility (SRP Scientifics Pvt. Ltd, Bangalore) is established at State Level Diagnostic Laboratory, Sri Venkateswara Veterinary University, Tirupati.

Apparatus Required

Biohazard Laminar air flow chamber (MAGNEHELIC), Hot air oven (Equitron) & PSM Horizontal autoclave (PSM) for sterilization, Bacteriological incubator (Equitron), Refrigerator (LG), Deep Freezer (Vestfrost), Centifuge & Refrigerated Centrifuge (Remi), Binocular Microscope (Magnus), Thermal cycler (Proflex PCR, Life technologies), Gel documentation system (Gel Doc XR, BioRad), electrophoresis apparatus (Tarsons) Water bath digital (Equitron), Vortex (Remi), Micropipettes (Eppendorf) ,forceps, scissors, sterile loop (Himedia), surgical blade (glass van), are established at State Level Diagnostic Laboratory, Sri Venkateswara Veterinary University, Tirupati to carry out the processing of samples.

II. Identification of Bacillus anthracis

Blood smear examination:

A presumptive diagnosis can be made if the characteristic bacteria are found in blood, other body fluids or tissue smears. Anthrax is often diagnosed by detecting *B. anthracis* in a blood sample from a carcass. Blood clots poorly in affected animals and samples may be obtained by making a small cut in an ear vein, or by collecting it with a syringe from any available vein. Bacteremia is rare in pigs, and a small piece of affected lymphatic tissue is often collected aseptically instead.

Polychrome methylene blue (Mc'Fadyean's Reaction) Staining procedure for capsule Visualization:

Preparation of polychrome methylene blue stain

- Methylene blue: 0.3 g
- Ethyl alcohol (95%): 30 ml
- Potassium hydroxide: 0.01 g
- Distilled water: 100 ml

This preparation is allowed to ripen for one year. The stain is kept in bottle, which is half filled and shaken at intervals to aerate thoroughly. The oxidation of methylene blue forms a violet compound that gives the stain its polychromic property. It may also be ripened quickly by the addition of 1 % potassium carbonate to the stain solution.

Procedure

- Make two thin blood smears (from ear vein) on a microscope slide.
- Air dry and fix by dipping the slide in absolute alcohol for 30–60 seconds.
- Slide should not be heat dried to avoid distortion of morphology of the capsule.
- Flood with polychrome methylene blue stain.
- Wait for 30–60 seconds.
- Wash off the stain with tap water and air dry.
- Examine under oil immersion.

Interpretation of result

- Irregular pink capsular material surrounding the blue stained bacilli is a characteristic morphological feature of *Bacillus anthracis* indicating positivity.(**M'Fadyean's Reaction)**

Giemsa staining Procedure

Preparation of the Giemsa Stain Stock solution (500ml)

- Into 250ml of methanol, add 3.8g of Giemsa powder and dissolve.
- Heat the solution up to ~60^{o}C
- Then, add 250ml of glycerin to the solution, slowly.
- Filter the solution and leave it to stand for about 1-2 months before use.

Preparation of Working solution

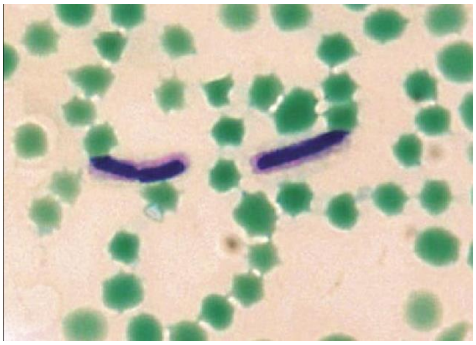
- Add 10ml of stock solution to 80ml of distilled water and 10ml of methanol

Staining Procedure

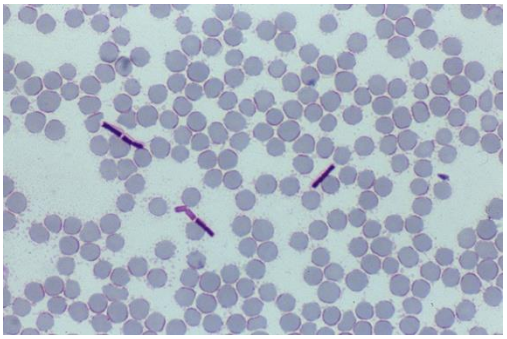
- On a clean dry microscopic glass slide, make a thin film of the specimen (blood) and leave to air dry.
- dip the smear (2-3 dips) into pure methanol for fixation of the smear, leave to air dry for 30seconds
- Flood the slide with 5% Giemsa stain solution for 20-30 minutes.
- Flush with tap water and leave to dry

Interpretation:

Giemsa stains the bacillus **purple and the capsule reddish-mauve** colour is a characteristic morphological feature of *Bacillus anthracis*



Polychrome Methylene blue staining



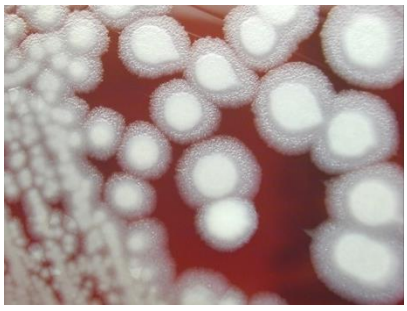
Giemsa staining

Isolation of B.anthracis from fresh specimens (swabs from blood and nostrils)

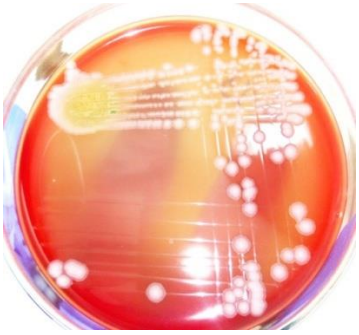
Reaction on Blood agar

Bacillus anthracis grows readily on most types of nutrient agar, however, 5–7% horse or sheep blood agar is the diagnostic medium of choice. Blood is the primary clinical material to examine.

- Swabs of blood, other body fluids or swabs taken from incisions in tissues or organs can be spread over blood agar plates.
- After overnight incubation at 37°C, B. anthracis colonies are grey-white to white, 0.3–0.5 cm in diameter, non-haemolytic, with a ground-glass surface, and very tacky when teased with an inoculating loop.
- Tailing and prominent wisps of growth trailing back toward the parent colony, all in the same direction, are sometimes seen. This characteristic has been described as a 'medusa head' or 'curled hair' appearance



Non-hemolytic, non-pigmented, dry ground glass from surface, edge irregular with comma projections, Agar"**Medusa Head" colonies**



Non-haemolytic colonies of *B. anthracis* environmental soil sample on Blood

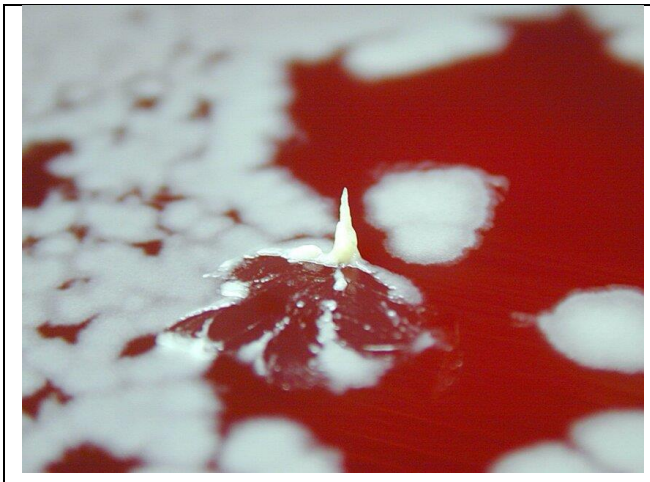
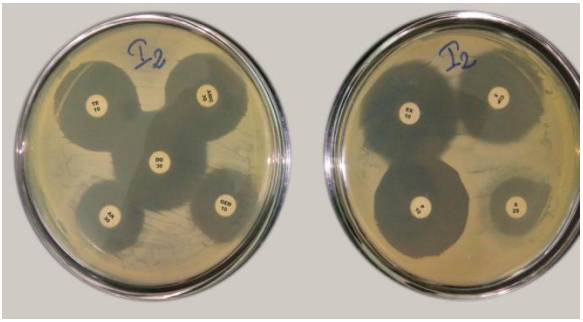
IV.Confirmation/ Identification of *Bacillus anthracis*

The recovered isolates either from blood agar or selective media like PLET agar and TSMP blood agar are used for further confirmation by the following tests

Two tests for confirming the identity of B. anthracis are

1. Gamma phage lysis
2. Penicillin susceptibility.

The typical procedure for these tests is to plate a lawn of suspect *B. anthracis* on a blood or nutrient agar plate and place a 10–15 µl drop of the phage suspension on one side of the lawn and a 10-unit penicillin disk to the other side. Allow the drop of phage suspension to soak into the agar before incubating the plate at 37°C. A control culture, e.g. the Sterne vaccine or the NCTC strain 10340, should be tested at the same time as the suspected culture to demonstrate the expected reaction for gamma phage lysis and penicillin susceptibility. If the suspect culture is *B. anthracis*, the area under the phage will be devoid of bacterial growth, because of lysis, and a clear zone will be seen around the penicillin disk indicating antibiotic susceptibility.

	
Gamma phage lysis	Penicillin susceptibility and antibiotic sensitivity

Processing of other Specimens

Identification of *B. anthracis* from old, decomposed specimens, processed materials, and environmental samples, including soil, is possible but these samples often have saprophytic contaminants that outgrow and obscure *B. anthracis* on non-selective agars.

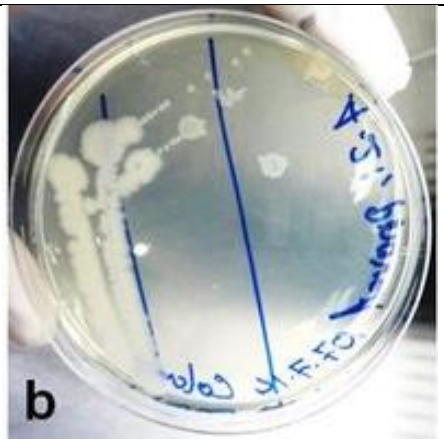
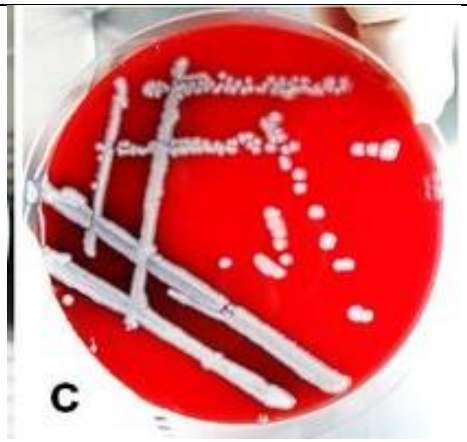
The following procedure is followed:

i) The sample is blended in two volumes of sterile distilled or de ionised water and placed in a water bath at 62.5 ± 0.5°C for 15–30 minutes. Turnbull et al. (2007) have demonstrated that heat activation of spores can be conducted at a temperature range of 60–70°C with holding times not exceeding 15–30 minutes for best recovery.

ii) Tenfold dilutions to 10–2 or 10–3 are then prepared. From each dilution, 10–100 µl are plated on to blood agar and optionally 250–300 µl on to PLET agar (polymyxin, lysozyme, EDTA [ethylene diamine tetra-acetic acid], thallous acetate) (Knisely, 1966; WHO, 2008). All plates are incubated at 37°C.

iii) Blood agar plates are examined for typical colonies as previously described after overnight incubation, and the PLET plates are examined after 40–48 hours. Confirmation of the identity of suspect colonies as *B. anthracis* is done as described above.

PLET medium (Knisely, 1966; WHO, 2008) is prepared by using heart-infusion agar base (DIFCO) made up to the manufacturer's instructions with the addition of 0.25–0.3 g/liter EDTA and 0.04 g/litre thallous acetate. The mixture is autoclaved and uniformly cooled to 50°C before adding the polymyxin at 30,000 units/liter and lysozyme at 300,000 units/liter. After mixing thoroughly, the agar is dispensed into Petri dishes.

	
PLET agar produced roughly circular and creamy white with ground glass appearance.	Blood agar plate- produced non-hemolytic, slightly convex, ground-glass in appearance and showed a characteristic "Medusa head" pattern of growth

Processing of ear piece/ hide samples

Collect earpiece samples from animals died of Anthrax and process for isolation of *Bacillus anthracis* according to the procedure followed by Melissa *et al*, 2005 and OIE, 2018 with slight modifications.

Preparation of sterile sand

Initially remove big particles of sand and other organic waste material manually from sand and dry it for a day under sunlight. Later sieve the sand and soak in 10% HCL solution overnight. Next day, pack the sand with muslin cloth, and wash under tap water for overnight. Later, in the the next morning, unpack the soil and dry under sunlight for a whole day before sterilizing in hot air oven at 160 ^{0}C for 2hrs.Finally store the sterile sand at room temperature for further use. Once the sterile sand is ready, the following procedure will be followed for the processing of tissue samples:

- Take 1gm of tissue sample with phosphate buffer saline (PBS) and cut into small pieces with sterile scissors and forceps.

- After cutting, transfer the tissue pieces into sterile mortar along with sterile sand and triturate for 15-20 minutes using a pestle.
- Filter the triturated contents into a sterile beaker with muslin cloth.
- Centrifuge the filtrate at 3,000 RPM for 10-15minutes.
- After centrifugation, carefully aspirate the supernatant into a test tube and boil in a water bath for 30 min at 60-65 ^{0}C.
- Then, prepare tenfold dilution of 10^{-1} to 10^{-5} processed sample with sterile PBS.
- Finally streak 100µl of sample from each dilution (from 10^{-2} and 10^{-3}) on blood agar plates and incubate at 37 ^{0}C for 24-48 hrs.

Molecular Diagnosis:

DNA extraction for conventional PCR and qPCR testing

All procedures related to sample aliquoting and DNA extraction were carried out in a class II biosafety cabinet at a bio containment level 3 facilities. Sterile filter pipette tips were used throughout all extractions.

DNA extraction:

DNA extraction was conducted using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Germany) spin column protocol, with initial sample preparation conducted as outlined below.

a) **<u>From Blood/ tissue Smears:</u>**

Scrapings were collected in a 1.5 ml microcentrifuge tube. After this, 200 µl PBS and 20 µl of 20 mg/ml proteinase K were added to the tubes. For blood samples, 20 µl proteinase K was pipetted into a 1.5 ml microcentrifuge tube. A 100 µl aliquot of the blood sample was transferred into the tube containing proteinase K, and the solution adjusted to 220 µl by adding 100 µl of phosphate buffered saline (PBS).

b) From swabs:

The sampled end was cut off and placed into a 1.5 ml microcentrifuge tube and soaked in 200 µl PBS with 20 µl proteinase K. The mixture was incubated at ambient temperature for at least one hour, vortexing the tubes mid-way and after incubation.

c) From skin and ear / muzzle tissue sample:

1gm of tissue sample was taken with phosphate buffer saline (PBS) and cut into small pieces with sterile scissors and forceps. After cutting, the tissue pieces were transferred into sterile mortar along with sterile sand and triturated for 15-20minutes using a pestle. These triturated contents were then filtered into a sterile beaker with muslin cloth. Later, the filtrate was centrifuged at 3,000RPM for 10-15minutes. After centrifugation, the supernatant was aspirated carefully into a test tube and boiled in a water bath for 30 min at 60-65 ^{0}C. Following this, 360 µl tissue lysis buffer (ATL buffer, included in the Qiagen kit) was added to each tube and vortexed. Proteinase K (40 µl) was added to the mixture and left to incubate at 56 ˚C for 6 to 8 hours or overnight until complete tissue lysis was achieved.

For the above three materials/ sample types the following is the common procedure after respective procedures of each one

For all these sample types, the supernatant (220 µl) was transferred to a new micro centrifuge tube and the DNeasy Blood & Tissue Kit spin column (Qiagen) protocol was completed according to the manufacturer's protocol. No-template controls were included in each extraction by taking only reagents through the extraction process. All DNA extracts were stored at -20˚C prior to use in PCR.

Conventional PCR

Confirmation of virulence can be carried out using the conventional PCR.

PCR for amplification of genus specific *rpoB* gene according to the method of Nazir *et al*, 2015 with some modifications.

Primer used for genus specific *rpoB* gene of Bacillus

Primer Code	Gene	Primer Sequence (5'-3')	Amplicon Size (bp)	Reference
BA-RF	*rpoB* (genus specific)	GACGATCATYTWGGAAACCG	359	Nazir *et al*, 2015
BA-RR		GGNGTYTCRATYGGACACAT		

Composition of PCR reaction mixture for amplification of genus specific gene of *rpoB* of Bacillus

Reagent	**Concentration (µl)**
Red Dye Master mix	12.5 µl
Forward primer (100µM)	1.0 µl
Reverse primer(100µM)	1.0 µl
Nuclease free water	7.5 µl
Template DNA	3 µl
Final reaction volume	**25 µl**

Thermo cycler conditions: initial denaturation at 95 ºC for 2 min, 35 cycles of denaturation at 95 ºC for 30 sec, annealing at 45 ºC for 30 sec, elongation at 72 ºC for 30 sec, final elongation at 72 ºC for 1 min and with hold temperature at 4 ºC.

PCR for amplification of species specific genes of *B.anthracis*

PCR for amplification of *PA* and *CAP* genes of *B.anthracis*

Primer used for PA gene and CAP genes of *B.anthracis*

Primer Code	Gene	Primer Sequence (5'-3')	Amplicon Size (bp)	Reference
PA 5	Protective antigen (*PA*)	TCC-TAA-CAC-TAA-CGA-AGT-CG	596	OIE, 2018
PA 8		GAG-GTA-GAA-GGA-TAT-ACG-GT		
CAP1234	Capsular antigen (*CAP*)	CTG-AGC-CAT-TAA-TCG-ATA-TG	846	OIE, 2018
CAP1301		TCC-CAC-TTA-CGT-AAT-CTG-AG		

Table-3b: Composition of PCR reaction mixture for amplification of *PA & CAP* genes of *B.anthracis*

Reagent	**Concentration (µl)**
Red Dye Master mix	12.5 µl
Forward primer (100µM)-PA 5	1.0 µl
Reverse primer(100µM)-PA 8	1.0 µl
Forward primer (100µM)-CAP 1234	1.0 µl
Reverse primer (100µM)-CAP 1301	1.0 µl
Nuclease free water	5.5 µl
Template DNA	3 µl
Final reaction volume	**25 µl**

Thermo cycler conditions: initial denaturation at 95 ºC for 5 min, 30 cycles of denaturation at 95 ºC for 30 sec, annealing at 55 ºC for 30 sec, elongation at 72 ºC for 30 sec, final elongation at 72 ºC for 5 min and with hold temperature at 4 ºC.

PCR for amplification of species specific *rpoB* gene of *B.anthracis*

Primer used for species specific *rpoB* gene of *B.anthracis*

Primer Code	**Gene**	**Primer Sequence (5'-3')**	**Amplicon Size (bp)**	**Reference**
BA-SF	*rpoB* (species specific)	TTCGTCCTGTTATTGCAG	208	Nazir *et al*, 2015
BA-RR		GGNGTYTCRATYGGACACAT		

Table-4b: Composition of PCR reaction mixture for species specific *rpoB* gene of *B.anthracis*

Reagent	**Concentration (µl)**
Red Dye Master mix	12.5 µl
Forward primer (100µM)	1.0 µl

Reverse primer(100µM)	1.0 µl
Nuclease free water	7.5 µl
Template DNA	3 µl
Final reaction volume	**25 µl**

The total volume of the reaction mixture was made up to 25µl with nuclease free water. The tubes were spinned for 10 seconds and PCR was carried out in thermal cycler (Proflex PCR, Life Technologies)

Thermo cycler conditions: initial denaturation at 95 ºC for 2 min, 35 cycles of denaturation at 95 ºC for 30 sec, annealing at 45 ºC for 30 sec, elongation at 72 ºC for 30 sec, final elongation at 72 ºC for 1 min and with hold temperature at 4 ºC.

PCR for amplification of *Ba813* gene of *B. anthracis*

Primers used for chromosomal *Ba813* gene of *B. anthracis*

Primer Code	Gene	Primer Sequence (5'-3')	Amplicon Size (bp)	Reference
R1	*Ba813*	TTAATTCACTTGCAACTGATGGG	152	Ramisse *et al*, 1996
R2		AACGATAGCTCCTACATTTGGAG		

Table-5b: Composition of PCR reaction mixture for amplification of Ba813 gene of *B. anthracis*

Reagent	**Concentration (µl)**
Red Dye Master mix	12.5 µl
Forward primer (100µM)	1.0 µl
Reverse primer(100µM)	1.0 µl
Nuclease free water	7.5 µl
Template DNA	3 µl

Final reaction volume	**25 µl**

The total volume of the reaction mixture was made up to 25µl with nuclease free water in 0.2mL micro centrifuge tube. The tubes were then spinned for 10 seconds and PCR was carried out in thermal cycler (Proflex PCR, Life Technologies).

Thermo cycler conditions: initial denaturation at 94 ºC for 5 min, 35 cycles of denaturation at 94 ºC for 30 sec, annealing at 58 ºC for 30 sec, elongation at 72 ºC for 30 sec, final elongation at 72 ºC for 1 min and with hold temperature at 4 ºC.

Analysis of PCR products:

Amplified PCR products were subjected to 2 percent agarose gel electrophoresis as described by Sambrook *et al*, (1989).

In brief, agarose gel (2%) was prepared by dissolving required amount of agarose in 1x TBE buffer (compostion given in appendix). Agarose was dissolved by heating in a microwave oven and cooled at 50 ^{0}C, added ethidium bromide at the concentration of 0.5 µg per ml and mixed properly. Then, the comb was placed so as to form wells. Then, 15µl of sample DNA was mixed with 3µl of loading dye and loaded in to the wells. The size of PCR products were ascertained with 100 bp standard molecular size marker (DNA ladder, Genei, Bangalore) and was loaded into the first well. Sterile distilled water was used as negative control. The electrophoresis was carried out at 80 volts for 60 minutes. PCR amplified products were visualized under U.V trans-illuminator and documented with gel documentation system (BIO-RAD, Gel Doc^{TM} XR+, DELL).

DNA sequence analysis

Amplified PCR products of genus specific Bacillus and species specific *B.anthracis* genes were sent for sequencing. Resulting sequences were submitted in FASTA format and analysed by

nucleotide database of the National Center for Biotechnology Information (NCBI) - Basic Local Alignment Search Tool available at http://www.ncbi.nlm.nih.gov/BLAST and compared with similar gene sequences available in Genbank. After confirmation of Bacillus and *Bacillus anthracis*, selected isolates sequences were submitted to genbank through Bankit submission portal for Accession numbers.

Multiple nucleotide alignment and Phylogenetic analysis

Multiple sequence alignment of nucleotide sequences was carried out using Clustal X 2.1 program to know percent identity and divergence. Phylogenetic analysis of received sequences of isolates were compared with analogous sequences of other *B.anthracis* isolates showing highest percent query coverage retrieved from Genbank, NCBI data base from different geographical regions to know the ancestral relatedness and evolutionary relationship of the isolates**.**

Real-time PCR

The procedure for DNA extraction from clinical samples and environmental samples is similar as mentioned in the conventional PCR. As per OIE manual the following methods were reported for detection of Anthrax.

Molecular beacons and multi-allelic real-time PCR for detection of and discrimination between virulent Bacillus anthracis and other Bacillus isolates (Hadjinicolaou *et al.*, 2009/ Journal of Microbiological Methods 78 (2009) 45–53)

Sequences of DNA oligonucleotides used as molecular beacons and primers used in the real-time PCR assay

Name	Oligonucleotide and molecular beacon sequence (5′–3′) a	Target allele[b]	Nucleotide position[c]	GenBank Accession no.
MBcapA	FAM-GCCTCGTGACGTCCCATCATAATGTCACCAACGAGGC-Dabcyl	capA	2144–2168	M24150

2113 (F)	CGTGAGAACGAAAAATTGACGATG	capA	2113–2136	M24150
2199 (R)	TGTACCGTAACGATTAACAATCTC	capA	2176–2199	M24150
MBcapB	FAM-GCCGAGAGCCTCTTTAACTACCCTGCGTTGCTCGGC-Dabcyl	capB	561–584	M24150
530 (F)	GCGCCGTAAAGAAGGTCCTAAT	capB	530–551	M24150
619 (R)	CATTCACAAATAAGTGCTTCTGCTTC	capB	594–619	M24150
MBcapC	FAM-GCCTGCAGGTAAAATACCTGTTCTTTCTGTAGCAGGC-Dabcyl	capC	1549–1573	M24150
1518 (F)	TAGTATTAGGAGTTACACTGAGCC	capC	1518–1541	M24150
1606 (R)	GAGTGCTAAATAACCAGGTACAAC	capC	1578–1606	M24150
MBlef	FAM-GCGGAGCTTTCTCAAGTAGCTTTTCTGCTGCCTCCGC-Dabcyl	lef	955–979	M30210
920 (F)	TAGAAGTAAAAGGGGAGGAAGCTG M30210	lef	920–943	M30210
1023 (R)	CTTTCCTCCAATTGCTTTATACATCTC M30210	lef	997–1023	M30210
MBpag	FAM-CAGCGGAAGTAGCAAATGTATATTCATCACTCCGCTG-Dabcyl	pag	356–372	AF306783
316 (F)	CAATCTGCTATTTGGTCAGGATTTATC AF306783	pag	316–342	AF306783
417 (R)	TTGGTCATCTACCCACATTGTTAC	pag	394–417	AF306783
MB16srRNA	FAM-CAGCCGTTACCTCACCAACTAGCTAATGCGACGGCTG-Dabcyl	16s rRNA	240–264	*EF062509*
211 (F)	TTCGGCTGTCACTTATGGATG	16s rRNA	211–231	EF062509
294 (R)	TCGGCTACGCATCGTTGCCTTG	16s rRNA	271–294	EF062509
MBIAC [d]	ROX-CGAGCCGCTACTCAGCAGAGGCTCCCTCGGGCTCG-Dabcyl			
302 (F)	TTGGCGATAGCCTGGCGGTG			
437 (R)	TGTTTACCGGGCATACCATCCAGAG			

DNA extraction: As per DNA Extraction kit from Qiagen

Thermal cycling conditions:

The cycling parameters were as follows: 1 cycle for 2 min at 95 °C followed by 50 cycles each consisting of the data collection step for 30 s and a second step for 10 s, starting at 80 °C and applying auto-incrementation of −1 °C per half-minute cycle until 31 °C were reached. The reaction consisted of a 25 µl solution containing 12.5 µl Platinum ® Quantitative PCR Supermix-UDG (Invitrogen, Carlsbad, CA), 1 µl (5 pmol/µl or 8 pmol/µl in the case of MBcapB) of the beacon probe with or without 100 pmol of a perfectly complementary single-stranded oligonucleotide target (Table), with distilled water making up the remaining volume. Changes in fluorescence were measured at 490 nm and the data collected at each temperature interval were plotted to form these thermal denaturation profiles and determine the optimal annealing temperature for the real-time PCR reactions

Isolation of Anthrax from Environmental Soil samples:

Classical method for isolation of B. anthracis:

The method used for the isolation of spores from environmental samples was that described in OIE Terrestrial Manual 2012 with some modifications. For culturing and isolation of B. anthracis the TSMP medium was used, consisting in the semi-selective Columbia blood agar added with trimethoprim (16 mg/lt), sulfamethoxazole (80 mg/lt), methanol (5 ml/lt) and polymyxin (300,000 units/lt). TSMP has the same efficacy of PLET in isolating B. anthracis. Briefly, to each 7.5 gram aliquot of soil sample were added 22.5 ml of deionized sterile water. After 30 minutes of washing by vortexing, the suspension was incubated at 64°C for 20 min to eliminate any vegetative forms of soil contaminants.

From each sample, 10 ml of supernatant were collected and dilutions of 1:10 and 1:100 were made using normal saline solution. Subsequently, 10 plates of TMSP were seeded with the undiluted suspension (100 µl/plate), 10 plates with the 1:10 dilution and 10 plates with the 1:100 dilution. After 24 and 48 hours of incubation at 37°C, each plate was examined for the presence of suspect colonies of B. anthracis and of contaminants. All colonies were counted. B. anthracis colonies were identified by Gram staining, colony morphology and anthrax-specific PCRs.

Ground anthrax bacillus refined isolation (GABRI) procedure:

To each 7.5 gram aliquot were added 22.5 ml of washing buffer consisting of deionized water containing 0.5% Tween 20. After 30 minutes of washing by vortexing, the suspension was centrifuged at 2000 rpm for 5 min to eliminate gross debris. The supernatant was harvested and then incubated, aerobically, at 64°C for 20 min to eliminate vegetative forms of *B. anthracis*. After incubation, 5 ml of supernatant were added to 5 ml of Tryptose Phosphate Broth containing 125 µg/ml of Fosfomycin. Then, from each sample, 10 plates of TMSP were seeded with 1 ml/plate of the mix and were incubated, aerobically, at 37°C. After 24 and 48 hours of incubation, each plate was examined and the colonies of *B. anthracis* and of contaminants were counted. *B. anthracis* colonies were identified by anthrax-specific PCRs.

Identification of Bacillus anthracis in environmental samples by Real time PCR
Citation: Irenge *et al*., 2010, Appl Microbiol Biotechnol (2010) 88:1179–1192

Primers and probes used in this study

Type	Target	Name	Sequence (5′ →3′)	Position	Reference
Primer	purA	PUR-ANT-FOR	CAACACTTAAAATTTGTGTTGCTTACAA	5208833-5208806	AE017225
Primer		PUR-ANT-REV	TCACATTTCGCTAAAATGTTTAAGTTTG	5208746-5208773	
Fluorogenic MGB-Probe		PUR-ANT-PRO	FAM-CGATGGGAAAGTTAT-NFQ-MGB	5208802-5208788	
Fluorogenic LNA-Probe		LNA-BA1	FAM-TCGATAACTTTCCCATCGCA-NFQ	5208785-5208804	
Non-fluorogenic C-probe		C-probe-BA	TGCGATGGMAAAGTTATCGATGAAGT	5208804-5208779	
Primer	ptsI	PTS-BAC-FOR	GCTTGACGGAAYTCATCAAGAGT	3902805-3902827	AE017225
Primer		PTS-BAC-REV	TATGYCTTGAWGARCAAGATGTGTTC	3902925-3902900	
Fluorogenic MGB-Probe			PTS-BAC-PRO VIC-ACAACTTCGTGCATT-NFQ-MGB	3902894-3902880	
LNA-Probe		LNA-BC2	YakimaYellow-GTACACAACTTCGTGCATT-NFQ	3902898-3902880	
Primer	pXO1	Lef-1-FOR	CATCGGTCTGGAAATAAAGGATGTA	150116-150092	AE011190
Primer		Lef-2-REV	GCACTACTTTCGCATCAATCCTTATA	150037-150062	AE011190
Fluorogenic MGB-Probe		pXO1-LEF-PRO	FAM-CTTTTTCGGATTGCTTA-NFQ-MGB	150067-150083	
Fluorogenic LNA-Probe		LNA-pXO1	FAM-TCTTTTTCGGATTGCTTA-BHQ1	150066 - 150083	
Primer	pXO2	CapA-1-FOR	TGACGATGGTTGGTGACATTATG	54939-54917	AE011191
Primer		CapA-2-REV	AATCTGTACCGTAACGATTAACAATCTC	54871-54898	
Fluorogenic MGB-Probe		pXO2-CAPa-PRO	VIC-TTTACGTGACGTCCCA-NFQ-MGB	54900-54915	
Fluorogenic LNA-Probe		LNA-pXO2	Yakima-Yellow-TTTACGTGACPTCCCATCA-BHQ1	54900-54918	

DNA extraction from environmental samples:

The PowerMax™ Soil DNA Isolation Kit (Mo Bio Laboratories, Inc, Carlsbad, CA, USA) was used to extract DNA from the 14 soil samples according to the manufacturer s instructions. DNA from other environmental samples (36 samples) was extracted using the NucliSens® miniMag semi-automated apparatus (Biomérieux Inc., Boxtel, The Netherlands), according to the manufacturer's instructions.

Real-time PCR assays:

Each duplex real-time PCR was carried out in 25 μL of a reaction mixture containing 2.5 μL of extracted DNA as template, 12.5 μL of TaqMan Universal PCR Master Mix (Applied Biosystems) containing dUTP and uracyl Nglycosylase (UNG), 300 nM of each primer and 100 nM of each MGB fluorogenic probe or 50 nM of each LNA Fluorogenic probe. Real-time PCR amplification was performed with an ABI Prism 7700 sequence detector (Applied Biosystems) under the following conditions: incubation at 50 °C for 2 min to activate UNG, initial denaturation at 95 °C for 10 min, and then 40 cycles of amplification with denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Each sample was tested in triplicate and data were recorded as Cycle threshold (Ct) on a TaqMan 7900HT Sequence Detection System (Applied Biosystems), using the analytical software from the manufacturer.

Other Serological methods in diagnosis of Anthrax

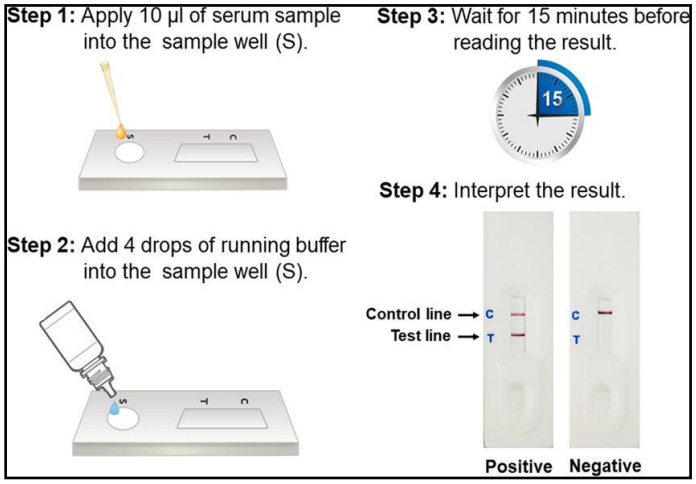
Immunological Detection and Diagnosis

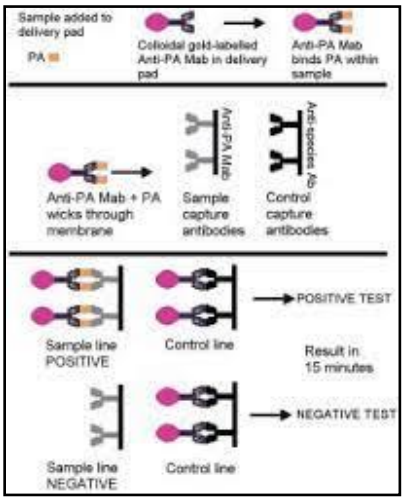
B. anthracis is antigenically very closely related to B. cereus, which is considered a ubiquitous component of the environmental microflora. The only unshared antigens that lend themselves to differentiating these two species by immunological approaches are the anthrax toxin antigens, produced during the exponential phase of growth, and the capsule of *B. anthracis*. This places considerable constraints on the extent to which immunological methods can be used in routine detection methodology.

Anthrax immunochromatographic test (AICT)

The anthrax immunochromatographic test (AICT) is a field test that detects a component of the anthrax toxin in blood. It is used in Australia to rapidly identify animals that have died recently of anthrax. The ICT was obtained as a kit from the United States Naval Medical Research Centre (Silver Spring, Maryland, USA). The kit contains colloidal gold bound with a monoclonal antibody to anthrax PA in an absorbent wick. The ICT had a specificity of 100% (98.5 to 100%; 95% CI).

The ICT is a simple test that requires only basic training in the performance and interpretation, with results available in 15 min. It could potentially be used by knackery staff for active surveillance of animals that die suddenly.





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