Manual for Laboratory Diagnosis of Anthrax
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Foreword

The use of microorganisms as a means of waging war or for generating terror is increasingly becoming a real possibility around the world. Any one of the thousands of biological agents that are capable of causing human infection could be considered a potential biological weapon. Only a small number of these, however, can be cultivated and dispersed effectively so as to cause death in numbers that will threaten the functioning of a large community. Of the recognized possible biological weapons, anthrax bacilli are rated the most lethal on account of their high case fatality rates; rapid transmission by aerosols, ease of growing in large quantities in the laboratory and their stability in the environment.

In the recent past, several Member Countries have perceived a threat of anthrax being used as a bioweapon. Many have made enquiries from WHO about the consequences of such an episode and the preparations they should make. While it is difficult to predict such events of bioterrorism, Member Countries of our Region can certainly enhance their preparedness to efficiently cope with such eventualities. An important part of this preparedness is the need to strengthen laboratory support services of national health systems.

Member Countries of the Region already possess a strong infrastructure in the form of institutions of public health and excellent laboratories. However, to safely handle anthrax and many other such organisms with high communicability, requires a specialized infrastructure. In the absence of facilities that conform to internationally accepted biosafety levels, it is not advisable for laboratories to process either clinical or environmental material.

Realizing that Member Countries in the South-East Asia Region do not have the requisite expertise to ensure appropriate laboratory infrastructure and methodology for laboratory support, WHO has developed this manual with technical inputs from a large number of international experts. It is hoped that this document will be useful in strengthening the capacity of Member Countries to effectively meet the challenge of anthrax.
Acknowledgement

World Health Organization wishes to acknowledge the services of Prof M.K. Lalitha, Department of Clinical Microbiology, Christian Medical College, Vellore, Tamilnadu, India for preparing the first draft of this document. She was ably assisted by Mrs. Umadevi Mukundan, retired Instructor. The document was extensively reviewed by a group of experts in a consultation held at Bangkok. The list of the experts is annexed and their contribution is gratefully acknowledged.
1. Introduction

The purpose of this manual is to provide practical guidance to laboratories for the diagnosis of anthrax in the event of naturally occurring or deliberately caused disease.

2. The Disease

Anthrax is an acute bacterial zoonoses caused by a Gram-positive bacterium, *Bacillus anthracis*. Human beings almost invariably contract anthrax from animals. Recent events, however, have shown the potential of this organism in deliberate causation of damage to human health, leading to considerable mortality, morbidity and panic amongst the communities.

3. Bacteriology

*B. anthracis* are gram-positive thick, long (3–8 μm x 1–1.5 μm) straight bacilli with square or truncated ends with parallel sides. In clinical specimens the organisms are usually found in pairs or chains of three or four bacilli. The chain of bacilli with truncated and swollen ends gives a characteristic “bamboo stick” appearance. A well-defined capsule surrounds an entire chain of bacilli. Spores are not formed *in vivo*. The bacilli are non-motile, a feature which is unusual among other *Bacillus* species. They are non acid-fast.

In laboratory media, the bacilli form long chains. Spores develop at the end of the log phase of multiplication. These are central / subterminal, ellipsoidal and non-bulging and resist Gram stain, appearing as unstained areas within the cell. With malachite green/safranine (or malachite green/
basic fuchsin) staining, the spores are stained green and the vegetative forms are pink. In the Ziehl-Neelsen staining, spores are pink and the vegetative forms are blue. Capsules are lost in normal culture. They can be induced either by growth in bicarbonate agar containing serum under a 5–20% carbon dioxide atmosphere or in defibrinated sheep or horse blood.

3.1 Cultural Characteristics

*B. anthracis* is a non-fastidious organism and can grow on simple laboratory media. They are facultative anaerobes. The optimum temperature for growth is 37°C and the pH, 7.0–7.4.

**Nutrient agar (NA)**

After overnight incubation at 37°C colonies are large 2–3 mm in diameter, irregular, raised, dull, opaque and grayish white with ‘frosted glass’ (ground glass) appearance. The edge of colonies may be curled or fringed edges with long interlacing chains of bacilli resembling curly locks. This is referred to as “medusa head appearance” but is not encountered as frequently as textbooks often suggest. The colony is membranous in consistency and hence not easily emulsifiable.

**Sheep blood agar (SBA)**

Lack of haemolysis is the norm. Weak haemolysis may be observed very rarely under areas of confluent growth, which should not be confused with beta haemolysis.

On sheep blood agar the colonies are non-haemolytic 2-3mm in diameter, irregular, raised, opaque and grayish white with a ‘frosted glass’ appearance. The edge of the colonies are curled or fringed having a ‘medusa head’ appearance.
Nutrient broth (NB)
No turbidity or very fineflocular turbidity is seen with flocular deposit. The deposit comes up as silky strands on shaking the broth gently, because of the tendency to form long chains in vitro and tenacious growth character of the organism.

PLET medium

This medium is used for the isolation of \textit{B. anthracis} from contaminated clinical materials or environmental samples. It consists of heart infusion agar with polymyxin, lysozyme, ethylene diamine tetra acetic acid (EDTA) and thallous acetate. After incubation at 37°C for 36–48 hours, the colonies of \textit{B. anthracis} are 1–3 mm, roughly circular, creamy white with ground-glass texture. Colonies are usually smaller in size on this medium compared to NA or SBA.

Polymyxin blood agar

This medium is useful for testing unheated suspensions of old decomposed or processed animal specimens or environmental specimens and reduces or prevents growth of many Gram-negative bacteria.

Bicarbonate agar

Colonies are mucoid in nature on this medium due to capsule formation.

3.2 Special Features

Susceptibility to penicillin G

\textit{B. anthracis} is almost always susceptible to penicillin, as shown by susceptibility to penicillin G 10 units discs on Mueller-Hinton agar. In contrast the non-pathogenic \textit{Bacillus} species are more generally resistant to penicillin.

Susceptibility to gamma bacteriophage

Gamma Phage has the ability to lyse \textit{B. anthracis} grown aerobically on blood or other nutrient agar and rarely lyses any other \textit{Bacillus} species.

Animal pathogenicity test

A definitive identity of a suspect \textit{B. anthracis} isolate can be done by inoculating the organism into a mouse or a guinea pig and confirming the cause of death by smear or isolation. However, for ethical reasons, animal inoculations are only done under exceptional circumstances.
4. Clinical features

Anthrax in humans can manifest itself in various clinical forms.

4.1 Clinical Forms

Cutaneous
Affects the skin, caused by bacteria coming in contact with cuts or abrasions on the skin. Starts as a pimple-like lesion followed by development of a ring of vesicles around the papule and leads to a black “eschar” within 1–7 days after contact. The lesion is surrounded by an extensive area of gelatinous oedema.

Inhalation/pulmonary
The most dangerous form is caused by inhalation of the bacteria or spores. Symptoms occur between two days to six weeks after contact. Early
symptoms include cough, fever, muscle pain and vomiting. Later, the patients develop worsening respiratory problems, generally with fatal systemic involvement. If untreated, this form of the disease has a 90% fatality.

Gastrointestinal
This is caused by consuming uncooked / partially cooked meat of infected animals. Symptoms include bloody diarrhoea and other abdominal complaints followed by massive ascites. In later stages, systemic involvement with toxaemia and shock may develop.

Septicaemic
Secondary to the primary form of the disease.

Meningeal 8
Meningitis and meningo-encephalitis may occur secondary to septicaemia or primary anthrax meningitis may occur due to inhalation of spores reaching the central nervous system directly.

4.2 Case Definition for Epidemiological Investigation

Suspected
A case that is compatible with the clinical description. In enzootic areas, all sudden death should be regarded as suspected anthrax cases.

Confirmed
A suspected case that is laboratory confirmed. A diagnosis based on clinical signs may be difficult, especially when the disease occurs in a new area.

Case definition based on laboratory evidence
All laboratory tests required to confirm the diagnosis of anthrax are not possible in all laboratories. The public health services may require immediate warning from the laboratory when clinical material is suspected of deriving from an anthrax case. To support such investigation and control activities, the following definitions of anthrax have been suggested:
5. Anthrax and the Microbiology Laboratory

The microbiology laboratory plays a vital role in establishing a diagnosis of anthrax by examining smears, and/or isolating the causative agent from clinical specimens, animal material and environmental samples.

5.1 Laboratory facilities

Laboratory facilities are usually classified as peripheral, intermediate and central laboratories in most of the countries of the SEA Region. The diagnostic services for anthrax can be provided to different extents based upon the facilities available.

Peripheral laboratory (district laboratory)

These laboratories can receive clinical samples and carry out Gram and polychromemethylene blue staining to establish initial “suspect” diagnosis of anthrax, so that immediate control measures can be instituted. Laboratories at this level should forward samples to an intermediate level laboratory as per the recommended procedure (Annex 1).

Intermediate laboratory (Regional/ medical college/ provincial laboratory)

These laboratories may be equipped to BSC level 2 and using the safest practices possible, should make a presumptive identification for anthrax by means of culture. If facilities are available, the intermediate laboratories...
can perform special tests and PCR. If necessary, the samples can be forwarded to the central (reference) laboratory for further confirmation. Isolates must be forwarded to the central reference laboratory for repository.

Central laboratory (reference laboratory — at least one per country)
This laboratory will reconfirm identification and may perform tests for genotyping, maintain a culture collection, serve as EQAS centre and carry out surveillance. It should also be charged with maintaining a national database as well as providing technical support to intermediate and peripheral laboratories.

5.2 Recommended Laboratory Infrastructure

According to the classification by WHO of microorganisms on the basis of their hazards B. anthracis belongs to hazard group 3.

Clinical specimens and materials
All clinical materials may be handled at lower hazard levels with the following safety precautions:
- Use of adequate protective clothing (gloves, masks, gowns with tight wrists and ties at the back and protective eye-shields). Masks (disposable, dust masks) are recommended in a peripheral laboratory, as they may lack appropriate containment facilities. B. anthracis may sporulate if the clinical material is left at the work bench.
- High quality, properly positioned facilities for hand washing.
- Careful dressing of skin abrasions.
- Proper handling of potentially contaminated articles.

Culture work
Manipulation of cultures or spores should be done in a safety cabinet, preferably a Class III cabinet.

Environmental samples
All suspect environmental specimens must be handled in a safety cabinet, again, preferably a Class III cabinet. A deliberately contaminated environmental sample is potentially very dangerous and the processing of suspect environmental samples should be restricted to a proper hazard level 3 laboratory in the central/reference laboratory.
5.3 Equipment and Materials

Peripheral laboratory

- Microscope
- Autoclave
- Microscope slides and cover slips, immersion oil, lens paper
- Test tubes/ bottle screw caps
- Sterile swabs
- Autoclavable discard bags
- Gloves, masks (disposable, dust masks), gowns
- Bunsen burner or spirit lamps to avoid contamination as the specimens may be referred to an intermediate or reference laboratory
- Specimen transportation kits
- Disinfectant spray “gun”

Reagent

- (Loeffler polychrome) methylene blue stain (quality controlled by reference laboratory for capsule staining)
- Gram stain

Disinfectant

- Sodium hypochlorite

Intermediate laboratory

As at peripheral laboratory plus:

- Biological safety cabinet, split air conditioner
- Incubator
- Centrifuge
- Waterbath
- CO₂ incubator/ candle jar
- Equipment for culture (loops, Bunsen burner, etc.)

Reagents

- (Loeffler polychrome) methylene blue stain (quality controlled by reference laboratory for capsule staining)
- Gram stain
- Malachite green/Ziehl-Neelsen stain
- Gamma phage (quality controlled by reference laboratory for efficacy)

Media
- Nutrient broth
- Blood agar
- Bicarbonate agar/ sheep or horse blood
- Polymyxin blood agar
- Penicillin disc and standard strain of *Staphylococcus aureus*

Central laboratory
In addition to equipment and materials in 5.3.1 and 5.3.2 the following should be provided:
- Hazard level 3 laboratory
- Biological class III safety cabinet
- Equipment and materials for PCR
- Media and materials for antimicrobial susceptibility testing
- Fumigation capability when necessary.

The ideal is a BSL (Biosafety level) 3 facility containing a class III cabinet. In some laboratories, a class II cabinet with respirator in a BSL 3 facility or a class III cabinet in a BSL 2 facility, has had to suffice and untoward accidents have not resulted.

Disinfectants
- Sodium hypochlorite
- Formalin (38-40 % formaldehyde)
- Formaldehyde degenerator

5.4 Biosafety Measures

Physical requirements
- A room separated from public areas by a door is required. There are no particular restrictions on locating the facility near public or heavily traveled corridors; however, doors should remain closed.
Coatings on walls, ceilings, furniture, and floors should be cleanable, e.g. by washing down with a hypochlorite solution. BSL 2 and 3 laboratories should have sealed windows and ventilation should be through extractable fans. Split airconditioning should be provided to maintain temperature in hot climates.

There are no special air handling requirements beyond those concerned with proper functioning of the biological safety cabinets, if used, and those required by building codes.

Hand washing facilities must be provided, preferably near the point of exit to public areas.

Separate hanging areas should be provided for street clothing and laboratory coats. An entry alcove with one door to the public area and inner door to the laboratory should be constructed. The hand-wash facility and coat hooks can be located in this, so that laboratory coats can be put on, taken off and stored here. On exiting, the laboratory coat is taken off, hung up and hands are washed before going into the public hallway.

Eye wash stations may be required by local statute.

In addition, where chemical disinfecting procedures are practised, effective concentrations and contact times must be employed. Chemical disinfectants used for decontamination of materials in the laboratory must be replaced regularly.

A biohazard sign with appropriate information must be posted at the entrance to the laboratory.

Laboratory furnishings and work surfaces should be impervious and regularly cleaned and disinfected.

Coat hooks must be provided for laboratory coats near the exit.

An autoclave must be available in or near the laboratory.

Laboratory doors should be self-closing.

Operational requirements

Biological safety cabinets (BSC) are required for all manipulations of agents, which may create infectious aerosols. The BSC must have been tested and certified within the previous 12 months according to accepted standards.
Air from these cabinets may be recirculated to the room only after passage through a high efficiency particulate air (HEPA) filter, or preferably a double HEPA filter.

Centrifugation must be carried out using closed containers sealed with parafilm and aerosol proof safety heads or cups. These should be opened after centrifugation only in the BSC.

An emergency plan for handling spills of infectious materials must be developed and be ready for use whenever needed. Laboratory workers must be educated and drilled in the emergency plans.

Vacuum lines used for work involving the agent must be protected from contamination by HEPA filters or equivalent. BSC level 2 cabinet which is equipped with evacuation and HEPA filter systems for decontamination of air. Safety is enhanced by the provision of heating facility for the discharged air. In the BSC 3 level facility, all vents should be fitted with HEPA filters and connected to a vacuum line which may not exit the containment parameters.

A laboratory coat to be worn only in the laboratory area is required. Coats that fasten on the front are permissible up to BSL 2. Gowns should be worn in BSL 3 laboratories. These coats/gowns shall not be worn outside the containment laboratory.

Special care should be taken to avoid contamination of the skin with infectious materials; gloves should be worn when skin may be exposed to infectious materials. Care should be taken to ensure that wrists are not exposed between gloves and sleeves of coats/gowns, especially for cabinet work.

Contaminated glassware must not leave the facility, except in an autoclave bin destined for the autoclave; for items not being autoclaved, decontamination must be carried out using procedures demonstrated to be effective.

Service personnel and cleaning staff who enter the facility must be informed of the hazards that might be encountered. Cleaning staff should clean only the floors. Cleaning of BSL3 laboratories is the responsibility of the laboratory staff; cleaners are excluded from these rooms unless they have been fumigated. The laboratory personnel have the responsibility for rendering the facility safe for routine cleaning. Periodic intensive cleaning must
be done at regular intervals. Cleaning and maintenance staff should receive immunization, and medical surveillance if appropriate.

5.5 Good Laboratory Practices

Laboratories handling specimens from anthrax cases should ensure that safety measures are appropriately implemented and enforced.

- Wear laboratory coats with elastic cuffing, disposable gloves and masks, head gear and protective eyewear.
- Use aerosol containment masks while handling biowarfare material and cultures, along with utilization of a level 3 type of facility.
- Avoid production of aerosols or dust.
- Mouth pipetting is strictly prohibited.
- Do not touch the areas outside the cabinet with gloved hands.
- Collect all materials removed from the hood including gloves and masks into an autoclavable bag used for collecting infectious waste for autoclaving and incineration. Autoclave, fumigate or otherwise sterilize reusable articles before cleaning.
- Wash hands thoroughly with soap and water. Use disposable towels or air drier after washing.
- Use leak-proof screw capped containers for specimens.
- For spills and splashes see 5.5.1 below.

Cleaning of laboratory work area

Regular

Dry sweeping of work area is contraindicated. Use wet mopping with 10,000 ppm hypochlorite solution and detergent. Vacuum cleaners should be avoided, but, if it is considered necessary to use one, it should be fitted with a HEPA filter.

Workbenches should be wiped down with hypochlorite solution containing 10,000 ppm available chlorine after the day’s work.

Spills and splashes on floor

Bench or apparatus should be flooded with 10,000 ppm hypochlorite solution for 5 minutes in case of fresh cultures and 30–60 minutes for
spore suspensions. Vertical surfaces should be washed down thoroughly with a cloth soaked in hypochlorite solution (5000–10,000 ppm). The mops/towels used for mopping should be autoclaved. If facilities are available, fumigation of the area may be done. The operator should wear gloves and safety spectacles while mopping the contaminated area.

Spills and splashes on clothing

Wear disposable laboratory gowns while working with B. anthracis or wear a plastic apron over the lab coat. Contaminated gowns should be placed in bags and autoclaved.

Spills and splashes on the skin

Wash the skin (contaminated area) with hypochlorite solution (5000 ppm) for one minute and wash thoroughly with soap and water. Where the skin is broken, wash with large quantities of water and contact a medical officer for further assistance.

Splashes in the eye

The eye must be flushed out with large quantities of water and a medical officer contacted.

5.6 Quality Assurance

To ensure the accuracy, reliability and reproducibility of various procedures followed in the laboratory, strict in-house (internal) quality control measures should be implemented on a regular basis. The laboratories should be encouraged to participate in the external quality assessment scheme (EQAS), wherever applicable.

An Internal Quality Control programme assures the quality of specimen processing, documents the validity of the test method, monitors the performance of test procedures, reagents, media, instruments and personnel and reviews test results for errors and clinical relevance.

QA of equipment

Log books containing working details of all equipment should be maintained.

E.g. Incubator
Refrigerator
Waterbath
Freezer

Daily checking and recording of temperature.
Record of cleaning, defrosting, etc. at regular intervals.
Autoclave

(a) Swabs / filter paper strips impregnated with spores of \textit{B. stearothermophilus} are placed randomly inside the autoclave to test the effectiveness of sterilization procedure. After the procedure the swabs are inoculated in a tube of nutrient broth. The tube is incubated at 56°C for seven days. The absence of growth indicates good performance of the autoclave.

(b) The Bowie-Dick tapes should be used to check the efficacy of the autoclave with every load. For details on interpretation, see the manufacturer’s recommendations.

Biological Safety Cabinet (BSC)

The cabinet should be completely free from microbes not only pathogenic but environmental also. The presence of bacteria can be tested by the use of “settle plates” as follows: Switch on the safety cabinet. Keep a blood agar plate exposed on the work surface of the cabinet for 30 minutes. Close the plate and incubate it at 35–37°C overnight. The presence of bacteria is indicated by the number of colonies found on the surface of the blood agar plate, which warrants the change of the bacterial filter fitted in the BSC. The reading of the manometer is also an indirect evidence of the clogging of filters. The validation of BSC should be undertaken at regular intervals as per instructions of the manufacturers.
5.7 Advantages and Disadvantages of Various Tests for Detection of B. anthracis

<table>
<thead>
<tr>
<th>Category of materials</th>
<th>Tests</th>
<th>Rapidity (hours)</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Interpretation for diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical specimens</td>
<td>Gram stain</td>
<td>1</td>
<td>Variable</td>
<td>Not specific</td>
<td>Suspect</td>
</tr>
<tr>
<td></td>
<td>Polychrome Methylene Blue</td>
<td>1</td>
<td>Variable</td>
<td>100%</td>
<td>Presumptive</td>
</tr>
<tr>
<td></td>
<td>Culture</td>
<td>18-48</td>
<td>High</td>
<td>100%</td>
<td>Confirmatory</td>
</tr>
<tr>
<td></td>
<td>PCR (PA DNA)</td>
<td>7-8</td>
<td>Variable</td>
<td>100%</td>
<td>Presumptive</td>
</tr>
<tr>
<td></td>
<td>ELISA (PA)</td>
<td>4</td>
<td>Variable</td>
<td>100% (fresh specimens only)</td>
<td>Presumptive</td>
</tr>
<tr>
<td></td>
<td>* ELISA (anti-PA)</td>
<td>4</td>
<td>Low</td>
<td>High</td>
<td>Useful for convalescent follow up</td>
</tr>
<tr>
<td>Environmental Materials</td>
<td>Malachite green (spore stain)</td>
<td>1</td>
<td>Low</td>
<td>Not specific</td>
<td>Suspect. Does not differentiate B. anthracis from other Bacillus species</td>
</tr>
<tr>
<td></td>
<td>Culture</td>
<td>24-48</td>
<td>High</td>
<td>100%</td>
<td>Confirmatory</td>
</tr>
<tr>
<td></td>
<td>ELISA(PA)</td>
<td>11-12</td>
<td>Variable</td>
<td>100%</td>
<td>Presumptive</td>
</tr>
<tr>
<td></td>
<td>PCR (PA DNA)</td>
<td>7-8</td>
<td>Variable</td>
<td>100%</td>
<td>Presumptive</td>
</tr>
</tbody>
</table>

* Protective antigen is available commercially; laboratories will need to standardize their own ELISA system. This is for seroepidemiology studies.
Model standard operating procedures

A. Fresh Clinical Sample Collection from Humans and Animals

B. Collection of Environmental Samples to Confirm the Presence of B. anthracis

C. Microscopic Examination for Anthrax

D. Bacteriological Diagnosis of Anthrax

E. Processing and Culture of Environmental Samples

F. ELISA for Detection of B. anthracis

G. PCR for Detection of B. anthracis
A. Fresh Clinical Sample Collection from Humans and Animals$^{1,2}$

1. Objective
To ensure correct/optimal collection of appropriate samples.

2. Samples to be collected (refer to 5.2)
Collection of samples from humans depends on the condition of the patient and stage of the disease.

<table>
<thead>
<tr>
<th>Clinical picture</th>
<th>Specimen</th>
<th>Quantity</th>
<th>Container</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cutaneous</td>
<td>Vesicular fluid</td>
<td>2</td>
<td>Sterile swabs</td>
</tr>
<tr>
<td>Inhalation/pulmonary</td>
<td>Blood</td>
<td>10 ml</td>
<td>Blood culture bottles</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>0.5 ml</td>
<td>Sterile screw capped container</td>
</tr>
<tr>
<td></td>
<td>Nasal swab</td>
<td>2</td>
<td>Sterile swabs</td>
</tr>
<tr>
<td>Gastro-intestinal</td>
<td>Blood</td>
<td>10 ml</td>
<td>Blood culture bottles</td>
</tr>
<tr>
<td></td>
<td>Ascitic</td>
<td>2 ml</td>
<td>Sterile screw capped container</td>
</tr>
<tr>
<td></td>
<td>Peritoneal fluid</td>
<td>2 ml</td>
<td>Sterile screw capped container</td>
</tr>
<tr>
<td>Meningitis</td>
<td>CSF</td>
<td>0.5 ml</td>
<td>Sterile screw capped container</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>10 ml</td>
<td>Blood culture bottles</td>
</tr>
</tbody>
</table>

Fresh animal blood or tissue samples should be collected into sterile containers with aseptic techniques.

The samples should be stored at 2–8 °C. If a long delay in the transportation of the sample is not expected, these can be transferred at room temperature and do not require cold chain.

3. Equipment and materials
   (a) Leakproof specimen collection containers
(b) Sterile swabs, forceps, scissors, syringe and needles [24-26 gauge], saline
(c) Alcohol swabs
(d) Sharps disposal containers
(e) Labels and markers or pens
(f) Adhesive tape
(g) Autoclavable discard bags

3.1 Safety

Personal protection equipment (PPE)

- Gowns, aprons or laboratory coats. Sleeves should belong, with elastic cuff.
- Disposable gloves
- Disposable face masks

Safety procedure

- Separate hanging areas should be provided for street clothing and laboratory coats. Gowns, apron and/or lab coats should be hung up before leaving laboratories.
- Hand washing facilities must be provided, preferably near the point of exit to public areas. Hands must be washed before leaving the laboratory.
- Overshoes or lab footwear should be used in BSL 3 laboratories.
- Eye wash stations may be required by local statute.

Discard, discharge and disposal

**Autoclave**

Basically all specimens and used tubes, petri dishes, bottles etc. should be autoclaved when finished with.

When working in safety cabinets, all specimens, culture plates, loops, tubes and other contaminated articles must be collected in autoclavable bags for infectious waste (appropriate colour coded) and autoclaved for 121°C for one hour preferably followed by incineration.

Contaminated surgical instruments should be put into autoclavable containers for autoclaving.
Microscope slides, coverslips and other sharp items should be placed in autoclavable sharps containers and autoclaved, preferably followed by incineration.

If working outside safety cabinets, pipette tips and other disposable items should be immersed overnight in hypochlorite solution (10,000 ppm) and then autoclaved, preferably followed by incineration. Laboratory clothing should be autoclaved before being sent to the laundry.

Fumigate
Equipment that cannot be autoclaved, boiled or immersed in disinfectant solutions should be fumigated.

Cabinets and rooms should be fumigated when suspect of being contaminated.

Hazardous substances
(a) Use hypochlorite solutions (10,000 ppm) prepared fresh every day.
(b) Handle sodium hypochlorite wearing gloves and goggles.
(c) Use formalin in well ventilated areas. It is injurious to skin and mucous membranes.
(d) Wear gloves and goggles or facemasks while handling formalin.

4. Procedure
(a) Use sterile leakproof, screw-capped containers for collection of samples.
(b) Follow aseptic techniques to protect the persons collecting the specimens and also to prevent contamination.
(c) Discourage persons with skincuts or abrasions from collecting specimens.
(d) Use disposable and impermeable gloves, aprons, disposable masks and disinfectable boots while collecting samples.
(e) Avoid spillage or aerosol formation as *B. anthracis* sporulates immediately on exposure to air.
(f) Label the container clearly and transport with proper precautions to avoid spillage.
(g) Collect specimens before the administration of antimicrobial therapy, as *B. anthracis* is highly susceptible to most of the commonly used agents.
B. Collection of Environmental Samples to Confirm the Presence of B. anthracis

1. Objective
To ensure that appropriate environmental samples associated with suspect cases or with deliberate release are collected correctly.

2. Equipment and materials
- Swabs and sterile water for moistening swabs
- Sterile spatulas or spoons
- Sterile containers for samples
- Secondary containers for sample containers (e.g. metal suitcase or trunk)
- Autoclavable discard bags for tools, clothing, boots, etc.
- Autoclavable discard bags for disposables
- Sharps containers
- Portable handwashing equipment

2.1 Safety

Personal protective equipment (PPE)
(a) Gowns, caps, [coveralls, disposable or re-usable, may be better than separate gowns and caps] quality tested respirator and goggles.
(b) Double disposable rubber gloves
(c) Boots, as necessary

Safety procedure
(a) Before specimen collection, put on the chosen clothing, including double gloving. Ensure disinfectant, disposal bags and handwashing equipment are ready.
(b) After specimen collection, rinse gloved hands in 10,000 ppm hypochlorite solutions and discard outer gloves.
(c) Discard used PPE into disposal bags, separating autoclavable
and non autoclavable items. Inner gloves should be discarded last. Sharps should be placed in sharps container.

(d) Wash hands.

Discard, discharge and disposal

(a) Autoclave autoclavable materials.

(b) Disinfect or fumigate non autoclavable materials (ref Section A.3.1).

Hazardous substances

(a) Prepare fresh hypochlorite solutions (10,000 ppm) each day.

(b) Handle sodium hypochlorite wearing gloves and goggles.

(c) Use formalin in well ventilated areas as it is injurious to skin and mucous membranes.

(d) Wear gloves and goggles or disposable dust masks while handling formalin.

3. Protective prophylaxis with antimicrobial agents

This should be offered if there is reason to believe that an individual has received a high exposure of aerosolized or ingested spores, or that spores may have penetrated the skin.

4. Procedure

(a) Containers containing suspected materials should be double bagged and sent to reference laboratory.

(b) Exposed surfaces should be swabbed with moisten swabs, double bagged and sent to the reference laboratory.

(c) Water should be collected by means of a syringe without needle and double bagged.

(d) Food samples must be collected in small sterile plastic containers and then double bagged.

(e) Soil samples should be collected into sterile containers and double bagged.
C. Microscopic Examination for Anthrax

1. Objective
Microscopic examination of clinical material for anthrax bacilli to establish a ‘suspect’ diagnosis of anthrax and of environmental materials for the presence of anthrax spores.

2. Equipment and materials
   (a) Binocular microscope with good oil immersion
   (b) Glass slides, cover slips
   (c) Staining jar
   (d) Stain bottles
   (e) Wash bottles
   (f) Stain tray with glass rod
   (g) Plasticine
   (h) Plastic autoclavable bag
   (i) Stains:
       - Gram stain
       - Loeffler (Polychrome) methylene blue
       - Absolute alcohol
       - Leishman stain
       - Geimsa stain
   (j) Immersion oil
   (k) Mounting fluid
   (l) Sodium hypochlorite 10%

3. Procedure
3.1 Preparation of smears
   (a) Make two thin smears of clinical/animal material by rolling over the swabs or spreading a small drop on a microscopeslide.
   (b) Air dry.
   (c) Fix by dipping in absolute alcohol for 30–60 seconds.
   (d) Do not heat dry to avoid distortion of morphology of the capsule.
(e) Re-dry.

(f) Stain one smear with polychrome methylene blue stain for the demonstration of capsule and the other with Gram stain.

3.2 Gram stain

(a) Follow the standard method.

(b) Observe the typical morphology of the bacillus:

In clinical material *B. anthracis* are Gram positive thick, long, straight bacilli with square or truncated ends with parallel sides found usually single, in pairs or chains of three or four bacilli. The chain of bacilli with truncated and swollen ends gives a characteristic "bamboo stick" appearance.

(c) Remember this is not a suitable stain for demonstration of capsule.

3.3 Polychrome methylene blue stain for capsule (M’Fadyean reaction)

This is the ideal method for demonstration of the capsule.

(a) Put a large drop of polychrome methylene blue on the smear to cover it completely.

(b) Leave for 30–60 seconds and air dry.

(c) Wash off (into hypochlorite solution, 10,000 ppm). A wash bottle is better than a tap.
(d) Examine under oil immersion.

The capsule is seen clearly as pink amorphous material surrounding the blue-black bacilli.

Leishman and Giemsa stains are recommended for staining CSF, other body fluids and blood, as the organisms are clearly visualized from the cellular background.

3.4 Giemsa stain
This is used especially for staining blood smears.
(a) Prepare fresh Giemsa stain by diluting stock stain 1:10 in buffer distilled water (pH 7.2).
(b) Air dry films. Fix in methanol for one minute.
(c) Wash off with wash bottle into hypochlorite solution.
(d) Flood the slide with Giemsa stain.
(e) Stain for 25–30 minutes.
(f) Run tap water on to the slide to float off the stain and to prevent deposition of precipitate on to the film.
(g) Drain, dry vertically.
(h) Examine under oil immersion.

The bacilli stain purple with red capsule.

3.5 Leishman stain
(a) Pour 8–12 drops of Leishman stain on the smear.
(b) Wait for two minutes.
(c) Dilute with double the volume of buffer water.
(d) Mix by rocking the slide or with a Pasteur pipette
(e) Keep for 7–10 minutes.
(f) Run tap water on to the slide to float off the stain.
(g) Drain and dry vertically in air.
(h) Examine under oil immersion.

The bacilli stain purple with a clear space around indicating capsule.
4. Safety measures (ref A.3.1)
   (a) Wash the stain off with water into a disposable tray/tray lined with plastic disposable bag containing sodium hypochlorite solution.
   (b) Discard the used slides and stains etc into the sharps container.
   (c) Dispose of the tray and the plastic bags as infectious waste.
   (d) Incinerate/autoclave the waste.

   The stained smears may be mounted using a clear mounting medium with neutral pH under a cover slip, for preservation. Polychromemethylene blue stained smears store much better if not mounted.

5. Quality control

Check all batches of stains prepared with control smears of known reactions or positive and negative reactions. In addition, include a smear of B. anthracis (Sterne strain) in each test.

<table>
<thead>
<tr>
<th>Staining method</th>
<th>Positive control</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram stain</td>
<td><em>Staphylococcus aureus</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>Capsule stain</td>
<td><em>Bacillus anthracis</em> (Wild strain)</td>
<td><em>Bacillus subtilis / Sterne strain</em></td>
</tr>
</tbody>
</table>
D. Bacteriological Diagnosis of Anthrax

1. Objective
Isolation and confirmation of *Bacillus anthracis* from clinical material.

2. Processing of specimens
Handle all specimens as per guidelines provided in sections/SOP for biosafety measures (see 5.2, 5.5)

2.1 Vesicular fluid
(a) Prepare two smears.
(b) Stain one by Gram stain and other by polychrome methylene blue stain.
(c) Follow the method and interpretation given in SOP C.
(d) Inoculate vesicle fluid onto sheep blood agar (SBA) and nutrient broth (NB) using swab or Pasteur pipette.
(e) Incubate plates at 37°C for 18–24 hours.
(f) Read the plates for colony characters:
   *B. anthracis* produces large, irregular, opaque, tenacious, non-haemolytic colonies with frosted glass appearance on SBA.
(g) Examine NB for floccular deposit and surface pellicle and use this suspension for checking motility of the isolate either by hanging drop method or by any other reliable method.

Hanging drop preparation
(a) Place a cover slip on a small piece of paper on the table inside the BSC.
(b) Place a small drop of mineral oil in each corner of the cover slip.
(c) Place a flat drop of the broth culture in the centre of the cover slip, taking care not to have contact with the oil.
(d) Invert a cavity slide over the cover slip and press gently to allow the oil to spread and form a complete seal.
(e) Turn the slide, so that the cover slip is on top.
(f) Inspect the drop to be sure it is “hanging” freely.
(g) Examine under the microscope, focussing first on the edge of the drop with low power and then switching to high power magnification.

(h) Ensure proper light adjustment by using light reflected by the concave mirror, partially closing the iris diaphragm and lowering the condenser, so as to have dim but adequate light to clearly view the hanging drop.

(i) Observe the motility:

*B. anthracis* is a non-motile organism. This characteristic is unusual among other *Bacillus* species.

(j) Discard the slide and cover slip into 10 % hypochlorite solution. A preliminary report on culture may be sent with presumptive diagnosis.

Examination of culture smears
(a) Pick up a very small portion of the suspected colony with a sterile loop.
(b) Mix on a slide with a loopful of sterile normal saline.
(c) Spread out gently to form a thin smear.
(d) Allow the smear to air dry and heat fix.
(e) Stain with Gram stain and malachite green.
(f) Observe spores.

2.2 Malachite green stain for spores

(a) Dry the films and fix with minimal flaming.
(b) Place the slide over a beaker of boiling water, resting it on the rim with the bacterial film uppermost.
(c) When, within several seconds, large droplets have condensed on the underside of the slide, flood it with 5% aqueous solution of malachite green. Leave to act for one minute and let the water continue to boil.
(d) Wash in cold water.
(e) Treat with 0.5% safranine or 0.05% basic fuchsin for 30 seconds.
(f) Wash and dry.

This method colours the spores green and the vegetative bacilli red.
2.3 Modified Ziehl-Neelsen stain for spores

(a) Air dry and heat fix the smear.
(b) Cover the smear with carbol fuchsin.
(c) Heat for 3-5 minutes.
(d) Do not allow the stain to boil.
(e) Wash with water.
(f) Decolourize with alcohol.
(g) Wash with water.
(h) Counterstain with methylene blue.
(i) Wash with water.
(j) Dry in air.
(k) Observe under oil immersion.

Spores will be stained red and vegetative forms blue.

2.4 Special tests

Induction of capsule formation

In blood

(a) Transfer a pinhead quantity of growth from a suspect colony to 2.5 ml defibrinated sheep or horse blood in a sterile test tube or small bottle.
(b) Incubate 5–18 hours at 37 °C.
(c) Transfer a drop with a 1 ml loop immersed to the bottom of the unshaken bottle or tube to a microscope slide and make a thin smear.
(d) Stain and examine as described in SOP C.

On bicarbonate agar plates

(a) Plate the suspect colony onto bicarbonate/saline agar.
(b) Incubate overnight at 37 °C under a 10–20% CO₂ atmosphere.
(c) Makesmears, stain and examine as described in SOP C. Although the capsule stains well when produced by this method, it does not appear so well circumscribed as when produced in vivo or in blood as described above.
Susceptibility to penicillin G
(a) Inoculate the organisms to be tested in 1.5 ml nutrient broth.
(b) Incubate for two hours at 37°C.
(c) Adjust the turbidity to Mc Farland’s 0.5 standard.
(d) The organism is inoculated on the entire surface of a Mueller Hinton agar/blood agar plate with a sterile swab.
(e) Let the inoculum dry for a period not exceeding 15 minutes.
(f) Place a penicillin G 10 units disc over it.
(g) Press the disc gently to ensure even contact with the medium.
(h) Use B. cereus as penicillin-resistant control and Staphylococcus aureus ATCC as penicillin-sensitive strain.
(i) Incubate the plate for 18 hours at 37°C.
(j) Measure the zone of inhibition around the disc.

Susceptibility to penicillin is indicated by a zone of inhibition of growth (≥29mm) around the disc.

Diagnostic gamma bacteriophage test
(a) Inoculate a blood agar plate evenly over its entire surface with a loopful of the test culture.
(b) Place a loopful or a small drop (10–15 µl) of phage suspension in the center of the plate.
(c) Allow to dry (do not spread).
(d) Incubate at 37°C for 6 hours to overnight.
(e) Observe for phage-induced bacterial lysis indicated by area of no growth.
(f) Include a culture of B. anthracis as a positive control (the Sterne strain will do).

CSF/ascitic fluid
(a) Do macroscopic examination.
(b) Do not centrifuge the fluids, as they are almost always haemorrhagic.
(c) Proceed with smear and culture as described in SOP D.3.1
Suggested procedure for isolation and identification of B. anthracis and confirmation of diagnosis

1. **Vesicular fluid, Ascitic fluid, Blood, CSF**
   - Smear
   - Gram stain
   - Polychrome methylene blue stain
   - Suspect/presumptive diagnosis

2. **Nutrient broth**
   - Motility (non-motile)

3. **Sheep blood agar**
   - Non-haemolytic grey colonies
   - Rough creamy-white colonies with ground-glass appearance
   - Capsule test
   - Polychrome methylene blue stain
   - Lysis by gamma phage
   - Susceptibility to penicillin G
   - PCR, if available

4. **Culture**
   - PLET (mixed flora)

5. **Confirmatory report**

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Manual for Laboratory Diagnosis of Anthrax
Suggested procedure for isolation and identification of B. anthracis and confirmation of isolate from environmental material

1. **Environmental material**
   - **Smear**
   - **Spore examination**
   - **Suspect identification**

2. **Nutrient broth**
   - **Motility** (non-motile)

3. **Sheep blood agar**
   - **Non-haemolytic grey colonies**
   - **Rough creamy-white colonies with ground-glass appearance**
   - **PLET (mixed flora)**

4. **Culture**
   - **Capsule test**
   - **Lysis by gamma phage**
   - **Susceptibility to penicillin G**
   - **PCR, if available**

5. **Confirmatory report**
Blood sample

(a) Incubate the inoculated blood culture bottle at 37°C.
(b) Examine the bottle after 18–24 hours incubation.
(c) Observe the growth as indicated by turbidity.
(d) With a sterile syringe and needle aspirate the broth (do not open the bottle) and make smears.
(e) Stain smears as described in SOP C.

If the smear is suggestive of *B. anthracis*, subculture on blood agar medium and proceed with identification as described in SOP D.3.1.

(a) If there is no growth in the broth, mix the broth gently and reincubate the bottle for three days.
(b) When growth occurs proceed with subculture and identification as in D.3.1.

4. Safety measures

(a) Perform all procedures in appropriate containment facility.
(b) Seal the plates and tubes before incubation.
(c) Discard the plates/tubes after appropriate disinfection/autoclave into autoclave bags. Autoclave and incinerate as in SOP A.3.1.
(d) Dispose of as infectious waste as in SOP A.3.1.

5. Quality control

Media are supplied by the manufacturers for use only after vigorous quality checking of their products. Still, all batches of media prepared, whether from dehydrated materials or from ingredients should be subjected to quality control for sterility and performance before use.
E. Processing and Culture of Environmental Samples

1. Objective
To confirm the presence of *B. anthracis* in the environmental samples.

2. Equipment and materials
   (a) Class II or III biological safety cabinet in BSL 3 laboratory or class III safety cabinet in BSL 2 laboratory.
   (b) Respirator while working in class II safety cabinet.

3. Procedure
   (a) Open the sample container in the safety cabinet and adopt the following procedures according to the type of sample.

3.1 Powder
Using a sterile wet swab, take a small amount of the powder and emulsify it in 1–2 ml sterile distilled/deionized water.

3.2 Surface and nasal swabs
Emulsify it in 1–2 ml sterile distilled/deionized water.

3.3 Food
Emulsify approximately 1 g of suspected food sample in 10 ml of sterile distilled/deionized water using pestle and mortar.

3.4 Soil
   (a) Mix approximately 1 g of suspected sample in 10 ml of sterile distilled/deionized water.
   (b) Prepare 10 ml volumes of 1:10 and 1:100 dilutions of the suspension in distilled water.

3.5 Water
   (a) Treat water as soil if the sample is too muddy.
   (b) Process drinking water according to standard procedures.
(c) Place the suspended sample in a 62–63°C water bath for 15 minutes to kill all vegetative forms and heat shock any spores present.

(d) Spread 100–200 µl on appropriate culture media. For soil and dirty water, use selective PLET agar; for water which appears clean and other samples unlikely to be heavily contaminated with non-anthrax bacteria, use blood agar. For food, depending on the condition of the sample, it may be best to use both PLET and blood agar.

(e) Incubate blood agar overnight and PLET agar for 36–48 hours at 37°C.

(f) The suspected colonies are further isolated and identified (see SOP D.3.1).

(g) In addition, a positive dot-blot ELISA and PCR tests (for protective antigen) may be helpful as a screening method for detection of *B. anthracis* (see SOP F)

4. Quality control

(a) Spread a sample (100–200 µl) of the “sterile” distilled/deionized water being used for sample suspension on a blood agar plate.

(b) Use a known positive sample, if available, as positive control. If not available, use a suspension of Sterne strain spores of known concentration (approximately $10^3$ per ml).
F. ELISA for Detection of B. anthracis

1. Objective
Presumptive diagnosis using antigen detection ELISA for protective antigen.

2. Equipment and materials
   (a) No special equipment required for performing the ELISA. All the necessary materials are supplied in the kits.
   (b) Class II biological safety cabinet with respirator for specimen preparation.

3. Procedure
Follow kit manufacturer’s instructions.

4. Quality control
Positive control: *B. anthracis* Sterne strain.
Negative control: Non-pathogenic *Bacillus* spp, *B. subtilis*/*B. cereus*.
G. PCR for Detection of B. anthracis

1. Objective
For presumptive diagnosis/detection from clinical specimens/environmental samples at intermediate or reference laboratory.

2. Equipment and materials

2.1 Equipment
Thermocycler, Gel electrophoresis apparatus, microfuge, UV transilluminator and Polaroid camera/gel documentation system, micropipettes, freezer, clean hoods [Class II for clinical specimens and DNA extraction; Class III biological safety cabinet or class II cabinet with respirator for handling environmental specimens], vortex mixer, heating block.

2.2 Materials and reagents
Filter tips, PCR grade water, primers, PCR tubes, appropriate extraction and running kits.

3. Procedure

3.1 Procedure with the clinical or environmental sample
A BSL 2 is required for the preliminary handling of clinical specimens and level 3 facilities for environmental specimen. The extracted DNA is non-hazardous and hence, the subsequent procedures are similar for both. A quantity of the sample as specified by the manufacturer of the kit being used is taken and the DNA extracted. The DNA extract is used as the template for amplification and detection.

3.2 Procedure with culture
Two to three colonies are picked and

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PCR for the detection of B. anthracis
Gel picture of B. anthracis DNA, PA and CAP
Lanes 1-positive control for PA, 5,6,7-positive for PA gene, 2&4 water blanks, 3-negative control, 8-Molecular weight ladder, 9-positive control for CAP, 13,14,15-positive for CAP gene, 10&12-water blanks, 11-negative control
suspended in sterile normal saline and adjusted to 1.0 McFarland units, then 200 µl of the suspension are taken and DNA extracted.

DNA extraction

As specified by the manufacturer in the protocol provided with the kits. Suitable primers for confirming the presence of the pX01 and pX02 plasmids are given in the following table.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer ID</th>
<th>Sequences 5'→3'</th>
<th>Product size</th>
<th>Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>PA-5 3048-3029</td>
<td>TCC TAA CAC TAA, CGA AGT CG</td>
<td>596 bp</td>
<td>1 mM</td>
</tr>
<tr>
<td></td>
<td>PA-8 2452-2471</td>
<td>GAG GTA GAA GGA, TAT ACG GT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capsule</td>
<td>1234</td>
<td>CTG AGC CAT TAA</td>
<td>846 bp</td>
<td>0.2 mM</td>
</tr>
<tr>
<td></td>
<td>1411-1430</td>
<td>TCG ATA TG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1301</td>
<td>TCC CAC TTA CGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2257-2238</td>
<td>AAT CTG AG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*References:*


**Carry out PCR using 25 µl reaction volume. PCR mix is as follows:**

- **PCR buffer**: 2.5 µl
- **MgCl₂**: 2.5 µl
- **DNTPs**: 4.0 µl
- **Primer 1**: 1 µl (50 pm)
- **Primer 2**: 1 µl (50 pm)
- **Amplitaq polymerase**: 0.25 µl
- **Nex DNAs**: 5 µl

Alternatively, pre-mixed, pre-dispensed, dried beads, stable at room temperature, containing all the necessary reagents, except primer and template, for performing 25 µl PCR reactions are commercially available ("Ready-to-Go". Pharmacia Biotech, Uppsala, Sweden. Product No 27-9555-01).

Use the following PCR cycles: 1 x 95°C for 5 min; 30 x (95°C for 0.5 min, followed by 55°C for 0.5 min, followed by 72°C for 0.5 min); 1 x 72°C 5 min; cool to 4°C.

After PCR, 10% of the reaction volume of tracking dye (0.02% xylene cyanol, 0.02% bromophenol blue and 50% glycerol) is added to each PCR
tube, and 10 µl loaded into wells on a 1% agarose gel in TAE and the gel
electrophoresed for 45 minutes at 120V followed by staining in ethidium
bromide solution for visualization under UV light. A 1-kilobase ladder
for size markers should be included in the outer wells of the gel.

4. Quality control

Positive control: A known virulent \textit{B. anthracis} clinical strain for PA and
CAP or alternatively \textit{B. anthracis} Sterne strain for PA only and Pasteur
strain. Negative control: Non-pathogenic \textit{Bacillus} spp., \textit{B. subtilis} / \textit{B. cereus}

A positive PCR test is \textit{confirmatory} for diagnosis of anthrax especially
for \textit{clinical materials} from sterile sites such as CSF and blood. It is considered
as screening for \textit{environmental samples}. 
Annexes

1. Specimen transport
2. Propagation of phage
3. Stains
4. Media
5. Disinfection and sterilization practices
6. Colour code for plastic disposal bags
7. List of experts who participated in consultation on finalization of Manual
Annex 1

Specimen transport

Transport all specimens at ambient temperature, as B. anthracis remains viable at this temperature.

The packaging requirements are determined by the UN and are contained in ICAO and IATA regulations in the form of packaging instructions (PI) 602 and 650. The current packaging requirements are described below. UN approved packaging systems are available commercially.

1. Basic triple packing system

This system consists of three layers as follows:

1. Primary receptacle. A labelled primary watertight, 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 that protects it and its contents from outside influences such as physical damage and water while in transit.

Specimen data forms, letters and other types of information that identify or describe the specimen and also identifies 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 4 litres-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 quantity limits do not apply to blood or blood products not believed to 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.

2. Hazard labels for dangerous goods
For all dangerous goods to be shipped by airfreight, specific hazard label(s) must be affixed to the outside of each package. The following hazards labels are of importance for the culture collections or other institutions shipping biological substances:

3. Hazard labels for infectious substances
Labelling of the outer package for shipment of infectious substances must include the elements listed hereafter.

   (1) The International Infectious Substance Label.
   (2) An address label with the following information:
       - The receiver’s (consignee) name, address and telephone number
       - The shipper’s (consignor) name, address and telephone number
       - The UN shipping name (Infectious Substances Affecting Humans or animals as the case may be) followed by the scientific name of the substance.
       - The UN number (Humans – UN2814, Animals UN2900)
       - Temperature storage requirements (optional)

   If the outer package is further packed in an overpack (with dry ice for instance) both outer pack and over pack must carry the above information, and the overpack must have a label stating “INNER PACKAGES COMPLY WITH PRESCRIBED SPECIFICATIONS”.

   (3) Required shipping documents — these are obtained from the carrier and are fixed to the outer package:
       - The shipper’s Declaration of Dangerous Goods
       - A packing list / proforma invoice which includes the receiver’s address, the number of packages, detail of contents, weight, value (note: state that there is “No commercial value” as the items are supplied free of charge)
       - An airwaybill, if shipping by air.
(4) An import and/or export permit and/or declaration if required.
(5) If the outer package contains primary receptacles exceeding 50 ml in combination of at least two “Orientation Labels” (arrows) must be placed on opposite sides of the package showing correct orientation of the package.

4. Requirements for airmail

Infectious substances and diagnostic specimens may be shipped by registered airmail. The basic triple packaging system is used with the same requirements as for other means of conveyance.

The address label must display the word “LETTRE” and the green Customs Declaration Label for Postal Mail is required for international mailing. Diagnostic specimens are to be identified with the violet ‘PERISHABLE BIOLOGICAL SUBSTANCES’ label. Infectious substances are to be identified with the International Infectious Substance label. Infectious substances must be accompanied with a shipper’s Declaration of Dangerous Goods form.

Because of local/international restrictions, prior contact should be made with the local post office to ascertain whether the packaged material will be accepted by the postal service.
Annex 2
Propagation of phage

1. Stage One
(a) Spread a blood agar plate of the Sterne vaccine strain of *B. anthracis*. Incubate at 37°C overnight.
(b) Inoculate approximately 10 ml of nutrient broth with growth from the blood agar plate and incubate at 37°C, on a shaking incubator if possible, for about until just cloudy, then refrigerate.
(c) Spread with a spreader 100 µl of the culture from step ii on three pre-dried blood agar plates and incubate at 37°C for approximately 30 minutes.
(d) With a spreader, spread 100 µl of the phage suspension to be amplified over the same plates. Incubate at 37°C overnight.
(e) Harvest the phage-lysed growth on the blood agar plate in 5 ml of nutrient broth followed by a second ‘wash’ of 5 ml nutrient broth. Make it up to 10 ml with nutrient broth. Incubate at 37°C, on a shaking incubator if possible, for 4–5 hours.
(f) Centrifuge at 2000 rpm for 30 minutes (preferably in a refrigerated centrifuge). Filter (0.45 µm). Label this ‘Stage One’ filtrate.

2. Stage Two
(g) This is essentially the same procedure as Stage One, only using the filtrate from step vi to harvest the phage from the plates.
(h) Prepare three Sterne strain lawns on blood agar, as in step (iii). Incubate at 37°C for approximately 30 minutes.
(i) Spread 100 µl filtrate from vi (Stage One filtrate). Incubate at 37°C overnight.
(j) Harvest the phage-lysed growth on the plates (from step viii) with 5 ml of the Stage One filtrate (vi), followed by a second 5 ml wash with the rest of the Stage One filtrate. The result should be approximately 9 ml of harvested material.
(k) Add 1 ml of 10 x nutrient broth.
(l) Incubate, on a shaker incubator if possible, at 37°C for 4–5 hours, centrifuge with appropriate precautions and filter (0.22 mm).

(m) The resulting filtrate is checked for sterility and titrated by spreading tenfold dilutions on lawns of the vaccine strain to determine the concentration of the phage. This should be of the order of $10^8$–$10^9$ plaque-forming units per ml. Keep refrigerated at a temperature of between 2°C and 8°C. Do not freeze.
Annex 3
Stains

1. Polychrome methylene blue
This is made by allowing Loefflers' methylene blue to "ripen" slowly. The stain is kept in bottles, which are half filled and shaken at intervals to aerate the contents. The slow oxidation of the methylene blue forms a violet compound that gives the stain its polychrome properties. The ripening takes 12 months or more to complete. It may be ripened quickly by the addition of 1% potassium carbonate to the stain.

2. Loeffler's methylene blue
Methylene blue
This stain is used to demonstrate M'Fadyean reaction of *B. anthracis*. The blue bacilli are surrounded by irregular purple capsular material.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylene Blue</td>
<td>0.2g</td>
</tr>
<tr>
<td>Absolute alcohol or rectified spirit</td>
<td>10.0ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>90.0ml</td>
</tr>
</tbody>
</table>

Dissolve the dye in alcohol and then add water. Filter through a filter paper.

3. Gram stain
A differential strain for gram positive (purple) and gram negative (red) bacteria.

Crystal violet

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystal violet</td>
<td>1.0g</td>
</tr>
<tr>
<td>5% sodium bicarbonate</td>
<td>1.0ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>99.0ml</td>
</tr>
</tbody>
</table>

Add 1 g of crystal violet and the sodium bicarbonate into a mortar and, using a pestle, grind until you get a good paste. Then add water and mix well. Filter through a filter paper.

Grams Iodine

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodine crystals</td>
<td>2.0gm</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>10.0ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>90.0ml</td>
</tr>
</tbody>
</table>

Add NaOH to the iodine crystals kept in a mortar. Grind with pestle to get a good paste. Add distilled water and mix well. Filter through a filter paper.
Acetone 100%:
Safranine

- Safranine 0.34g
- Absolute alcohol or rectified spirit 10.0ml
- Distilled water 90.0ml
Grind the dye in alcohol and then add water. Filter through filter paper.

4. Modified Ziehl-Neelsen stain for spores
Carbol fuchsin

- Basic fuchsin (powder) 1.6g
- Phenol liquefied 22.5ml
- Alcohol (95% or absolute) 50ml
- Distilled water 422.5ml
Dissolve the fuchsin in alcohol using a mortar and pestle. Mix phenol with water and add to the dissolved dye. Filter the mixture before use. Warm phenol at 45°C in a water bath and measure with a warm pipette.

Acetone 100% or alcohol 95%

Loeffler's methylene blue

5. Giemsa stain: stock solution

- Giemsa powder 0.3g
- Glycerine 25.0ml
- Methanol, acetone free 25.0ml
The stock solution is diluted 1 in 10 in distilled water before use.

6. Leishman’s stain

- Leishman powder 0.15g
- Methanol, acetone free 100.0ml

Solution no. I

- Sodium hydroxide 8g
- Distilled water to make 1,000ml

Solution no. II

- Potassium dihydrogen phosphate 27.2g
- Distilled water to make 100ml
Mix 23.7 ml of solution no. I with 50 ml of solution no. II. Add 20 ml of this to 1000 ml distilled water. The pH of this should be 6.8.
Annex 4

Media

1. Nutrient broth (NB)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1.0g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>0.3g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100ml</td>
</tr>
</tbody>
</table>

Weigh out all the ingredients as above, peptone should be taken last, because it sticks to the paper on exposure. Mix the ingredients and dissolve them by heating. When cool, adjust the pH to 7.4–7.6. Distribute in tubes, bottles or flasks and sterilize by autoclaving.

2. Bicarbonate agar

Reconstitute enough nutrient agar base powder for 100 ml of agar in 83 ml of water. Autoclave and cool to 50 ºC in water bath. Add 7 ml horse serum or lysed horse blood (filter sterilized if necessary) and 10 ml of filter sterilized 7% solution of sodium bicarbonate (both the serum/lysed blood and the bicarbonate should be brought to 50 ºC before adding to the molten agar). Mix and pour into petri dishes.

Use: Capsule formation can be induced on this medium when incubated in a candle jar or CO₂ incubator (preferably 10–20 % CO₂ atmosphere).

3. Blood agar

Sterile defibrinated sheep blood 7.0ml
Nutrient agar (melted) 100.0ml

Add blood (5–7%) to melted NA, which is cooled to 45–50°C. DO NOT USE HUMAN BLOOD. Mix well and pour as plates or slopes.

Use: an enriched medium, most pathogens grow in it. Serves as a differential medium for haemolytic organisms.

4. Polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar (pH 7.35)

Heart infusion agar/broth

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>0.3g/litre</td>
</tr>
<tr>
<td>Thallous acetate</td>
<td>0.4g/litre</td>
</tr>
</tbody>
</table>

Sterilize by autoclaving at 121°C for 15 minutes.
Cool to 50°C and add:

- Polymyxin B sulfate 30000 units/litre
- Lysozyme 300000 units/litre

It is important that the molten agar is at 50°C throughout its bulk before the polymyxin and lysozyme are added. When large volumes are being prepared in order to pour a large number of plates, the molten agar must be held in the water bath for an extended period. Mix thoroughly by swirling and pour as plates.

Use the best selective medium for isolation of *B. anthracis* from animal and environmental specimens contaminated with other organisms including other *Bacillus* species.
Annex 5
Disinfection and sterilization practices

1. Disinfectants used for destroying spores of B. anthracis

   (1) 2–10% formalin 30 min at 40°C
   (2) 0.25% formalin 6 hrs at 60°C
   (3) 3% peracetic acid 8 litres (2 gallon)/ square metre 10 min.
   (4) Sodiumhypochlorite 5–10 min at room temperature (10,000 ppm)
   (5) Iodoform in 100–300 ppm 1–5 min at room temperature

2. Use of heat for destroying B. anthracis
For small volumes or quantities with light contamination, autoclave at 121°C for 30 min.
Destruction autoclaving (discard bins, autoclave bags, etc. containing cultures, used and contaminated laboratory materials, etc. autoclave at 121°C for 60 minutes

3. Fumigation:
Note: Formaldehyde is a gas and is usually generated by boiling solutions of the gas in water (formalin) or heating solid paraformaldehyde strongly. Water becomes saturated at approximately 37%, so concentrated formalin is 37% formaldehyde

3.1 Biosafety cabinets
Close the cabinet completely or the windows and other outlets if it is a room should be sealed with heavy adhesive tape. For cabinets of 1–3 m³ formaldehyde gas is generated by boiling off 15–30 ml (depending on size of cabinet) of 37% formaldehyde solution diluted 2-3 fold with water. The temperature should be ≥ 8°C. Exposure time should be at least four hours; usually overnight is convenient. The doors are sealed and left closed for 12–48 hours. The formaldehyde vapour may be neutralized with ammonia before venting. Neutralization of formalin vapour is done with ammonia by keeping 300 ml of ammonia (in an open vessel) for 1000 cubic feet space for 4-8 hours.
3.2 Rooms

Properly functioning chemical respirators and a formaldehyde meter must be on hand for this procedure.

For rooms up to 25-30 m$^3$, 4 litres containing 400 ml of concentrated formalin may be boiled off in an electric kettle. For larger rooms, the volumes are scaled up. The rooms should be well sealed and the temperature should be $\geq 15^\circ$C. The fumigation time should be at least 4 hours; usually overnight is convenient. Spore-impregnated disks placed in the room before fumigation and subsequent culture for viable bacteria after the procedure can be used to check the effectiveness of fumigation procedure.

Personnel should not enter the room without a respirator until the formaldehyde meter shows the concentration of the formaldehyde in the air to be $\geq 2$ ppm.
Annex 6

Colour code for plastic disposal bags

<table>
<thead>
<tr>
<th>Yellow</th>
<th>Black</th>
<th>Pink</th>
<th>Blue</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Infective material only</td>
<td>Non-infective material</td>
<td>Infectious plastic items only</td>
<td>All sharps Sharps should always be discarded into official sharps containers</td>
</tr>
</tbody>
</table>

* Waste contaminated or potentially contaminated with blood or body products. Do not discard non-infectious items here.
Annex 7
List of experts who participated in consultation on finalization of Manual

Dr Md Moyez Uddin
Virologist,
Head of Microbiology Laboratory (Incharge)
Institute of Public Health,
Mohakhali
Dhaka
Bangladesh
e-mail: banlab@bdonline.com

Mr Karma Tshering
Laboratory In-charge, Microbiology
JDWNR Hospital
Thimphu
Bhutan
e-mail: Karma_sang@hotmail.com

Dr Veena Mittal
Joint Director (Microbiology)
National Institute of Communicable Diseases
22, Sham Nath Marg
Delhi-110 054
India
e-mail: surbhimittal@vsnl.net

Dr M.K. Lalitha
Professor of Microbiology
Department of Clinical Microbiology
Christian Medical College and Hospital
Vellore-632 004
India
e-mail: mkl_micro@yahoo.com
Dr H.V. Batra  
Head of Microbiology  
Defense Research Development Establishment  
Gwalior  
India  
e-mail: H_V_batra@rediffmail.com

Dr Gunawan Yamin  
Head, Division of Microbiology,  
Directorate of Health Labs, Ministry of Health  
J L H.R. Rasuna Said,  
Jakarta  
Indonesia  
e-mail: gyamin@cbn.net.id

Ms Shareefa Manike  
Assistant Director Laboratory Services  
Indira Gandhi Memorial Hospital  
Male  
Maldives  
Phone: 313980  
e-mail: shareefamanike@hotmail.com

Dr Chandrika Devi Shrestha  
Sr. Pathologist  
Nepal Public Health Laboratory  
Ministry of Health  
Teku,  
Kathmandu  
Nepal  
e-mail: adsh@ntc.net.np

Dr Pranitha Somaratne  
Medical Research Institute  
Colombo  
Sri Lanka  
Phone: 693532 Fax: 691495  
e-mail: kiyo@sti.lk
Mrs Surang Dejsirilert  
Head of Miscellaneous Bacteriology Laboratory  
National Institute of Health  
Department of Medical Sciences  
Ministry of Public Health  
Tiwanon Road  
Nonthaburi 11000  
Thailand  
Phone  662-589-9850 ext 9404  
e-mail: sudejsi@health.moph.go.th

Dr Peter Turnbull  
Arjemptur Technology Ltd  
C/O 86 St Mark’s Avenue  
Salisbury  
Wiltshire  
SP1 3DW  
United Kingdom  
e-mail:peterturnbull@tesco.net

Dr Ottorino Cosivi,  
CDS/CSR/EPH  
WHO  
Avenue Appia  
Geneva  
Switzerland  
e-mail: cosivio@who.int

Dr Rajesh Bhatia  
Regional Office WHO South East Asia  
IP Estate  
New Delhi 110 002  
India  
Phone  23370804 ext 26553  
e-mail: bhatiaraj@whosea.org

Mr Narintr Tima  
Programme Monitoring & Evaluation Officer  
WR Thailand Office  
Nonthaburi
References


