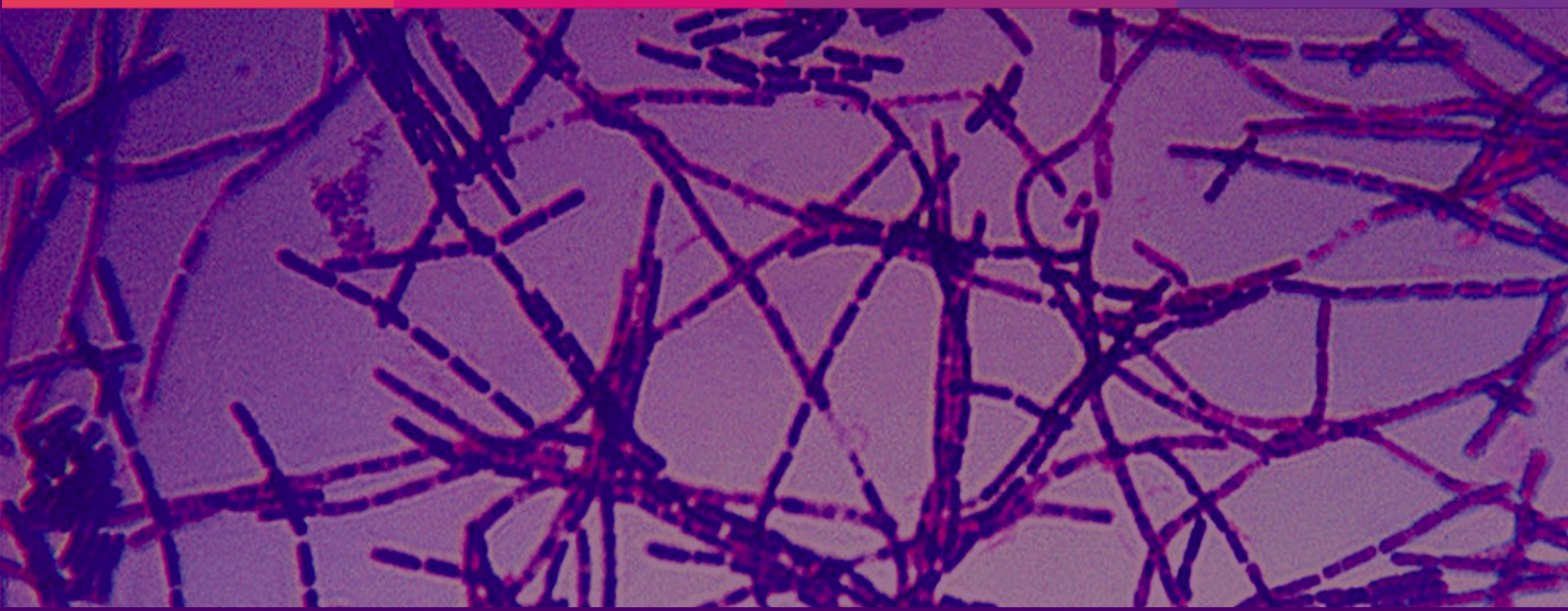
सत्यमेव जयते
Ministry of Health & Family Welfare
Government of India

Standard Operating Procedure

ANTHRAX

Guidance document for Laboratory Diagnosis of Anthrax



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2. Centre for Arboviral and Zoonotic diseases, National Center for Disease Control (NCDC), Ministry of Health and Family Welfare

ICMR, New Delhi
Standard Operating Procedures for Anthrax. 1st edition

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FOREWORD

Anthrax is a neglected disease with a potential for natural or manmade outbreaks. The extraordinary stability of *B.anthracis* spores that survive in soil for around 200 years and their resistance to many of the disinfection procedures demands extremely careful handling of samples or bacterial cultures and following stringent decontamination procedures.

Anthrax remains a remarkably under-reported disease due to failure to suspect, detect and diagnose the disease in many parts of India. The Indian Council of Medical Research (ICMR) has been at the forefront of addressing public health challenges and in providing technical support to fill the aforementioned gaps in biomedical research. This Standard Operating Procedure (SOP) is a step towards such endeavor. It is a practical manual intended to be used as a reference guide by various laboratories in different parts of the country. The manual describes the safe and scientific methods of specimen collection, transport, culture, identification and molecular testing for diagnosing Anthrax.

I applaud the diligent efforts of the authors, editorial board members and the coordination team who have worked hard to bring this publication to its present form. I am hopeful that the users of this manual will be able to appreciate its value in their respective fields of work. The SOP will further evolve for clinical as well as research purposes through periodic revisions and updates.

I convey my best wishes to all.

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MESSAGE

The devastating effects of COVID-19 pandemic globally have taught us several lessons on public health preparedness to prevent, protect against and respond to health emergencies. Anthrax being a lethal bacterial disease with additional potential of *Bacillus anthracis* being used as a bioweapon, merits due attention from such perspective. It is also important to recognize that persistent hot spots of anthrax with repeated outbreaks have been reported in the past from different parts of India. Awareness among the communities and training of healthcare workers and veterinary officers are therefore recommended as an essential component of preparedness for future emergencies.

Effective control of anthrax depends on timely diagnosis and reporting, which presently remains as major lacunae in several endemic regions of the country. This resource material has been developed as a reference manual to respond to such observations and to guide laboratories on standardized uniform protocols for diagnosis.

The team at the Indian Council of Medical Research (ICMR) has prepared this Standard Operating Procedure (SOP) in partnership with the relevant stakeholders. We believe that this SOP will be of tremendous use as a national guideline for handling suspected human, animal and environmental specimens towards diagnosing and managing anthrax. It will also add fillip to the existing biosafety measures in the country and facilitate responsible handling of agents of biothreat potential.

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CONTENTS

ICMR
Standard Operating Procedure for Anthrax

I. Human Anthrax

1. Important standard precautions

1.1. Safety measures :

All laboratory personnel should be well trained in biosafety and biosecurity before commencing work. Laboratory personnel should ensure adherence to safety protocols.

BSL-2 practices, containment equipment, and facilities are recommended for primary inoculation of cultures from potentially infectious clinical materials.

BSL-3 practices, containment equipment, and facilities are recommended when work involves high potential for aerosol formation, producing quantities of the organism or activities with antibiotic-resistant strains. Biosafety level may be considered based on the risk assessment of the activities performed.

General Good Laboratory Practices (GLP) must be followed as per the World Health Organization (WHO) guideline for Anthrax, 4th edition 2008.

Laboratory personnel should wear gown or laboratory coat with long sleeves and elastic cuffings and disposable gloves.

Appropriate disinfectant (usually 10% hypochlorite solution) should be prepared freshly on regular basis.

Screw capped non-breakable containers should be used for transport of specimens, cultures, etc. Autoclavable carriers or secondary containers should be used for moving cultures within the laboratory.

1.2. Storage instructions :

Storage of samples and *B.anthracis* isolates in the laboratory are strictly prohibited

- Samples and isolates should be kept only till the results are reported. All samples should be stored at 2-8^{o}C

- After reporting, the samples and isolates should be autoclaved and decontaminated as per protocol of the World Health Organization (WHO).

- Isolates may be sent to the reference laboratory for confirmation if required
  National reference laboratories: Department of Clinical Microbiology, Christian Medical College, Vellore (CMC), Tamilnadu and Center for Arboviral and Zoonotic Diseases (CAZD), National Center for Disease Control (NCDC), New Delhi.

1.3. Disinfection :

- Laboratory accessories like pipettes, tips, loops, spreaders etc. should be autoclaved (e.g. autoclavable bags).
- Biosafety cabinet should be disinfected after use with 10% sodium hypochlorite solution.
- Contaminated items should be kept in strong leak proof containers preferably within autoclavable bags.
- Hands should be thoroughly washed with soap and water and dried before leaving the facility.

1.4. Decontamination :

- All materials used including labware should be decontaminated by autoclaving at 121°C for 1 hour.
- Microscopic slides, cover slips and other sharp items should be placed in autoclavable sharp containers and autoclaved, preferably followed by incineration.
- Infectious disposable waste should be autoclaved followed by incineration.
- Non-autoclavable materials should be disinfected or fumigated.
- Laboratory clothing should be autoclaved before being sent to the laundry.

1.5. Spillage management :

- If accidental spillage or exposure to anthrax spore soccurs in the laboratory (including spills of infectious substances like blood or body fluids), PPE should be worn before cleaning the spillage (Gloves, protecting clothing including face and eye shield).
- The spill should be covered with absorbent cloth or paper towels soaked with disinfectant (10% hypochlorite).
- The disinfectant should be applied concentrically beginning at the outer margin of the spill area and working towards the centre.
- The spill should be allowed to soak in the disinfectant for about one hour.
- The materials should be cleared using forceps. Broken glass or other sharps should be placed in autoclavable sharps container for disposal.
- After autoclaving, the material has to be incinerated.
- Refer **Annexure II** for detailed information on disinfection and decontamination

.

1.6. Final disposal

- All cultures of *Bacillus anthracis*, specimens and disposable labware used for isolation, identification and performing molecular characterization should be decontaminated by placing in double autoclave bags for autoclaving followed by incineration.
- The disposable material should be autoclaved twice followed by incineration before final disposal.

- Fumigation should be carried out for non-disposable items.

- The decontaminated material should be sent for final disposal after securely bagging the contents kept in a covered trolley to the disposal area.

Instruction for disposal of Anthrax Carcasses (Refer Guidelines for Prevention and Control of Anthrax, Joint publication by Zoonosis Division, National Institute of Communicable Diseases and World Health Organization (WHO), Country office for India, 2006**-Annexure III)**

- AVOID USING LIME or other calcium products on carcasses or contaminated ground.

- Carcasses disposal should be done by incineration. However, deep burial is also an acceptable method.

- Performing an autopsy is prohibited when anthrax is being considered.

- Carcasses are inadvertently opened for post mortem examination or scavenging. Ensure that all body openings (e.g. anus, mouth, nose etc) are plugged with absorbent material, non-perforated towel, cloths etc., to prevent leakage.

- Head should be covered with heavy duty plastic bag and tied to prevent scavenging and spreading of spores by insects, birds and animals.

- Finally, incineration should be done. Incineration or burning of carcasses by Pyre or Pit is the preferred method of disposal..

- Ensure that an adequate amount of fuel is added to completely reduce the carcass to ash.

- If incineration is not possible deep burial may be a viable option.

In case of prolonged rains or logistical problem, carcass disposal may be delayed. Under these circumstances, cover the carcass and surround the area with 10% formalin or hypochlorite solution. This should be protected from scavenging and then finally incinerated.

Refer **Annexure III** for detailed information on carcass disposal

Note to the Clinicians :

Clinicians are encouraged to provide adequate volume of specimen and a second specimen to improve diagnostic yield. Swabs are generally discouraged when adequate purulent material or vesicular fluid is available. Two swabs are needed, one for smear and the other for culture. Clinicians are encouraged to contact the lab prior to sending suspected anthrax specimens. Refer **Annexure I** for Case definition for human and animal Anthrax.

2. Lab Diagnosis

2.1. Specimen collection :

Specimens for human Anthrax:

Pus (exudate) aspirate, swab, fluid from bleb, ascitic fluid, Peritoneal fluid, Bronchial lavage, CSF, blood, sputum or stool.

(a) Cutaneous Anthrax: Vesicular exudate from the lesions is collected by sterile swab at early stage or material from underneath the eschar after lifting with sterile forceps in later stage

(b) Intestinal Anthrax: A faecal specimen can be collected if patient is not severely ill. If patient is severely ill, ascitic fluid (peritoneal fluid) can be collected.

(c) Pulmonary Anthrax: Sputum can be collected if patient is not severely ill. If patient is severely ill, bronchial lavage can be collected.

Specimen	Container	Expected sample amount*	
Aspirated pus (exudate)	Universal disposable sterile container (wide mouthed, screw capped, leak proof)	≥0.5 ml	
Pus swab# (when pus cannot be aspirated)	Sterile tube with two sterile swabs	Two swabs; Each should be insinuated beneath the scab and rotated 2-3 times each	
Fluids (bleb, ascitic, peritoneal etc.)	Universal disposable sterile container (wide mouthed, screw capped, leak proof)	≥0.5 ml	
CSF	Sterile Eppendorf (given in the lumbar puncture set)	≥0.5 ml	
Blood	BacT / Alert blood culture bottles	Maximum:10 ml	
Sputum & Gastric aspirate	Universal disposable sterile container (wide mouthed, screw capped, leak proof)	≥0.5 ml	
Stool	Universal disposable sterile container (wide mouthed, screw capped, leak proof)	Maximum: 1/3 of container	

2.2. Specimen Transport and Processing :

All specimens are collected in appropriate leak proof, break resistant (preferably screw capped) containers and secured.

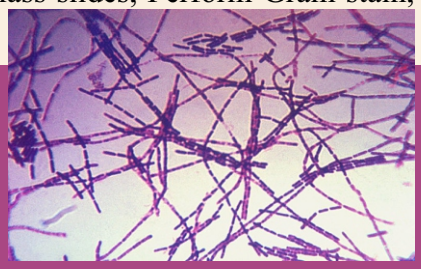
The container should be wiped with disinfectant solution (sodium hypochloride), labelled and tripled packaged.

The sample container is placed in a clear plastic cover with biohazard label, sealed and immediately transported to the laboratory (hand carried).

Personal Protective Equipment (PPE) : Wear gloves, N95 mask, apron, visor and boot cover during sample processing and follow-up.

Processing of sample : Samples are processed in the bio-safety cabinet BSLII type B2 with Biosafety Level 2 practices. BSL3 standards should be used when the work involves producing quantities of the organism, activities with high potential for aerosol production or activities with antibiotic-resistant strains.

2.3. Microscopy : Make 2 smears each on 3 separate glass slides, Perform Gram stain, polychrome methylene blue and methylene blue staining.



Gram stain :

Procedure :

Prepare the smear :

1. Use clean grease free and scratch free slides.
2. The slides are labeled with diamond marker or white pencil to ensure identification.
3. Use an inoculating loop or sterile pipette, or needle and syringe to place the liquid specimen (aspirate/fluid/pus/broth) at two point of a labeled slide.
4. Smear using swabs: Press the swab a few times onto the glass slide in a circular manner (impression smear on the surface of the slide).

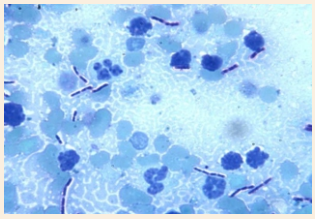
   Note: For specimens from suspected anthrax cases, two smears are prepared/clean glass slide.
5. To prepare smears from colonies growing on agar media by placing a small drop of saline onto the center of a labeled slide. Touch the center of a colony with a sterile smear loop and transfer a small amount of the bacterial colony to the drop of saline and mix the bacteria with the saline. Spread the mixture over an area approximately 2 sq.cm.
6. Allow smear to air dry.
7. Heat fix the smear. Mark the position of the smear as the reverse of the slide.
8. Note: If lot of purulent material is present, use methanol to fix the smear
9. Flood the fixed smear with the crystal violet allowing the stain to remain for one minute.
10. Pour off crystal violet and rinse slide in water using a wash bottle or gently flowing tap water until clear.
11. Flood the slide with iodine solution for one minute; rinse with gently flowing tap water or water from a wash bottle.
12. While holding the slide at a tilted angle, apply a few drop of decolorizer to the upper end of the slide, and allow the decolorizer solution to flow over the smear for 2 to 5 seconds.

1. Stop the decolorization after 2 - 5 seconds with a gentle flow of water. Do not apply decolorizer until the color stops running off because that will over decolorizer the bacteria.
2. Rinse with gently flowing tap water or water from the wash bottle.
3. Apply the counter stain safranine for 30 seconds and remove the excess stain by washing with gentle flowing tap water or water from the wash bottle water from a wash bottle.
4. Drain slide or air dry do not blot dry.
5. Examine the smear microscopically using the oil immersion objective (100x).

Gram stain report:

- Gram positive large thick rectangular bacilli
- Size 4x1µm
- Box car Shaped
- The ends of the bacilli are truncated giving a "Bamboo-stick" appearance
- Spores-Non bulging oval central or sub terminal spores
- In tissue single or short chains of bacilli without spores
- In culture long chains spores are seen

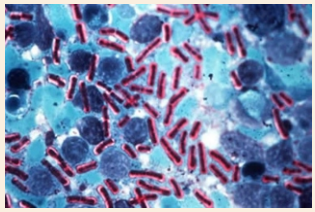
Methylene Blue Staining :



Purpose :

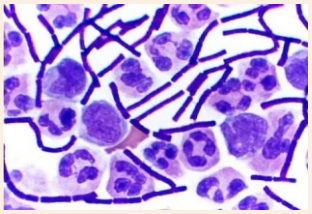
Methylene blue is a simple direct stain used to reveal the morphology of bacteria and to visualize the presence of capsule.

Expected Result :
Bacteria stain deep blue and leukocytes stain blue against a light blue staining background. Capsule of *B. anthracis* stains pink (this may not be seen often)



Polychrome Methylene blue (PCMB) staining protocol :
Purpose :
Demonstration of capsulated *Bacillus anthracis* in clinical specimens from patients suspected to have anthrax or after capsule induction test.

Mc Fadyean's reaction :



In Polychrome methylene blue stains *B. anthracis* capsule appears as amorphous purple material surrounding blue bacilli known as (Mc Fadyean'sreaction).

Media Inoculation and Culture Characteristic :
Media inoculation :
Nutrient agar (NA), Blood agar (BA), Nutrient broth (NB)
(seal all the culture plates and tubes with adhesive tape).

Incubation : Blood agar (BA) 5-10% CO_2 incubator at 37ºC
Nutrient agar (NA), Nutrient broth (NB) aerobic incubation at 37º C

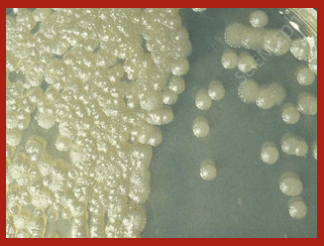
Duration of incubation :

Plates (BA & NA) : 48 hours

2.4. Culture characteristics :

Nutrient agar

Colonies are 2-3mm in size irregular, raised dull greyish whitewith frosted glass (ground glass)appearance.



Medusa head appearance :

When colonies are viewed low power microscope the edge of the colony which is composed of long interlacing chains of bacilli appears as locks of matted hair, giving medusa head appearance, may have fringed edge or put out curled protrusions (tailing).

Colonies have tacky consistency.

Blood Agar :

Non-hemolytic dry white or greyish white wrinkled irregular, low convex colonies with tailing along the streak line.

Blood Agar with penicillin : Concentration of penicillin 0.05-0.50 units

Solid medium with Penicillin: Colonies have a string pearl appearance look due to the cells becoming larger and spherical because of the weaker cell walls under the action of penicillin, and cells tend to occurs in chain on surface of agar.

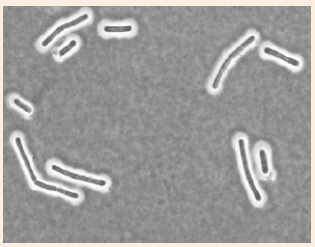
Nutrient broth : Floccular deposit with supernatant clear or slightly turbid

Do a hanging drop preparation from the overnight incubated nutrient broth (NB).

B anthracis is non-motile.

Note : *B anthracis* will be often observed in chains in NB, look for motility amongst bacilli which are singly placed or in pairs before reporting. If in doubt, re-inoculate into a fresh NB, incubate for 2 hours and then do a hanging drop for motility.
Keep the slide in a petridish and autoclave (DO NOT discard the cover-slip in discard jar).

Capsule induction test :



The capsule induction test is performed for quick confirmation of *B. anthracis* from Blood agar (BA) & Nutrient agar (NA) plates.

When is it performed: When long, thick Gram Positive Bacilli with sharp ends, with spores, are seen in Gram stained culture smears.

Procedure :

Inoculate a single colony into 5 ml of sterile sheep blood, seal with adhesive tape and incubate in 5% CO_2 incubator for 12-24 hrs.

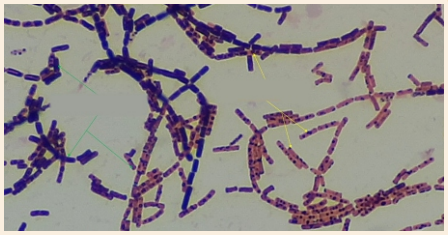
1. After 12-24 hrs incubation make smear of the blood using swab.
2. Discard the swab in 10% hypochlorite solution but NOT IN LYSOL.
3. Stain with polychrome methylene blue & methylene blue stain and look for the McFadyean's reaction.

Note: After the completion of the test autoclave the test tube with blood.

Susceptability to penicillin G:

1. Make a lawn culture of *Bacillus anthracis* on nutrient agar or blood agar.
2. Place 10 units of penicillin disk and incubate at 37°C for 16-18 hrs.
3. Zone of inhibition will be visible around the penicillin disk confirms susceptibility to penicillin.

Culture Smear :



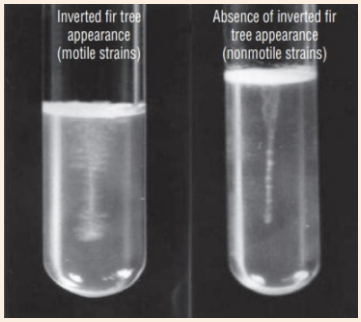
Gram-staining : Reveals bamboo stick appearance, i.e. long chain of gram-positive bacilli with non-bulging spores (appears as empty space)

Spores :

They can be demonstrated using special stains, such as hot 5% malachite green(Ashby's method)or 0.25% sulphuric acid used in acid fast staining for spores.

Spores appears as pink in colour bacilli appears as blue.

2.5. Biochemical Test :



Catalase Test :

- Catalase produce should be done inside the petri plate

Gelatin Liquefaction Test:

- Slow liquefaction of gelatin Growth occurs as "**Inverted Fur tree appearance"**(due to liquefaction of gelatin which occurs maximum at the surface, and then slows down towards the bottom)

Nitrate Test:

- Nitrate are reduced to nitrite.

Carbohydrate fermentation Test:

- Glucose, Maltose,Sucrose,Trehalose dextrin - fermented without gas.
- Salicin not fermentated.

Safety Measures :

- **Place petri dishes (or other culture containers) in a purpose-designed carrier or secondary container, such as a sandwich box, for movement around the laboratory. The carrier or container should be labelled with the agent, the operator's ID and date.**
- **Discard the plates/tubes into autoclave bags. Autoclave, preferably followed by incineration.**
- **Discard used slides in 10% hypochlorite and autoclaved**
- **Other sharp items into the sharps container which is autoclaved and then, preferably, incinerated also.**
- **Incinerate/autoclave other used disposable items of equipment.**
- **Double Autoclave the recyclable item.**
- **Fumigate or otherwise decontaminate non-disposable items of equipment which cannot be autoclaved.**
- **Decontaminate the safety cabinet after use with 10% sodium hypochlorite solution appropriate disinfectants.**

3. Serology :

Antibodies appear in convalescent sera and can be detected by ELISA or immunodiffusion in gel method.

Direct Demonstration :

3.1. Direct Immunofluorescence test :

It detects capsular and cell wall polypeptide antigens by using fluorescent tagged monoclonal antibodies. It is used for confirmation of the diagnosis during bioterrorism outbreaks.

3.2. Detection of anti-PA antibodies

Purpose:

To detect anti-PA (protective antigen) antibodies, which are suggestive of exposure to *Bacillus anthracis*

Principle:

Indirect ELISA; IgG antibody to protective antigen (anti-PA) if present in the serum will bind to the antigen coated on the micro-titre plate. The amount of antibody present will be proportional to the intensity of color generated when enzyme linked conjugate (anti-species antibody) is added.

Sample: Canine serum

Procedure: As per the prescribed kit insert

Kit used: Anthrax Protective Antigen IgG ELISA

Calculations :

1. Check Calibrator Factor (CF) value on the calibrator bottle. This value might vary from lot to lot. Make sure you check the value on every kit.
2. Calculate the cut-off value: Calibrator OD x Calibrator Factor (CF).
3. Calculate the Ab (Antibody) Index of each determination by dividing the O.D. value of each sample by cut-off value.

Validation criteria: All 3 criteria given below have to be satisfied for every ELISA run before declaring test results.

The three validation criteria to be met are:

1. The O.D. of the Calibrator should be >0.250.
2. The Ab index for Negative control should be <0.9.
3. The Ab Index for Positive control should be >1.2.

Interpretation:

<0.9 No detectable antibody to PA IgG by ELISA
0.91.1 Borderline positive. Follow-up testing is recommended if clinically indicated.
>1.1 Indicative of vaccination, current or previous Anthrax infection.

Quality assurance:

- Kit controls (PC & NC) should give expected results
- Results of the in-house QC and split sample testing should be within range.

PPE : As serum is not containing anthrax bacilli (only antibodies), standard PPE should be worn while testing

4. Antimicrobial Susceptibility Testing : (AST)

Antimicrobial Susceptibility Test is performed and interpreted as per the protocol described in Table 2K of CLSI M-100 S-20 (Vol 30; No.1); January 2010.

The gold standard for determining antimicrobial susceptibility for Anthrax is the conventional broth microdilution (BMD) and E-test method which is based on the Clinical and Laboratory Standards Institute guidelines.

(Note : DD (disk diffusion) is not the recommended method for *B. anthracis*)

MIC is performed for Penicillin, Tetracycline, Doxycycline, Ciprofloxacin and Levofloxacin. by broth microdilution (BMD) and E-test method

4.1. Broth Microdilution Test :

Broth microdilution tests are done in microtiter plates with a final volume of 0.1 ml. Requirements :

1. U-bottom or V-bottom 96 well microtiter plates
2. Sterile graduated pipettes – 10 ml
3. Sterile Pasteur pipettes
4. Overnight culture of test and control organisms
5. Required antibiotic in powder form (either from the manufacturer or standard laboratory accompanied by a statement of its activity in mg/unit or per ml. Clinical preparations should not be used for reference technique)
6. Required solvent for the antibiotic
7. Sterile distilled water - 500 ml
8. Suitable nutrient broth medium (caution adjusted Muller Hinton Broth Medium).
9. Micropipettes 20 – 200 µl, 10 µl with sterile tips

Procedure:

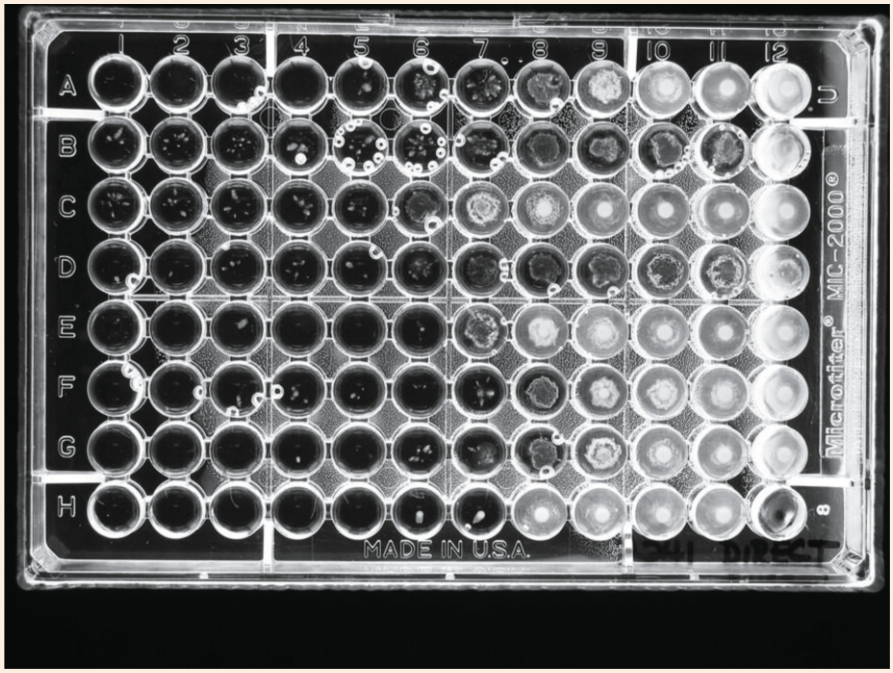
1. Prepare antimicrobial stock and serial two-fold dilution of the antimicrobial range to be tested in broth as in macrobroth dilution.
2. Add 0.1 ml of the antibiotic into the wells
3. Include one sterility well (only broth, no antimicrobial/inoculum) and one growth control well (broth with inoculum, no antimicrobial) in each tray
4. Inoculum preparation:
   a. Prepare 0.5 McFarland matched suspension of the organism either by direct suspension or growth method
   b. Dilute 1:20 in water-tween 80 diluent (2ml in 40 ml diluent)
5. Add 0.01 ml of the standardized inoculum preparation in all the wells except the sterility well using prongs or with micropipettes.
6. Seal the plate with a plastic tape or in a plastic bag
7. Check purity of inoculum by subculturing onto non-selective agar plate
8. Inoculum verification is done as follows:
   a. Take 0.01 ml of the growth control and dilute in 10 ml sterile saline
   b. Plate 0.1ml onto non-selective plate
   c. Count colonies after overnight incubation
   d. Approximate count of 50 indicates an inoculum density of 5 x 105 CFU/ml
9. Incubate the trays stacked no higher than four plates at 35 ± 2 °C for 16 to 20 hours in ambient air

Reading of plates:

1. Check purity of inoculum
2. Check sterility control well. It should be clear/non-turbid
3. Check growth control for adequate growth of at least 2mm button formation
4. The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the microdilution wells as detected by the unaided eye
5. When single skipped well is seen, read the highest MIC
6. Do not report results if more than one skipped well is present
7. For trimethoprime and sulfonamides, read the end point at the concentration in which there is = 80% reduction in growth as compared to the control

Recording template for microbroth dilution MIC:

		1	2	3	4	5	6	7	8	9	10	11	12
	Drug	--	--	--	--	--	--	--	--	--	--	--	
	conc.	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	Nil
A	Iso. 1												GC
B	Iso. 2												GC
C	Iso. 3												GC
D	Iso. 4												GC
E	Iso. 5												GC
F	Iso. 6												GC
G	Iso. 7												GC
H	Iso. 8											SC	GC



Broth microdilution test

4.2. E-test method :

The E test which is a quantitative method for antimicrobial susceptibility testing applies both the dilution of antibiotic and diffusion of antibiotic into the medium. A predefined stable antimicrobial gradient is present on a thin inert carrier strip. E test can be used to determine Minimum Inhibitory Concentration.

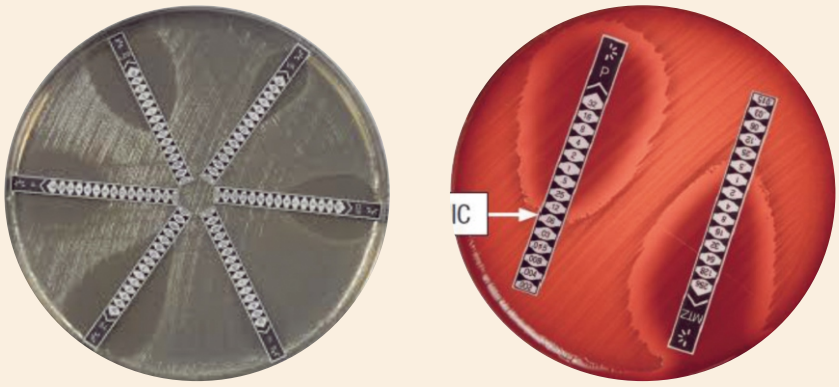
When this E test strip is applied onto an inoculated agar plate, there is an immediate release of the drug. Following incubation, a symmetrical inhibition ellipse is produced. The intersection of the inhibitory zone edge and the calibrated carrier strip indicates the MIC value over a wide concentration range (>10 dilutions) with inherent precision and accuracy.

Requirements :

1. E-test strips
2. Media (Mueller Hinton Blood Agar)
3. 0.5 McFarland turbidity standard
4. Cotton swabs
5. Forceps/ e-test applicator
6. Overnight culture of QC and test organisms

Procedure :

1. Remove E-test strips from freezer and allow to come to room temperature.
2. 0.5 McFarland standard matched suspension of the organism is prepared by growth method or direct colony suspension.
3. Streak a lawn culture of the standardized inoculum as in disk diffusion testing within 15 minutes of inoculum preparation
4. Allow excess moisture to be absorbed into the media
5. Apply strips onto agar surface with the forceps making sure the graduated surface is facing up.
6. Place only one strip for 90 mm petridish.
7. Do not change position of the strip once placed on the plate.
8. Incubate at 35± 2°C for 16 to 20 hours in ambient atmosphere.



Placement of multiple e-test strips on 150 mm and 100 mm petridishes

Reading:

1. Read MIC as the point where the inhibition ellipse intersects the scale.
2. Refer E-test reading guide

ETEST® READING GUIDE **ERG 001**

Aerobic Bacteria[1)]

ORGANISM EFFECTS

Ignore haemolysis (e.g. strep)
Read growth; **0.032** µg/mL

Ignore swarming (e.g. *Proteus* spp.)
Read growth edge; **0.064** µg/mL

S. maltophilia – trim/sulfa
Ignore haze in ellipse; **0.19** µg/mL

Pneumococci – ß-lactams
Read all growth; **4** µg/mL

Pneumococci – ß-lactams, read haze/inner colonies; **1.5** µg/mL

DRUG EFFECTS

Bactericidal drugs – read hazes, microcolonies; **1.5** µg/mL

Bactericidal drugs – read macro/microcolonies; **≥32** µg/mL

Bacteriostatic drugs – read at 80% inhibition; **0.032** µg/mL

Tigecycline – read at 80% inhibition; **0.032** µg/mL

Linezolid – read at 90% inhibition; **1** µg/mL

Intrinsic activity, clavulanate
Extrapolate curve; **3** µg/mL

ß-lactams – paradoxical effect
Read all growth; **≥256** µg/mL

Glycopeptides – slim ellipse
Read end of dip; **1** µg/mL

Polypeptides – slim ellipse
Read bottom of dip; **0.38** µg/mL

Polypeptides – read colonies in the dip; **3** µg/mL

RESISTANCE EFFECTS

GISA/hGISA – vancomycin
Read all growth; **8** µg/mL

ORSA – oxacillin
Read all growth; **64** µg/mL

KPC – carbapenems
Read all colonies; **8** µg/mL

ß-lactamase induction by clavulanate; **≥256** µg/mL

Small colony variants – bactericidal drugs; **32** µg/mL

TECHNICAL AND HANDLING

Between markings – read upper value; **0.19** µg/mL

Etest strip placed upside down
Invalid; repeat the test

Uneven – read upper value; if >1 dilution, repeat the test

Ignore line of growth alongside strip; **0.25** µg/mL

Distorted ellipse – wet surface, invalid; repeat the test

[1)] Please see CIS 006 (cidal/static chart), CIS 007 (reading endpoints) and product package inserts for additional reading information (www.abbiodisk.com/ETM).

AB BIODISK Tel. +46-(0)8-730 07 60, Fax. +46-(0)8-83 81 58, etest@abbiodisk.se, www.abbiodisk.com

E-test reading guidefrom AB Biodisk

Interpretative criteria:

Anti-microbial agent used	Susceptible MIC (µg/ml)	Non-susceptible MIC (µg/ml)	Comments
Penicillin	≤0.12	≥0.25	Can be extrapolated to amoxicillin
Tetracycline Doxycycline	≤1 ≤1	--	Non-susceptible strains are very rare*
Ciprofloxacin Levofloxacin	≤0.25 ≤0.25	--	

*If MIC value suggests isolate is not susceptible, the following has to be done

1. Confirm the identity of the isolate (as *B. anthracis*)
2. Confirm the AST results
3. Save the isolate
4. Submit the isolate to a reference laboratory for confirmation of ID & susceptibility

5. Confirmation of *B. anthracis*:

1. "Bamboo stick" appearance in Gram stain (GPB with non-bulging spores in culture isolates)
2. MacFadyean's reaction in polychrome methylene blue stain
3. Non-motile bacilli
4. Non-haemolytic colonies on blood agar after overnight incubation
5. Medusa head appearance on NA/BA
6. Capsule induction test positive
7. Penicillin susceptible (often)
8. PCR confirmation (detection of PX01 and PX02 plasmid)

6. Antimicrobial resistance in Anthrax

Rapid initiation of appropriate antibiotic therapy is crucial in the treatment of anthrax particularly in the systemic manifestations. Penicillin had been the drug of choice for all types of anthrax since 1940s, but naturally occurring strains are increasingly reported to be resistant. *B. anthracis* is sensitive to a broad range of antibiotics including tetracyclines, macrolides, aminoglycosides, fluoroquinolones, carbapenems, linezolid, clindamycin, rifampin, quinupristin-dalfopristin, daptomycin, and first-generation cephalosporins. However , second and third generation cephalosporin, cotrimaxozole to be avoided in the management of Anthrax, as they are relatively resistant to these drugs.

Most strains of naturally occurring *B. anthracis* have a chromosomally mediated, weak, inducible Beta-lactamase and cephalosporinase, and there have been rare reports of the development of resistance during therapy with penicillin, especially if subtherapeutic doses may have been administered. Being the most potent bioweapon the organism is expected to be ineffective to penicillin treatment due to the reason that the penicillin resistance can be induced in laboratory conditions. The guidelines for bioterrorism-associated anthrax recommend use of fluoroquinolones, carbapenems, and doxycycline until resistance testing is available, as β-lactam resistance in such strains is presumed to be likely to be ruled out.

The gold standard for determining antimicrobial susceptibility for Anthrax is the conventional broth microdilution (BMD) method which is based on the Clinical and Laboratory Standards Institute guidelines. This method is growth- dependent and requires an incubation period of 16 to 20 h for *B. anthracis*. Other methods commonly used for AST are agar dilution and diffusion-based assays such as the disc diffusion and the E test. These alternative methods, although easier to handle, require similar incubation times since visible growth is required for the interpretation of the results. A comparison of standard broth micro-dilution and E-test agar diffusion on *B. anthracis* isolates, conducted by the CDC, found that there was no statistically significant difference between both methods for any of the antimicrobial agents tested, except for penicillin in which the E-test method was two-fold dilutions lower than the standard broth microdilution method

Reports of antimicrobial susceptibility profile of *B. anthracis* are scarce. This is probably due to the less number of human anthrax cases reported. Long-term antibiotic therapy, as would be administered for post exposure prophylaxis in anthrax, might induce antimicrobial resistance by the selection of resistant mutants. The possibility of an inducible beta-lactamase activity should be taken into consideration under clinical conditions where high numbers of organisms are to be expected. Though fluoroquinolones were highly active, strains that are resistant to fluoroquinolone has been isolated by Price et al invitro studies due to development of mutations in gyrA, parC and gyrB.Beta-Lactam-resistant strains have been attributed to the derepression of cephalosporinase. Doxycycline resistance was conferred on *B. anthracis* by transfection with a pBC16 plasmid carrying a tetracycline resistant gene, tet.

Lightfoot et al evaluated nine antimicrobial agents with 33 epidemiologically distinct isolates by the agar dilution method. 90% of the strains were determined to be sensitive to penicillin, amoxicillin, gentamicin, streptomycin, erythromycin, tetracycline and ciprofloxacin. Doganay et al also used the agar dilution method for testing thirty antibiotics with 22 *B. anthracis* strains. They identified additional beta-lactams, aminoglycosides, clindamycin, vancomycin, and ofloxacin as antibiotics having activity.

Antibiotic susceptibility profile of various Human Anthrax outbreaks reported globally

Human Outbreak Cases	Number of isolates	Year of Isolation	Susceptiblity Profile			
			Pencillin	Fluoroquinolones	Erythromycin and Clindamycin	Tetracycline
Turkey	22	1981-1988	100%	100%	-	-
Ukraine	Case report	1992	0%	100%	100%	100%
France	96	1994-2000	85%	100%	95-100%	100%
India	Case report	1997	0%	100%	100%	100%
Romania	21	2000-2004	90%	100%	100%	100%
Florida	11	2001	95%	100%	98%	100%
Atlanta, Georgia	65	2004	96%	100%	100%	100%
Zambia	5	2011	100%	100%	100%	-
Kyrgyzstan, Central Asia	138	2005-2015	78%	>95%	--	>95%

7. Molecular Diagnosis:

PCR with specific primers can be used for further confirmation.

Molecular Typing: It is used for epidemiological studies to trace the source of infection.

Various methods available are as follows :

- MLVA (Multiple locus variable number of tandem repeat analysis)
- AFLP (Amplified fragment length polymorphisms)

7.1. Real-time PCR for anthrax

Purpose :
To detect DNA of *B anthracis* directly form samples and from cultures

Principle :
A segment of DNA unique to the pXO1 and pXO2 gene of *B anthracis* is detected by real-time PCR using a commercial assay kit.

DNA extraction :
Samples : Pus, purulent tissue, aspirates, fluids and stool

Amount of sample :
About 25 mg of purulent tissue (which is hand-ground in a glass mortar and pestle) or 200 µl of aspirate or fluid specimen or 200mg of stool.

Pre-treatment (to improve DNA yield): Add 100 µl of Lysozyme. Pulse vortex for 15 seconds; Incubate at 37° C for one hour.

DNA from tissues and aspirates/fluids or cultures is extracted using the DNeasy Blood & Tissue Kit or Blood Mini kit as per manufacturer's instructions after the pre-treatment procedure

The DNA Stool extraction kit is used to extract DNA from lysozyme treated stool using manufacturer's instructions.

The spin column protocol is used for extracting DNA from all samples. DNA purity is measured using 260/280 nm in a NanoDrop 2000 and the ratio is expected to be above 1.5.

The eluted DNA is stored in 3 or 4 aliquots (20-25 µl/ aliquot) at -70° C pending PCR amplification.

Real time PCR :
Kit based test used : *Bacillus anthracis* Detection Kit as per kit insert

Targets amplified :
The targets amplified are pXO1 and pXO2 gene respectively and an internal positive control (IPC)

Components of the kit are as follows : Kit to be stored 20°C

1. *Bacillus anthracis* Target Assay Mix, pXO1
2. *Bacillus anthracis* Target Assay Mix (10X), pXO2
3. Negative control
4. Fast PCR Master Mix (2X)

Note : pXO1 & pXO2 probes are labelled with FAM while the IPC is labelled with VIC

Real-time PCR equipment used :

- Real time PCR Machines
- All samples and controls are run in duplicate
  Workflow as described in the kit insert
- Prepare PCR master mix

Component	Contains	pXO1 master mix/reaction	pXO2 master mix/reaction
Fast PCR Master Mix (2X)	Polymerase & PCR buffer with $MgCl_2$	15 µl	15 µl
Target Assay Mix (10X)	Forward & reverse primer plus TaqMan probe	3 µl	3 µl
Total volume		18 µl	18 µl

Note : When preparing the master-mix, always prepare for n+1 reactions. This will ensure adequate master mix is dispensed for all reactions.

1. Example if, 10 samples including controls have to be run including controls, master mix is prepared for 22 reactions (as all samples and controls are run in duplicates).

- Distribute 18 µl into each 0.1 ml tube or strip
- Set up amplification (create and store run file before-hand)
  PCR amplification parameters

Stages		Temperature	Time	Cycles
Stage 1	Enzyme activation	95°C	20 seconds	1 cycle
Stage 2 (PCR)	Denaturation	95°C	3 seconds	45 cycles
	Annealing & extension	60°C	30 seconds	
Stage 3	Hold	4°C	∞	

- Amplification performed on "Fast" run mode
- After the run is completed (sample temperature has reached 4°C) the amplification plots for all samples are viewed

2. Set the baseline: Baseline is set by default above 0.1

3. Check each sample for a FAM™ dye (target-specific) signal and a VIC® dye (IPC) and note the Ct value

The threshold Ct value for a positive result is <35 for both duplicates. A negative result is given when the Ct value is >40 for both duplicates

If value is between 35 and 40 for any sample, assay is repeated, and if Ct<35 it is reported as positive. If Ct is still between 35 & 40 it is reported as borderline signal.

Interpretation

FAM dye signal (target)	VIC® dye signal (IPC)	Result
+	+	Positive
+	-	Positive
-	+	Negative
-	-	Troubleshoot*

Note : * Please refer kit insert or manual

II. Animal Anthrax

8. Introduction

- Herbivorous animals such as cattle, sheep and elephant are susceptible. Horses and pigs are less susceptible.
- Infection occurs in susceptible animals by ingestion of spores present in the soil
- Animal develops Fatal septicemia, localized cutaneous lesions and discharge large number of bacilli from mouth, nose and rectum
- These bacilli sporulate in soil and remains a source of infection for animals and humans

8.1. Specimen collection and Transport :

Legislation in most countries forbids postmortem examination of animals that have died of anthrax. Animals that have died suddenly and unexpectedly should not be necropsied unless anthrax has been ruled out as the cause of death.

Blood stained fluid may exude from one or more body orifices. Petechiae and ecchymoses are often present in unpigmented or hairless areas of the skin.

Unclotted blood from nasal, buccal or anal orifice, body fluids or spleen (if it is an open carcass) for carcasses 1 or 2 days old are collected. Blood from veins from ear pinnae and swabs from the lesion may be collected. Tissues or any blood stained material maybe collected for old putrefying carcasses.

8.2. Specimen for transport ("double-bagging")

- The specimens should be collected into sterile containers.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.

Generally, specimens should be stored at 28 °C. 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 12 hours.

8.3. Specimen Processing

The sample is processed in the BSL level II laboratory. BSL III practices are recommended for aerosol producing activities.

Personal Protective Equipment (PPE): Wear gloves, N95 mask, apron , visor and boot cover; The mandated Personal Protective Equipment is to be worn during sample processing and follow-up.

For microscopy culture and confirmation of anthrax please refer methods given for Human Anthrax.

III. Environmental sampling:

9. Sample collection :

9.1. Specimen Processing :

Sample collected : Soil sampling
Sample collection areas : The soil samples in anthrax endemic areas especially in pastures where cattle graze (in endemic/non-endemic/suspected anthrax is reported) will be sampled for anthrax using the more efficient ground anthrax bacillus refined isolation (GABRI) procedure, described by Fasanella et al.

When a suspected outbreak is reported, soil samples corresponding to the head and tail area of the carcass should be collected. These are spots most likely to have been contaminated by spore contaminated blood (Braun et al 2022). Using appropriate PPE, samples should be scooped from the surface (50-70g; ~half full 50 ml conical tube/flask).

Data collected : Data on soil type, pH, precipitation and temperature should be noted in-addition to the GIS co-ordinates.

9.2. Culture :

Soil processing for culture :
Ground anthrax bacillus refined isolation (GABRI) procedure (as described by Fasanella et al, 2013) :

1. A 7.5g aliquot of soil will be added to 22.5 ml of washing buffer consisting of deionized water containing 0.5% Tween 20.

2. After 30 minutes of washing by vortexing, the suspension will be centrifuged at 2000 rpm for 5 min to eliminate gross debris.

3. The harvested supernatant is incubated, aerobically, at 64°C for 20 min to eliminate vegetative forms of *B.anthracis.*

4. After incubation, 5 ml of supernatant is added to 5 ml of Tryptose Phosphate Broth containing 125 µg/ml of Fosfomycin.

5. Then, from each sample, 10 plates of TMSP are seeded with 1 ml/plate of the mix and incubated, aerobically, at 37°C.

6. **Medium Use :**
   TSMP medium (Trimethoprim Sulfamethoxazole Methanol (5 ml/lt) PolymyxinMedium): The TSMP is nothing but Columbia blood agar with trimethoprim (16 mg/lt), sulfamethoxazole (80 mg/lt), methanol (5 ml/lt) and polymyxin (300,000 units/lt).

   PLET medium It consists of polymyxin, Lysozyme, Ethylene diamine tetra acetic acid (EDTA) and Thallons acctate added in heart infusion agar. It has been devised to isolate *B.anthracis* from mixture of other spore forming bacilli.

7. After 24 and 48 hours of incubation, each plate is examined and the colonies of *B.anthracis* appears 2-3 mm rough, circular creamy white with ground glass appearance.

8. Colony morphology of *B. anthracis* should be follow as per the SOP in human anthrax

10. Appendix

10.1. Gram Stain :

Purpose :
Gram stain is most commonly used differential stain which divides bacteria into two major groups as Gram positive and Gram negative.

Ingredients and preparation :
Crystal Violet Stain:

Crystal violet	1.0 gm
Sodium bicarbonate 5%	1.0 ml
Distilled water	99.0 ml

- Add 1gm of crystal violet into a mortar.
- Using a pestle grind it well.
- Then add sodium carbonate little by little to get a smooth paste.
- Finally add water and mix well.
- Filter through a filter paper into a stoppered bottle.

Gram's Iodine :

Iodine crystal	2.0 gm
1N Sodium hydroxide	10.0 ml
Distilled water	90.0 ml

- Add NaOH to the iodine crystals, kept in a mortar.
- Grind the paste to get a smooth paste.
- Add distilled water and mix well
- Filter through a filter paper into a stoppered brown bottle.
- Keep away from sunlight.

Acetone 100%

Safranine Stain :

Safranine	0.34 gm
Absolute alcohol/rectified spirit	10.0 ml
Distilled water	90.0 ml

- Add absolute alcohol to the powder kept in a mortar.
- Grind the paste into a smooth paste.
- Add distilled water and mix well
- Filter through filter paper into a stoppered bottle.

Use:
Widely used in diagnostic bacteriology to differentiate Gram positive and Gram negative organisms.

Quality Control :

Positive control : a heat fixed smear of *S. aureus*, Gram positive violet/purple cocci in clusters.

Negative control : a heat fixed smear of *E. coli,* Gram negative pink/red bacilli.
If the control slide is improperly stained, repeat with a new control slide and a slide prepared

from clinical material. Because most problems are due to personal techniques, adjust staining time and technique. However, if proper results are not obtained, after those adjustments, consider a possible problem with the stains.

Notify supervisor if unable to obtain correct results.

10.2. Loeffler's Methylene Blue :

Ingredients :

Methylene blue	0.2 gm
Absolute alcohol/rectified spirit	10.0 ml
Distilled water	90.0 ml

Preparation :

- Weigh methylene blue and put it in the mortar
- Add absolute alcohol little by little and grind the powder into a smooth paste with the mortar.
- Add distilled water and mix it thoroughly.
- Filter through a filter paper into a stoppered bottle.

Use :

This simple stain is used to make out clearly the morphology of organisms e.g. *H.influenzae* in CSF, *N.gonorrhoeae* in urethral pus.

Quality Control :

Usea heat fixed smear of *Staphylococcus* aureus and *H.influenzae*

10.3. Polychrome Methylene Blue :

Preparation :

This is made by allowing Loeffler's methylene 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.

NOTE : The ripening takes 12 months, or more to complete or it may be ripened quickly by the addition of 1% potassium carbonate to the stain.

Expected Results :

The capsule of *B. anthracis* is seen clearly as pink amorphous material surrounding the blue - black bacilli (M'Fadyean reaction)

10.4. Malachite Green staining 5% :

- Make a smear and fixation can be done by heat or alcohol fix
- Place the slide over a beaker of boiling water, resting it on the rim with the bacterial smear uppermost;
- Cover with 5% aqueous solution of malachite green
- Stain for 5 minutes , adding more stain solution if the stain covering the smear starts to dry

Or

- Place the slide in a moist chamber (a petri dish with moistened filter paper will do)
- Cover with 5% aqueous solution of malachite green
- Leave to act for 60 minutes

Then, following either procedure

- Wash of stain with water using wash bottle (into hypochlorite solution)
- Counterstain with 0.5% safranin or 0.05% carbolfuchsin for 30 seconds
- Wash again (into hypochlorite solution) and allow to dry

Expected results : Spore appear green and the vegetative bacilli red

11. Culture Media :

11.1. Nutrient Broth (NB)

Peptone	1.0 gm
Beef extract (Lab Lemco)	0.4 gm
Sodium chloride	0.5 gm
Distilled water	100.0 ml

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.

Use :
This is a basal medium and is also used to grow non festidious organisms for various purpose.

11.2. Nutrient agar (NA)

Agar powder	1.5 to 1.8 gm
KH2PO4	0.05 gm
NaH2PO4	0.05 gm
Nutrient broth	100.0 ml

Mix the agar in nutrient broth and heat to dissolve. When cool adjust the pH to 7.5 0 7.6. Sterilize by autoclaving. Pour as plates or slopes. To make deeps, reduce agar concentration to 0.5%.

Use :
This is used as a base for many media. Only nonfastidious organisms will grow on this.

11.3. Blood agar (BA)

Sterile defibrinated sheep blood	10 ml
Nutrient agar (melted)	100.0 ml

Pour about 10 ml of melted Nutrient agar, as a base, into sterile petri dishes and allow setting. This forms a thin base for pouring blood agar. Add steriledefibrinated sheep blood (5 10%) to nutrient agar, the latter should be cooled to about 45 50°C before blood is added. Mix well and pour about 15 ml of blood agar over the base in each petri dish. Human blood is not recommended for the preparation of blood agaras it may contain certain antibacterial substances and hence unsuitable for use in media preparation.
Alternately blood agar may be made with no agar base.

Use :
It serves as an enriched medium and a differential medium for haemolytic organisms. Most common pathogens grow on it.

Quality control :
Viridans Streptococci- good growth with á-haemolytic colonies Group A *Beta-haemolytic Streptococci*- good growth with Beta-haemolytic colonies

11.4. Mueller Hinton Blood agar (MHA with 10% sheep blood)

MHA is made from commercially available dehydrated medium Difco TM MHA.
Catalogue No: 225250, (BD, Sparks, MD, USA).

38 gms of the powder provided is dissolved in 1 liter of dist. water mixed thoroughly boiled for 1 minute to completely dissolve. Adjust pH to 7.3+ 0.2.Autoclave at 121°C at 15 mts.Do not over heat.

Approximate formula per liter is as given below.

Beef extract	2.0 gm
Acid digest of casein	17.5 gm
Starch	1.5 gm
Agar	17.0 gm
Distilled water	1000 ml

Use :
Standard medium for antimicrobial susceptibility testing - fastidious organism.

12. Biochemicals

12.1. Carbohydrate Fermentation Test :

Purpose :
To determine the ability of an organism to ferment a specific carbohydrate incorporated in the basal medium, producing acid or acid and gas.

Test medium and materials :

Prepare sugar solutions as described below for different groups of organisms and dispense in 3 4 ml quantities into test tubes (12 x 100 mm). Introduce Durham's tubes into glucose broth for the detection of gas production. Autoclave at115°C for 10 minutes. Disaccharides like lactose and sucrose are better filtered and added to sterile basal medium.
(Glucose, sucrose, maltose, trehalose, dextrin, and salicin)

Sugar	0.5 gm
Nutrient broth base	100.0 ml
Bromthymol blue indicator (0.2% alcoholic)	1.2ml

Procedure : Inoculate the test organism to the carbohydrate medium incubate at 37° C aerobically for 24-28 hours

Result : Positive-acid production with yellow colour
Negative- no change in colour

Carbohydrate sugars	Positive control	Negative control
Glucose	ATCC 25922 *E. coli*	*Acinetobacter baumannii complex*
Sucrose	ATCC 25922 *E. coli*	*Acinetobacter baumannii complex*
Maltose	ATCC 25922 *E. coli*	*Acinetobacter baumannii complex*
Trehalose	ATCC 25922 *E. coli*	*Acinetobacter baumannii complex*
Dextrin	*Cornybacterium diptheriae*	Diphtheroids
Salicin	ATCC 700603 *Kleb. pneumoniae*	Acinetobacter baumannii complex

12.2. Catalase Test :

Purpose :
To detect the ability of an organism to produce the enzyme catalase which breaks down Hydrogen peroxide to water and nascent oxygen.
To differentiate between *Staphylococci* and *Streptococci.*

Test material :

Growth on Nutrient agar.
3% hydrogen peroxide
A young culture of *S. aureus* on NA agar.

Test procedure :

- Emulsify part of a colony in sterile saline on a clean glass slide.
- Add a drop of 3% H2O2.
- Inoculate a known positive control always

Reading and interpretation :

Appearance of gas bubbles immediately after the addition of H2O2 is to indicates the presence of catalase enzyme.

Eg: *S.aureus* positive
Streptococci negative

Note :

- Donot use Nichrome wire as this may give false positive reaction
- Do not use growth on BA to test catalase as RBCs might give false positive reaction.

12.3. Gelatinase Test :

Purpose :

To test the ability of an organism to produce a proteolytic enzyme gelatinase which liquefies gelatin.

Test medium :

Gelatin medium :

Nutrient broth	100.0 ml
Gelatin	6.0 gms

Adjust pH 7.2 7.4

- Heat ingredients other than gelatin, to dissolve in a water bath.
- Then add gelatin little by little to get a uniform solution.
- Dispense 5.0 ml per tube.

Autoclave at 121°C for 15 mts.

Test procedure :

- Inoculate the test organism as a stab into nutrient gelatin.
- Incubate at 37°C for 48hrs to a few days.

Reading and interpretation :

Keep the gelatin cultures in a beaker of ice or in the refrigerator before taking reading.
Liquefication of gelatin i.e. absence of setting of gelatin at low temperature indicates gelatinase activity.

E.g., *S. aureus*, *Pseudomonas spp.* Positive
S. epidermidis, E.coli Negative

Note :

Gelatin normally melts at about 24 - 27°C and sets below 20°C.

12.4. Nitrate Reduction Test :

Purpose :

To test the ability of an organism to reduce nitrate to nitrite and occasionally to gas, molecular nitrogen.

This is used for the identification of Enterobacteriaceae.

Test medium and reagents :

Potassium Nitrate Broth :

Potassium Nitrate (KNO_3) nitrite free	0.2 gm
Peptone	5.0 gm
Distilled water	1000 ml

Transfer into tubes in 5 ml amounts and autoclave.

Test reagent :

Solution A :

Sulphanilic acid	8.0 gm
5N acetic acid	1000 ml

Dissolve sulphanilic acid in acetic acid.

Solution B :

Beta-naphthylamine	5.0 gm
5N acetic acid	1000 ml

Dissolve Alpha-naphthylamine in acetic acid.

Test procedure :

- Inoculate the organism into potassium nitrate medium.
- Incubate at 37°C for 24 96 hrs.
- Immediately before use, make the test reagent by mixing equal volumes of solutions A and B.
- Add 0.1 ml of the test reagent to the test culture.

Reading and interpretation :

A red color developing within a few minutes indicates the presence of nitrite and hence the ability of the organism to reduce nitrate to nitrite.
E.g. *Enterobacteriaceae*

If red color does not develop, a pinch of zinc powder is added and shaken. If a red color develops on addition of zinc, it confirms that the test was negative
E.g. *Erwinia spp*

If no color develops following addition of Zinc, it confirms that nitrite was further reduced to nitrogen.
E.g. *Ps. aeruginosa.*

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ANNEXURE I

Case Definition

Recommended case definition : Humans

Suspected

A case that is compatible with the clinical description

AND

- Has an epidemiological link to confirmed or suspected animal cases (bleeding from natural orifices or bloated carcass) OR exposure to contaminated animal products.

- with or without Gram positive spore forming bacilli (1.5 to 3-4ìm in size), arranged end to end in chains(bamboo stick appearance).

Confirmed :

A suspected case that is laboratory confirmed by one or more of the following:

- Isolation of *B.anthracis* from a clinical specimen (e.g., blood, lesions, discharges)

- Demonstration of *B.anthracis* in a clinical specimen by microscopic examination of stained smears (vesicular fluid, blood, CSF, pleural fluid, stools)

- Positive serology (ELISA, Western blot, toxin detection, chromatographic assay, FAT)

Source : WHO Recommended Surveillance Standards (WHO/CDS/CSR/ISR/99.2)

Clinical description :

- Cutaneous anthrax (most common after direct exposure): Skin lesion begins as a painless, pruritic papule on exposed parts (hands, feet and neck) which develops into a vesicle (usually 1-3 cm in diameter) and then a painless ulcer with a characteristic black necrotic (dying) area in the centre surrounded by erythema and edema. Systemic symptoms are mild and may include malaise and low-gradefever. There may be regional lymphangitis and lymphadenopathy. Occasionally more severe form of cutaneous anthrax may occur with extensive local oedema, induration and toxaemia.

- Gastrointestinal anthrax: There are two clinical forms of intestinal anthrax - Symptoms include nausea, vomiting, fever, abdominal pain, hematemesis, bloody diarrhoea and massive ascites. Unless treatment starts early toxaemia and shock develop resulting in death. Oropharyngeal anthrax clinical features are sore throat,dysphagia,fever,lymphadenopathy in the neck and toxaemia.

- Pulmonary (inhalation): brief prodrome resembling acute viral respiratory illness, followed by rapid onset of hypoxia, dyspnea and high temperature, with X-ray evidence of mediastinal widening.

Source : Updated by Zoonosis Division NCDC on 02.07.2019

ANNEXURE II

DISINFECTION AND DECONTAMINATION

Bacillus anthracis tend to form spores when conditions are not conducive to growth and multiplication. The spore forms are highly resistant to extremes of heat, cold, pH, desiccation, chemicals (and thus to disinfection), irradiation and other such adverse conditions. Spores will germinate outside an animal if they fall into appropriate conditions, i.e. a temperature between about 8oC and 45°C, a pH between about 5 to 9, a relative humidity greater than 95% and the presence of adequate nutrients. The recorded survival time of spores ranges from 6 months to 71 years under different condition. To break the local cycle of anthrax infection, disinfection, decontamination and correct disposal of infected/contaminated materials are of considerable importance in preventing transmission of infection.

The details of the disinfectants for decontamination of various infected materials are as follows :

A. Spore form by using either of following:

- 2% Glutaraldehyde
- 5-10% Formaline 5% Lysol
- 5-10% Sodium hydroxide At least for 2 hours
- 3% peracetic acid
- 1 in 5000 solution of bichloride of mercury
- 1% Formic acid
- 2% Hydrochloric Acid
- Ethylene oxide vapour for 10 hours
- Moist heat 121°C for 30 minutes
- Dry heat 120° - 140°C for 3 hours
- Flame gun - on floors and crevices

B. Vegetative form

- If carcass is unopened, bacilli lasts for 3 days at 25-30°C, and for weeks at 5-10°C and for few minutes at 60°C.

C. Manure/Dung/Bedding

- By incineration
- By autoclaving at 121°C for 30 minutes
- Or immersion in 4 % formaldehyde for 12 hours

D. Floor space/shed/vehicle

- Preliminary disinfection using 10% formaldehyde; (1-1.5 It/ sq.m.) or 4% glutaraldehyde for at least 2 hours Cleaning - by washing or scrubbing with hot water Final disinfection by one of the following disinfectants applied for atleast 2 hours.
- 10%formaldehyde
- 4% glutaraldehyde
- 3% hydrogen peroxide or
- 1% peracetic acid

E. Closed rooms/ cabinets

- Fumigation - Boiling of water containing concentrated formalin in an electric kettle and leaving overnight or at least for 4 hour

F. Spills/Splashes/Accidents

- Floor - by Hypochlorite solution containing 10,000ppm available chlorine.
- Clothes - should be autoclaved or fumigated.
- Eyes - should be flushed out with copious quantity of water immediately. Rubbing to be avoided. Medical help to be taken.
- Skin - should be bathed in hypochlorite solution containing 5000 ppm available chlorine for one minute and washed with soap and water. In broken skin bleeding to be encouraged, washed with water. Medical help to be taken at the earliest.
- Mouth - Mouth pipetting should not be done in any case. If accidently done, mouth should be thoroughly washed with hypochlorite solution containing 2000 ppm available chlorine.

G. Liquid Manure

- By treating per m3of slurry with 37% formaldehyde solution (approx. 50-100 kg. of formalin). The mixture is left for 4 days.

H. Sewage sludge

- By 5% formaldehyde as 8% of dry matter for 10 hours
- Or 3% peracetic acid for 30 minutes

I. Water

- By autoclaving, filtration or by 5-10% formaldehyde for 10 hours.

J. Soil

- By incineration/heat treatment 121°C for 30 minutes. 5% formaldehyde as 50 lts/sq.m. or covering with concrete/ tarmac

K. Other materials

- By incineration 9
- Autoclaving
- Overnight soaking in 4% formaldehyde/2% glutaraldehyde
- Or by fumigation (Ethylene oxide/formaldehyde)

L. Wool and Hair

- By duckering process (five stages) i.e.
- Immersion in 0.25-0.3% soda liquor
- Immersion in soap liquor;
- Two immersions in 2% formaldehyde solution; and
- Rinsing in water

M. Hide and Skin

- By formaldehyde or ethylene oxide fumigation

N. Bone, Hoof and Horn

- No statutory regulations exist, but sterilization is essential before making feed ingredients and fertilizers.

Source : Guidelines for Prevention and Control of Anthrax, Joint publication by Zoonosis Division, National Institute of Communicable Diseases, Directorate General of Health Service and World Health Organization (WHO), Country office for India, 2006.

ANNEXURE III

DISPOSAL OF CARCASSES

Sporulation of *B.anthracis* requires oxygen and therefore does not occur inside a closed carcass. Regulations forbid post mortem examination of animals when anthrax is suspected. The methods of disposal of an anthrax carcass are incineration, rendering or burial.

A. Incineration

Incineration must be done with appropriate care to ensure complete burning from beneath.

I. Pit Method

For a large animal, a pit about 0.5m deep and exceeding the length and breadth of the carcass by about 0.25m on each side should be dug near the carcass. A trench approximately 0.25m wide by 0.25m deep should be dug along the length of the centre of the pit extending beyond the ends by about 0.75m; this serves the purpose of allowing air for the fire under the carcass. The bottom of the pit and the trench should be covered with straw which is then soaked in kerosene.

Above the kerosene-soaked straw, place a few pieces of heavy timber (or other type of beams which will hold the carcass well above the bottom of the pit) across the pit and then scatter thin pieces of wood over beams and straw. Then add larger pieces of wood and, if available, coal, until the pit is filled upto top ground level. Saturate all the fuel with kerosene. The carcass can then be drawn onto the pyre, preferably propped up so that it is on its back. Further kerosene should be poured over the carcass. The fire is started at either end of the longitudinal trench.

The approximate quantities of fuel that will be needed for a large domestic animal are 20 kg of straw, 10 liters of kerosene, and either 2 tonnes of wood or 0.5 tonnes of wood and 0.5 tonnes of coal. It will be necessary to decontaminate the ground where the carcass lay and from where it was removed to the pit and also the ground, equipment, etc. contaminated during this moving process.

II. Raised carcass method

This method may be appropriate when labour is scarce or the ground unsuitable for the construction of a pit.

Place straw over a 2 x 1.5 metre area. Place two wooden beams (approximately 2m lengths of small tree trunks, railway sleepers, etc.) over the straw parallel to each other and about 1.2-m apart and aligned with the direction of prevailing wind. Soak the straw with kerosene and cover with thin and thick pieces of wood and coal if available. Place further stout cross-pieces of wood or other material across the two main beams to support the carcass. The fuel (wood or coal) is banked up either side (but not at the ends where the air must be allowed to enter upon the carcass) of the carcass and fuel and carcass further doused with kerosene.

More fuel may be required than with the pit method. For a large domestic animal, an estimate is 0.75 tonnes coal + 0.5 tonnes wood or, if coal is unavailable, approximately 3 tonnes of wood, plus 20 kg straw and 20 liters of kerosene.

As with pit method, it will be necessary to decontaminate the site where the carcass lay before incineration and the ground and equipment contaminated in moving it from there to the pyre.

B. Rendering

Rendering is essentially a cooking process which results in sterilization of raw materials of animal origin such as parts of carcass which may be utilized safely for subsequent commercial purposes. In general, the raw materials are finely chopped and then passed into a steam heated chamber and subjected to temperatures ranging from 100°C to 150°C for 10-60 minutes.

C. Burial

Where neither incineration nor rendering is possible, for example due to lack of fuel, burial is the alternative. Deep burial (2m) of carcass covered with lime and soil in 1:3 ratio. Burial should be discouraged in favour of incineration or rendering wherever possible. Periodic reports of viable anthrax spores at burial sites of animals which died many years back have testified to the unreliability of burial procedures for long term control of the disease.

Disturbance of such sites, for example by ploughing, or laying drainage, brings the spores to the surface; even without site disturbance, spores can work their way up to the soil surface. In either case, this may result in new live stock cases. Further disadvantages to the burial sites are that scavengers may dig down to reach the carcass and in dry, dusty areas, the digging process can spread the contaminated soil extensively.

Source : Guidelines for Prevention and Control of Anthrax, Joint publication by Zoonosis Division, National Institute of Communicable Diseases, Directorate General of Health Service and World Health Organization (WHO), Country office for India, 2006.

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