Standard Operating Procedures

Characterization and authentication of *Bacillus anthracis*

Project

‘‘DBT Network Programme on Anthrax Diagnosis and Control in India’’

National Centre for Veterinary Type Cultures,

ICAR-National Research Centre on Equines

Hisar, Haryana, India-125001

List of Contents

Contact Diary of NCVTC components under project DBT Network Programme on Anthrax Diagnosis and Control in India

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Details of sender institutes;

S. No.	Institutes	In-charge	Email	Mobile No.
1	Odisha University of Agriculture and Technology, Bhubaneshwar, Odisha	Dr. Niranjan Sahoo		
2	Institute of Animal Health and Veterinary Biologicals, Bengaluru, Karnataka	Dr. S M Byregowda		
3	Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh	Dr. D. Rani Prameela		
4	Institute other than project components			
Note: Actively contact a minimum of 15 days before sending culture. Culture was not sent in March please.				

1. Receiving Anthrax culture

a. Information from the sender, do all necessary arrangements to receive and process the culture.
b. Culture to be received by the Principal Investigator/Co-PI or SRF under this project (By name).
c. Culture will be placed under the lock in Refrigerator.

d. Inform the sender after receiving samples/send a copy to the sender RECIEPT IN RECIPT REGISTER.
e. OPENING: On arrival, all lab work is performed in a BSL-3 laboratory using a Class II Biosafety cabinet.
f. NOTE Check the leakage or any other damage to the **outer (tertiary) packaging container**.
g. NOTE Check the leakage or any other damage to the **secondary packaging container**.
h. Remove the documents from the container and make the required entries in the receiving register and provide the receiving register number to all the culture isolates.
i. **Open** the secondary container in Class II BSC using BSL-3 instructions.
j. **Primary container is now** carefully opened, and quality of the container and gross exam checked.
k. Complete all the entries of the preliminary quality register.
l. Discard all the rough materials, including secondary packaging taking all bio-safety measures.
m. Store the culture tubes in the sample cabinet of the refrigerator and process further for bacteriology study.

   Note: Screw-capped non-breakable containers should be used for transport of cultures, etc.
n. Complete the logbook of the refrigerator, and after locking, keys submitted to P.I.

Culture Receipt Register

S. No.	R.R. No.	Receiving Date	Sending Date	Deposited by & Institute	Description of packaging	Remark	Received by (Name & sign with date)	Sign of P.I.

Preliminary Quality Check Register

S. No.	R.R. No.	Date	Deposited by & Institute	Secondary container remark	Culture data sheet and	Primary container remark	Viability and purity	No of Vials of each culture	Processed by (Name & sign with

					details provided				date)

Culture Processing Register

R.R. No.	Date	Morphology SBA/NA/PLET agar	Staining	Biochemical test	Penicillin susceptibility test	Result	Remark	Sign of P.I.

Culture Authentication Record Register

S. No.	R.R. No.	Date	Deposited by & Institute	Morphology	Staining	Biochemical test	Penicillin susceptibility test	Remark	Sign of P.I.

Accession Register

S. No.	R.R. No.	Date of Authentication	Deposited by & Institute	Details	Accession Number	Remark	Sign of P.I.

Preservation Register

S. No.	R.R. No.	Accession Number	Deposited by & Institute	Date of Preservation	No. of vials	Location in Refrigerator	Remark	Sign of P.I.

Molecular analysis record Register

S. No.	Accession Number	DNA isolation	Conventional PCR	RT PCR	Nest PCR	16s r RNA PCR	Sequencing	Remark	Sign of P.I.

Culture Distribution Register

S. No.	Accession Numbe r	Sen t Dat e	Send to Scienti st &	MTA recor ds	Purpo se (s)	No. of isola te &	Culture sending medium (Slants/Glyc	Sen d ing ID	Receiving confirmat ion	Sig n of P.I.

			Institute			**vials**	**erol stock)**	**& via**		

2. Identification and authentication of *Bacillus anthracis*

Biosafety Level-III laboratory

The biosafety level-III laboratory facility is established at ICAR-National Research Centre on Equines, Sirsa Road, Hisar, Haryana, 125001.

2.1 Culture on sheep blood agar

a. All required material except culture was placed in a Bio-safety cabinet and sterile with UV.
b. The primary container opens in the Class 2 BSC and is examined.
c. Sterile disposable loops use for inoculums of the pure culture on the sheep blood agar plate.
d. After streaking, the plates were sealed with a Petri plate seal.
e. We placed the streaked SB agar plates at 37°C for 24 h Incubator.
f. The next day, the microbial growth was examined, and the *B. anthracis* colonies were white, gray–white and nonhaemolytic, 2–4 mm in diameter, slightly moist, matte in appearance, with a ground-glass surface and very tacky when teased with an inoculating loop. Fringed edges or tailing is sometimes seen with nutrient agar. This characteristic has been described as a ‘medusa head’ or ‘curled hair’ appearance.
g. Take a picture of the culture plate and save the file with the name “VTCC/DBT/BA/BA00”
h. These type colonies were selected and future stained and streaked on nutrient agar plates.
i. All the examination entries fill in the preliminary quality register.
j. After work clean the cabinet and dispose of the material as per BSL3 requirements.

2.2 Culture staining

2.2.1 Gram Staining

Gram staining of the colony from streaked SB agar plates following the standard protocol of Gram staining as follows:

a. A clean, grease-free slide was taken.
b. The slides are labeled with markers to ensure identification.

c. Prepare the smear of suspension on the clean slide with a loopful of the culture.
d. Air dry and heat fix.

Note: The above steps were carried out inside laminar airflow.

e. Crystal violet was poured and incubated for approximately 30 seconds to 1 minute and rinsed with water.
f. Flood the gram's iodine for 1 minute and wash with water.
g. Then, the sections were washed with 95% alcohol or acetone dropwise for approximately 10 seconds and rinsed with water.
h. Add safranin as a counter stain for approximately 1 minute and wash with water.
i. The samples were air dried and observed under an oil immersion lens microscope.

Note:

- The samples were washed off into hypochlorite solution (10,000 ppm) at each stage.
- Drain slide or air dry does not blot dry.
- The microscope lens was cleaned with lens paper.
- In Gram-stained preparations, the developing spores appear as unstained areas within the cell.

j. Observe the typical morphology of the *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 3 or 4 bacilli. The chain of bacilli with truncated and swollen ends gives a characteristic "bamboo stick", square-ended (box-car) or "vertebrate" shape.
k. Take a picture of the stained slide and save the file with the name "VTCC/DBT/GS/BA00".

2.2.2 Polychrome methylene blue (Mc'Fadyean's Reaction) capsule staining

a. Thin smears were made on a microscope slide.
b. The slides were air dried and fixed by dipping them in absolute alcohol for 30–60 seconds.
c. The slide should not be heat dried to avoid distortion of the morphology of the capsule.
d. A large drop of polychromylene methylene blue was placed on the smear to cover it completely.
e. Wait for 30–60 seconds.

f. The stain was washed off with water using a wash bottle (in hypochlorite solution, 10,000 ppm) and allowed to dry.
g. Examine under oil immersion.
h. When dry, examine under the 10x lens. The anthrax bacilli can be seen as tiny short threads. Switch to oil immersion and look for the capsule, which is seen clearly as pink amorphous material surrounding the blue–black bacilli.
i. Take a picture of the stained slide and save the file with the name "VTCC/DBT/CS/BA00"

2.2.3 Malachite green spore staining

a. Make a thin film on a microscope slide.
b. Dry the films and heat or alcohol fix.
c. Place the slide over a beaker of boiling water and rest it on the rim with the bacterial smear uppermost.
d. Cover with a 5% aqueous solution of malachite green
e. Staining was performed for 5 minutes, and more stain solution was added if the stain covering the smear started to dry.
f. Wash off the stain with water using a wash bottle (into hypochlorite solution).
g. Counterstaining with carbol fuchsin for 30 seconds
h. Wash again (into hypochlorite solution) and allow drying.
i. When dry, examine under the oil immersion and look for the spore, which appears green and the vegetative bacilli red.
j. Take a picture of the stained slide and save the file with the name "VTCC/DBT/SS/BA00"

2.3 Culture characteristics on nutrient agar

a. All required material except culture was placed in a Bio-safety cabinet and sterile with UV.
b. Sterile disposable loops use for inoculums of the pure culture on the nutrient agar plate.
c. After streaking, the plates were sealed with a Petri plate seal.
d. We placed the streaked nutrient agar plates at 37°C for 24 h Incubator.

e. The next day, the microbial growth was examined, and *B. anthracis* colonies were 2-3 mm in size irregular, raised dull grayish white with a frosted glass (ground glass) appearance.
f. When colonies are viewed under a low-power microscope, the edge of the colony, which is composed of long interlacing chains of bacilli, appears as locks of matted hair, giving a medusa head appearance, and may have a fringed edge or put out curled protrusions (tailing).
g. Colonies have tacky consistency.
h. Take a picture of the stained slide and save the file with the name "VTCC/DBT/NA/BA00"

2.4 Biochemical Test

2.4.1 Catalase test via the test tube method

a. Pour 1-2 ml of hydrogen peroxide solution into a sterile test tube.
b. Using a sterile wooden stick, take several colonies of the 18- to 24-hour test organism and immerse them in hydrogen peroxide solution.
c. Observe for immediate bubbling.
d. Positive reactions are evident by immediate effervescence (bubble formation).

Note: Be careful not to pick up any agar. This is particularly important if the colony isolate was grown on agar containing red blood cells. Carryover of red blood cells into the test may result in a false-positive reaction

2.4.2 Oxidase test via the swab method

a. Dip swab into reagent (Kovac's oxidase reagent) and then touch an isolated suspect colony
b. Observe for color change within 10 seconds.

2.5 Culture characteristics on PLET agar

a. All required material except culture was placed in a Bio-safety cabinet and sterile with UV.
b. Sterile disposable loops use for inoculums of the pure culture on the PLET agar plate.
c. After streaking, the plates were sealed with a Petri plate seal.
d. We placed the streaked agar plates at 37°C Incubator.

e. After incubation at 37°C for 36–48 hours, the colonies of *B. anthracis* were 2–3 mm, roughly circular, creamy-white with ground-glass texture.
f. Colonies are usually smaller in size on this medium than on nutrient or blood agar and lack tackiness. Tailing edges are not seen.
g. Take a picture of the stained slide and save the file with the name "VTCC/DBT/PA/BA00"

Note: Thallous acetate is highly toxic and environmentally unfriendly in terms of disposal.

2.6 Penicillin susceptibility test

a. All required material is laminar (except culture) and sterile with UV.
b. In its simplest form, this involves spreading a portion of a nutrient or blood agar plate with the culture under test.
c. A 10 U penicillin disc was placed at some point within the area of spread.
d. After streaking, the plates were sealed with a Petri plate seal (Abdos, U10901).
e. The zone of susceptibility will be visible after overnight incubation at 37°C.
f. Three quality control strains, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, and *Escherichia coli* ATCC 25922, were tested daily.

3. Preservation of *Bacillus anthracis*

a. The bacterial cultures will be allowed to grow overnight in autoclaved nutrient broth (4 ml).
b. On the next day, we centrifuged the enriched bacterial culture and discarded the supernatant, followed by re-suspension in 2 ml of fresh autoclaved N.B.
c. Then, we centrifuged the re-suspended bacterial culture again at the same time and discarded the supernatant.
d. We added 2 ml N.B. to the pellet and dissolve it with pipetting followed by the addition of 1 ml 80% glycerol.
e. Mix it well. Dispense 1.5 ml in 2.0 ml capacity cryovial. Then, we placed it at room temperature for 30 min than at 4°C for 4 hrs followed by -20°C for 4 hrs.
f. For long-term storage, we will store the cultures at -80°C in deep freezers at the NCVTC bacterial culture repository.

4. DNA isolation in BSL-3

DNA extraction will be conducted using the Zymo Research Quick-DNA Fungal/Bacterial Mini prep Kit (D6005). No-template controls were included in each extraction by taking only reagents through the extraction process. DNA extraction will be done in replica. All DNA extracts were stored at -20°C prior to use in PCR.

- Viable *B. anthracis* is an infectious agent and must be handled with care by trained personnel working under appropriate conditions.
- All procedures involving manipulation of live organisms (i.e., inoculums preparation, making dilutions, and inoculations of media) must be performed in a BSC in a BSL-3 laboratory using BSL-3 practices.
- All biological materials and used tips/tubes will be discarded into disinfectant solution (10000 ppm hypochlorite solution).
- Use appropriate PPE (scrubs, gown, gloves, dedicated shoes, shoe covers and hair cap).
- All containers and equipment must be sprayed with disinfectant before removing from the BSC.
- Tubes should be contained within aerosolve containers or similar biosafety devices for all centrifugation steps. Following centrifugation, these containers should be transferred into the BSC (microfuge is used in the BSC) before they are opened.
- Recommended set up for BSC: Items are arranged in the BSC to avoid contaminated items being passed over clean items. Items or equipment in direct contact with the etiologic agent remain in the BSC until surface decontaminated.
- All biological waste is placed in a discard pan lined with a biohazard bag for autoclaving.

Requirement-

1. 2ml eppendorf-1
2. ZR Bashing bead lysis tube-1
3. Zymo-Spin™ III-F Filters-1
4. Zymo-Spin™ IICR Columns-1

5. Collection Tubes-3
6. 1.5 ml eppendorf- 1
7. 1000µL micro tips
8. 200µL micro tips
9. Vortex
10. Centrifuge
11. Marker
10. Tough Tags

Protocol for DNA Extraction from Zymo Research Quick-DNA Fungal/Bacterial Mini prep Kit (D6005): -

1. Prepare overnight grown culture by inoculating bacteria in 6 ml of Nutrient broth and incubate in shaking incubator.
2. Take 2 ml Eppendorf tube and add 1.5 ml overnight grown culture and centrifuge it at 11,000 rpm for 4-6 minutes to harvest cells.
3. If culture is on plate, then mix the culture with PBS and centrifuge it.
4. Discard the supernatant through micro tips and add 750 µl bashing bead buffers to Eppendorf.
5. Mix the pellet and buffer properly.
6. Now, add the solution to ZR Bashing bead Lysis Tubes and vortex it for 5 minutes.
7. Centrifuge the solution at 12,000 rpm for 1 min.
8. Transfer 400 µl supernatant to Zymo-Spin™ III-F Filters applied to collection tubes.
9. Centrifuge it at 8,000 rpm for 1 min.
10. Discard the filters and add 1200 µl Genomic Lysis Buffer in collection tube (400+1200=1600 µl)
11. Transfer 800 µl mixtures to Zymo-Spin™ IICR Columns applied to new collection tubes.
12. Centrifuge at 10,000 rpm for 1 min. Discard the flow through from collection tube through micropipette.
13. Again add 800 µl mixtures to same column and centrifuge.
14. Discard the collection tube and place the column in new collection tube.
15. Add 200 µl DNA Pre-wash buffer to the column and centrifuge at 10,000 rpm for 1 min.

16. Discard the solution from collection tube through micropipette, add 500 µl g-DNA Wash Buffer to column and centrifuge it at 10,000 rpm for 1 min. (Centrifuge for 2 min. for air dry).
17. Discard the collection tube, transfer the column to 1.5 ml eppendorf and add 50 µl DNA elution buffer. Wait for 4 min. and centrifuge for 1 min at 10,000 rpm.
18. Again add 50 µl DNA Elution Buffer to column. Wait for 4 min. and centrifuge it for 1 min at 10,000 rpm.
19. Discard the column and store the ultra-pure DNA in 1.5 ml Eppendorf.

5. Biochemical Characterization of strains by API kits

API 50 CHB/E Medium is intended for the identification of *Bacillus* and related genera, as well as Gram-negative rods belonging to the *Enterobacteriaceae* and *Vibrionaceae* families. It is a ready-to-use medium which allows the fermentation of the 49 carbohydrates on the API 50 CH strip to be studied.

The API 20 E strip may be used in association with the API 50 CH strip to provide supplementary tests.

Reagents and material required

- API 50 CHB/E Medium (Ref. 50430)
- API 50 CH strip (Ref. 50 300)
- API 20 E strip (Ref. 20 100)
- API 20 E reagent kit (Ref. 20 120)
- **api*web*™** identification software (Ref. 40 011)
- API NaCl 0.85 % Medium, 5 ml (Ref. 20 230)
- Mineral oil
- McFarland Standard
- Pipettes
- Ampoule rack
- Small and large ampoule protectors
- Sterile distilled water or sterile saline, 1 ml

Procedures

Select the pure culture from the nutrient agar plate.

Preparation of the strips as per the package inserts for API 50 CH and API 20 E.

Preparation of the inoculums;

A. Suspension for inoculation of the API 20 E strip:
    a. Open a tube containing 1 ml of sterile saline.
    b. Pick up all the bacteria from the culture using a swab.
    c. Prepare a heavy suspension (S) in the tube.
    d. Open an ampoule of API NaCl 0.85 % Medium (5 ml).
    e. Prepare a suspension with a turbidity equivalent to 2 McFarland by transferring a certain number of drops of suspension into the ampoule: record this number of drops (n).
B. Suspension for inoculation of the API 50 CH strip :
    a. Open an ampoule of API 50 CHB/E Medium.
    b. Inoculate the ample of API 50 CHB/E Medium by transferring twice the number of drops of suspension (i.e. 2n) into the ampoule.
    c. Homogenize.
    d. The solutions must be used immediately after preparation.

Inoculation of the strips;

a. Fill the tubes (not the cupules) with the inoculated API 50 CHB/E Medium.
b. NOTE: The addition of mineral oil is optional; it is not however recommended for strict aerobic bacteria.
c. Inoculate the first 12 tests only of the API 20 E strip, as the last 8 tests are duplicated on the API 50 CH strip.

Incubate the strip at 29°C ± 2°C for 24 hours (± 2 hours) and 48 hours (± 6 hours).

Read the results after 24 hours (± 2 hours) and 48 hours (± 6 hours) of incubation.

For the API 50 CH strip:

- A positive test corresponds to acidification revealed by the phenol red indicator contained in the medium changing to YELLOW.
- For the esculin test (tube no. 25), a change in color from red to BLACK is observed.
- Record the results on the result sheet.

NOTE: If a positive test becomes negative at the second reading, only the positive result should be taken into account (this is caused by an alkalinization due to the production of ammonia from peptone).

For the API 20 E strip:

- The reagents are added just before the last reading.
- To read the tests, refer to the API 20 E package insert.
- The results of the first 11 tests and the NIT reaction in the GLU test should be recorded for final interpretation.

The biochemical profile obtained for the strain after the final reading can be identified using the identification software with the database.

6. Safety measures

- All laboratory personnel should be well trained in biosafety and biosecurity before commencing work.
- Laboratory personnel should ensure adherence to safety procedures.
- All laboratory procedures should be performed in the Biosafety Level 3 containment facility (BSL III) inside Biosafety cabinet Class III to minimize the production of aerosols.
- General Good Laboratory Practices (GLPs) must be followed as per the World Health Organization (WHO) guideline for Anthrax, 4th edition 2008.
- Laboratory personnel should wear personal protective equipment with long sleeves and elastic cuffing and disposable gloves.
- Place Petri plates (or other culture containers) in a secondary container for movement around the laboratory.
- The container should be well labeled with the agent, the operator's name and the date.

6.1 Disinfection

- Laboratory accessories such as pipettes, tips, loops, spreaders, etc., should be autoclaved (e.g., in autoclavable bags).
- The biosafety cabinet should be disinfected after use with a 10% sodium hypochlorite solution.

- Contaminated items should be kept in strong leakproof containers, preferably within autoclavable bags.
- Hands should be thoroughly washed with soap and water and dried before leaving the facility.
- Discard the plates/tubes into autoclave bags. Autoclave, preferably followed by incineration.
- Slides were discarded in 10% hypochlorite and autoclaved.
- Other sharp items were placed into the sharps container, which was autoclaved and then preferably incinerated.
- Double Autoclave the recyclable item.
- Fumigate or otherwise decontaminate non disposable items of equipment that cannot be autoclaved.

6.2 Decontamination:

- All materials used, including labware, should be decontaminated by autoclaving at 121°C for 30 minutes to 1 hour, preferably followed by incineration.
- Microscopic slides, coverslips 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.

6.3 Spillage management

- If accidental spillage or exposure to anthrax spores occurs in the laboratory, 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 toward the center (PERIPHERY TO CENTER).
- The spill should be allowed to soak in the disinfectant for approximately one hour.
- The materials should be cleared using forceps. Broken glass or other sharps should be placed in an autoclavable sharps container for disposal.

- After autoclaving, the material is incinerated.

6.4 Final Disposal

- All cultures of *Bacillus anthracis*, specimens and disposable labware used for isolation, identification and performing molecular characterization should be decontaminated by placing them 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.