

Genus Bacillus

Bacillus anthracis

Historical Importance *Bacillus anthracis*

- Discovery of Anthrax bacillus is credited to Davaine and Rayer (1863-1868).
- Pasteur established germ theory of disease.
- First pathogenic bacteria to be observed under microscope (Pollender 1849).
- Greenfield & Pasteur -First bacterial vaccine-Anthrax.
- First bacteria used to prepare attenuated vaccine.
- First communicable disease transferred by inoculation of blood (Davaine 1850).
- First bacillus to be isolated in pure culture & shown to possess the spores.

Hosts: Cattle, sheep, pigs, horses, humans etc.

Morphology

- Largest pathogenic bacteria.
- Gram positive and non-acid fast.
- Non-motile.
- Rod shaped bacilli with truncated ends or often concave and somewhat swollen.
- Size: 1-1.5 μ X 4-8 μ .
- Chain of bacilli presents a bamboo stick appearance (also called as Box-car bacillus-looks like rail carriages).



- Capsule: The capsule is polypeptide in nature, being composed of a polymer of d(-) glutamic acid. Capsules are not formed under ordinary conditions of culture but only if the media contain added bicarbonate or are incubated under 10-25% CO₂. If grown in media containing serum, albumin, charcoal or starch, capsule formation may occur in the absence of CO₂.

McFadyeans reaction (Polychrome methylene blue staining):

- When blood films containing bacilli are stained with polychrome methylene blue for few seconds and examined under the microscope, an amorphous purplish material is noticed around the bacilli. This represents the capsular material and is characteristic of the Anthrax bacilli. This is called McFaydeans reaction. Bacilli takes dark blue colour and the capsule takes purplish pink colour.
- (When methylene blue is kept in dark for about 6 months or 1 year with occasional shaking, slow oxidation reaction takes place which dissociates the methylene blue into azur A and azur B Which combines with d(-)glutamic acid present in Anthrax bacilli.)
- 0.3 g of methylene blue is dissolved in 30 ml of 95% ethanol; 100 ml of 0.015 potassium hydroxide (KOH) is mixed with the methylene blue solution. Addition of 15 potassium carbonate ripens the stain quickly.

Procedure:

1. Prepare a thin, small smear from small drops of blood or tissue fluid.
2. After fixing and drying, a small (approximately 20 µl) drop of stain is placed on the smear and spread over it with an inoculating loop.
3. After 1 minute the stain is washed with water into a hypochlorite solution (10,000 ppm available chlorine).
4. The slide is blotted, air-dried and observed initially using the 10X objective lens under which the short chains appear like short hair; once found, these can be observed under oil immersion(1000X).

Observation: The cells are found in pairs or short chains and are often square-ended. The gram and regular Giemsa stains do not reveal the capsule.

Spores:

- This bacterium is spore forming. The spores are ellipsoidal, situated centrally in the cell, and are 0.7µ-0.8µ to 1.5µ in size. Spores are NOT formed in the animal body but are formed in the culture or in the soil.
- Spores are central or subterminal, elliptical or oval in shape, and are of the same width as the bacillary body so that they do not cause bulging of the vegetative cell. Spores do not stain by ordinary methods but can be stained with Sudan black B.
- Sporulation takes place at an optimum temperature of 25°C-30°C. Sporulation occurs under unfavourable conditions for growth and is encouraged by distilled water, 2% NaCl or growth in oxalated agar. Oxygen is required for sporulation, but not for germination. Sporulation is inhibited by calcium chloride and anaerobic conditions.

Cultural Characteristics *Bacillus anthracis*

- Aerobes, Facultative anaerobes.
- Optimum temp-37 °C
- Optimum temp for sporulation-25-30 °C
- Optimum pH: 7-7.4
- **Nutrient Agar**- irregularly round colonies are formed, 2-3 mm in diameter, raised, dull, opaque, grayish white, with a frosted glass appearance. Under the low power microscope, the edge of the colony is composed of long, interlacing chains of bacilli, resembling locks of matted hair. This is called the 'Medusa head appearance' or 'Curled hair lock' appearance .

As the colony ages, "vesicles" may appear on the surface, giving it a contoured appearance.

- Virulent capsulated strains form rough cultures, while avirulent or attenuated strains form smooth colonies. In medium containing iron salts, virulent *anthracis* produces pink or purple coloured pigmented colonies.
- **On gelatin stab culture**- a characteristic inverted fir tree appearance is seen, with slow liquefaction commencing from the top. Fine filaments of growth develop laterally along the line inoculum. The growth nearer to the surface of the medium is the longest and then progressively shorter when there is less oxygen, resembling a inverted fir tree.
- **On blood agar**- The colonies are non-hemolytic, though occasional strains produce a narrow zone of hemolysis.



- In broth - Growth occurs as floccular deposits, with little or no turbidity.
- **Selective medium (PLET medium)**- It consists of polymyxin, lysozyme, ethylene diamine tetra acetic acid(EDTA) and thallous acetate added to heart infusion agar, has been devised to isolate *anthracis* from mixtures containing other spore bearing bacilli. Thallous acetate inhibits growth of anthracoids and polymyxin and lysozyme inhibits growth of gram negative bacteria.
- It is prepared by using heart-infusion agar base with the addition of 0.25-0.3 g/litre EDTA and 0.04g/litre thallous acetate.

Pearl string test: Charlton,1980

When *Bacillus anthracis* is grown on the surface of a solid medium containing 0.05-0.50 units of penicillin/ml, in 3-6 hours the cells become large, spherical, and occur in chains on the surface of the agar, resembling a string of pearls. Due to the impairment of cell wall the bacilli becomes spherical. The 'string of pearls reaction' clearly differentiates *Bacillus cereus* and other aerobic spore bearers from *Bacillus anthracis*.

Procedure

1. To 100 ml of molten agar, add required sodium benzyl penicillin and mix carefully.
2. Pour into petridishes and allow to set. With a scalpel cut a block about 1.6 cm² from the penicillin agar plate and place it on a microscopic slide in petridish containing a small piece of moistened absorbent cotton wool to prevent agar from drying out.
3. Use a young colony to streak the center of the agar block.
4. Place a clean coverslip on the agar block and incubate the petridish at 37 °C.
5. After 2 hours, remove the slide and examine the inoculum microscopically by oil immersion for the string of pearls growth.

Physical Properties & Resistance:

- Vegetative bacilli – 60 °C 30 minutes.
- Spores 100 °C in 10 minutes.
- Spores Dry heat 150 °C for 60 minutes.
- 4% potassium permanganate kills them in 15 minutes.
- 5% phenol doesn't kill them and they survive in there for weeks.
- HgCl₂ in a 1/1000 solution may fail to kill anthrax spores in less than 70 hours.
- Destruction of the spores in animal products imported into non-endemic countries is achieved by '**duckering**' in which formaldehyde is use as a 2% solution at 30-40 °C for 20 min. for disinfection of wool and as 0.25% at 60 °C for 6 hrs for animal hair and bristles.

Biochemical Tests

Sugar	Glucose	Sucrose	Maltose	Dextrin	Mannitol	Glycerol	Lactose	L-arabinose
<i>B.anthraxis</i>	+	+	+	+	-	-	-	-

- *Bacillus anthracis* ferments glucose, sucrose, maltose & dextrin (**GSuMaDe**). *B.anthraxis* forms acid but no gas from the fermentable sugars. Some strains produce slight acid in glycerol and sialicin. There is no fermentation of lactose, galactose, arabinose, rhamnose, mannose, raffinose, inulin, mannitol, dulcitol, sorbitol, inositol and adonitol.
- The organism is methyl red MR positive and voges-proskauer VP positive.
- Catalase Positive, H₂S and Indole Negative
- Nitrates are reduced to nitrites; ammonia is produced.

Antigenic Structures

- Capsular Antigen-Non-Protective
- Somatic Antigen-Non-protective
- Complex Protein/Anthrax toxin:

Three components: PA -Protective Antigen, EF -Edema Factor & LF -Lethal Factor

Action of Toxin

- Act synergistically
- PA-Binds to surface receptors on sensitive eukaryotic cells thereby allowing translocation of EF & LF into the cell. The antibody to PA is protective because it blocks the first step in toxin activity, namely, its binding to target cells.
- EF is an adenylate cyclase, capable of generating high level of cAMP in the cytoplasm of susceptible cells leading to edema.
- LF-PA+LF Induces cytokine release and death of target cell (Cytotoxic) by unknown mechanism. Formation of ion permeable pores in membranes leading to hemolysis.
- Loss of the plasmid which encodes the F toxin renders the strain avirulent. This is believed to have been the basis for the original Anthrax vaccine developed by Pasteur.

Anthrax – Clinical Signs

- *Bacillus anthracis* is pathogenic for cattle, sheep(except Algerian), horses, mules, swine, dogs and cats.
- Anthrax appears as a septicemia in the cattle, sheep, horses and mules in **Peracute, Acute, Subacute** and rarely in chronic form.

Sudden Death ,
Haemorrhages from the natural orifices,
Incomplete rigor mortis



- Acute form: Depression, fever, inappetance, rapid respiration , increased heart rate, congested mm. oedematous swellings
- Sub acute form: Depression, fever, inappetance , weakness, prostration and death.
- Chronic : Swelling localised, fever, enlarged LN, respiratory distress death.
- **In horses:** Acute form is very common and death may take place one day after edematous swelling of the throat and neck region. There may be symptoms of colic. In less acute, oedematous swelling become generalized and death occurs after 2-3 days.
- **In sheep:** Disease is acute and death appears rapidly after convulsions. They have a greater natural resistance than herbivores.
- The usual signs are oedematous swellings in the region of throat and neck interfering normal respiration, enteritis and rise in temperature. The disease is not always fatal.
- **In swine:** The disease is manifested by an acute pharyngitis with extensive swelling and hemorrhage of the throat region.
- **In dogs:** Pharyngeal or oral anthrax produces swelling about the head and throat; alimentary involvement is typically manifested as severe gastroenteritis. Generally older canines are less susceptible.
- **Poultry:** They are highly resistant to anthrax, a characteristic that is attributed to their higher body temperature.
- Other birds, reptiles, and fish are not susceptible under natural conditions.

On postmortem examination:

Anthrax is characterized by

- Delayed or incomplete rigor mortis.

Why is rigor mortis absent in anthrax?

- Rigor mortis is dependent on ATP in order for it to occur. However, the anthrax bacilli have a toxin called edema factor (EF) or calmodulin dependent adenylate cyclase, which is a toxin that inhibits the stiffening of muscles by constantly stimulating the release of cAMP, so that the cAMP level in body is never low. Thus even after death it supplies the muscles with adequate cAMP to be relaxed, even after a prolonged period.
- Rapid decomposition; and bloating.
- Dark, blood-stained urine.
- The blood is dark red, tarry (deoxygenated blood) and does not clot; these facts have led the French to call this disease “charbon”. Blood is seen exuding out of the natural orifices.
- Hemorrhages in various parts of the body, especially in serous membranes.
- Edematous infiltrations in the subcutis.
- The mononuclear phagocytic system, especially the spleen, serves as the principal defense against circulating bacilli, with pronounced splenomegaly. There is marked swelling of the spleen, which is dark red and soft (raspberry-jam consistency). This characteristic spleen has given rise to terms “splenic fever” and “milzbrand”(German) by which the disease is often known.

***Bacillus anthracis* in Human**

- **Cutaneous Form** – Pruritis-Small papule-vesicle containing hemorrhagic fluid-Breaks down-Black eschar-Black eschar is surrounded by ring of vesicles containing serous fluid-develops area of edema and induration – Malignant pustule. This disease was known as the hide porter's disease (the disease used to be common in dock workers carrying loads of hides and skins on their bare backs).
- **Intestinal Anthrax** – This is rare and occurs mainly in primitive community who eat improperly cooked carcasses of animals dying of anthrax- A violent enteritis with hemorrhagic diarrhoea occurs-rapid death.
- **Pulmonary form** – 'Wool Sorter's Disease'. This is called so because it used to be common among workers in wool factories, due to inhalation of dust from infected wool. This is a hemorrhagic pneumonia with a high fatality rate. Hemorrhagic meningitis may occur as a complication.

Diagnosis of Anthrax:

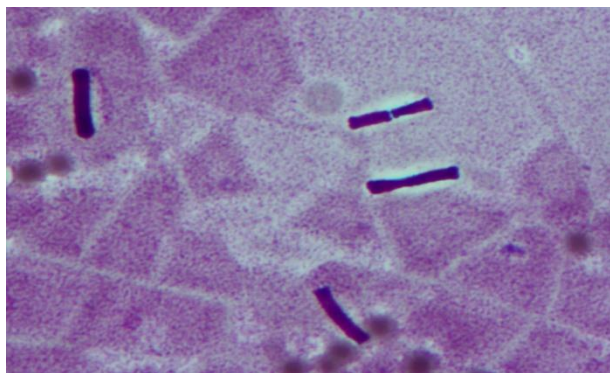
Clinical sample collection:

Since the blood of animals succumbing to anthrax clots poorly, blood or edema fluid samples may be drawn from carcasses for analysis. Blood is collected by taking a small fresh cut on the skin, puncturing the superficial vein of the ear or in the region of the foot. Care should be taken to seal the injection site by placing cotton soaked in sodium hypochlorite (10,000 ppm).

I. Blood Smear Examination:

Microscopic examination of stained blood smears reveals large numbers of encapsulated bacilli, either single or in short chains. The capsules surrounding the bacterial cells can be visualized microscopically in smear stained with Wrights, methylene blue, or Giemsa stain.

Note: In case of horses and pigs since peripheral blood contains fewer organisms, smears should be made from the edematous fluid or lymph nodes.



Blood smear: Bacilli with truncated ends and capsule

II. Isolation & Identification of *Bacillus anthracis* :

Media preferred	PLET medium /Nutrient agar: - Irregularly round colonies are formed, 2-3 mm in diameter, raised, dull, opaque, grayish white, with a frosted glass appearance. Under the low power microscope, the edge of the colony is composed of long, interlacing chains of bacilli, resembling locks of matted hair - 'Medusa head appearance' or 'Curled hair lock' appearance Blood agar: Non-hemolytic colonies
Microscopic	Gram positive rods arranged in chains with truncated ends, sporeforming, non-motile, capsulated bacteria, MacFadyeans reaction when stained with methylene blue.
Biochemical Tests & Sugar Fermentation Test	• <i>Bacillus anthracis</i> ferments glucose, sucrose, maltose & dextrin (GSuMaDe). <i>B. anthracis</i> forms acid but no gas from the fermentable sugars. Some strains produce slight acid in glycerol and sialicin. There is no fermentation of lactose, galactose, arabinose, rhamnose, mannose, raffinose, inulin, mannitol, dulcitol, sorbitol, inositol and adonitol. • The organism is methyl red MR positive and voges-proskauer VP positive. • Catalase Positive • Nitrates are reduced to nitrites; ammonia is produced. • H₂S and Indole Negative

Animal Inoculation Test:

- Sub-cutaneous inoculation of suspected material into guinea pig or mouse result in death within 48 hours with lesions like gelatinous haemorrhagic oedema at the inoculation site, congested viscera, dark red blood and enlarged darkened spleen.
- Smears from splenic pulp if stained by Gram's method will reveal typical Gram positive bacilli.

Ascoli's Test (Ascoli 1911):

- Grind up the organ or blood of suspected animal and suspend in 5-10 parts of saline and boil for 15 minutes.
- Filter through filter paper and allow it to cool. Place 0.5ml of anti-anthrax serum (1:50) (rabbit antiserum) in a small test tube and overlay with 0.5 ml of clear filtrate. Let it stand at room temperature for 15 minutes.
- Result: A white ring of precipitation indicates a positive reaction.

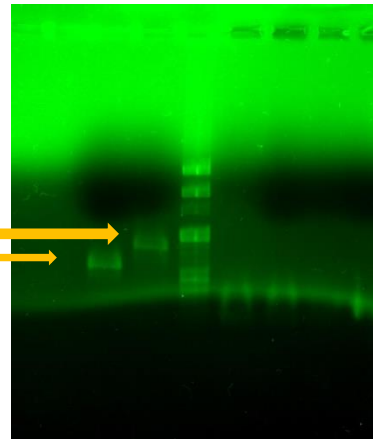
This test is used for the confirmation of *B. anthracis* in a suspected case when the sample taken is of putrified tissue or is from an animal that died 2-3 days back.

Enzyme Linked Immunosorbent Assay.

Polymerase Chain Reaction:

pX01 and pX02 Gene

pX02 Cap gene 846 bps
pX01 PA gene 596 bps



Control of Anthrax:

1. Post-mortem NOT recommended.

When the cadaver is opened for performing necropsy, the bacteria begin to form spores. Oxygen is required for sporulation, but not for germination. Sporulation is inhibited by anaerobic conditions. Because sporulation of *B. anthracis* requires oxygen and therefore does not occur inside a closed carcass, regulations in most countries forbid postmortem examination of animals when anthrax is suspected. Most, if not all the vegetative *B. anthracis* cells in the carcass are killed in a few days by putrefactive processes. Nevertheless, with the characteristic (though not invariable) terminal serosanguinous exudates from the nose, mouth and anus, contamination of the environment around an anthrax carcass can still be expected. The spores are highly resistant to drying, heat, cold and disinfectants. Spores remain viable for many years in soil and water as long as 60 years. These spores may be ingested by other healthy animals and they may contract infection.

2. Proper disposal of carcass died of anthrax.

Deep burial or incineration.

3. Vaccine:

- Sterne's Anthrax Spore Vaccine: Single Vaccine –One Year Immunity. This vaccine contains spores of non-capsulated avirulent mutant strain.
- Mazucchi vaccine: This vaccine contained spores of stable attenuated carbazoo strain in 2% saponin.
- Pasteur's vaccine was anthrax bacillus attenuated by growth at 42-43 C.

Public Health aspects:

Distribution and transmission:

- The organism is spread by the soil-contaminated feeds, and by water. Pasture lands remain contaminated for years. The spores of the organism are spread by water which has flooded over contaminated ground.
- Tanneries, wool-sorting establishments, rendering plants, and fertilizing plants have also been incriminated as sources of anthrax which has been spread by stream flow.

- Carnivorous animals, dogs, cats and hogs may contract anthrax by feeding on dead carcasses.
- Birds spread the infection by carrying bits of flesh or by eating infected meat and eliminating spores in their faeces.
- Bloodsucking flies have been incriminated in the spread of the organism among animals, and in some cases from animals to man.
- Man contracts the disease by contact with infected animals, by sorting wool, from shaving brushes, from contaminated furs and hair cushions, and from insect bites.
- There is need of great care in performing necropsy on animals.
- Infections most often result from spores entering through injuries to the skin causing cutaneous anthrax.
- Spores are present in soil, hair, hides, wool (wool-sorter's disease), faeces, milk, meat and blood products.

Anthracid Bacilli

Many members of the genus *Bacillus*, other than the anthrax bacillus, have occasionally caused human infections. Of them, the most important is *cereus*, which from 1970 has been recognised as a frequent cause of food-borne gastroenteritis. It has also been associated with septicemia, meningitis, endocarditis, pneumonia, wound infections and other suppurative lesions, particularly as an opportunist pathogen. *B. subtilis*, *B. licheniformis* and a few other species have also occasionally been isolated from such lesions.

These and a large number and variety of non-pathogenic aerobic spore bearing bacilli that appear as common contaminants in cultures and have a general resemblance to the anthrax bacilli have been collectively called pseudo-anthrax or anthracoid bacilli.

Differentiating features between anthrax and anthracoid bacilli:

Sr.No.	Anthrax bacilli	Anthracid bacilli
1	Non-motile	Generally motile
2	Capsulated	Non-capsulated
3	Grow in long chains	Grow in short chains
4	No turbidity in broth	Turbidity in broth
5	Inverted fir tree in gelatin	Atypical or absent
6	Slow gelatin liquefaction	Rapid gelatin liquefaction
7	Methylene blue reduced weakly	Methylene blue reduced strongly
8	Haemolysis absent	Haemolysis well marked
9	Sialicin fermentation negative	Sialicin fermentation usually positive
10	Susceptibility to gamma phage	Not susceptible
11	Produce toxin, neutralized by B.anthraxis	Not neutralized
12	Growth inhibited by chloral hydrate	Not inhibited
13	Pathogenic to laboratory animals	Not pathogenic
14	No growth in penicillin agar(10 units/ml)	Grows usually
15	Medulla head colony is seen	Not present
16	No growth at 45 C	Grows usually

Bacillus subtilis

- Commonly known as the “hay bacillus” or “grass bacillus”.
- It is a normal gut commensal in humans.
- The organism is found in the soil; hence it is on most vegetation.
- It is also found in the gastrointestinal tract of ruminants.
- It is spread by water, wind, and normal traffic in feeds, and is present all over the world.

History:

Bacillus subtilis was first described by Ehrenberg as *Vibrio subtilis* in 1938.

Morphology:

- Shape and size: Cylindrical rods. They are straight or slightly curved, with rounded ends, singly or in chains and size is 3μ-4μ.
- It is gram positive.
- They form a tough, protective endospore.
- It is a facultative anaerobe.
- They are actively motile with peritrichic flagella.
- Catalase positive bacterium.

Growth requirements and characteristics:

- *Bacillus subtilis* is easily cultured on any nutrient medium.
- Surface colonies on agar are small, grayish, amoeboid with crenate margin and a wool-like edge. The surface is finely granular and dull. The growth is membranous, slightly sticky, and is emulsified with difficulty.
- In broth: A thick ring pellicle which usually sinks within 24 hours is seen.

Biochemical properties:

- Acid is formed from i.e. ferments glucose, sucrose, and maltose.
- Indole negative.
- Methyl red negative.
- Vogues - Proskauer positive.
- It reduces methylene blue.
- Nitrates are also reduced by the bacterium.

Resistance:

- The bacillus is extremely resistant to heat by the virtue of the spores. Spores remain viable for years in the dry state.
- Boiling kills it in 2 hours, or death may be brought about at 120 °C in 15 minutes.
- It is sensitive to penicillin. Ordinary chemical disinfectants kill by prolonged contact.

Antibiotic produced: Subtilin.

Bacillus cereus

Widely distributed in nature and may be readily isolated from soil, vegetables and a wide variety of foods including milk, cereals, spices, meat and poultry.

History:

Colonies of *Bacillus cereus* were originally isolated from an agar plate left exposed to the air in a cow shed.

Hosts: Humans, cattle

Morphology:

- Gram positive.
- Large (1 x 5-10 μm) motile rods, centrally located endospores can sometimes be seen as uncolored regions after Gram staining of cells from older cultures.
- An aerobe, facultative anaerobe.
- *B. cereus* is generally motile but non-motile strains may occur.
- Non-capsulated.
- Can produce protective endospores. It is not susceptible to gamma phage.

Not pathogenic to laboratory animals.

Cultural characteristics: Large, opaque, grey-yellow, granular, flat colonies (diameter 5-10 mm). Most strains produce a clear broad zone of hemolysis on blood agar.

B. cereus produces a wide zone of haemolysis around the colonies. It is a beta haemolytic bacterium.

Virulence factors: Include cereolysin and phospholysin C.

Bacillus licheniformis

Bacillus licheniformis is a bacterium commonly found in the soil. It is found on bird feathers, especially chest and back plumage, and most often in ground-dwelling birds (like sparrows) and aquatic species (like ducks).

Morphology:

It is a gram-positive bacterium.

It is rod shaped (straight or slightly curved rods)

Size: 1.5-3.0 X 0.6-0.8 μm in diameter.

Motile by peritrichous flagella.

No capsule present.

Cultural characteristics:

Aerobic, facultatively anaerobic.

Colonial morphology is variable and may give a appearance of the mixed culture.

On agar media: Colonies become opaque with dull to rough surface, hair-like outgrowths ("licheniform"), attached strongly to agar.

On blood agar: Non-hemolytic.

Growth in 2-7% NaCl.

Temp. Requirement: Mesophilic (temperature optimum: +30°C) and may

Biochemical properties:

Citrate + Vogues-Proskauer + Oxidase variable. Ecology: • *Bacillus licheniformis* forms spores in soil. A pathway that leads to endospore formation is initiated when the bacterium is starved. • It is a mesophilic bacterium. Its optimal growth temperature is around 30 °C, though it can survive at much higher temperatures. • Growth in the presence of lysozyme variable. •

Bacillus subtilis and *Bacillus pumilus* bacteria are commonly known to cause food poisoning and food spoilage. *Bacillus licheniformis* also is known for contaminating dairy products. Food borne outbreaks usually involve cases of cooked meats and vegetables, raw milk, and industrially produced baby food contaminated with *B. licheniformis*.

Antibiotics produced: Bacitracin.
