

BACILLUS

Learning objectives

To know in detail about,

- The diseases caused by Bacillus species in domestic animals and human
- Morphology, cultural and biochemical characteristics of *B.anthraxis*
- Toxins of *B.anthraxis*
- Pathogenesis, control and prevention of anthrax
- Different diagnostic methods of *B.anthraxis*
- Explain the role of PLET agar in isolation of *B.anthraxis*
- Mcfadyen's reaction, Ascoli's precipitation and string of pearl's test
- Difference between *B.anthraxis* and anthracoids

SYSTEMATICS

Domain	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacilliales
Family	Bacillaceae
Genus	Bacillus
Species	<i>B.anthraxis</i> , <i>B.cereus</i> , <i>B.subtilis</i> , <i>B.mycooides</i> , <i>B.megaterium</i> , <i>B.mesentericus</i>

- *B.anthraxis* causes Anthrax in animals and Wool sorter's disease, hide porter's disease, Malignant Pustule in humans.
- *B.cereus* causes food poisoning in humans.
- Other bacilli in this group are non-pathogenic and they are called as anthracoids.
- *B.licheniformis* is an emerging pathogen and it is implicated in sporadic abortions in cattle and sheep.

HISTORY

- Discovery of the anthrax bacillus is credited to Davaine and Rayer (1863 –1868).
- Considerable historic interest is attached to anthrax bacilli.
- Pollender 1849 – Anthrax bacillus was the first pathogenic bacterium observed under the Microscope
- Davaine 1850 – first communicable disease shown to be transmitted by inoculation of infected blood
- Koch 1863 – first bacillus to be isolated in pure culture
- Pasteur 1881 – used for the preparation of attenuated vaccine.

HABITAT

- *Bacillus anthracis* spores remain viable for many years in soil, water and animal hides and products.
- Spores have been isolated from naturally infected soil as long as 60 years.

MORPHOLOGY

- Members of the family are Gram +ve large rods, aerobic (facultative anaerobic), endospore forming, capsulated, mostly catalase positive and fermentative organisms. They are motile by peritrichous flagella.
- The anthrax bacillus is one of the largest pathogenic bacteria. They are Gram +ve, straight, rod shaped, non-motile organisms measuring 4-8 μm x 1-1.5 μm .
- In cultures the bacilli are arranged end to end in long chains. The ends of the bacilli are truncated or often concave and somewhat swollen.
- Chain of bacilli presents a bamboo stick appearance (and also called as Box car bacillus - looks like linked rail carriages).
- In tissues or in blood smear, it is found singly, in pairs or in short chains, the entire bacilli being surrounded by 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 contains serum, albumen, charcoal, starch or bicarbonates with reduced partial pressure of carbon dioxide.
- When blood films containing bacilli are stained with polychrome methylene blue for a 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 asreaction. This reaction depends on the degree of heat employed for fixation of a blood film.
- Sporulation occurs readily outside the body in the presence of oxygen. Spores are formed in culture or in the soil, but never in the animal body during life.
- Sporulation occurs under unfavourable conditions for growth and is encouraged by distilled water, 2% NaCl or growth in oxalated agar. Sporulation takes place at an optimum temperature of 25-30°C and in atmosphere containing low partial pressure of oxygen. Sporulation is inhibited by anaerobic conditions and by CaCl₂
- Spores are central, 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. The spores do not stain by ordinary methods. But can be stained with Sudan black B.

CULTURAL CHARACTERISTICS

- Grows readily under aerobic and as facultative anaerobic at an optimum temperature of 35-37°C.
- On agar plates, irregular, round, raised, dull, opaque, grayish white, 2-3mm in diameter frosted glass appearance colonies are produced.
- Under the low power microscope, the slightly serrated edge of the colony is composed of long, interlacing chains of bacilli, resembling locks of matted hair. This is referred to as medusa head or judge's wig or woman's curling hair type of growth.
- *B. licheniformis* produces characteristic hair-like outgrowths from streaks of the organisms on agar media. Colonies become brown with age.
- The name of this species derives from the similarity of its colonies to lichen.
- In medium containing iron salts, virulent *B.anthraxis* produces pink or purple coloured pigmented colonies.
- Virulent capsulated strains form rough colonies, while avirulent attenuated strains form smooth colonies.
- In gelatin stab, fine filaments of growth develop laterally along the line of inoculum.
- The growth nearer to the surface of the medium is the longest and then progressively shorter where there is less oxygen, resembling inverted fir tree appearance.
- On haemolysis, the *B.anthraxis* produces slight haemolysis with capsule production, compared with anthracoid organisms.
- *B. cereus* produces a wide zone of complete haemolysis around the colonies.
- A selective medium (PLET) consisting of polymyxin, lysozyme, EDTA and thallos acetate added to heart infusion agar is useful for isolating *B.anthraxis* from mixtures containing other spore-bearing bacilli.

BIOCHEMICAL PROPERTIES

- *Bacillus anthracis* organisms are catalase +ve .
- They ferment glucose, maltose, saccharose, trehalose and dextrin, produce acid but no gas.
- Nitrates reduced to nitrites. Indole is negative.

RESISTANCE

- The vegetative bacilli are destroyed at 60°C in 30mts.
- In the carcasses of animals, the bacilli remain viable in the bone marrow for a week and in the skin for two weeks.
- Normal heat fixation of smears may not kill the bacilli in blood film.
- The spores are highly resistant to drying, heat, cold and disinfectants. Spores remain viable for many years in soil, water and animal hides and products.
- Spores have been isolated from naturally infected soil as long as 60years.
- They resist dry heat at 140°C for 2-3hrs and boiling for 10mts.
- They survive in 5% phenol for weeks. Spores can be killed at 120°C for 10min and 4% KMnO₄ treatment for 15mts.
- Destruction of the spores in animal products is achieved by HCHO.
- Treat 2% solution of HCHO at 39-40°C for 20mts for disinfection of wool and as 0.25% at 60°C for 6 hrs for animal hair and bristles. This process is called as duckering.
- The anthrax bacillus is susceptible to sulphonamides, penicillin, erythromycin, streptomycin, tetracycline and chloramphenicol.

ANTIGENICITY

- The complex antigenic structure includes a capsular polypeptide, a somatic protein and a somatic polysaccharide antigen.
- The capsule contains poly-D-glutamic acid, which is only detectable in virulent strains, having antiphagocytic property.
- The somatic protein is protective antigen present in the edema fluid.

TOXINS AND VIRULENCE FACTORS

- *B. anthracis* produces an extracellular toxin which is composed of three components, Factor I, II & III namely oedema factor (EF), protective antigen (PA) and lethal factor (LF) respectively.
- Factor I consists of chelating compound containing phosphorus with protein and carbohydrate moieties.
- Factor II & III consists of proteins.
- All three factors are essential to exhibit their toxic properties.
- They are not toxic individually but the whole complex produces local oedema and generalized shock.

PATHOGENESIS OF BACILLUS

- Anthrax can occur in virtually all mammalian species but birds are highly resistant.
- The main routes of entry of endospores are by ingestion, from soil when grazing or in contaminated food, and by infection of wounds.
- Inhalation of spores occurs in man, but to a lesser extent in animals.
- Transmission by biting insects may be important, especially during an outbreak of anthrax.
- Cattle, sheep and goats are most susceptible to infection, while horses and humans occupy an intermediate position and pigs and carnivores are comparatively resistant, but can succumb if the infective dose is high.

PATHOGENICITY

CLASSIFICATION

Symptoms

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.

Cattle

- Bulls are more susceptible than cows. They have a mortality rate of 90%.
- There are three clinical causes of bovine anthrax.
- In peracute septicemia death occurs within 2 hours after animal collapsing with convulsions, sudden death in animals that appeared normal is common.
- In acute septicemia death occurs within 48 to 96 hours clinical signs include fever, anorexia, ruminal stasis, hematuria and blood tinged diarrhea.
- Pregnant animals may abort and milk production often abruptly decreases.
- Terminal signs include severe depression, respiratory distress and convulsions.
- In chronic cases, clinical signs are manifested for more than 6 days and are rare.
- *B.licheniformis* infection is associated with the feeding of contaminated silage and is responsible for abortion in cattle and sheep.

Sheep

- Disease is acute and death occurs rapidly after convulsions.

Pigs

- 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 .

Dogs

- The throat will be inflamed and swollen, gastroenteritis **Lesions**
- The carcass of animals will putrify rapidly and develop incomplete rigor mortis.
- The blood is dark, (tarry colored), clots poorly & exudes from the natural orifices.
- The spleen is greatly enlarged, dark and friable. The spleen reveals black cherry jam consistency.
- The LN (lymph node) at the region of initial infection site is hemorrhagic and edematous.
- Ecchymotic hemorrhages on the serosal surface of the abdomen, thorax, epicardium and endocardium are common.
- Subcutaneous edematous swellings are present on the ventral aspect of the neck **Note**
- When suspected for anthrax care should be taken not to open the carcass. Muzzle piece or ear piece is usually sent for examination.

DIAGNOSIS

Clinical symptoms

- Blood films from dead animals made by 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 alcohol and ignited.
- The smears are heat fixed and stained by Wright's or Giemsa's stain to reveal *B. anthracis* as large blue rods with characteristic dark pink or purple coloured capsules.
- In case of horses and pigs since peripheral blood contains fewer organisms, smears should be made from the edematous fluid or LN's.

Cultural examination

- Swabs from blood are inoculated on to blood agar plates and incubated at 37°C for 24 hrs and examined for their typical growth.
- Swabs are inoculated in agar enriched with blood or serum and incubated for 6 hrs at 37°C and examined by stained smears.

Bacteriological examination of hair, wool, hide, bone, bone meal & others

- Samples are added to cold saline and shaken intermittently for 3 hours.
- The supernatant fluid is then heated to 70°C for 10 minutes.
- Then they are filtered through two layers of muslin cloth, added to melted agar, and poured into petridishes, allowed to set and incubated at 37°C.
- After 12 hours incubation, plates are to be examined for characteristic colonymorphology.

Ascoli's Precipitation 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 0.5 ml of anti-anthrax serum (1:50) in a small test tube and overlay with 0.5 ml of clear filtrate.
- Stand at room temperature for 15 minutes. A white ring of precipitation indicates a positive reaction.

String of Pearl's test: (Charlton,1980)

- *B.anthraxis* produces swollen round cells in chains (string of pearls) when incubated for 3-6 hrs on tryptose agar containing 0.05 –0.5 I.U of penicillin/ml.
- To 100ml of molten nutrient agar, add required Sodium beznyl penicillin and mixcarefully.
- 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 a petridish containing a small piece of moistened absorbent cotton wool to prevent the agar drying out.
- Use a young colony to streak the center of the agar block.
- Place a clean cover slip on the agar block and incubate the petridish at 37°C.
- After 2 hrs, remove the slide and examine the inoculum microscopically by oil immersion for the string of pearls growth.
- Differentiation from non-pathogenic *Bacillus* sp. It is considered reliable but needs experience.

- Cherry gamma phage is used. It can be propagated on *B. anthracis* strain 14, which makes a suitable control.
- On one half of blood agar plate, streak *B. anthracis* strain 14 culture as control and on the other half streak the culture to be identified.
- Add drops of phage preparation (1:10 dilution) on both halves. Incubate the plates in the upright position for 8-12 hrs.
- Zones of clearing will be seen on control culture and on the suspected half if it is *B. anthracis*.

Animal Inoculation

- Guinea pigs & mice are highly susceptible. Materials are inoculated by dermal scarification, subcutaneous or intramuscular.
- Death occurs in 2-3 days and the organisms will be readily identified in the blood & tissues.
- Apart from this laser pyrolysis, gas-liquid chromatography and mass spectrometry are used for detection of anthrax toxin productions.

• DIFFERENTIAL DIAGNOSIS

Difference between *B. anthracis* & Anthracoid organisms in culture

<i>B. anthracis</i>	Anthracoid organisms
Non motile	generally motile
Capsulated	non capsulated
Grows in long chains	Grows in short chains
No turbidity in broth	Turbidity in broth
Inverted fir tree in gelatin	Atypical or absent
Methylene blue reduced weakly	Reduced strongly
Haemolysis weak	Strong
Liquefaction of gelatin is slow	Rapid
Lecithinase reaction is weak	Rapid
Ferments salicin slowly	Rapid
Produces toxin, neutralised by <i>B. anthracis</i> antitoxin	not neutralized
Pathogenic to g.pigs & mice	non pathogenic
Susceptible to gamma phage	not susceptible

Difference between *B. anthracis* & Anthracoid organisms in blood smear stained with Polychrome methylene blue stain

<i>B. anthracis</i>	Anthracoid
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Dark pink or Purple coloured capsules	No capsule
Organism rod stained blue	Organism rod stained blue
Ends truncated	Ends bulged and rounded
Single, pair or short chain	Usually long chain
Absence of spores	Spores may be present

TREATMENT, IMMUNITY AND PUBLIC HEALTH ASPECTS

Treatment

- The organism is susceptible to penicillin-G, tetracyclines, erythromycin and chloramphenicol.

Immunity

- Prevention of anthrax in animals is aided by active immunization.
- Initially Bail demonstrated that oedema fluid and tissues obtained from anthrax lesion, which had been freed from viable organism, had protective properties.
- He termed the active substances as "aggresins".
- Pasteur's vaccine was anthrax bacillus attenuated by growth at 42 –43° C .
- As the spore is the common infective form in nature, vaccines consisting of spores of attenuated strains were developed.
- The strene vaccine contained spores of noncapsulated avirulent mutant strain.
- It should be given 1 month before anticipated outbreaks.
- The Mazucchi vaccine contained spores of stable attenuated carbazoo strain in 2% saponin.
- It gives good protection when given subcutaneously.
- Protective humoral antibodies develop in 7-10 days against Factor II of the exotoxin complex and lasts about one year.

Public Health aspects

- There is need for 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-Pulmonary anthrax*), faeces, milk, meat and blood products.
- The skin lesion (*cutaneous anthrax*) is usually solitary, painless, seropurulent, necrotizing, hemorrhagic and ulcerous.
- It leaves a black scar (*anthrax=coal*), which accounts for the name malignant pustule.