Genus Mycobacterium

Historical

Villemin	1865	Conclusive proof of infectiousness of tuberculosis. Specific disease caused by inoculable agent transmitted from man and cattle to rabbits, G. pigs.
Robert Koch	1882	Discovered the cause of tuberculosis Isolated the organism on coagulated blood serum. Inoculated the culture in in guinea pig. Koch's Phenomenon. Prepared the glycerin broth extract of tubercle bacilli. i.e, tuberculin.
		Henle-Koch's postulates: Different investigators were reporting causative agents of various infectious diseases as, there should be some criteria for the claims. First indicated by Jacob Henle were enunciated by Koch Henle-Koch's postulates. According to Koch's postulates microorganisms can be accepted as the causative agent of infectious disease only if the following conditions are satisfied: 1.Bacteria should be constantly associated with the lesions of disease. 2.Should be possible to isolate the bacteria in pure culture. 3.Inoculation of such pure culture into suitable lab animals should reproduce the lesions of the disease. 4.Should be possible to re-isolate the bacteria in pure culture from the lesions produced in the experimental animal. 5.Additional criteria: Specific antibodies to the (subsequently introduced) infection in serum. **Koch's phenomenon:** When tubercle bacillus (or its protein) was injected into a guinea pig already infected with mycobacteria - an exaggerated response i.e., hypersensitivity reaction known as Koch's phenomenon was recorded. Koch's phenomenon experiment: Injection of virulent tubercle bacilli s/c – developed lesion at the site of injection after 10-15 days – lesion softened and ulcerated. Similar dose was given to pre-sensitized (infected at least a week earlier) G. pig – local reaction developed within 1-2 days-ulceration was milder. (Due to Allergic reaction-Delayed type of hypersensitivity).
Paul Ehrlich	1882	Acid fast characteristic staining.

Genus *Mycobacterium*

- Mycobacteria are straight or slightly curved rods
- Acid fast
- Non-motile
- Non-spore forming bacteria.
- Aerobic and micro-aerophillic
- Some (Saprophytes) grows rapidly whereas others (Pathogenic) grow slowly
- Cell wall contain abundant lipids i.e., Mycolic acid.
- Mycobacteria are rich in lipids. **Cord factor** (trehalose 6,69-dimycolate [TDM]), a surface glycolipid is present in *Mycobacterium tuberculosis*. TDM is a pleiotropic molecule against the host and participates in the pathogenesis of tuberculosis.

Important *Mycobacterium* Species

- Important pathogenic species:

  Mycobacterium tuberculosis
  Mycobacterium bovis
  Mycobacterium avium
  Mycobacterium avium subspecies *paratuberculosis*
  Mycobacterium leprae

- Commonly known saprophytic bacteria:

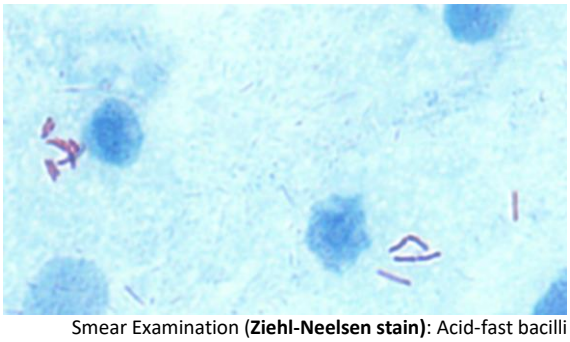
  Mycobacterium phlei
  Mycobacterium smegmatis
  Mycobacterium microti
  Mycobacterium ulcerans

Transmission

- Tubercle bacilli are distributed throughout human and bovine populations in most parts of the world.
- Animal stabled together or kept in close contact in pens, crowding appears to be a definite factor in the prevalence of tuberculosis i.e., aerosol route.
- Among cattle through feed, water, pulmonary exudates in cows-swallowed-organisms pass with faeces - contaminate ground and feed-contaminate water tank.
- In human - M.O. is transmitted by sputum/other exudates containing bacilli. Close contact during coughing, sneezing, spitting, contaminated food, water.
- Human tubercle bacilli may be transmitted to dogs, swine and cattle.
- Handling of cooking utensils by a person with open lung lesions of tuberculosis.
- Bovine type is pathogenic to man- caused by drinking of milk from a cow with tuberculos mastitis. Swine may be infected.
- Tuberculosis is spread through avian family by the medium of feed and water contaminated with faecal material .

Morphology

Mycobacterium tuberculosis: Slender rod, 0.2-0.6um X 1.5-4.0um in size.
Mycobacterium bovis: Shorter and thicker than above.
Mycobacterium avium: More pleomorphic, from filamentous to coccid form.
Non-spore forming, Non-motile.
Extremely pleomorphic, Acid fast bacilli.



Smear Examination (**Ziehl-Neelsen stain**): Acid-fast bacilli take pink red colour against blue background. (Photo AB)

Growth requirements and characteristics

Aerobic, atmosphere with 5% CO2 aids growth.

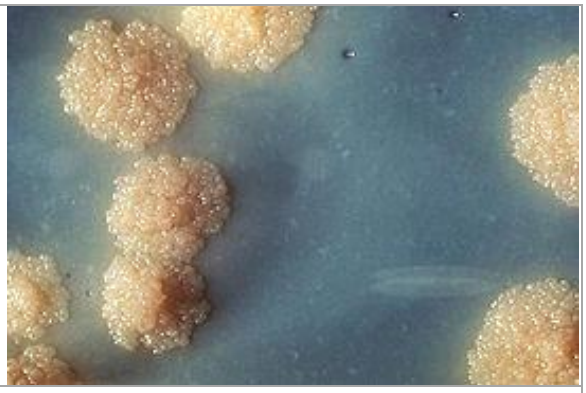
- Optimum temperature 37^0C. Avian tuberculosis bacteria grows best-25^0C to 45^0C.
- Optimum pH:
  Mycobacterium tuberculosis: pH- 7.4 to 8.0
  Mycobacterium bovis: pH- 5.8 to 6.9
  Mycobacterium avium: pH- Slightly alkaline.

Colony Characteristics

- Media used for isolation:
  Lowenstein-Jensen medium
  Stonebrinks medium
  Dubos & Davis Medium
  Petragnani medium
  Prausker & Beck Medium
  Middlebrooks Medium
- ***Mycobacterium tuberculosis****:* Thicker, wrinkled, cream buff, white heaped up, crumb like. Cord factor (trehalose 6,69-dimycolate [TDM]), a surface glycolipid, causes *M. tuberculosis* to grow in serpentine cords *in vitro*. Virulent strains of *M. tuberculosis* have TDM on their surface.
- ***Mycobacterium bovis***: Small, shining, grey & later coalesce to form buff white with fine granular surface.
- ***Mycobacterium avium****:* Moist creamy, smooth, no surface pellicle.
- ***Mycobacterium leprae***- Do not grow on artificial media, can be grown using animal inoculation method - Nine banded Armadillo or Foot pad of mice.

  Eugonic: Mycobacterium whose growth is enhanced by glycerin is known as Eugonic.
  Dysgonic: Used especially in reference to the slow and relatively poor growth of cultures of the bovine tubercle bacillus (*Mycobacterium bovis*) in the presence of glycerine.

- Good growth of Mycobacterium is seen in 4-6 weeks.
- Generation time-12-18 hours.
- Iron is essential for the growth Mycobacterium add chelating agents i.e., exochelins and mycobacterins - are synthesized by all cultivable mycobacteria except *Mycobacterium avium* subspecies *paratuberculosis*.
- Mycobacterin- Water insoluble lipids in cell wall of mycobacterium.
- Exochelins - Low molecular weight peptides secreted in external environment and are water-soluble.

	
Mycobacterium tuberculosis on Lowenstein Jensen Medium - Image CDC	*Mycobacterium bovis* on Middlebrooks medium

Biochemical and sugar fermentation test

- Little is known.
- Catalase positive.
- H2S positive (slightly).
- MR, VP-Negative.
- Slight acid reaction-In glucose, maltose, trehalose and glycerol.

Transmission

Bovine tuberculosis is contagious and spread by contact with infected domestic and wild animals. The usual route of infection is by inhaling infected droplets which are expelled from the lungs by coughing. Calves and humans can also become infected by ingesting raw milk from infected cows. Because the course of disease is slow, taking months or years to kill an infected animal, an animal can spread the disease to many other herd mates before it begins to manifest clinical signs. Therefore, movement of undetected infected domestic animals and contact with infected wild animals are the major ways of spreading the disease.

Pathogenicity

Tuberculosis is a chronic infection with *M. tuberculosis* complex, including *M. tuberculosis, Mycobacterium avium* and *Mycobacterium bovis*, that is characterized morphologically by granulomatous inflammation, a compact organized collection of macrophages and their derivatives, such as epithelioid and giant cells, at the site of infection.

The pathogenicity of *M. tuberculosis* is related to its ability to escape killing by macrophages and induce delayed-type hypersensitivity (DTH) induces lesions characterized by chronic granulomatous inflammation**.**

Cord factor in *M. tuberculosis* participate in pathogenesis. Virulent strains of *M. tuberculosis* have TDM on their surface, and injection of purified TDM into experimental animals TDM, a surface glycolipid derived from the cell walls of virulent strains of *M. tuberculosis*, plays a critical role in the process. Taken together with the previous reports that mycobacterial TDM can induce apoptosis and angiogenesis.

- ***Mycobacterium tuberculosis:*** Mycobacterium tuberculosis is pathogenic to animals i.e., bovine, swine, horses, sheep, goats, dog and parrot. Other birds are resistant.
- ***Mycobacterium bovis:*** Primarily pathogenic for cattle, swine readily get infected, man is susceptible (public health problem). Children less than 16 years of age are susceptible to non-pulmonary type of tuberculosis. Avian birds are resistant.
- ***Mycobacterium avium:*** Primarily pathogenic for birds. Sheep and horse-moderately susceptible. Cow and goat-slightly susceptible. Dog and cat are resistant.

Development of primary tuberculosis

Inhalation of mycobacterium

↓

Engulfed by alveolar macrophages. Initial lesion formed

↓

Transported by macrophages to lymph node

↓

Lymphatics and hematogenous dissemination to other parts of lung and other organs such as liver, spleen, bone marrow

↓

After 10 days of infection

↓

T cell mediated immunity:CD8 suppressor kills macrophages infected with mycobacterium
CD4 helper T cells secrete interferon gamma
Activates macrophages to kill intracellular mycobacterium (Epithelloid cells)

↓

Form compact cluster around the foci

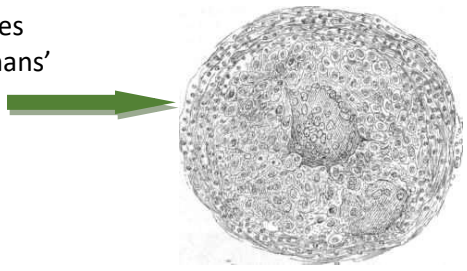
↓

Anoxia

↓

Immuno-compromised animals-reactivation of primary lesion

Photomicrograph showing typical tubercles with epithelioid cells, lymphocytes, Langhans' giant cells with central caseous necrosis.



Clinical Signs of Bovine Tuberculosis

- TB usually has a prolonged course, and symptoms take months or years to appear.
- The clinical signs include:
    - Intermittent hacking cough & Dyspnoea- Acute Respiratory distress ,
    - Prominent enlarged Lymph nodes,
    - Extreme emaciation
    - Intermittent diarrhoea, constipation, loss of appetite, fluctuating fever

Immunity

- Bacille-Calmette-Guerin BCG Live vaccine (Attenuated *Mycobacterium bovis*)
- Used in calves and children without any harmful effect.

Treatment:

- Animals tested positive for tuberculosis are not recommended to be treated. It is recommended to cull such positive animals.
- In case of human beings **DOTS Therapy**- Directly Observed Therapy-Short course (DOTS) for the treatment of Tuberculosis is widely recommended and is proven to be effective.
- Anti-TB **Bactericidal drugs**: Rifampicin, Isoniazid, Pyraziamide and Streptomycin.
- Anti TB **Bacteriostatic drugs**: Ethambutol, Hiacetazone.

Diagnosis

1. Direct smear examination

Clinical material collection: Sputum, faecal material, pulmonary exudates. Smear from pulmonary exudate in case of animals and sputum in case of human being, is prepared and stained with Ziehl-Neelsen method. Presence of acid fast bacilli under microscope, followed by isolation and identification of bacteria confirms the disease. Use Petroff's method for preparing the sample for isolation.

Petroff's Method: To overcome contamination, while isolating the mycobacterium following procedure can be followed to overcome the contamination.

- Take 3% solution of NaOH + tissue/exudates(suspected)-Equal volume.
- Shake the tube vigorously.
- Stand for 30 mins.
- Neutralize by 3N Hcl.
- Centrifuge & discard the supernatant.
- Collect the sediment and is 'seed' over the surface of slants of suitable culture medium

2. Isolation and identification

Cultural Characteristics

- ***Mycobacterium tuberculosis****:* Thicker, wrinkled, cream buff, white heaped up, crumb like.
- ***Mycobacterium bovis***: Small, shining, grey & later coalesce to form buff white with fine granular surface.
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Microscopic

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Non-spore forming, Non-motile.
Extremely pleomorphic, Acid fastbacilli.

Biochemical Tests & sugar fermentation test:

- Catalase positive,
- H2S positive (slightly).
- MR, VP-Negative.
- Slight acid reaction-In glucose, maltose, trehalose and glycerol

3. Tuberculin test (Intradermal)

- Shave and clean the area on the skin of mid neck region of suspected animal.
- Hold the skin fold on mid neck region, and record thickness of skin fold by Vernier's calliper in mm.
- Inject intradermally 0.1 ml of *Mycobacterium bovis* Purified Protein Derivative (PPD) (Bovine Tuberculin).
- Keep the animal under observation for next 72 hours.
- Measure the thickness of skin at the site of injection again after 72 Hours.
- In positive case, at the site of injection- diffuse or extensive oedema, exudation, necrosis, pain or inflammation of the lymphatic ducts in that region or of the lymph nodes is recorded with increase in skin thickness by more than 4mm.

OIE guidelines for interpretation of Tuberculin test

The tuberculin test is usually performed on the mid-neck, but the test can also be performed in the caudal fold of the tail. The skin of the neck is more sensitive to tuberculin than the skin of the caudal fold. Delayed hypersensitivity may not develop for a period of 3–6 weeks following infection.

As the sensitivity of the test is less than 100%, eradication of tuberculosis positive animal achieved with only a single tuberculin test should be confirmed with two other recommended tests. It should be recognised that when used in chronically infected animals with severe pathology, the tuberculin test may be unresponsive.

The dose of tuberculin injected must be no lower than 2000 International Units (IU) of bovine or avian tuberculin. A correct injection is confirmed by palpating a small pea-like swelling at each site of injection.

clinical signs, such as diffuse or extensive oedema, exudation, necrosis, pain or inflammation of the lymphatic ducts in that region or of the lymph nodes. The reaction is considered to be inconclusive if none of these clinical signs is observed and if the increase in skin-fold thickness is more than 2 mm and less than 4 mm. The reaction is considered to be positive if clinical signs, as mentioned above, are observed or if there is an increase of 4 mm or more in skin-fold thickness. Moreover, in M.-bovis-infected herds, any palpable or visible swelling should be considered to be positive. Animals that are inconclusive by the single intradermal test should be subjected to another test after an interval of 42 days to allow desensitisation to wane (in some areas 60 days for cattle and 120 days for deer are used).

2. Opthalmic test: Instillation of 1-2 drops of Tuberculin in one of the eye and kept under observation for next 24-72 hours; In positive case, reddening i.e., congestion, ocular discharge and all signs of inflammations are evident.

3. Nucleic acid amplification by Polymerase Chain Reaction.
4. ELISA: Sensitive.
5. Latex agglutination test: Sensitive and specific.
6. Animal inoculation test: Demonstration of Koch's Phenomenon in Guinea pig.

**

JOHNE'S DISEASE (Paratuberculosis)

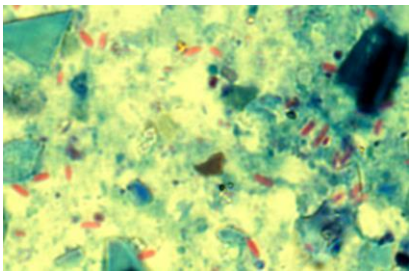
Heinrich Albert Johne Germany (1895)

Paratuberculosis (Johne's disease) is a chronic enteritis of ruminants caused by *Mycobacterium avium subsp. paratuberculosis (M. paratuberculosis)* characterized by slow progressive wasting and long term chronic intermittent diarrhoea, along with thickening and corrugation of small intestine, necrosis and calcification of MLN.

Mycobacterium avium subsp. paratuberculosis

Morphology

Short acid fast rod, (0.5 x 1.5 microns)
Fcultative intracellular, Acid-fast bacillus.
The organism grows in characteristic clumps caused by a network of intercellular filaments.
Non-spore forming, Non-motile.



Processing faecal sample

The faecal sample can be frozen at -70°C.

i) Suspension and decontamination of faeces
- 1 g of faeces is transferred to tube containing 20 ml of sterile distilled water.
- The mixture is shaken for 30 minutes & large particles are allowed to settle for 30 minutes.
- The uppermost 5 ml of faeces suspension is transferred to a 50 ml tube containing 20 ml of HPC(**Hexadecylpyridinium chloride**) . The tube is inverted several times to assure uniform distribution and allowed to stand undisturbed for 18 hours at room temperature.

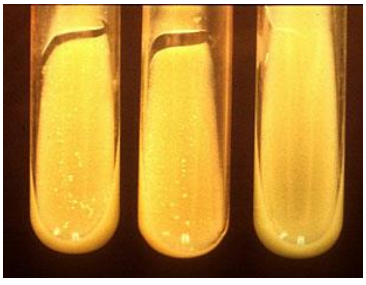
ii) Inoculation of culture media: 0.1 ml of the undisturbed sediment is transferred to each of four slants of Herrold's medium, three with mycobactin and one without mycobactin. A smear should be made from the sediment and stained by Ziehl-Neelsen method.

Cultural Characteristics

Herrold's egg yolk medium with mycobactin
Modified Dubos's medium
Middlebrook 7H9, 7H10 and 7H11
Löwenstein-Jensen medium with mycobactin

Small 1-5 mm, firm, glistening, white (LB) /brown (MB) rough-smooth colonies. Colonies of *Mycobacterium avium subspecies paratuberculosis* grown on Middlebrook agar media without Tween are rough in appearance

Mycobacterium avium subspecies paratuberculosis on Herold Egg yolk Medium with Mycobactin
JCourtesy: Shoor Veer Singh



Growth of slow-growing, small, white-yellow colonies only on media containing mycobactin (two left tubes) is indicative of *Mycobacterium avium subsp. paratuberculosis*

Transmission

Johne's disease is introduced into a herd when healthy but *Mycobacterium avium subspecies paratuberculosis* (MAP) infected animals (subclinical infection) are purchased by herd owners. Cattle usually become infected as calves when feces contaminated with MAP are ingested.

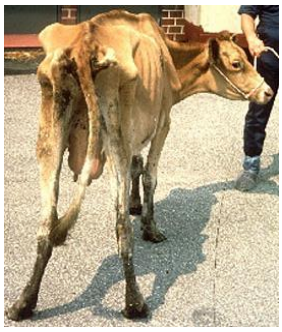
Other possible modes of transmission include

- ingestion of contaminated milk and/or colostrum,
- insemination using contaminated semen and
- intrauterine transmission to bovine fetuses.

Incubation Period of Johne's Disease: 12 months to several years

Clinical Signs

Slow Progressive wasting (loss of condition) &
Chronic intermittent diarrhoea
Typically, affected animals remain bright and alert, without fever, and eating well.



Lesions:

Thickening & corrugation of the small intestine
Mesentric lymph nodes edematous enlarged,
Necrosis & Calcification of Mesentric lymph node

Diagnosis

1. **Isolation & Identification** of *M. avium subsp. paratuberculosis*.

2. **Rectal Pinch Smear Examination**:
i. Insert hand per rectally and pinch out the mucous membrane of the rectum.
ii. Wash the mucous membrane with sterile saline.
iii. Place the mucous membrane on the slide and munch with another slide.
iv. Dry the smear and stain with Ziehl-Neelsen staining method.
v. Acid fast bacilli against blue background are seen under microscope in positive cases.

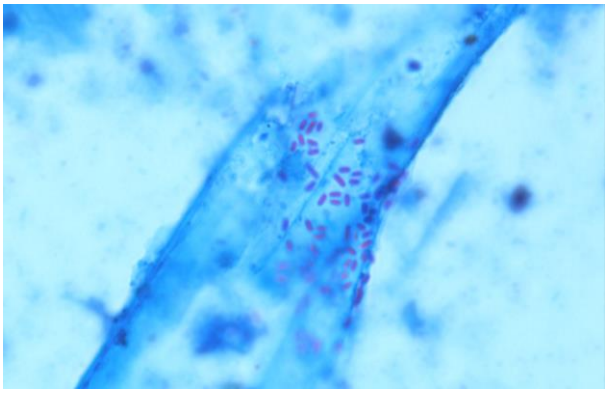


Photo: AB

3. **Complement Fixation Test**
4. **Enzyme Linked Immuno-sorbent Assay**
5. **Agar Gel Precipitation test**
6. **Johnin Test**

The test is carried out by the intradermal inoculation of 0.1 ml of Purified Protein Derivative (PPD) of *Mycobacterium avium subspecies paratuberculosis* (Johnin) into a clipped or shaven site, usually on the side of the middle third of the neck.

- The skin thickness is measured with calipers before and 72 hours after inoculation.
- Increases in skin thickness of over 2 mm should be regarded as indicating the presence of DTH.

A herd test gives only an indication of the number of sensitised animals and may thus be used only as a preliminary test prior to the initiation of a control programme.

7. Nucleic acid amplification: The DNA after extraction from suspected sample/bacteria, should be subjected to IS*900* specific PCR, positive DNA yields 229 bp long fragment as amplified product, confirmed as MAP.

Control strategies for Johne's Disease in India

1. Test & Cull: Is being practiced on some goat farms only. Should be applied at farmer level.
2. Mass Vaccination in goats where there is history of MAP.
3. Sero-monitoring: Susceptible host especially dairy cattle, goat should be sero-monitored for Antibodies titer. Samples of other domestic animals will also be included.
4. DIVA ELISA test: To differentiating vaccinated & infected animals.
5. Molecular Epidemiology of MAP: Livestock should be monitored for MAP genotypes.
6. Screening of breeding males and females: For shedding status of MAP in semen and vaginal secretion.
7. Practicing improved hygiene and
8. Appropriate husbandry & grazing practices.

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