Genus Mycobacterium

- Aerobic, non-motile, non-spore forming, slow growing (2-40 days) rod-shaped (bacilli) bacteria that are characteristically acid fast.
- Mycobacteria are widely distributed in nature (over 40 species), mostly saprophytic or opportunistic in nature and few of them pathogenic for man and animals (causing human and bovine tuberculosis and human leprosy) and others pathogenic for cold-blooded animals.
- They containing mycolic acids in cell wall (like Nocardia, Rhodococcus, and Corynebacterium).
- The name mycobacterium, meaning fungus-like bacterium, is derived from the mould-like appearance of Mycobacterium tuberculosis when grown in liquid media.

- They are mostly slow growers; need incubation for 3-8weeks in human and bovine types, avian type 2-6 weeks. M. avium subsp. Paratuberculosis (MAP) needs up to 16 weeks.
- Acid fastness refers to the ability of mycobacterial cells to bind phenol-based dyes (e.g. Basic fuchsin in 5% phenol), with heating while staining; the dye is retained when the smear is decolorized with acidified alcohol.
- Complex lipids in mycobacterial cell wall include the mycolic acids; peptidoglycan and glycolipids. This cell wall composition is also responsible for resistance of Mycobacteria to drying, extremes of pH, and other environmental stresses.

On the other hand, protects the organism in the phagolysosome with survival of Mycobacteria in macrophages. Components of the cell wall are immunostimulatory, and are the basis for Adjuvants, including Freuud's complete .

It is divided into:

- Those associated with tuberculosis (M. tuberculosis complex or **MTC**); such as M. tuberculosis, M. bovis, **BCG**, M. africanum and M. microti,
- and other mycobacteria that may be associated with human disease (atypical, tuberculoid or Mycobacteria other than tuberculosis bacilli; **MOTT**).
- M. leprae is in separate group.

Mycobacteria of medical and veterinary significance

- Person-to-person spread is common, especially with M. tuberculosis.

- Infection with M. bovis, a well-recognized animal pathogen, can be acquired zoonotically, via infected cow's milk or from other animals such as deer.

Species pathogenic for man and animals:

1- M. tuberculosis Var hominis (M. tuberculosis Or **Human type**) Affected mainly human, transmitted to animals.

2- M. tuberculosis Var bovinus (M. bovis Or **Bovine type**) affected mainly cattle transmitted to man.

3- M. tuberculosis Var avium (M. avium Or **Avian type**) cause of tuberculosis of birds.

4- M. avium subsp. Paratuberculosis (**M. paratuberculosis**) cause paratuberculosis (John's disease) in adult cattle, sheep and goat causing **chronic bacillary dysentery**.

5- M. leprae cause **leprosy** in man only.

Mycobacteria which are pathogenic for animals and man.

Mycobacterium sp.	Main host	Species occasionally infected	Diseases
M. tuberculosis	Man, captive primates	Dogs, cattle, canaries	Tuberculosis
M. bovis	Cattle	Deer, man, cats, other mammals	Tuberculosis
M. africanum	Man	-	Tuberculosis
M. avium complex	Most avian species	Cattle	Tuberculosis
M. marinum	Fish	Man, aquatic mammals, amphibians	Tuberculosis
M. leprae	Man	Chimpanzees	Leprosy
M. avium subsp. Paratuberculosis	Cattle , sheep, goat, deer	Other ruminants	Paratuberculosis (Johne's disease)
Unspecified acid fast bacteria	Cattle	-	Associated with skin tuberculosis
M. farcinogenes *M. senegalense*	Cattle	-	Implicated in bovine farcy

Species of cold blooded animals:

1- M. piscium in fish, frogs, lizards, snakes, turtles.

2- M. cheloni affects lung of turtles.

3- M. thamnophes affects snakes.

Many MOTTs are found in the environment but they can also colonize man (e.g. in part of a previously damaged respiratory tract) and cause clinical infection. Their isolation form clinical samples has to be interpreted with caution; isolation form a single sample on a single occasion is unlikely to be significant, except from blood cultures, and repeat samples should be obtained. Mixed infection with two or more species of mycobacteria can occur; cultures should be inspected carefully to check for such a possibility.

Saprophytic species (Atypical Mycobacteria):

_ They are found in soil, dust, air, water. They are rapid grower; grow easily on nutrient agar especially when 5-10% glycerin is added.

_ Also they are acid water fast (resist acid dissolve in water) but not acid alcohol fast.

_ Nowadays these microorganisms produce localized lesions in different lymph nodes and organs in animals specially pigs, camels and cattle in addition to human beings.

_ These microorganisms sensitive to most antibiotics but resist drugs used in treatment of tuberculosis in man as neomycin, para-aminosalicylic acid (PAS) and rifamycin. _ In camels cause pulmonary ., Pigs digestive localized forms (due to garbage ingestion).

Runyon classification:

Atypical mycobacteria were divided according to pigment production and rate of growth into:

A. Photochromogenes: produce yellow pigment after exposure to light e.g. **M. kansasi and M. marinum**.

B. Scotochromogenes: produce yellow to orange pigment in the absence of light e.g., **M. gordonei**.

C. Non chromogenes: Not produce pigment e.g. **M. xenopi and M. avium complex.**

D. Rapid growers: grow within 3-5 days e.g **M. fortitum, M. phlei and M. chelonae.**

Boenicka classification

Atypical mycobacteria were classified into 72 types according to biochemical tests mainly niacin test, aryl-sulphatase test, iron uptake test, growth at different temperatures (10-45°C).

Morphology.

- Mycobacteria are **Gram positive rods** but very difficult to stain with Gram stain due to thick complex cell wall. They are aerobic, non-spore forming, non-motile.
- Slender rods sized 2.5-3.5x0.3-0.5µm arranged singly or in clumps.
- Human type appears long, thin, curved and beaded while bovine type appears short, thick, straight and stubby and the avian type appears pleomorphic (some short and thick stubby, others long thin beaded, the majorities are filamentous).

Mycobacteria are acid-fast (resist de-colourization by 20% H_2SO_4 in distilled water or 3% HCl in alcohol when stained by **Ziehl Neelsen's** stain mainly hot method composed of 5% strong carbol fuchsin; basic fuchsin dissolved in phenol) and appear as **red bacilli** with blue back ground.

Tan Thiam Hook stain use malachite green with methylene blue without heat. Tubercle bacilli appear **green** and others are blue.

Fluorochrome oramin "O" stain is a new technique in which stain attaches to mycolic acid so the tubercle bacilli appear **orange red** against black background.

Culture characters.

_ Mycobacteria are aerobic bacteria. Mammalian types need optimal temperature of 37°C and while avian type needs 40°C. M. marinum and M. ulcerans grow at 30 but not at 37°C.

Few species e.g. M. phlei and M. xenopi grow at of 45°C or more.

_ They are not grow on ordinary media but require specific media containing egg such as

Dorset egg (whole egg), **Lowenstein Jensen** (whole egg + potato + starch + mineral salts), **Petregnani's** medium (whole egg + potato + starch + mineral salts + milk) and **Dubos Oleic** media (whole egg).

Gruft Lowenstein media: Lownestein media modified by Gruft by addition of nalidixic acid, penicillin and malachite green.

_ For MAP; Supplementation of media with mycobactin J is required and the media used for isolation are **Middlebrook 7H 10 and Middlebrook 7H 11 agar media.**

_ Pathogenic Mycobacteria grow slowly; primary cultures usually acquire from 3-8 weeks in human and bovine types and 2-6 weeks in avian types before colonies can be detected by naked eye (in case of MAP, cultures are not discarded as negative before 16w . In contrast, the colonies of rapidly growing saprophytes are visible within days.

o **The human type:** colonies appear luxuriant (eugonic), dry, tough, irregular wrinkled raised appear first brown then brick brown on aging and difficult to break down colonies.

o **The bovine type:** difficult in growth than human type, less luxuriant (dysgonic) moist, granular, non-pigmented flat colony and easily broken up.

o **The avian type:** more luxuriant (is faster in growth; more eugonic), moist, granular, glistening elevated with pigmentation varied from grayish, yellow, or orange.

_ Addition 5-10% glycerin or sodium pyruvate to the media, enhance the growth of human and avian types but have no effect (or may be inhibitory) on bovine type.

Biochemical reactions.

- Mycobacterium tuberculosis inactive (weak enzymatic activity)

- Slight acid formation from glucose, trehalose, maltose and glycerol and dose not ferment lactose (grows in milk without any changes).

- Catalase positive, MR-VP negative.

- Nitrate reduction and niacin production is characteristic for human type.

- Tween hydrolysis is characteristic for MOTT.

Resistance of Mycobacteria.

_ Mycobacteria are highly resistant to the environmental conditions.

_ When protected from heat and light they remain viable in soil for long time.

_ They resist dryness for a long period reach 2-3 weeks.

_ They are destroyed by 5% phenol for 48h.

Typing of Mycobacterium tuberculosis:

Typing based on morphology and cultural characters is not accurate. Typing based on inoculation of lab animals is most accurate, using G. pigs, rabbits, chickens in duplicates

(due to the long time of the experiment 6-8 weeks). These lab animals must be tested before inoculation with avian type and have to be negative.

Site of injection: **G. pigs are injected I/P**, **rabbits are injected I/V** in ear vein and **chickens dose is divided into 3 portions the 1st portion is orally, the 2nd is I/M in the pectoral muscles and the 3rd is S/C.**

- The 1st group of lab animals should be sacrificed after 6 weeks.
- The 2nd group of lab animals should be kept for 8 weeks if the 1st group was negative.

- Mammalian types were differentiated from avian type by G. pig (generalized; miliary tuberculosis highly susceptible to human and bovine types while not susceptible to the avian type) and chicken inoculation (avian type only causes generalized form in chickens.
- The bovine type is differentiated from human type in rabbit as bovine type gives generalized form in rabbit but human type gives localized from in sub maxillary lymph node. s

Lab animal	Bovine type	Human type	Avian type
G. pig	+++ (high susceptible)	+++ (high susceptible)	--- (unsusceptible)
Rabbit	+++	+	+/-
Chickens	---	---	+++

Tuberculosis in cattle

Transmission of M. bovis is mainly through aerosols generated by infected cattle. Calves can become infected by ingesting contaminated milk. The virulence of M. bovis related to its ability to survive and multiply in host macrophages and migration of macrophages containing viable Mycobacteria can disseminate infection.

The complex lipid and waxy composition of the mycobacterial cell wall contributes in association with **tuberculoproteins** to the immunogenicity on which the development of the host responses and the lesions depends.

N.B. Human tuberculosis is associated with chronic cough with sputum. In late stages, bloody sputum,

Laboratory diagnosis of Mycobacteria:

Sample collection: According to the disease and the form. Samples include lymph nodes, tissue lesions, sputum and milk. Aspirates, urine or any body fluids should be centrifuged; the sediment is taken for examination. Feces are suspended, then centrifuged use the sediment for examination. In calcified or caseated lymph nodes, the samples taken from caseated but not from calcified portion.

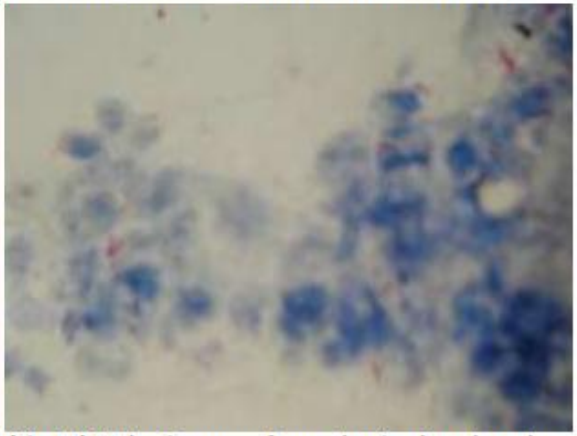
1- Direct microscopical examination:

Smears were prepared from different samples and stained with ZN to show acid fast bacilli. In most cases the direct microscopical examination is not accurate, may give negative results except in cases with open form.

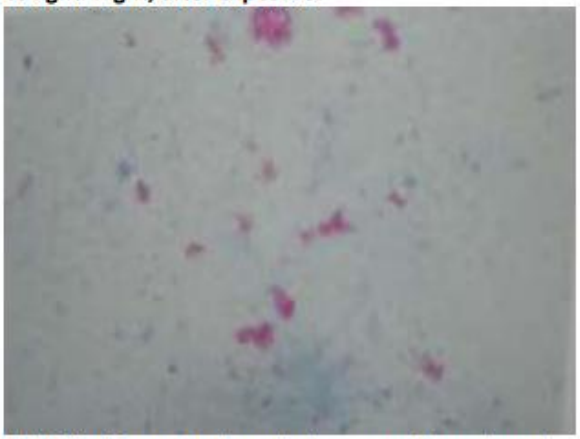
(Fig.23) Mycobacteria, on Hohn's medium. Left, *M. tuberculosis*, eugenic, dry firm. Middle, *M. bovis* dysgonic. Right, *M. avium*, eugenic; moist, waxy.

(Fig.24) M. kansasii, on Löwenstein-Jensen medium showing photochromogenes. Left, before exposure to light. Right, after exposure.

(Fig.25) Atypical Mycobacteria, from right to left: *M. vaccae, M. smegmatis, M. fortuitum* and *M. scrofulaceum*, various incubation times.



(Fig.26) M. bovis, smear from a bovine lymph node, Ziehl-Neelsen stain, red acid-fast rods.



(Fig.27) M. paratuberculosis smear from lymph node of ox, ZN stain, acid-fast rods in groups.

2- Isolation by cultivation:

Before cultivation treatment (purification) of the sample is a necessary

(if the microorganism is scanty or if pure culture is required or in case of presence of contaminants, and dissolving all the cells leaving the intact tubercle bacilli for a certain period of time).

The treatment is done by:

i. Antiformin method:

_ Part of the sputum or suspected material is added to 3-4 its volume antiformin; consisting of equal portions of 15% NaOH & 15% Na chlorinated, (antiformin must be diluted by 1:6 sterile saline solution). _ Left in incubator for one hour till homogenization.

_ Then centrifuged at 3000 rpm for 15 min. then, take the sediment and wash with sterile saline solution. _ Repeat centrifugation and washing twice to get rid of antiformin which may affect the tubercle bacilli.

_ Finally take the sediment and divide into three portions: 1st portion; for direct smears by ZN method.

The 2nd portion; for cultivation on selective or non-selective media.

The 3rd portion; for injection into lab animals for typing of TB.

ii. Petroff's method:

_ Take the sample then ground and add equal volume of 4% NaOH and left for 30 min. then discard NaOH.

_ Add 8-10% HCl for 30 min. then discard the supernatant.

_ Add sterile saline solution, centrifuge at 3000 rpm for 10 min.

_ Take the sediment and adjust the pH of the sediment using pH indicator.

_ Finally this sediment is used in direct smear, isolation and animal inoculation.

iii. By using 5% oxalic acid or 10% trisodium phosphate.

3- Biochemical tests.

4- Animal pathogenicity.

5- Serological identification: ELISA for detecting circulating antibodies.

6- Molecular techniques:

DNA probes, complementary to species-specific sequences of rRNA, are commercially available.

Nucleic acid amplification procedures such as PCR are sensitive and rapid methods.

DNA restriction endonuclease analysis is used in epidemiological studies.

7- Field diagnosis: (Tuberculin test):

The **tuberculin test, based on delayed-type hypersensitivity to mycobacterial tuberculoprotein, is the standard ante mortem test in cattle.** Reactivity in cattle is usually detectable 30-50 days after infection.

Types of tuberculin:

i. Koch's old tuberculin (KOT):

It is prepared by growing mycobacteria in glycerol broth in flat bottom flask for about 10 weeks then steamed for 2 hrs to kill the organism and make extract then filtrate. The filtrate either evaporated to 1/10 its volume to prepare concentrated tuberculin or carbolized with 0.5% phenol to prepare diluted tuberculin.

This preparation has disadvantage of containing a protein of media in addition to the protein of the organism.

ii. New tuberculin “Seibert and Long” (PPD):

It is used to produce an extract of tubercle bacilli, using a synthetic media free from protein. This tuberculin may be prepared in dry powder form which dissolved in borate or phosphate saline just before use.

Mycobacteria are cultivated on synthetic media in flat bottom flask for 8 weeks then steamed for 3 hrs to kill the microorganism and make extract then filtrate and add 1.2% glycerin to its original volume in addition to 0.5% phenol act as preservative then centrifuge. Take the supernatant then add 10% tri-chloro-acetic acid and centrifuge again.

The supernatant is either dispersed in 10ml ampoules or lyophilized in dry state in vials.

iii. K38DA tuberculin: prepared by radiation and mostly used in man.

*** Two main methods of tuberculin testing are employed:

A. Single intradermal (caudal fold) test, 0.1 ml of bovine PPD is injected intradermal into the caudal fold of the tail. The injection site is examined 72 hrs later and a positive reaction is characterized by a hard or oedematous swelling.

B. Comparative intradermal test, 0.1 ml of avian PPD and 0.1 ml of bovine PPD are injected intradermal into separate clipped sites on the side of the neck about 12 cm apart. Skin thickness at the injection sites is measured with calipers before injection of tuberculins and after 72 hrs. An increase in skin thickness at the injection site of bovine PPD which exceeds that at the avian PPD injection site by 4 mm or more is interpreted as evidence of infection and the animal is termed a reactor.

_ Non-specific reactions of tuberculin test:

** False positive test results: Cattle may react positive to tuberculin but fail to show any specific lesions during P.M. examination, this may be due to:

_ Animal may be in early stage of disease lead to reaction of tuberculin without P.M. lesions.

_ The animal may be sensitized to Mycobacteria other than M. bovis as human or avian types.

_ The presence of skin lesions in the form of painless nodules; containing atypical mycobacterium.

_ The animal may be vaccinated.

_ Liver fluke infestation

** False negative test results: may be recorded due to:

_ Cattle tested before delayed-type hypersensitivity to tuberculoproteins develops (at about 30 days post infection) do not react.

_ In some cattle an unresponsive state, referred to as anergy, may accompany advanced tuberculosis. The mechanisms involved are incompletely understood.

_ A transient desensitization may follow injection of tuberculin. Reactivity usually returns within 60 days.

_ Cows may be unresponsive to the tuberculin test during the early post-partum period.

Immunization.

1. BCG (Bacille Callmette et Guerin)

Bovine strain attenuated by 2 methods; by growing on potato glycerin bile media (bile act as antagonistic for tuberculosis) or several passage 99 times. It is used in immunization of young children, young calves 1-15 days before any chance of natural infection.

Disadvantages of BCG are retrogressive lesions which are difficult to differentiate from infection.

2. Raw vaccine: Human type killed by heat but low potency (less immunogenic)

3. Diaplyte vaccine: Human type from which fat has been extracted with formalin.

4. Vole bacillus vaccine: This vole (type of rats) is usually infected with M. murius which when injected in G. pigs it produce immunity against both bovine and human types. The immunization power is similar to BCG but not used up till now.

5. Spahlinger vaccine: Human type grown onto body fluid such as ascitic fluid and left naturally to die. It is of low potency.