



20

MYCOBACTERIUM

20.1 INTRODUCTION

Mycobacterium are slender rods that sometimes show branching filamentous forms resembling fungal mycelium. In liquid cultures they form a mould-like pellicle. Hence the name 'mycobacteria', meaning fungus like bacteria. They do not stain readily, but once stained, resist decolourisation with dilute mineral acids. Hence they are called 'Acid fast bacilli'. They are aerobic, nonmotile, noncapsulated and nonsporing.



OBJECTIVES

After reading this lesson, you will be able to:

- describe the morphology of Mycobacterium tuberculosis & M. leprae
- describe the characteristics of Mycobacterium tuberculosis & M. leprae
- explain about pathogenesis of Mycobacterium tuberculosis & M. leprae
- explain the laboratory diagnosis Mycobacterium tuberculosis & M. leprae

The first member of this genus to be identified was Lepra bacillus discovered by Hansen. Koch (1882) isolated the mammalian tubercle bacillus and proved its causative role in tuberculosis. In humans tuberculosis is caused by mycobacterium tuberculosis and also by bovine type called Mycobacterium bovis.

The second human pathogenic mycobacterium is the lepra bacillus causing Leprosy. The third group of mycobacterium is a mixed group from varied sources like birds, cold-blooded and warm blooded animals, from skin ulcers, soil, water and other environmental sources. They are called as atypical mycobacteria. They are opportunistic pathogens and can cause many types of diseases.

20.2 MYCOBACTERIUM TUBERCULOSIS

Morphology

M. tuberculosis is a straight or slightly curved rod, about 3 X 0.3 μm in size, occurring singly, in pairs or as small clumps. *M. bovis* is usually straighter, shorter and stouter.

Tubercle bacilli have been described as Gram positive, even though after staining with basic dyes they resist decolourisation by alcohol even without the effect of iodine. When stained with carbol fuchsin by Ziehl-Neelsen method or by fluorescent dyes they resist decolorisation by acids such as 20% Sulphuric acid as well as by alcohols. The unsaponifiable wax (mycolic acid) forms a semipermeable membrane around the cell that makes it acid fast.



Notes

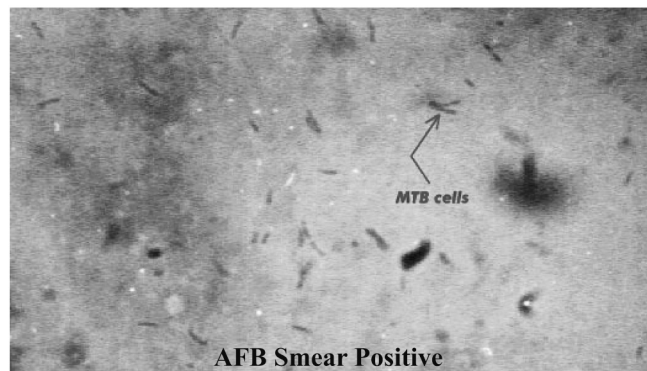


Fig. 20.1: AFB smear

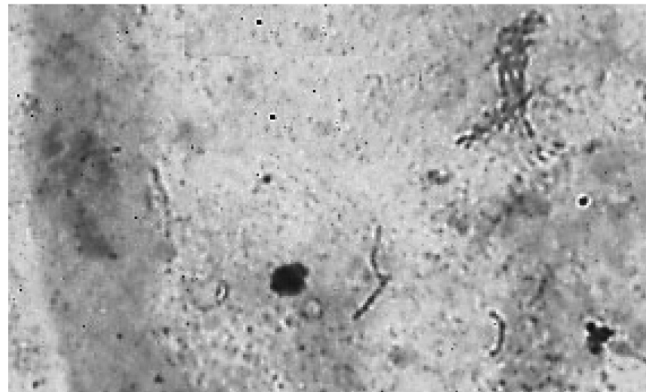


Fig. 20.2: Mycobacterium tuberculosis Acid-Fast stain

Culture characteristics

The bacilli grow and invitro time for generation is 14-15 hours. The optimum temperature is 37°C and growth does not occur below 25°C or above 40°C. Optimum pH is 6.4-7.0. *M. tuberculosis* is an obligate aerobe while *M. bovis* is microaerophilic on primary isolation. *M. tuberculosis* grows luxuriantly in culture as compared to *M. bovis* which grows sparsely.

Mycobacterium

On solid media, dry, rough, raised, irregular colonies with a wrinkled surface are seen and they are creamy white, becoming yellowish coloured on further incubation. *M. bovis* forms flat, smooth, moist, white colonies that break up easily.



INTEXT QUESTIONS 20.1

1. Mycobacteria means
2. Mycobacteria are bacilli
3. Presence of around cells makes it acid fast
4. Mycobacteria tuberculosis is an aerobe

Resistance

Mycobacteria are not heat resistant, being killed at 60°C in 15-20 minutes. Cultures may be killed by exposure to direct sunlight for two hours. But bacilli in spectrum may remain alive for 20-30 hours. Bacilli are relatively resistant to chemical disinfectants, surviving exposure to 5% phenol, 15% sulphuric acid, 3% Nitric acid, 5% oxalic acid and 4% sodium hydroxide. They are sensitive to formaldehyde and glutaraldehyde.

Biochemical reactions

Niacin test: Human tubercle bacilli form niacin when grown on an egg medium. When 10% cyanogens bromide and 4% aniline in 96% ethanol are added to a suspension of the culture, a canary yellow colour indicates a positive reaction. The test is positive with human type and negative with bovine type.

Aryl sulphatase test: This test is positive only with atypical mycobacteria. The bacilli are grown in a medium containing 0.001 M tripotassium phenolphthalein disulphate. 2N NaOH is added drop by drop to the culture and pink colour indicates a positive reaction.

Neutral red test: Virulent strains of tubercle bacilli are able to bind neutral red in alkaline buffer solution.

Catalase-Peroxidase tests: this is used to differentiate tubercle bacilli from atypical mycobacteria. Most atypical mycobacteria strains are catalase positive while tubercle bacilli are weakly positive. Tubercle bacilli are peroxidase positive but not atypical mycobacteria.

MODULE

Microbiology



Notes



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A mixture of equal volumes of 30 volumes of H_2O_2 and 0.2% catechol in distilled water is added to 5 ml of test culture and are allowed to stand for few minutes. Effervescence indicates catalase production and browning indicates peroxidase activity.

Amidase tests: The ability to split amides has been used to differentiate mycobacteria. A 0.00165 M solution of the amide is incubated with the bacillary suspension at 37°C and 0.1 ml $\text{MnSO}_4 \cdot 4 \text{H}_2\text{O}$, 1.0 ml of phenol solution and 0.5 ml hypochlorite solution are added. The tubes are placed in boiling water for 20 minutes. A blue colour indicates a positive test.

Nitrate reduction test: this is positive with *M tuberculosis* and negative with *M bovis*.

Antigenic properties: Antigens have been identified in mycobacteria. Group specificity is due to polysaccharides and type specificity to protein antigens. Delayed hypersensitivity develops following an infection of tubercle bacilli to the bacillary protein. *M tuberculosis* stains are antigenically homogeneous but is not useful in diagnosis or in immunity.

Bacteriophage: Mycobacteriophages have been isolated from soil, water and other environmental sources as well as from lysogenic strains.

There are four phage types A,B,C and an intermediate type between A & B as I, which is common in India.

Molecular typing: DNA fingerprinting provides a method for differentiating between strains of tubercle bacilli. Restriction endonuclease treatment yields nucleic acid fragments of varying lengths and the pattern are strain specific. This restriction fragment length polymorphism (RFLP) is used in strain typing.

Pathogenesis: Open case of pulmonary tuberculosis is the source of infection, which is most common in India. One open case may infect 25 contacts. The mode of infection is by direct inhalation of aerosolized bacilli in droplet nuclei of expectorated sputum. Coughing, sneezing and speaking releases numerous droplets as many as 3000 infectious nuclei per cough. Dried bacilli in dust are much less infectious.

The majority of inhaled bacilli are arrested by natural defenses of the upper respiratory tract and which reaches the lungs are ingested by alveolar macrophages. Number and virulence of the infecting bacilli, host factors including genetic susceptibility, age, immunocompetence, stress, nutrition and coexisting illness influence the outcome of the infection.

Humans have effective defence against the infection as only a tenth of the infected develop active tuberculosis. Cell mediated immunity appears to be

effective, whereas humoral immunity is irrelevant. The key cell is the activated CD4+ helper T cell which develops as Th-1 or Th-2 cells, releasing cytokines such as interferon γ (gamma) interleukins 1 and 2, toxic necrosis factor α (alpha) and others exerting different biological effects. Th-1 dependent cytokines activate macrophages resulting in protective immunity and containment of the infection. Th-2 cytokines induce delayed type hypersensitivity (DTH), tissue destruction and progressive disease.

The essential pathology in tuberculosis is the production in infected tissues of a characteristic lesion the tubercle, this is an avascular granuloma composed of a central zone containing giant cells with or without caseation and a peripheral zone of lymphocytes and fibroblasts.

Tuberculosis may be classified as primary and post primary.

Primary tuberculosis is the initial infection by tubercle bacilli. In endemic countries like India this usually occurs in young children, the bacilli engulfed by alveolar macrophages multiply and give rise to a subpleural focus of tuberculous pneumonia, commonly in upper lobe, the Ghon factor. The hilar lymph nodes are involved. The Ghon focus together with enlarged hilar lymph node constitutes primary complex. This occurs about 3-8 weeks from the time of infection and is associated with the development of tuberculin hypersensitivity. In most of the cases the lesion heals spontaneously in 2-6 months leaving a calcified nodule and a few bacilli may survive and remain latent. In children with impaired immunity or other risk factors they may cause miliary, meningeal or other forms of disseminated tuberculosis.

The post primary type of tuberculosis is due to reactivation of latent infection or exogenous reinfection. It affects mostly in the upper lobes of the lungs, the lesions undergoing necrosis and tissue destruction, leading to cavitation. The necrotic materials are released through airway, to expectoration of latent sputum, which is the main source of infection.



INTEXT QUESTIONS 20.2

1. Niacin test is negative in
2. Aryl sulphatase test is positive in
3. In molecular typing is used in stain typing
4. Mycobacteria gets transmitted by infection
5. Primary complex constitutes of &





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Laboratory Diagnosis: Tuberculosis may be demonstrated in the lesion by microscopy, culture isolation and molecular methods.

Pulmonary Tuberculosis

The sputum is tested for pulmonary tuberculosis. The bacterial shedding in the sputum is abundant in cases with caseation, but scanty in lesions that do not communicate with airways. Sputum is best collected in the morning before any meal. If scanty, a 24-hour sample may be tested and sputum sampling on three days increases the chances of detection. Laryngeal swabs or bronchial washings may be collected and in children gastric lavage can be examined.

Microscopy

Direct or concentration smears of sputum are examined. Sputum microscopy is the most reliable single method in the diagnosis and control of tuberculosis. New slides should be used for smears and should not be reused as acid fast bacilli may not always be removed from slides by cleaning.

Smear should be prepared from the thick purulent part of the sputum. Smears are dried, heat fixed and stained by Ziehl-Neelsen technique. This smear is covered with strong carbol fuchsin and gently heated to steaming for 5-7 minutes, without letting the stain boil and become dry. The slide is then washed with water and decolourised with 20% sulphuric acid till no more stain comes off and then with 95% ethanol for two minutes. Decolourisation may be carried out as a single step with acid alcohol. After washing, the smear is counter stained with Loeffler's methylene blue, 1% picric acid or 0.2% malachite green for one minute. Under the oil immersion objective, acid fast bacilli are seen as bright red rods while the background is blue, yellow or green depending on the counter stain used. Atleast 10,000 acid fast bacilli should be present per ml of sputum for them to be readily demonstrable in direct smears. A negative report should not be given till at least 300 fields have been examined, taking about 10 minutes. A positive report can be given only if two or more typical bacilli have been seen. Smears are seen depending on the number of bacilli seen.

No. of AFB	Seen in (oil immersion field)	Report
0	300 F	AFB not seen
1-2	300 F	Doubtful, repeat smear
1-9	100 F	1 +
1-9	10 F	2 +
1-9	1 F	3 +
10 or more	1 F	4 +

When several smears are to be examined daily, fluorescent microscopy is used. Smears are stained with auramine phenol or auramine rhodamine fluorescent dyes and when examined under ultraviolet illumination, the bacilli will appear as bright rods against a dark background.

Concentration methods

(i) Petroff's method

This method is widely used. Sputum is incubated with an equal volume of 4% sodium hydroxide solution at 37°C with frequent shaking till it becomes clear. It is then centrifuged at 3000 rpm for 20 minutes and the sediment neutralized with N/10 HCl and used for smear, culture and animal inoculation.

A simpler method like, treating the sputum with an approximately equal volume of a sterile solution containing 20 g cetrimonium bromide and 40 g of NaOH per litre of distilled water. The contents are mixed with cotton swab and left to stand for five minutes. About 0.2 ml of the inoculum is smeared firmly with the swab over the entire surface of acid buffered medium.

Culture

Culture is a very sensitive diagnostic technique for tubercle bacilli, detecting as few as 10 to 100 bacilli per ml. The concentrated material is inoculated into atleast two bottles of IUAT-LJ medium. If the specimen is positive by microscopy a direct drug sensitivity test may be done. Cultures are examined for growth after incubation at 37°C for four days, for rapid growing mycobacteria, fungi and contaminant bacteria and atleast twice weekly thereafter. A negative report is given if no growth occurs after 8-12 weeks. Any growth seen is smeared and tested by Ziehl Neelsen staining. For routine purposes, a slow growing, non pigmented, niacin positive acid fast bacillus is taken as *M.tuberculosis*. Confirmation is by biochemical studies

Sensitivity tests

As drug resistance is an important problem in tuberculosis it is desirable to have sensitivity of isolates tested as an aid to treatment and they are of three types. The first is absolute concentration method in which a number of media containing serial concentration of the drugs are inoculated and the minimum inhibitory concentrations calculated

The second is resistance ratio method in which two sets of media containing graded concentrations of the drugs are inoculated. One set with the test strain and other with a standard strain of known sensitivity

The third is proportion method which indicates average sensitivity of the strain.

**Notes**



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Allergic test – Mantoux test

0.1ml of Purified Protein Derivative (PPD) containing 5 TU is injected intradermally on the forearm with a tuberculin syringe causing a wheal. The injection should not be given subcutaneously but in between the layers of the skin, intradermally. The site is examined 48-72 hrs later and induration measured at its widest point transversely. Induration of diameter 10mm or more is considered positive, 5mm or less is considered negative and 6-9mm equivocal.

A positive tuberculin test indicated hypersensitivity to tuberculo-protein denoting infection with tubercle bacilli or BCG immunization, recent or past with or without clinical disease

**INTEXT QUESTIONS 20.3**

1. is the most reliable single method in the diagnosis of tuberculosis
2. technique is used in demonstration of tubercle bacilli
3. is the common concentration method in diagnosis of tubercle bacilli
4., & are the common sensitivity tests in the diagnosis of tubercle bacilli
5. is the allergic test used in the diagnosis of tuberculosis
6. is injected in allergic test

20.3 MYCOBACTERIUM LEPRAE

Leprosy is a disease recognized since vedic times in India. The person suffering with leprosy is considered 'unclean' and a social outcast. The lepra bacillus was first observed by Hansen in 1868 and hence it is also called as Hansen's disease.

Morphology

M leprae is a straight or slightly curved rod, 1-8 X 0.2-0.5 μm in size, showing considerable morphological variation. It is Gram positive and stains more readily than tubercle bacillus. It is acid fast, but less so than tubercle bacillus. Hence 5% sulphuric acid instead of 20% is used for a decolourisation after staining with carbol fuchsin. In stained smears, live bacilli appear solid and uniformly stained, while the dead bacilli are fragmented and granular.

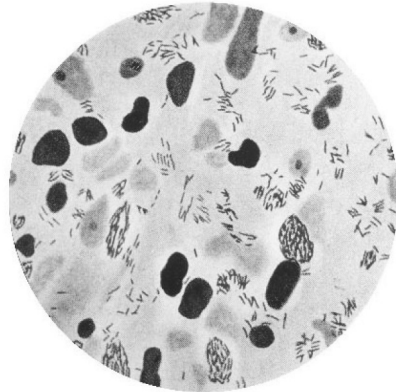


Fig. 20.3

The bacilli are seen, singly and in groups, intracellularly or lying free outside the cells. Mostly they appear as agglomerates, the bacteria being bound together by a lipid-like substance known as 'globi'.

Resistance:

Leptra bacilli have been found to remain viable in a warm humid environment for 9-16 days and in moist soil for 46 days. They survive exposure to direct sunlight for 2 hours and ultraviolet light for 30 minutes.



INTEXT QUESTIONS 20.4

1. Leprosy is caused by
2. Leprosy is also called as disease
3. Bacteria being bound with a lipid like substance known as
4. Leprosy is a chronic disease of humans

Leprosy

Leprosy is a chronic granulomatous disease of humans primarily involving the skin, peripheral nerves and nasal mucosa but capable of affecting any tissue or organ.

The disease may be classified into four types namely Lepromatous, tuberculoid, dimorphous and indeterminate.

Lepromatous type is seen where the host resistance is low. The bacilli are seen in large numbers or as globi inside lepra cells or extracellularly. This is known as 'multibacillary disease'. Superficial nodular lesions (lepromata) develop which consist of granulation tissue containing a dense collection of vacuolated cells in different stages of development from mononuclear cells to lepra cells. The



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nodules ulcerate, become secondarily infected and cause distortion and mutilation. Bacilli invade the mucosa of the nose, mouth and upper respiratory tract and are shed in large numbers in nasal and oral secretions. Cell mediated immunity is deficient and the lepromin test is negative. Lepromatous type is more infective than the other types.

Tuberculoid leprosy is seen in patients with high degree of resistance. The skin lesions are few and sharply demarcated, consisting of macular anesthetic patches. Neural involvement occurs early leading to deformities of hand and feet. Bacilli are scanty in the lesions and infectivity is minimal and this is known as 'paucibacillary disease'. Cell mediated immunity is adequate and the lepromin test is positive.

Borderline or dimorphous type refers to lesions possessing characteristics of both tuberculoid and lepromatous types. It may shift to the lepromatous or tuberculoid part of the spectrum depending on chemotherapy or alterations in host resistance.

The indeterminate type is the early unstable tissue reaction which is not characteristic of either the lepromatous or tuberculoid type.



INTEXT QUESTIONS 20.5

1. Bacilli seen in large number is known as disease
2. is more infective than other types
3. Neural involvement develops early in leprosy
4. Tuberculoid leprosy is also known as

Lepromin test

Lepromin test first described by Mitsuda, is a skin test for delayed hypersensitivity. The response to the intradermal injection of lepromin is typically biphasic, consisting of two separate events. The first is the early reaction consists of erythema and induration developing in 24-48 hours and usually remaining for 3-5 days. The second and more meaningful is the late reaction starting in 1-2 weeks, reaching a peak in four weeks and gradually subsiding in the next few weeks. The late reaction is a indication to measure cell mediated immunity induced by injected lepromin.

The lepromin test is not used to diagnose leprosy, nor does it indicate prior contact with lepra bacillus. The test is used for following purposes:

- To classify the lesions of leprosy patients. The lepromin test is positive in tuberculoid, negative in lepromatous and variable in dimorphous and indeterminate types of disease.

Mycobacterium

- To assess the prognosis and response to treatment. A positive reaction indicates good prognosis and a negative reaction indicates bad prognosis. Conversion to lepromin positivity during treatment is evidence of improvement
- To assess the resistance of individual to leprosy. It is desirable to recruit only lepromin positive persons for work in leprosaria as Lepromin-negative persons are more prone to develop the disease

Laboratory diagnosis

Bacteriological diagnosis is easy in the lepromatous but difficult in tuberculoid cases. The diagnosis is of demonstration of acid fast bacilli in the lesions. Specimens are collected from the nasal mucosa, skin lesions and ear lobules. A blunt narrow scalpel is introduced into the nose and intestinal septum scraped sufficiently to remove a piece of mucosa membrane, which is transferred to a slide and teased out into a uniform smear. Skin is pinched up tight to minimize bleeding and a cut about 5mm with scalpel. Blood or lymph, is wiped and the blade is turned transversely to scrape the slides and bottom of the cut so as to obtain a little tissue pulp which is uniformly smeared on the slide. About 5-6 different areas of the skins should be sampled, including the skin over buttocks, forehead, chin, cheek and ears. The smears are stained using Ziehi-Neelsen technique using 5% instead of 20% sulphuric acid for decolourisation

Smears are graded based on the number of bacilli

1-10 bacilli in 100 fields	1+
1-10 bacilli in 10 fields	2+
1-10 bacilli per field	3+
10-100 bacilli per field	4+
100-1000 bacilli per field	5+
More than 1000 bacilli clumps	6+

The bacteriological Index (BI) is calculated by totaling the number of +s scored in the smears and divided by the number of smears. Thus, if eight smears examined have a total of sixteen pluses, the BI will be 2. For calculating this a minimum of four skin lesions, a nasal swab and both the ear lobes have to be examined

Detection of antibody against M.Leprae phenolic glycolipid antigen has been claimed to be a specific diagnostic test. Microscopic demonstration of lepra bacilli and histology remain the most useful diagnostic procedures

MODULE

Microbiology



Notes



Notes



INTEXT QUESTIONS 20.6

1. is the skin test used for demonstration of delayed hypersensitivity
2. Bacteriological diagnosis is easy in cases
3. is used in demonstration of M.leprae bacilli
4. Specific diagnostic test in diagnosis of M.leprae is detection of



WHAT YOU HAVE LEARNT

- Mycobacteria tuberculosis is an obligatory aerobic, nonmotile, nonsporing, rod shaped bacterium which stains poorly by the Gram stain because its cell wall contains abundant amount of lipids. It retains Carbol Fuchsin dye during attempted decolourisation with acid and alcohol in Ziehl-Neelsen staining technique. M.tuberculosis is acid and alcohol fast by ZN staining method. It grows very slowly, taking several weeks to form a visible colony on enriched culture media.
- M. leprae is an obligate intracellular organism that gains access to skin and peripheral nerve tissue. Least severe form is tuberculoid (TT) and the most severe form is lepromatous



TERMINAL QUESTIONS

1. Describe the Laboratory Diagnosis of mycobacteria Tuberculosis and Leprae.
2. Explain Lepromin and Mantoux test.



ANSWERS TO INTEXT QUESTIONS

20.1

1. Fungus like bacteria
2. Acid fast bacilli
3. Mycolic acid
4. Obligate

20.2

1. M. bovine
2. Atypical mycobacteria
3. Restriction Fragment Length Polymorphism (RFLP)
4. Droplet
5. Ghon focus & hilar lymph nodes

20.3

1. Sputum microscopy
2. Ziehl-Neelson
3. Petroff's method
4. Absolute Concentration method, Resistance ratio method & Propotion method
5. Mantoux test
6. Purified Protein Derivative (PPD)

20.4

1. Mycobacterium leprae
2. Hansen's
3. Globi
4. Granulomatous

20.5

1. Multibacillary
2. Lepromatous type
3. Tuberculoid
4. Paucibacillary disease

20.6

1. Lepromin test
2. Lepromatous
3. Ziehl-Neelson technique
4. Antibodies



Notes