# 38 Mycobacterium I: M.tuberculosis



## INTRODUCTION

Mycobacteria 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, due to the presence of mycolic acid in their cell wall. They are called acid fast bacilli (AFB). Mycobacteria are slow-growing, aerobic, non-motile, non-capsulated and non-sporing.

The genus *Mycobacteria* contains three groups: **obligate parasites**, **opportunistic pathogens** and **saprophytes**.

### Obligate parasites

***Mycobacterium tuberculosis* complex**: Koch (1882) isolated the mammalian tubercle bacillus and proved its causative role in tuberculosis by satisfying certain basic principles of infectious agents (known as **Koch's postulates**). Tuberculosis in humans was subsequently shown to be caused by two types of the bacillus: the human and bovine types, designated *Mycobacterium tuberculosis* and *M.bovis*, respectively. *M.tuberculosis* complex includes two other mammalian types: *M.africanum*, causing human tuberculosis in tropical Africa; and *M.microti*, causing disease in voles and other small mammals. Recently, three more species have been added: *M.canetti* (similar to *M.africanum*), *M.caprae* (another cattle pathogen) and *M.pinnipedii* (a pathogen of seals).

***Mycobacterium tuberculosis***

**Clinical Case 1** A 50-year-old man presented with a history of low-grade fever with an evening rise in temperature and productive cough for the previous two months. He sought medical advice, since he had started coughing blood-tinged sputum for the past three days. His history revealed loss of appetite and a weight loss of 10 kgs over the previous four months. A chest x-ray revealed a nodular infiltrate in the apical area of the right upper lobe. The sputum smear was positive for AFB and the culture grew *M.tuberculosis*. He was started on DOTS therapy.

**Clinical Case 2** A 12-year-old boy complained of low-grade fever and mild headache for the previous two weeks, which worsened over the last two days. He also had vomiting, confusion and stiffness in the neck, for which his parents sought medical attention. History revealed that his father was a known case of pulmonary TB but had defaulted on treatment. CSF examination showed a mild increase in cell counts with predominant lymphocytes. Proteins were raised and glucose was low. ZN or Gram stain of CSF did not reveal any organisms. PCR assay of the CSF targeting the unique sequence of *M. tuberculosis* was positive. Two weeks later, the culture by automated system was also positive for this organism. A drug sensitivity test showed that the strain was resistant to rifampicin and INH. He was treated with second-line drugs, Ofloxacin, PAS Cycloserine and Ethionamide.

***Mycobacterium leprae***: The second human pathogenic mycobacterium is the lepra bacillus causing leprosy discovered by Hansen in 1868. Though described first, its properties are poorly understood due to it being non-cultivable in vitro.

## Opportunistic pathogens

**Non-tuberculous mycobacteria (NTM)**: This is a mixed group of mycobacteria from diverse sources: birds, cold-blooded and warm-blooded animals, from skin ulcers, and from soil, water and other environmental sources. They are broadly categorised as photochromogens, scotochromogens, non-photochromogens and rapid growers, based on their growth rates and pigmentation in the presence or absence of light. They are opportunistic pathogens and can cause many types of disease especially in immunocompromised individuals.

## Saprophytes

**Saprophytic mycobacteria:** These were isolated from a number of sources and include *M.phlei* from grass and *M.smegmatis* from smegma. *M.smegmatis* (seldom found in smegma, along with other rapidly growing mycobacteria) frequently contaminate urine cultures.

# MYCOBACTERIUM TUBERCULOSIS

## Morphology

*M.tuberculosis* is a straight or slightly curved rod, about 3 μm × 0.3 μm in size, occurring singly, in pairs or as small clumps. The size depends on conditions of growth. Long, filamentous, club-shaped and branching forms may sometimes be seen. *M.bovis* is usually straighter, shorter and stouter.

### Acid fast staining

They differ in their staining property from other bacteria. When stained with carbol fuchsin by the Ziehl–Neelsen method or by fluorescent dyes (auramine O, rhodamine), they resist decolourisation by 20% sulphuric acid and are therefore called acid fast. Acid fastness has been ascribed to the presence of an unsaponifiable lipid-rich (mycolic acid) wax material in the cell wall or to a semipermeable membrane around the cell.

Staining may be uniform or granular. Beaded or barred forms are frequently seen in *M.tuberculosis*, but *M.bovis* stains more uniformly.

Electron micrographs of thin sections show a thick cell wall composed of three layers enclosing a trilaminar plasma membrane. Spheroplasts and L forms are formed when grown in the presence of lysozymes.

*M.tuberculosis* is also **alcohol fast** (resists decolourisation with 3% hydrochloric acid in 95% alcohol), which differentiates it from saprophytic mycobacteria which are only acid fast.

## Cultural characteristics

The organism is a slow grower with generation time of 14–15 hours. Colonies appear between two weeks to eight weeks. Optimum temperature is 37°C. Temperatures below 25°C or above 40°C do not favour growth. Optimum pH is 6.4–7.0. *M.tuberculosis* is an obligate aerobe (Table 38.1).

Tubercle bacilli are highly susceptible to traces of toxic substances like fatty acids in culture media. The toxicity is neutralised by serum albumin or charcoal.

Several media, both solid and liquid, have been described for the cultivation of tubercle bacilli:

### Solid media

**Lowenstein–Jensen (LJ)** is the most widely employed media for routine culture. This consists of coagulated hen's eggs, mineral salt solution, asparagine and malachite green, the last acting as a selective agent inhibiting other bacteria (Fig. 38.1).

Other solid media used are those containing egg **(Petragnini, Dorset)**, blood **(Tarshis)**, serum **(Loeffler)** or potato **(Pawlowsky)**.

### Liquid media

Among the several liquid media described, **Dubos', Middlebrook's, Proskauer and Beck's, Sula's and Sauton's** are more common. Diffuse growth is

Table 38.1 *Some common mycobacteria and their habitats*

| | | |
|---|---|---|
| **Obligate parasites** | *M.tuberculosis, M.bovis, M.leprae* | Species always considered pathogens. |
| **Opportunistic pathogens** | *M.scrofulaceum, M.kansasii, M.marinum, M.ulcerans, M.avium*-intercelluar complex (MAC) | Uncommon causes of human disease. Infect immunocompromised individuals |
| **Saprophytes** | *M.smegmatis, M.gordonae, M.flavescens* | Found in soil and water. Produce environmental contamination. |


Fig. 38.1 *M.tuberculosis* on LJ medium (left); LJ medium without growth (right)

obtained in Dubos' medium containing **Tween-80** (sorbitan monooleate). Virulent strains tend to form long serpentine cords in liquid media, while avirulent strains grow in a more dispersed manner. In automated culture systems, liquid media is used.

### Newer methods of cultivation

Several **automated culture methods** have been introduced to detect early growth.

- **BACTEC 460**: This method uses radioistopes to detect growth. It is not preferred presently due to the use of radioisotopes.
- **BacT Alert**: This method uses colorimetric method of growth detection, due to production of $CO_2$ as a result of bacterial metabolism during growth.

**BACTEC MGIT**: This is an automated **mycobacteria growth indicator tube (MGIT)**. It is a rapid growth detection method, which uses 7H9 Middlebrook medium with fluorometric detection technology via $O_2$ consumption. An added advantage is that the incorporation of Pyrazinamide (PZA) in the medium detects resistance to this drug.

- **ESP system**: This is a continuous monitoring system for detecting growth of mycobacteria. It detects the pressure changes above the level of the medium either due to gas consumption or gas liberation resulting from bacterial growth. Recently this has been improved to detect drug susceptibility to mycobacteria.

## Resistance

Mycobacteria are killed at 60°C in 15–20 minutes. Bacilli in sputum may be viable for 20–30 hours and in droplet nuclei up to 8–10 days under suitable conditions. Cultures remain viable at room temperature for 6–8 months and may be stored for up to two years at –20°C.

**Susceptibility of mycobacteria to commonly used disinfectants in the hospital**

Mycobacteria can survive exposure to 5% phenol, 15% sulphuric acid, 3% nitric acid, 5% oxalic acid and 4% sodium hydroxide. They are killed by formaldehyde and glutaraldehyde. They are destroyed by tincture of iodine in five minutes and by 80% ethanol in 2–10 minutes. Ethanol is a suitable disinfectant for skin, gloves and clinical thermometers.

## Biochemical reactions

Several biochemical tests have been described for the identification of the mycobacterial species:

**Niacin test:** Human tubercle bacilli form niacin when grown on an egg medium. When 10% cyanogen bromide and 4% aniline in 96% ethanol are added to a suspension of the culture, a canary-yellow colour indicates a positive reaction. This test differentiates *M.tuberculosis* (positive) from *M.bovis* (negative).

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

**Catalase–peroxidase tests:** These help in differentiating tubercle bacilli from atypical mycobacteria and indicate sensitivity of the strain to isoniazid. Tubercle bacilli are only weakly positive for catalase and strongly for peroxidase. Catalase and peroxidase activities are lost when the tubercle bacilli become INH-resistant. A mixture of equal volumes of 30 vol. $H_2O_2$ and 0.2% catechol in distilled water is added to 5 ml of the test culture and allowed to stand for a few minutes. Effervescence indicates catalase production and browning indicates peroxidase activity.

**Nitrate reduction test**

This is positive with *M.tuberculosis* and negative with *M.bovis*. Addition of sulphanilamide and n-naphthylethylene to the suspension of bacteria in a nitrate

medium changes the colour into red; nitrate is interpreted as positive (Fig. 38.2).

### Amidase tests

The ability to split amides namely, acetamide, benzamide, carbamide, nicotinamide and pyrazinamide, helps to differentiate mycobacteria.

### Pyrazinamidase test

The enzyme pyrazinamidase hydrolyses pyrazinamide to ammonia and pyrazinoic acid which is detected by adding ferric ammonium sulphate. This is positive in *M.tuberculosis* and negative in *M.bovis*.

### Inhibition by thiophene-2 carboxylic acid (T2H)

This is also used to differentiate *M.tuberculosis* from *M.bovis*, the former being resistant. The organism is resistant if growth on T2H medium is >1% of the growth in control (Table 38.2).

### Neutral red test

Virulent strains of tubercle bacilli can bind neutral red in an alkaline buffer solution, while avirulent strains cannot.

### Tween-80 hydrolysis

A positive test is indicated by a change in the colour of the medium, from yellow to red at pH 7. It is useful in differentiating non-tuberculous mycobacteria (NTM).

## Antigenic properties

Several antigens have been identified in the mycobacterial cell wall and the cytoplasm. The cell wall contains

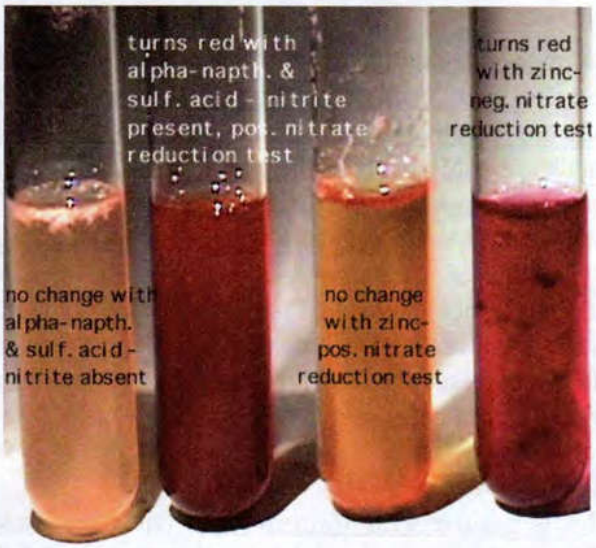


Fig. 38.2 Nitrate test

Table 38.2 *Characteristics differentiating* M.tuberculosis *and* M.bovis

| Test | *M.tuberculosis* | *M.bovis* |
|---|---|---|
| Niacin | + | – |
| Nitrate | + | – |
| Oxygen preference | Aerobic | Microaerophilic |
| Growth in T2H | + | – |
| Host | Human | Bovine |
| Culture | Eugonic | Dysgonic |
| 0.5% glycerol | Helps growth | No effect |
| Sodium pyruvate | Helps growth | Helps growth |
| P-nitrogenic acid | No growth | No effect |
| Colony character | Dry, rough, raised, irregular, wrinkled surface; Not emulsifiable | Flat, smooth, moist, white; Break up easily |
| Tween hydrolysis | ± | – |
| Pyrazinamidase 4 days | + | – |

three major antigens which are responsible for virulence of the organism:

- **Lipids:** The mycobacterial cell wall is rich in long-chain fatty acids called mycolic acid. Mycolic acids play a role in pathogenesis and, when complexed with peptidoglycan, are responsible for granuloma formation. The pattern of lipids on gas chromatography has been used to classify different species. Another factor called cord factor is also responsible for the virulence of bacteria.
- **Proteins:** These induce delayed type hypersensitivity and elicit tuberculin reaction. There is also some antigenic relationship between lepra and tubercle bacilli.
- **Polysaccharides:** Group specificity is due to polysaccharides. Their role in pathogenesis is not clear but they can induce immediate type of hypersensitivity.

Antibodies against the polysaccharide, protein and phosphatide antigens of tubercle bacilli have been demonstrated in the sera of patients. They have no protective or diagnostic relevance.

## Typing methods

They are used to define geographically the routes of transmission and dissemination in the environment and the source of infection in man and animals.

### Molecular typing

**Most typing methods currently are based on** DNA fingerprinting, which is a powerful epidemiological

tool for differentiating between strains of tubercle bacilli.

- **IS6110 Restriction Fragment Length Polymorphism (RFLP) typing:** The IS6110 is a target sequence in several methods currently used for molecular typing of *M. tuberculosis*. Restriction endonuclease treatment yields nucleic acid fragments of varying lengths, the patterns of which are strain-specific and can be used as fingerprinting (IS6110-based PCR Finger printing).
- **Spoligotyping (spacer oligotyping):** This is based on polymorphism in the direct repeat (DR) locus. This is the region present in all MTBC in a unique locus which contains well-conserved sequences. This is more useful in strains that have no or very few copies of IS6110. A great advantage of this method is its ability as a typing tool in non-viable cultures, AFB slides, and paraffin embedded tissues.
- **Insertion-sequence-based typing of non-tubercular mycobacteria (IS-based typing of NTM):** This method is based on differences in the insertion sequences between strains of avium complex. It can also be useful in typing *M.ulcerance*, *M. gordonae*, etc.

### Phenotypic methods

These were used earlier for epidemiological studies in determining strain relatedness. They have low discriminatory power, hence, are no longer used.

- **Bacteriophage typing:** Tubercle bacilli have been classified into four phage types: A, B, C and a type intermediate between A and B, designated I (for 'intermediate').
- **Bacteriocin typing:** *M.tuberculosis* can be typed by means of bacteriocins produced by rapidly growing mycobacteria.

## Host range

*M.tuberculosis* causes natural infection in humans, other primates, dogs and other animals which have close contact with humans. Experimentally, guinea pigs and hamsters are highly susceptible to the infection (mice are only moderately susceptible), and the infection develops progressively following intraperitoneal, intravenous or intracerebral inoculation. Extrapulmonary isolates are less virulent.

*M.bovis* produces tuberculosis in cattle, humans, other primates, carnivores including dogs and cats, badgers, swine, parrots and some birds of prey. Experimentally, guinea pigs are highly susceptible to this pathogen while rats are moderately susceptible. BCG, the tuberculous vaccine, is an attenuated strain of *M.bovis*.

## TUBERCULOSIS

**Tuberculosis is a potentially fatal infection, caused mainly by *M.tuberculosis* complex (MTC), that can affect any part of the body, with lungs being the most common organ involved.**

**Source:** The source of infection is usually an open case of pulmonary tuberculosis. It is estimated that an open case of tuberculosis in India may infect, on an average, 25 contacts before death or cure. The mode of infection is by direct inhalation of aerosolised bacilli contained in the droplet nuclei of expectorated sputum. Coughing, sneezing and speaking release numerous droplets—as many as 3000 infectious nuclei per cough. Dried bacilli in dust are much less infectious. The disease spreads most often among household or close and prolonged contacts of open cases (whose sputum may contain minimum 10,000 bacilli per ml). Infection also occurs infrequently by ingestion, for example, through infected milk, and rarely by inoculation.

The inhaled bacilli are arrested by the natural defences of the upper respiratory tract. Those that escape reach the lungs and are phagocytosed by the alveolar macrophages. Several factors including the number and virulence of the infecting bacilli, host factors including genetic susceptibility, age, immunocompetence, stress, nutrition and co-existing illness influence the outcome of the infection.

**Immunology:** Various components of the bacillus have been shown to possess different biological activities which may influence pathogenesis, allergy and immunity in the infection. Humans are evidently able to mount an effective defence against the infection as only about a tenth of those infected develop active tuberculosis. **Cell-mediated immunity** is the specific immune mechanism that plays a major role in tuberculosis. Humoral immunity has little or no role in protection or pathogenesis. The key cell is the activated CD4+ helper T cell which can develop along two different paths: the Th-1 and Th-2 cells, releasing cytokines such as interferon γ (gamma) interleukins 1 and 2, toxic effects of tumor necrosis factor α (TNF α) 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.

**Allergy and immunity:** Infection with the tubercle bacillus induces cell-mediated immunity which manifests as delayed hypersensitivity (allergy) and resistance to infection (immunity). The resultant of these two processes determines the course of the infection. Allergy can be induced by infection with virulent as well as avirulent tubercle bacilli.

**Koch's phenomenon**
This is of historical interest. Robert Koch demonstrated that when virulent tubercle bacilli are injected into a healthy guinea pig, it develops a nodule at the site of inoculation, which develops into an ulcer that persists, with caseation of draining lymph nodes. On the other hand, if the bacilli is injected into an already infected guinea pig (infected 4–6 weeks earlier), the lesion develops rapidly within one or two days, into a shallow ulcer which heals completely without involving the draining lymph nodes.

**Tuberculin tests:** This was originally prepared by Robert Koch, and is known as **Old Tuberculin (OT)**. Seibert modified it to produce the **purified protein derivative (PPD)**. Subsequently, highly purified preparations of PPD have since been developed which are currently in use.

**Pathology:** The essential pathology in tuberculosis is the production of a characteristic lesion, the **tubercle**, in infected tissues (Fig. 38.3). 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. Tuberculous lesions are primarily of two types: exudative and productive.

- The **exudative type** is an acute inflammatory reaction with accumulation of edema fluid, polymorphonuclear leucocytes, and later of lymphocytes and mononuclear cells. This is typically seen when there are plenty of virulent bacilli and the host response is DTH than of protective immunity.
- The **productive type** of lesion is predominantly cellular, associated with protective immunity.

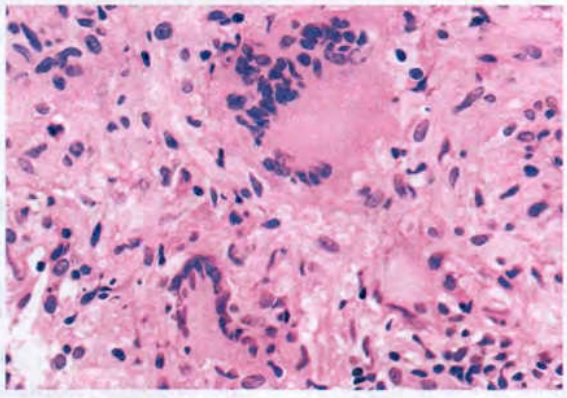

Fig. 38.3 Tubercle or granuloma with caseation

## Classification

Depending on the time of infection and the type of response, tuberculosis may be classified as primary and post-primary.

- **Primary tuberculosis** is the initial infection by the mycobacteria in a host. In endemic countries like India, young children usually are more susceptible. Alveolar macrophages engulf the bacilli which multiply intracellularly. They give rise to a subpleural focus of tuberculous pneumonia, commonly located in the lower lobe or the lower part of the upper lobe (**Ghon focus**). The hilar lymph nodes are involved. The Ghon focus together with the enlarged hilar lymph node constitutes the **primary complex**. This occurs about 3–8 weeks from the time of infection and is associated with the development of tuberculin hypersensitivity. In most cases, the lesion heals spontaneously in 2–6 months, leaving behind a calcified nodule. However, a few bacilli may survive in the healed lesion and remain latent. In a few, particularly in children with impaired immunity or other risk factors, the primary lesion may enlarge and cause miliary, meningeal or other forms of disseminated tuberculosis (*Case* 2).
- **Post-primary (secondary or adult) tuberculosis** is due to reactivation of latent infection (post-primary progression, endogenous reactivation) or exogenous re-infection and differs from the primary type in many respects. It affects mainly the upper lobes of the lungs, the lesions undergoing necrosis and tissue destruction, leading to cavitation. Lymph node involvement is unusual. The necrotic materials break out into the airways, leading to expectoration of bacteria-laden sputum, which is the main source of infection to contacts. In the immunodeficient, cavity formation is unusual. Instead, there is widespread dissemination of lesions in the lungs and other organs (*Case* 1).

## Epidemiology

Tuberculosis is an ancient disease. It is estimated that a third of the world's population (two billion), is infected with the tubercle bacilli. Every year, between eight and nine million new cases of tuberculosis appear, and three

million persons die from the disease. The large majority of the cases and deaths are from the poor nations. India is one of the worst affected countries. More than 40 per cent of the population is infected and around 15 million suffer from tuberculosis. Over three million of these are highly infectious, open cases. Half a million people die from the disease every year in India—one every minute.

**Factors of spread:**

- **Poverty** and tuberculosis go hand in hand. Tuberculosis has declined rapidly in the affluent nations due to improvement in the standard of living, but continues unabated in the poorer countries.
- Currently, with the AIDS pandemic, tuberculosis has become a problem for developed nations as well, with outbreaks among the HIV-infected individuals. **A close relationship has emerged between tuberculosis and HIV.** Not only does HIV infection reactivate latent tuberculosis but it also makes the disease more serious and renders treatment ineffective. Tuberculosis may, in turn, hasten the development of HIV infection into active disease.
- A third complication that has made the situation more grave is the emergence and spread of **multiple drug resistance** among tubercle bacilli. So serious is the global threat of tuberculosis combined with multidrug resistance and concomitant HIV infection that the World Health Organization in 1993 declared tuberculosis a global emergency.

Human infection with *M.bovis* used to be common in the early part of this century before pasteurisation of milk was widely practised. In many developed countries, such as in the UK, it has been almost eliminated by its control in cattle. The infection spreads to animals through aerosolised bacilli in moist cough sprays. An infected cow sheds the bacilli in milk, which is infectious to humans when consumed raw. The primary infection, mostly in children, would occur in the cervical and mesenteric lymph nodes, from where it could spread to the bone and joints and other extrapulmonary sites. Human infection with *M.bovis* is prevented by drinking only pasteurised or boiled milk. Person-to-person transmission of *M.bovis* is very rare.

## Laboratory diagnosis

A person is diagnosed as having tuberculosis by any one or more of the following diagnostic tests:

- Demonstrating the bacilli in the lesion, by microscopy
- Isolating the bacilli in culture
- Using molecular diagnostic methods to detect DNA or RNA of the bacilli from clinical specimen
- Demonstrating hypersensitivity to tuberculoprotein
- Animal experiment: This involves transmitting the infection to experimental animals.

The specimen collected would depend on the site of the lesion, whether pulmonary or extrapulmonary.

## Pulmonary tuberculosis

### Specimen and collection

- **Sputum**: Bacillary shedding in the sputum is abundant in caseation, but relatively scanty in organised lesions that do not communicate with airways. Sputum is best collected in the morning before any meal. If sputum is scanty, a 24-hour sample may be tested. Sputum sampling on three days increases the chances of detection.
- Where sputum is not available, **laryngeal aspirates** or **bronchial washings** may be collected.
- In small children who tend to swallow the sputum, **gastric lavage** can be examined.

Direct sputum sample smears maybe prepared from the thick part of the sputum in the peripheral laboratories and stained.

### Decontamination and concentration of specimens

Specimens from non-sterile sites and sputum need prior treatment so that microorganisms other than mycobacteria may not overgrow during prolonged incubation. Also, the sputum samples contain an organic matrix which may trap mycobacterial cells. Therefore, liquefaction, decontamination and concentration improve the yield. Concentration methods that do not kill the bacilli and that can be used for culture and animal inoculation have been described; these are used for the homogenisation and concentration of sputum and other specimens. All such methods should be done in Class II biosafety cabinets.

- **Petroff's method:** This simple 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, on an average for 20 minutes. It is then centrifuged at 3000 rpm for 20 minutes and the sediment neutralised with N/10 HCl and used for smear, culture and animal inoculation. Excessive exposure to alkali is deleterious and should be avoided.
- **NALC (N acetyl cysteine) combined with 2% NaOH:** This method is considered better than

Petroff's. Here, **N acetyl cysteine** is used for liquefaction of sputum. NaOH kills the contaminating bacteria. The sample is then neutralised with buffer and concentrated by centrifugation. This method is also compatible with culture in automated systems.

### Microscopy

Sputum microscopy is the most reliable single method in the diagnosis and control of tuberculosis. Smears should be prepared from the thick purulent part of the sputum.

**Staining:**

- **Ziehl–Neelsen technique (ZN):** Direct or concentrated smears of sputum are examined for AFB. Thick, purulent sputum needs to be digested and homogenised prior to staining. Two commonly used techniques are Petroff's method using 4% ACL, or NALC (N-Acetyl Cysteine) with 2% NaCl (Fig. 38.4). Smears are dried, heat-fixed and stained by the Ziehl–Neelsen technique. There are several modified techniques. The smear is covered with strong carbol fuchsin and gently heated to steaming for 5–7 minutes, without letting the stain boil and become dry.
- **Kinyoun's modification of acid fast staining:** This is a modified cold method where heating of the stain is not employed. It requires increasing the concentration of phenol acid and duration of staining. The slide is then washed with water and decolourised with 20% sulphuric acid till slide becomes colourless followed by decolourisation with 95% ethanol for two minutes. The two steps can be combined using acid alcohol (3% HCl in 95% ethanol). After washing, the smear is counterstained 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 counterstain used. **At least 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 graded based on Revised National TB Control Program (Table 38.3).
- **Auramine rhodamine:** When several smears are to be examined daily, it is more convenient to use fluorescent microscopy. Smears are stained with auramine phenol or auramine rhodamine fluorescent dyes and examined under ultraviolet illumination or where source is LED (light emitting diode). Bacilli appear as bright rods against a dark background. Because of the contrast, the bacilli can be seen even under the high dry objective, enabling large areas of the smear to be screened rapidly.

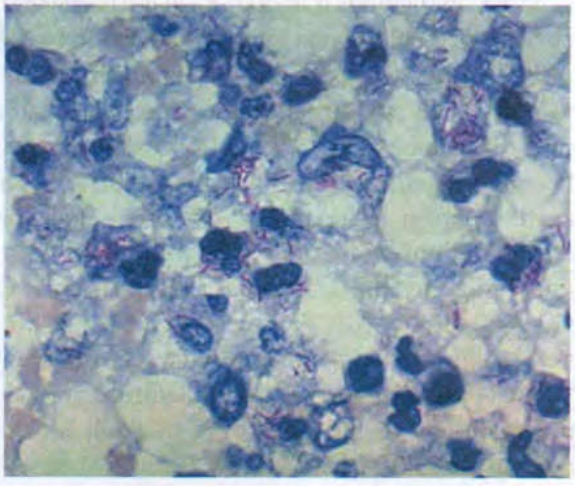

Fig. 38.4 *Mycobacterium tuberculosis* (acid fast bacilli on ZN stain)

**Differences between *M.tuberculosis* and saprophytic mycobacteria on staining**: Microscopic demonstration of acid fast bacilli provides only presumptive evidence of tuberculosis, as even saprophytic mycobacteria may present a similar appearance. Saprophytic Mycobacteria stain uniformly without barred or beaded appearance, and are usually only acid fast. Saprophytic mycobacteria may be present in tap water, rubber tubes, cork or bark, and can contaminate clinical materials. Saprophytes may pose problems with gastric aspirates, feces and urogenital specimens.

### Culture

Culture is the gold standard for diagnosis of tuberculosis, detecting as few as 10 to 100 bacilli per ml.

Table 38.3 *ZN smear evaluation and AFB report as per RNTCP guidelines*

| | RNTCP | | |
|---|---|---|---|
| | *Result* | *Grading* | *No. of fields* |
| >10/field | +ve | 3+ | 20 |
| 1–10/field | +ve | 2+ | 50 |
| 10–99/100 field | +ve | 1+ | 100 |
| 1–9/100 field | +ve | scanty * | 100 |
| No. of AFB in 100 | -ve | | 1000 |

*Record actual no. of bacilli seen in 100 fields.

- **Solid media:** The concentrated material is inoculated into at least two bottles of LJ medium. If the specimen is positive by microscopy, a direct drug sensitivity test may also be set up. Cultures are examined for growth after incubation at 37°C for four days (for rapid growing mycobacteria, fungi and contaminant bacteria) and at least twice weekly thereafter for 8–12 weeks, following which a negative report is given if no growth occurs. A smear is made from any growth, and stained by ZN method. A slow-growing, non-pigmented, niacin-positive, acid fast bacillus is taken as *M.tuberculosis*. When the isolate is niacin-negative, a battery of tests may be needed for identification, including growth at 25°C and 45°C, animal pathogenicity and biochemical tests (Fig. 38.5, Table 38.4).
- **Liquid media:** A liquid medium, Middlebrook 7H9, is available but its use has now become limited due to the increasing use of liquid culture medium (Mycobacteria Growth Indicator Tube (MGIT) for drug susceptibility.
- **Automated systems:** Continuously monitoring systems using BACTEC MGIT, BACTEC 9000MB and BacT/ALERT are slowly replacing solid culture methods, because of rapid indication of growth. They use the fluorescence quenching system. Another system uses a colorimetric carbon dioxide sensor in each bottle to detect growth.

The use of liquid media with radiometric growth detection such as BACTEC 460 with simplified culture and anti-tubercular drug sensitivity enabled results to be given in 2–3 weeks. However, they were based on radioisotopes, hence, have been replaced by non-radiometric methods as mentioned above.

All these systems use broth similar to 7H9 supplemented with a variety of growth media and antimicrobial agents.

**Anti-tuberculosis drug sensitivity tests:** As drug resistance is an important problem in tuberculosis, it is desirable to test the sensitivity of isolates as an aid to treatment. Sensitivity tests for *M.tuberculosis* are carried out using the following methods:

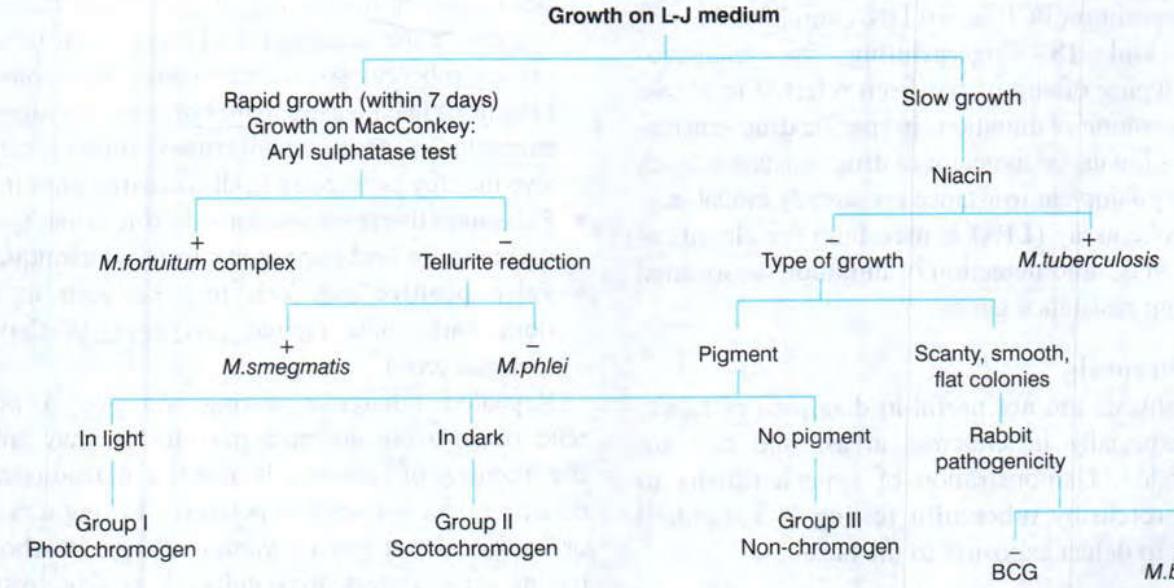


Fig. 38.5 Algorithm for identification of tubercle bacilli and related mycobacteria

Table 38.4 *Tests used for identification*

| *Type* | *Niacin* | *Nitrate reduction* | *Oxygen preference* | *Growth in TCH* | *Phage type* |
|---|---|---|---|---|---|
| Human (classical) | + | + | Aerobic | + | ABC |
| Asian type | + | + | Aerobic | – | I |
| African type | +/– | Variable | Microaerophilic | – | A |
| Vole | +/– | Variable | Microaerophilic | – | ? |
| Bovine | – | – | Microaerophilic | – | A |

*TCH=Thiophene-2-carboxylic acid hydrazide (5 mg/l)

- In the **absolute concentration method**, a number of media containing serial concentrations of the drugs are inoculated and the minimum inhibitory concentrations calculated.
- In the **resistance ratio method**, two sets of media containing graded concentrations of the drugs are inoculated, one set with the test strain and the other with a standard strain of known sensitivity.
- In the **proportion method**, the average sensitivity of the strain is indicated, taking into account the fact that any population will contain cells with varying degrees of sensitivity to a drug.
- **Automated systems**, as described above, are used more commonly now as the turnaround time is short.

### Molecular methods

- Polymerase chain reaction **(PCR)** and ligase chain reaction **(LCR)** are replacing culture methods especially for extrapulmonary tuberculosis.
- Transcription-mediated amplification **(TMA)** targeting ribosomal RNA has been introduced as an improvement on PCR-based DNA amplification.
- **RFLP and IS fingerprinting** for epidemiological typing of strains has been referred to above. Demonstration of mutations in specific drug sensitivity genes is a useful indicator of drug resistance. Such tests for rifampicin resistance are already available.
- Line probe assay **(LPA)** is used both for identification of MTC and detection of mutations associated with drug resistance genes.

### Immunodiagnosis

Serological tests are not useful in diagnosis of tuberculosis, especially in endemic areas, and are not recommended. Demonstration of hypersensitivity to tuberculoprotein by **tuberculin testing** is a standard procedure to detect exposure to the bacilli.

**Methods:**

- **Mantoux test**: This test uses purified protein derivative (PPD) and has been used routinely since 1910. In this test, 0.1 ml of PPD containing 5 TU (tuberculosis unit) is injected intradermally (between layers of skin and not subcutaneously) on the flexor aspect of the forearm with a tuberculin syringe, raising a wheal. The site is examined 48–72 hours later and the induration of 10 mm or more, measured at its widest point transversely to the long axis of the forearm, is taken as positive. Erythema is not taken into account. Induration of 5 mm or less is considered negative and 6–9 mm equivocal. A PPD dose of 1 TU is used when extreme hypersensitivity is suspected. An increased dose of 10 or 100 TU is used when 5 TU test is negative.
- **Heaf test:** Multiple puncture testing is used for screening and surveys, but it is not accurate enough as a diagnostic test.
- **Tine test:** Disposable prongs carrying dried PPD are also available for individual testing.

**Interpretation:**

- **Positive**: A positive tuberculin test indicates hypersensitivity to tuberculoprotein, denoting infection with the tubercle bacilli or prior immunisation with BCG. The test becomes positive 4–6 weeks after infection or immunisation. Tuberculin allergy wanes gradually and disappears after 4–5 years in the absence of subsequent contact with the mycobacteria. In endemic areas, the allergy is maintained by repeated contacts with the bacilli.
- **Negative**: Persons who have never had contact or been exposed to the tubercle bacilli are tuberculin-negative. False negative tests (anergy) may be seen in miliary tuberculosis, convalescence from some viral infections like measles, lymphoreticular malignancy, sarcoidosis, severe malnutrition, immunosuppressive therapy or impaired cell-mediated immunity.
- **False negative** results may also be due to inactive PPD preparations and improper injection technique.
- **False positive** reactions may be seen in infections with some related mycobacteria ('atypical' mycobacteria).

Repeated tuberculin testing will give a positive reaction in a non-infected person, but may enhance the intensity of response in reactive individuals. This booster effect is useful in persons showing a negative or equivocal test due to waning allergy, in whom re-testing after a week may induce a positive response (**'two-step testing'**). Re-testing is done at a site different from the earlier one.

**Uses:** Tuberculin testing may be used as an aid in diagnosing active infection in infants and young children. It also helps to determine prevalence of infection in an area, or as an indication of successful vaccination. Tuberculin testing of cattle has helped in the control of bovine tuberculosis.

**Interferron gamma release assay:** This test uses *Mycobacterium tuberculosis* antigen CFP10 which

reacts with T-lymphocytes of the patient to release γ interferon. This test is not very specific for pulmonary TB, hence, is not recommended any longer.

**Animal inoculation:** The concentrated material is inoculated intramuscularly into the thigh of two healthy guinea pigs about 12 weeks old. Subcutaneous inoculation is not recommended as it leads to a local ulcer which may be infectious. The animals are weighed before inoculation and at intervals thereafter. Progressive loss of weight is an indication of infection. Infected animals show a positive tuberculin skin reaction. One animal is killed after four weeks and autopsied. If it shows no evidence of tuberculosis, the other is autopsied after eight weeks.

## Diagnosis of extrapulmonary tuberculosis

The general procedure is as for pulmonary tuberculosis. The **specimen** depends on the site of infection: urine, CSF, joint fluid, biopsy material, blood or any other body fluid. **Microscopy** and **culture** (**animal inoculation is very rarely done now**) are used for the diagnosis of extrapulmonary tuberculosis, though it is difficult to obtain conclusive results as the bacilli are present in far fewer numbers in these lesions than in pulmonary disease. This has led to the use of molecular techniques for diagnosing extrapulmonary tuberculosis.

- CSF from tuberculous meningitis often develops a spiderweb clot on standing, examination of which may be more successful than of the fluid. The use of PCR and DNA probes may be more efficient in detecting the bacilli.
- Bone marrow and liver biopsy specimens from miliary tuberculosis and blood from those with HIV co-infection are useful for culture. Pus from tuberculous abscess often yields positive results in smear and culture.
- Pleural effusion and other exudates may be collected with citrate to prevent coagulation. They may be directly cultured after centrifugation. If other bacteria are present, prior concentration is necessary.
- Urinary excretion of bacilli in renal tuberculosis is intermittent. Hence, it is advisable to test 3–6 first whole voided morning samples of urine. Each sample is centrifuged at 3000 rpm for 30 minutes and the sediment used for culture after concentration.

## Prophylaxis

### General measures

For the prevention of tuberculosis, general measures such as adequate nutrition, good housing and health education are as important as specific antibacterial measures.

### Immunoprophylaxis

The **BCG (Bacille Calmette–Guerin) vaccine**, administered by intradermal injection of the live attenuated vaccine, was developed by Calmette and Guerin (1921). This is a strain of *M.bovis* attenuated by 239 serial subcultures in a glycerine–bile–potato medium over a period of 13 years. Following BCG vaccination, a tuberculin-negative recipient is converted to a positive reactor. The immunity may last for 10–15 years and is similar to the immunity following natural infection, except that it does not carry any risk of disease due to reactivation, as in the latter case.

**Safety measures:** The Lubeck disaster, in which several children developed fatal tuberculosis following oral immunisation, faced severe criticism. This was later found to be due to live, virulent tubercle being given instead of BCG by mistake.

Stringent safety measures have been enforced in the manufacture of the BCG vaccine. The recognised complications of this vaccine are as follows:

- **Local:** Abscess, indolent ulcer, keloid, tuberculides, confluent lesions, lupoid lesions, lupus vulgaris
- **Regional:** Enlargement and suppuration of draining lymph nodes
- **General:** Fever, mediastinal adenitis, erythema nodosum, tendency to keloid formation, and, very rarely, non-fatal meningitis. Very few cases of progressive tuberculosis reported are believed to have been in immunodeficient subjects.

**Efficacy:** The consensus opinion is that BCG may not offer protection from the risk of tuberculosis infection, but gives protection to infants and young children against the more serious types of the disease, such as meningitis and disseminated tuberculosis. The recommendation, therefore, is that in endemic countries such as India, the BCG vaccine be administered to babies by intradermal injection on the deltoid immediately after birth, or as early as possible, before the age of 12 months. The vaccine need not be administered after the age of two years. BCG should not be given to infants and children with active HIV disease, though it may

be given with benefit to asymptomatic HIV-positive cases. Babies born to mothers with AFB-positive sputum should not be given BCG at birth, but only after a course of preventive chemotherapy.

**Added advantages of BCG vaccine**
BCG induces non-specific stimulation of the immune system, providing some protection against leprosy and leukemia. Multiple injections of BCG have been tried as adjunctive therapy in some malignancies. Some workers have reported that BCG is superior to PPD for tuberculin testing.

Restoration of cellular immune capacity by '**transfer factor**' had been shown, many years ago, to help recovery in immunodeficient patients. A vaccine containing heat-killed *M.vaccae*, an environmental mycobacterium from Uganda, is being tested as an immunomodulator for stimulation of Th-1 cells which promote protective immunity.

**Chemoprophylaxis or preventive chemotherapy:** Administration of anti-TB drugs (usually only isoniazid) to persons with

- Latent tuberculosis (asymptomatic, tuberculin-positive)
- High risk of developing active tuberculosis
- Uninfected, exposed to high risk of infection
- Infants of mothers with active tuberculosis
- Children living with a case of active tuberculosis in the house
- HIV-infected contacts of active tuberculosis

The drug of choice is isoniazid 5 mg/kg daily for 6–12 months as the usual course. Trials have shown that this reduces the risk of developing active disease by 90 per cent.

## Treatment

**Chemotherapy** has revolutionised the management of tuberculosis in such a way that the earlier concept of sanatorium regimens, bed rest, fresh air and rich food, as well as operative interventions, such as artificial pneumothorax and thoracoplasty are no longer essential for cure, if domiciliary treatment with effective anti-tuberculosis drugs are given in optimal dose and duration.

Anti-tuberculosis drugs are of two types:

- **Bactericidal:** Of these, rifampicin (R) and pyrazinamide (Z) are called sterilising drugs because they effectively kill the bacilli in the lesions. On the other hand, bactericidal drugs, isoniazid (H) is effective only against replicating bacilli and streptomycin (S) only against extracellular bacilli and so are not by themselves able to sterilise the lesions.
- **Bacteriostatic:** Ethambutol (E), along with the other bactericidal drugs, constitutes the first-line drug in anti-tuberculosis therapy. The old practice of daily administration of drugs for two years or so has been replaced by short-course regimens of 6–7 months, which are effective and convenient. A typical example of such a schedule for a new smear-positive case is a combination of four drugs (HRZE) given three times a week during an initial intensive phase of two months, followed by 4–5 months of continuing phase with only two drugs (HR) three times a week. The regimen of treatment has undergone modifications over the years and now the treatment provided by the RNTCP follows the **Directly Observed Treatment-short course (DOTS).**

### Drug resistance

Drug-resistant tuberculosis has become a problem in high TB burden countries, including India. This is due to mutations, with an approximate rate of 1 in $10^8$ cell divisions. This may have been effectively prevented by the strategy of combination drug therapy, which had been introduced for this purpose. Unfortunately, this was improperly implemented. Multiple factors have led to the emergence of MDR-TB. Lapses in prescribing practices, drug delivery and patient compliance have led to build-up of resistance in the bacilli, over the years, reducing the efficacy of treatment.

Drug resistance can be:

- **Primary** (pre-treatment, initial), when the patient is infected with a strain of the tubercle bacilli which is already resistant,
- **Acquired** (secondary, post-treatment), when the infecting strain initially sensitive becomes resistant, usually as a result of improper or inadequate treatment. This is the more common type of resistance.

When acquired, resistant strains become increasingly common in an area; the chance of new patients presenting with primary resistance increases.

When an infecting strain acquires resistance to one drug, the chance of it becoming resistant to other drugs increases, unless the treatment schedule contains an adequate number of effective drugs.

### Multidrug-resistant tuberculosis (MDR-TB)

A very serious consequence of unchecked drug resistance has been the emergence and spread of **multid-**

**rug-resistant tuberculosis (MDR-TB).** Though the term multidrug resistance means only resistance to two or more drugs, in the context of tuberculosis, it specifically refers to **resistance to rifampicin and isoniazid**, with or without resistance to one or more other drugs. This is because R and H form the sheet anchor of short-term chemotherapy and any strain resistant to both these drugs is unlikely to respond to treatment.

**MDR-TB** is a global problem, menacing the poor and rich nations alike. It may be primary or acquired. Its presence in those with concomitant HIV infection makes it more dangerous. When first-line drugs become ineffective, second-line drugs must be tried. Large numbers of old and new drugs are being used: quinolones, aminoglycosides, macrolides, para aminosalicylic acid, thiacetazone, cycloserine, capreomycin and others. They are unsatisfactory, being much less effective, costlier, more toxic and requiring prolonged treatment schedules.

Extensively drug-resistant MTB **(XDR TB)** are extensively resistant strains. It is defined as multidrug-resistant tuberculosis (MDR-TB) that is resistant to isoniazid and rifampicin, plus any fluoroquinolone and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin).

## Revised National Tuberculosis Control Program (RNTCP)

RNTCP was implemented in India in 1992. The aim was to provide standardised treatment and proper diagnosis facilities. This was based on Directly Observed Treatment, Short Course (DOTS) strategy of WHO. The treatment is started following diagnosis made primarily by morning and spot sputum microscopy. This is made available free of cost to patients at designated microscopy centres (DMC). Treatment is provided under direct observation by a DOT Provider at the DOTS centre near the patients' home. This strategy can prevent emergence of drug resistance by ensuring the patient's compliance.

India DOTS is the fastest expanding program in the world. The treatment purposes and regimens are given based on sputum smear positivity and seriousness of the disease as category I, II and III of the treatment. The algorithms for laboratory diagnosis and treatment strategies are standardised. It also identifies accredited laboratories for drug susceptibility testing.

DOTS relies on treatment with first-line drugs rifampicin and INH.

**DOTS-Plus** refers to DOTS programmes that add components for MDR-TB diagnosis and treatment using quality-assured culture and drug susceptibility testing. Proper triage of patients for Culture and DST testing and management under DOTS-Plus is done in coordination with National and Supra-National Reference Laboratories.

**RNTCP—Standardised Treatment Regimen (Cat IV)**: This regimen is for the treatment of MDR-TB cases (and those with rifampicin resistance) under the RNTCP programme (Table 38.5). Cat IV regimen comprises 6 drugs—kanamycin, ofloxacin (levofloxacin), ethionamide, pyrazinamide, ethambutol and cycloserine during 6–9 months of the Intensive Phase— and 4 drugs—ofloxacin (levofloxacin), ethionamide, ethambutol and cycloserine during the 18 months of the Continuation Phase. *p*-aminosalicylic acid (PAS) is included in the regimen as a substitute drug if any bactericidal drug (K, Ofl, Z and Eto) or 2 bacteriostatic (E and Cs) drugs are not tolerated.

(Ref: Revised National Tuberculosis Control Programme DOTS-Plus Guidelines)

**Table 38.5** *Alternative method of grouping anti-TB agents*

| | |
|---|---|
| Group 1: First-line oral anti-TB agents | Isoniazid (H); Rifampicin (R); Ethambutol (E); Pyrazinamide (Z) |
| Group 2: Injectable anti-TB agent | Streptomycin (S); Kanamycin (Km); Amikacin (Am); Capreomycin (Cm); Viomycin (Vm). |
| Group 3: Fluoroquinolones | Ciprofloxacin (Cfx); Ofloxacin (Ofx); Levofloxacin (Lvx); Moxifloxacin (Mfx); Gatifloxacin (Gfx) |
| Group 4: Oral second-line anti-TB agents | Ethionamide (Eto); Prothionamide (Pto); Cycloserine (Cs); Terizadone (Trd); para-aminosalicylic acid (PAS) |
| Group 5: Agents with unclear efficacy (not recommended by WHO for routine use in MDR-TB patients) | Clofazimine (Cfz); Linezolid (Lzd); Amoxicillin/Clavulanate (Amx/Clv); Thioacetazone (Thz); Imipenem/Cilastatin (Ipm/Cln); high-dose Isoniazid (high-dose H); Clarithromycin (Clr) |

As per Revised National Tuberculosis Control Programme DOTS-Plus guidelines

**The Stop TB Strategy of World Health Organization**

To reduce the global burden of TB and for **a TB-free world**, in line with the Millennium Development Goals and the Stop TB Partnership targets, WHO has laid out the following objectives:

- Achieve universal access to high-quality care for all people with TB.
- Reduce the human suffering and socioeconomic burden associated with TB.
- Protect vulnerable populations from TB, TB/HIV and multidrug-resistant TB.
- Support development of new tools and enable their timely and effective use.
- Protect and promote human rights in TB prevention, care and control.

## RECAP

- *Mycobacterium tuberculosis* is an obligatory, aerobic, non-motile, non-sporing, rod-shaped bacterium which stains poorly by the Gram stain because its cell wall contains an abundance of lipids (mycolic acids). It retains strong carbol fuchsin dye during decolourisation with acid and alcohol in the Ziehl–Neelsen (ZN) staining technique (*Mycobacterium tuberculosis* is acid and alcohol fast by this staining technique). It grows very slowly, taking several weeks to form a visible colony on enriched culture media.
- *Mycobacterium tuberculosis* causes tuberculosis (TB) in humans; this is the leading cause of bacteria-related deaths worldwide.
- TB is transmitted by aerosols from an infected individual. Inhaled bacteria penetrate the alveoli and are ingested by alveolar macrophages. Bacteria grow intracellularly and slowly. The general health and robustness of the immune system of the individual determine whether organisms:
  - Are killed and cleared
  - Remain viable but controlled in a granuloma for many years, undergoing 're-activation' when the individual ages or immune status changes
  - Continue to grow, cause damage to the lungs, spread, and destroy other organs
- Cell-mediated immunity is the primary immune response that destroys the organism inside macrophages. Individual susceptibilities to TB reflect differences in the efficacy of an individual's cell-mediated response to infection.
- Early morning sputum is generally collected for diagnosis by:
  - Staining by the ZN method; acid and alcohol fast bacilli appear as long, thin, pink (sometimes beaded) rods.
  - Culture of sputum on Lowenstein–Jensen or Middlebrook medium; this may take up to 6–8 weeks to yield positive results.
- Automated systems have improved the turnaround time of culture and sensitivity:
  - The tuberculin skin test is a sign of exposure to the organism.
  - PCR can be used to detect *Mycobacterium tuberculosis* DNA in sputum and other specimens.
- Individuals suspected to have the disease should be treated with multiple antibiotics. MDR and XDR strains are a cause of concern in treatment.
- DOTS under RNTCP in India ensures proper therapy to patients.