Review of Literature
REVIEW OF LITERATURE

“A Salute to the Genius”

:- ROBERT KOCH :-

(1843-1910)

<table>
<thead>
<tr>
<th>Name</th>
<th>Robert Koch (Heinrich Hermann Robert Koch)</th>
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<tbody>
<tr>
<td>S/o</td>
<td>A Mining Engineer</td>
</tr>
<tr>
<td>Date of birth</td>
<td>11th December, 1843</td>
</tr>
<tr>
<td>Place of birth</td>
<td>Clausthal, Germany</td>
</tr>
<tr>
<td>Medical Studies</td>
<td>Gottingen University, 1862.</td>
</tr>
<tr>
<td>Marriage</td>
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<tr>
<td></td>
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<tr>
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<td>Remarried: Hedwing Freiburg, 1893.</td>
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<td>Work Done</td>
<td>M.D. Thesis on Succinic Acid, 1866</td>
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<tr>
<td></td>
<td>District Physician at Wollheim, 1872</td>
</tr>
<tr>
<td>Year</td>
<td>Achievement</td>
</tr>
<tr>
<td>--------</td>
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<tr>
<td>1877–80</td>
<td>Bacteriological Research, Breslau</td>
</tr>
<tr>
<td></td>
<td>Director of Imperial Health Office, Berlin</td>
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<tr>
<td></td>
<td>Professor of Bacteriology, Berlin</td>
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<tr>
<td></td>
<td>Recognition</td>
</tr>
<tr>
<td></td>
<td>Koch’s Institute, 1891</td>
</tr>
<tr>
<td></td>
<td>Noble Prize, 1905</td>
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<tr>
<td>27th May, 1910</td>
<td>Demise</td>
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**ACHIEVEMENTS:**

<table>
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<tr>
<th>Year</th>
<th>Description</th>
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<tbody>
<tr>
<td>1876</td>
<td>Aetiology of Anthrax – B.anthracis Procedures of Investigation,</td>
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<tr>
<td></td>
<td>Preservation &amp; Photograph Bacteria.</td>
</tr>
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<td>1878</td>
<td>Aetiology of Wound Diseases</td>
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<td>1880/81</td>
<td>Development of Bacterial Methodology</td>
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<td></td>
<td>Production of Pure Cultures</td>
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<td>Practice of Disinfection</td>
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<td>1882</td>
<td>Aetiology of Tuberculosis</td>
</tr>
<tr>
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<td>Discovery of Tubercle Bacillus</td>
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<td>1883/84</td>
<td>Discovery of Cholera vibrio in India.</td>
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<td>1885/90</td>
<td>Application of Bacteriology for Public Health</td>
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<td>1890</td>
<td>Tuberculin</td>
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<td>1892/93</td>
<td>Organisation of Cholera Control</td>
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<td>1896</td>
<td>Fight against Rinderpest in South Africa</td>
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<td>Year</td>
<td>Events</td>
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<tr>
<td>1897</td>
<td>o Investigations on Black Water Fever – Malaria</td>
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<td></td>
<td>o Texas Fever and Tsetse Disease</td>
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<td>o Plague expedition to India</td>
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<td>o Combating Leprosy in Memel</td>
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<td></td>
<td>o New Tuberculins</td>
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<td>1898 / 99</td>
<td>o Malaria Expedition to Italy etc.</td>
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<td>o Children Malaria</td>
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<td>o Quinine Prophylaxis</td>
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<td>1901</td>
<td>o Separation of Human &amp; Cattle TB</td>
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<td>1902</td>
<td>o Typhoid control in Germany</td>
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<td>1903 / 05</td>
<td>o Investigation on Coast Fever and Horse Deaths in South Asia</td>
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<tr>
<td>1906 / 08</td>
<td>o Sleeping sickness Expedition to East Africa</td>
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<tr>
<td>1908 / 10</td>
<td>o Continuation of TB Research</td>
</tr>
<tr>
<td>1880 / 1910</td>
<td>o Advisor to Reich and Prussia in the control of Epidemic Diseases.</td>
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**LIFE HISTORY:**

Heinrich Hermann Robert Koch, born on December 11, 1843 later became a German Physician & Discoverer of Tubercle bacillus (1882), Cholera bacillus (1883), development of Koch’s postulates & many other stunning investigations in the field of Medical Bacteriology. He studied Medicine under Jacob Henle at the University of Gottingen and graduated in 1866. He soon started working in a laboratory looking for cause of diseases.\(^\text{15}\)

In 1876, he succeeded in growing a pure culture of anthrax bacillus and then worked out a method of preventing anthrax in cattle through inoculation of this culture. By this time, Koch had perfected the methods of steam and dry heat sterilization as well as discovered the ‘Cholera vibrio’ responsible for Asiatic cholera. In 1878, he published his path-breaking thesis on infections following injuries. Koch’s many discoveries had caught the imagination of all the physicians in Germany and those in Europe, in general. In 1885, Koch was appointed as a Professor in Berlin University.\(^\text{14, 15}\)

Tuberculosis is known to be most ancient disease of the man kind. Well before Koch many other scientists have described the various aspects of the disease. However the question still remained was; whether the TB caused by microorganisms. Who would be the first to solve it?\(^\text{14}\)

This puzzle was solved by Robert Koch on **March 24\(^{\text{th}}, 1882\)**, when he discovered the tubercle bacilli which he called as ‘Beautiful Blue Bacilli’. On 24 March 1882, Koch presented his groundbreaking lecture on the etymology of tuberculosis to the **Physiological Society of Berlin**. The experiments Koch described in his lecture
defined tuberculosis as an infectious disease according to the postulates that are now known as the Koch-Henle postulates. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, is an organism that is particularly difficult to work with, making this lecture meritorious even today.\(^\text{14}\)

Koch began the lecture with a step-by-step account of his identification of the microbes. Tubercle bacilli are difficult to stain by conventional staining methods. Koch succeeded in staining them by first applying methylene blue and then vesuvin (Bismarck’s brown), resulting in a brown appearance of the host tissue and dark blue staining of the microbes. The methylene blue staining procedure lasted about 1 day at room temperature but, as Koch noted, the time of incubation could be considerably shortened by heating at 40 °C. The consistent identification of microbes in affected tissue fulfilled the first Koch-Henle postulate.\(^\text{14}\)
The next step was to isolate the bacilli and culture them in pure form. Here again, Koch had to develop a specific method appropriate for the slow-growing mycobacteria. He used cattle or sheep serum that had been sterilized by treatment at 58 °C for 1 hour on 6 successive days. The serum was then heated to 65 °C for several hours, after which it solidified. Because Koch wanted the material to have a translucent appearance, he took care not to heat it above 65–70°C, this being the temperature at which the solidified serum became opaque. To increase the surface area for bacterial growth, Koch slanted the tubes in which the bacilli were grown. Once he had inoculated the tubes, Koch had the patience to wait. It took 10 days for small dry spots to become visible. The successful isolation and culture of the bacteria fulfilled the second Koch-Henle postulate.14

Koch summarized this part of his talk as follows: "All these factors together allow me to note that the bacilli present in the tuberculous lesions do not only accompany tuberculosis, but in fact cause it. These bacilli are the true agents of tuberculosis."14

He demonstrated these bacteriological techniques at 7th International Medical Congress at London. After his great work in the field of tuberculosis he announced the discovery of TB bacillus at a monthly evening meeting of Berlin Physiological Society. People forgot to applaud, as audience was stunned. The disease which had ravaged and mystified for so long had now at long last divulged its secret. It was a major event in the history of medicine, a turning point in our understanding and conquest of that deadly disease which plagued mankind for millennia. Koch proved conclusively that the cause of the disease was infection by a specific organism which is isolated.15 Koch’s lecture created great interest in the medical community and was published on 10th April 1882, less than 3 weeks afterward, in the Berliner Klinische Wochenschrift (Berlin Clinical Weekly). World TB day celebrated on March 24th as
advocated by WHO, on the eve of discovery of TB bacillus by Robert Koch, we pay tribute to the father of the modern scientific approach to the management of TB.\textsuperscript{15,17}

In 1890, Koch prepared an extract of heat-killed tubercle bacilli and called it tuberculin. His idea was to use tuberculin for the prevention and treatment of tuberculosis without realizing that he was completely on the wrong track this time. His reputation suffered a lot when scientists realized his mistake, which left him disillusioned and bitter at the fag end of this career and life. In India, he conducted researches on bubonic plague. In 1891, he founded the institute of infectious diseases in Berlin and was its founder director. Koch is not only considered the “\textbf{Father of Tuberculosis}” but the “\textbf{founder of bacteriology}” as a separate discipline. Till today, \textit{M. tuberculosis} is also called Koch’s bacillus and the disease Koch’s disease.\textsuperscript{14,15}

In 1905, he was awarded with Nobel Prize in the field of Medicine. In his Nobel Prize lecture he gave his opinion concerning the problem of combating Tuberculosis. He postulated the conditions favouring settlement of TB bacilli in the body. It’s breeding in bad overcrowded housing of the poor. He felt that disease should be fought in both medical and social fields. He also recommended prevention of infection by isolation of patient in hospital or by screening at home.\textsuperscript{17}

He advised & advocated disinfection of patient’s excretions, care of the patient in organized dispensaries, information and health education of the population of patients and their families. He suggested the compulsory registration of all cases as a basis of coverage for statistical purposes. He considered dispensaries as one of the important tool in fight against TB.\textsuperscript{17,18}

In 2\textsuperscript{nd} volume of reports of Imperial Health Office he presented a classic paper on Aetiology of tuberculosis which was a masterpiece of medical literature. In this he
described his well known postulates. The stringent criteria which an organism must fulfill before it can be considered to be the causative of an infectious disease.\textsuperscript{18}

It is exhausting merely to mention his achievements. One can really wonder how he might have slogged through his life time. One need not hesitate to declare that this endless war of medical research will never see a greater medical technician than him. On this occasion we salute Robert Koch whose contribution to bacteriology and tuberculosis place him among medical immortals.\textsuperscript{15, 17, 18}

His life constitutes tons of hard work and achievements till his last breath at the age of 67 in 1910 at Baden. Without doubt his life was fully devoted to the investigations & discoveries in the field of Medical Bacteriology. His work & devotion solved many problems in this field & his discoveries regarding the tuberculosis have remained one of the greatest tools even today in our ‘fight against tuberculosis’. His institution has also produced so many great, in fact rare scientists.\textsuperscript{14, 17, 18}

Without consideration towards such a great scientist, any work in the field of tuberculosis can never be accomplished. Hence we \textit{“Salute the Pioneer”} in this regards.\textsuperscript{14, 15, 18}
HISTORY OF TUBERCULOSIS

“As a destroyer of Mankind, tuberculosis has no equal.”

V.A. Moore

Tuberculosis has been a major cause of suffering and death since times immemorial. Thought it to be one of the oldest human diseases, the history of tuberculosis is at least as old as the mankind. Over the years, not only the medical implications but also the social and economic impact of tuberculosis has been enormous.

There have been reference to this ancient scourge in the Vedas (vide infra) and it was called “rajaya kshma” (meaning ‘wasting disease’). Hippocrates (460-377 B.C) called the disease “Pthisis” a Greek word which meant “to consume” “to spit” and “to waste away”. The word “tuberculosis” is a derivative of the Latin word “tubercula” which means “a small lump”. Several names have been used to refer to tuberculosis in the years gone by. Acute progressive tuberculosis has been reported to as “galloping consumption”. Pulmonary tuberculosis has been referred to as “tabes pulmonali”. Tuberculosis cervical lymphadenitis has been called as “Scrofula”, “King’s Evil”, “Stroma” Abdominal tuberculosis has been called as “tabes mesenterica.” Cutaneous tuberculosis has been called “lupus vulgaris,” vertebral tuberculosis has been called as “Potts disease”.

Oliver Wendell Holmes referred to the disease as “White Plague”. While scores of other disease like small pox and plague killed millions of people, their reign has been relatively short-lived. Tuberculosis has been ever present and is resurging with a vengeance.

It is thought that tuberculosis probably existed in cattle before the advent of man. In Sanskrit the disease has been called “rajaya kshma” “ksayah” and “Sosa” changes
resembling those caused by tuberculosis has been described in the skeletal remains of Neolithic man. Terms such as “lungs cough” and “lungs fever” have been used in ancient Chinese literature to describe a disease which may have been tuberculosis. There have been references to what could have been tuberculosis in the code of Hammurabi of the Babylonian era. Evidence of tuberculosis lesions of bone have also been found in Egyptian mummies date back to 3400 B.C.\textsuperscript{16}

During the middle ages, there are records of healing touch of monarchs being used to treat scrofula. By around 1629, death certificates in London specified the disease as “consumption” which was a leading cause of death. By this time the contagious nature of tuberculosis was strongly believed though there were people who contested this opinion.\textsuperscript{16}

- **Diagnosis**

In the early days, diagnosis of tuberculosis was based on symptoms and signs. In Charaka Samhita, heaviness in the head, coughing, dyspnoea, hoarseness of voice, vomiting of phlegm, spitting of blood, pain in the sides of the chest, grinding pain in the shoulder, fever, diarrhoea and anorexia have been described as the eleven symptoms of tuberculosis. Furthermore, a physician who is well versed in the aetiology, clinical presentation and premonitory symptoms of consumption was considered to be a ‘Royal Physician’.\textsuperscript{16, 17, 18}

The earliest classical descriptions of tuberculosis in Greek Literature date back to the writings by Hippocrates, Aretaeus the Cappodocian (50 B.C) in his book “The causes and symptoms of chronic diseases” gave a very accurate description of tuberculosis and mentioned that fever, sweating, fatigue and lassitude were symptoms of tuberculosis. He suggested testing the sputum with fire or water was of diagnostic value.\textsuperscript{16, 17}
Galen described that patients with consumption manifest cough, sputum, wasting, chest pain and fever and considered haemoptysis to be pathognomonic of the disease. Following the pioneering efforts by Andreas Vesalius (1514-1564). Postmortem examination was performed frequently. This method of study facilitated the understanding of the pathological findings such as lung cavities, empyema among others. John Jacob Manget in 1700 gave the description of classical milliary tuberculosis. The clinical presentation of consumption was described in detail by Thomas Willis (1621-1675).\textsuperscript{16-18}

Fracastorius (1443-1553) is credited to have originated the ‘germ theory’ and believed that tuberculosis was contagious. In 1720, the English physician, Benjamin Marten conjectured in his publication “A new theory of consumption” that tuberculosis could be caused by “certain species of Animalcula or wonderfully minute living creatures, which once they had gained a foothold in the body, could generate the lesions and symptoms of the disease. For unknown reason the work of Marten went into oblivion for a long time.\textsuperscript{16}

On 24\textsuperscript{th} March 1882, Robert Koch announced the discovery of the tubercle bacillus during the monthly meeting of the Berlin physiological society. In 1884, he published a more comprehensive paper “Die a etiologic der tuberculose” in the second volume of the ‘Reports of the Imperial Health office ‘. In 1905, he was awarded the Nobel Prize of his contributions in the yield of tuberculosis research.\textsuperscript{16-18}

It was Robert Koch who finally demystified the ‘secret of the cause’ of tuberculosis and after thousand of years, the organism finally revealed itself to humans. Robert Koch’s discovery set the wheel in motion for further research in prevention and control of the disease.\textsuperscript{16-18}
Struggle to combat TB

In the Yajurveda, there are references to soma performing a “yagna” (sacred offering) seeking cure from tuberculosis. Since ancient times amulets, invocations, charms, Royal touch and prayers have been used to treat tuberculosis. Chemicals such as arsenic, sulphur, calcium, several vegetable, plant and animal products including excreta of humans and animals, blood letting have been used over the centuries in the fond hope of curing tuberculosis.16,18

Despite the fact that TB is a disease of antiquity and it is probably one of the illnesses most dealt with in the literature, there had been surprisingly little sound knowledge of the disease through the course of the disease which has not helped contemporary efforts to combat the illness. From the time of Hippocrates (460-377 BC) until the nineteenth century, the infectious nature of the disease was not even acknowledged rather, TB was considered a hereditary disorder. However, air a common vehicle for the transmission of live germs was included among the interpretation of the possible origin of the disease. For this reason the dietary regimen proposed by Hippocrates and Galen (130 -200 AD) remained the basis of treatment until the Renaissance.17

Only towards the latter half of the nineteenth century, did the infectious nature of TB become apparent, as a result of the studies by Villemin (1865) and particularly Robert Koch (1843-1910).17 Koch was the first to suggest the possibility of controlling this endemic disease, with the presentation of the results of his research in 1882 that showed that TB was a contagious disease. He not only isolated bacterium which was later named after him ‘Koch’s bacillus’ from the sputum of infected patients but also proposed that the principle measure for controlling the disease in the community would be to isolate affected patient. This suggestion pared the way for the “sanatorium” era of TB, during which prolonged confinement of patients in
sanatorium was believed to be the only effective way to cure TB and control its transmission.\textsuperscript{16, 18}

Based on the above, and throughout the prolonged history of the disease, it can be seen that the human host defences were the only means to counter \textit{M. tuberculosis}. In this confrontation between the micro organism and the immune defence system, the latter tended to prevail as a result of which only a very small proportion of infected individuals eventually developed the disease.\textsuperscript{16} However, when the disease became more established, the prognosis became very bleak in most cases with a mortality of more than 50\% five years after the onset of the disease. In turn 25\% of infected patients died within eighteen months. Cure was achieved only in 25\% to 30\% of cases the rest remaining chronically ill while continuing to spread the disease throughout the community. This extremely poor prognosis led to the development of various treatment attempts, most of which were empirical in nature and which proved to be ineffective.\textsuperscript{17, 18}

In the eighteenth century, treatment recommendations included moving to the countryside and partaking in moderate activities. There was still special attention to diet, with medications reserved for the initial or “inflammatory” stage of the disease. Thus during the initial phase, treatment involved bleeding, antiemetic agents and a light diet, whereas treatment in the “ulcerative” phase of the disease involved balsamic products, expectorants and opium.\textsuperscript{16, 17}

In the early part of the nineteenth century, the practice of bleeding became more common after the “initiative” doctrine developed by Broussais who introduced the use of leeches as therapy for TB in the first third of that century. The debate over what constituted appropriate treatment continued over the subsequent years, during which
the notion of the disease being associated with “impure air” regained popularly. Thus climate, exercise and diet were again regarded as fundamental to TB therapy. Accordingly, patients were sent to places where they could exercise outdoors while observing a ‘proper’ diet and medication regimen all under strict medical supervision. In this way sanatorium for patients with TB were created and became the standard treatment in all rich countries during the second half of the nineteenth century and the first half of the twentieth century.\textsuperscript{17}

Another important period in the history of TB management involved the use of different surgical procedures to heal the disease. As early as the second century Galen pointed out that the main difficulty in healing lung ulcerations in TB was the impossibility of keeping the lung at rest because of continuous breathing movements. Consequently it was proposed to collapse the lung to allow it to rest and thus aid healing. This theory led to the development of \textbf{Surgical Procedures} such as chondrotomy of the first rib, thoracoplasty, resection surgery, filling the extrapleural space with substances such as abdominal fat, paraffin, air, polyethylene sponges, Lucite pellets, ox spleen capsules or wax and especially pneumothorax induction. The last approach marked the beginning of the surgical era in the treatment of TB and was the most widely used methodology from the late nineteenth century till mid-twentieth century.\textsuperscript{16-18}

Therapy for TB changed dramatically, with the introduction of antibiotics for the management of infectious diseases. In fact, each newly introduced antibiotic was tested against TB the main health care problem in the world at the time. Sulphanilamide was the first sulpha drug in 1938 to be used against TB. However this agent was found to be ineffective in humans, although it had an inhibitory effect on TB in guinea pigs. A similar lack of efficacy was observed with more complex sulpha
agents such as promanide (1943) and penicillin, which Alexander Fleming began to use in clinical practice in 1941. All this changed with introduction of streptomycin by Waksman and Schatz in 1943, which has been used against TB since 1944. Since then other effective antituberculous agents have been developed leading to TB finally becoming a treatable disease in the mid-1950s.\textsuperscript{17, 18}

\section*{EPIDEMIOLOGY}
Tuberculosis is an ancient disease which continues to haunt us even as we step into the next millennium. Tuberculosis has been for many centuries the most important of human infections in its global prevalence devastating morbidity and massive mortality.\textsuperscript{19} It has been called the “white plague” and “the captain of all the men of death.” TB is a world-wide public health problem despite the fact that the causative organism was discovered more than 100 years ago and highly effective drugs and vaccine are available, making TB a preventable and curable disease.\textsuperscript{20} It is the most common cause of death due to a single infectious agent in adults and accounts for over a quarter of the avoidable deaths worldwide.\textsuperscript{20, 21}

\section*{Global Burden of Tuberculosis}\textsuperscript{19, 21, 23}
According to the recently published short update to the March 2009 report of the Global Tuberculosis Control, in 2008, there were an estimated 9.4 (range, 8.9-9.9 million) million incident cases (equivalent to 139 cases per 100,000 population) of tuberculosis (TB) globally. This is an increase from the 9.3 million TB cases estimated to have occurred in 2007, as slow reductions in incidence rates per capita continue to be outweighed by increases in population. Provisional analyses indicate that women account for an estimated 3.6 million cases (range, 3.4-3.8 Million). Most
of the estimated number of cases in 2008 occurred in Asia (55%) and Africa (30%), with small proportions of cases in the Eastern Mediterranean Region (7%), the European Region (5%) and the Region of the Americas (3%). The 22 high-burden countries (HBCs, defined as the countries that rank first to 22nd in terms of absolute number of cases and which have received particular attention at the global level since 2000) account for 80% of all estimated cases worldwide. **The five countries that rank first to fifth in terms of total number of incident cases in 2008 are India (1.6-2.4 million), China (1.0-1.6 million), South Africa (0.38-0.57 million), Nigeria (0.37-0.55 million) and Indonesia (0.34-0.52 million).** India and China alone account for an estimated 35% of TB cases worldwide. Of the 9.4 million incident cases in 2008, an estimated 1.2-1.6 million (13-16%) were human immunodeficiency virus (HIV) positive, with a best estimate of 1.4 million (15%). Of these HIV-positive cases, 78% were in the African Region and 13% were in the South-East Asia Region.

The estimated epidemiological burden of TB in the world in 2008 is shown in Table-1.
<table>
<thead>
<tr>
<th>Country</th>
<th>Population</th>
<th>Mortality</th>
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<th>Prevalence</th>
<th></th>
<th>Incidence</th>
<th></th>
<th>TB / HIV(%)</th>
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<tr>
<td></td>
<td></td>
<td>Best</td>
<td>Low</td>
<td>High</td>
<td>Best</td>
<td>Low</td>
<td>High</td>
<td>Best</td>
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<tr>
<td>Afghanistan</td>
<td>27 206 324</td>
<td>9 201</td>
<td>3 923</td>
<td>17 964</td>
<td>73621</td>
<td>41 568</td>
<td>117 413</td>
<td>51 456</td>
</tr>
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<td>Bangladesh</td>
<td>160 000 128</td>
<td>79 252</td>
<td>31 463</td>
<td>152 003</td>
<td>659 586</td>
<td>418 373</td>
<td>982 401</td>
<td>359 671</td>
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<tr>
<td>Brazil</td>
<td>191 970 504</td>
<td>7 284</td>
<td>2 714</td>
<td>15 249</td>
<td>55 698</td>
<td>12 407</td>
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<td>22262</td>
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<td>58 019</td>
<td>154 174</td>
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<td>64 083</td>
<td>329 249</td>
<td>1 175 048</td>
<td>408 980</td>
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<td>DR Congo</td>
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<td>49 417</td>
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<td>267 368</td>
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<td>553 196</td>
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<td>385 055</td>
<td>323 486</td>
<td>554 256</td>
<td>3 809 656</td>
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<td>4 473 415</td>
<td>2 828 485</td>
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<td>29 135</td>
<td>24 186</td>
<td>41 611</td>
<td>221 354</td>
<td>181 300</td>
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<td>584 354 906</td>
<td>115 137</td>
<td>78 633</td>
<td>195 852</td>
<td>929 166</td>
<td>702 873</td>
<td>1 342 886</td>
<td>674 585</td>
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<td>EUR</td>
<td>889 169 869</td>
<td>55 688</td>
<td>44 905</td>
<td>76 173</td>
<td>322 310</td>
<td>236 661</td>
<td>597 14</td>
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<td>NEAR</td>
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<td>321 214</td>
<td>804 372</td>
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<td>11 955 750</td>
<td>9 607 465</td>
<td>13 367 187</td>
<td>9 369 038</td>
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</table>

- a Mortality excluding HIV, according to ICD-10.
- b Percentage of incident TB Cases that are HIV-Positive.
- c Estimates are provisional, pending further analyses and data collection in 2010.

Indicates data not available.
The distribution of TB Incidence rates in different countries of the world is depicted in figure 1.

![Fig. 1: Showing the distribution of TB incidence rates in different countries of the world.](image)

**TB Burden In India**\(^{19, 20, 24}\)

India is the highest TB burden country in the world, accounting for 21% of the global incidence and two-thirds of the cases in Southeast Asia. In the year 2008, the incidence of TB was reported to be 1.982 million (1.586 – 2.379 million) with prevalence of 2.186 million (1.044-3.739 million). The prevalence of TB had been estimated at 3.8 million bacillary cases for the year 2000 by an expert group of government of India. About 40% of the Indian population is infected with TB bacillus. The incidence of TB in India is estimated based on the findings of the nationwide annual risk of tuberculosis infection (ARTI) study conducted in 2000-2003.
The national ARTI being 1.5%, the incidence on smear – positive TB cases in the country is estimated as 75 new smear-positive cases per 100,000 populations. Currently, another round of ARTI survey is being carried out throughout the country, the result of which is expected later in 2010. On a national scale the high burden of TB in India is illustrated by the estimation that TB accounts for 17.6% of deaths from communicable disease and for 3.5% of all causes of mortality (WHO 2004). More than 80% of the burden of TB is due to premature death, as measured in terms of disability-adjusted life years (DALYs) lost. The World Health Organization (WHO) estimated TB mortality in India as 276,512(24/100,000 population) in 2008. With Revised National Tuberculosis Control Programme (RNTCP) implementation there is 43% decline in death due to TB in India by 2008 as compared to 1990. It was estimated that TB mortality was over 5 million annually at the beginning of RNTCP. Data from specific surveys, however, suggest that case fatality rates prior to RNTCP were generally greater than 25%. In RNTCP era, case fatality has remained less than 5% for new cases registered under the program.

The estimated burden of TB in India is summarized in Table 2.
Table 2: Shows the estimated burden of TB in India

<table>
<thead>
<tr>
<th>Incidence (2009 WHO estimate)</th>
<th>Number (Millions) (95% CI)</th>
<th>Rate Per 100,000 Persons (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Cases</td>
<td>1.982 (1.6-2.4)</td>
<td>168</td>
</tr>
<tr>
<td>AFB smear – Positive</td>
<td>0.885</td>
<td>75</td>
</tr>
<tr>
<td>Period Prevalence (2000 Gol estimate)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFB Positive</td>
<td>1.7 (1.3-2.1)</td>
<td>165 (126-204)*</td>
</tr>
<tr>
<td>Bacillary*</td>
<td>3.8 (2.8-4.7)</td>
<td>369 (272-457)*</td>
</tr>
<tr>
<td>Prevalence, all cases</td>
<td>4.468</td>
<td>443</td>
</tr>
<tr>
<td>Prevalence, all cases</td>
<td>3.304</td>
<td>283</td>
</tr>
<tr>
<td>Prevalence, all cases</td>
<td>2.186 (1.044-3.739)</td>
<td>185</td>
</tr>
</tbody>
</table>

* Defined as a person with at least one AFB smear positive by Sputum microscopy, or at least one sputum culture positive for M.tuberculosis

+ Prevalence rate calculated from estimated number of person with disease in 2000, divided by 2000 population estimate

**Epidemiological Indices**

Indices or parameters are needed to measure the tuberculosis problem in a community as well as for planning and evaluation of control measures. Indices are also required for international comparison. Following epidemiological indices are generally used.

a) **Prevalence of Infection**

It is the percentage of individuals who show a positive reaction to the standard tuberculin test. When the test is done in defined age-groups, it yields age-specific prevalence which is a far superior indicator than the mere percentage of positive reactors in the total population. It may be mentioned that the interpretation of tuberculin test has become complicated in countries with a high coverage of BCG
vaccination at birth, since most of the vaccines become positive reactors to tuberculin test. This presents a problem in identifying true prevalence of infection. Further cross-sensitivity to atypical mycobacteria, where it occurred has also caused the prevalence to be over-estimated. Despite these limitation, tuberculin testing is widely used for estimating the prevalence of tuberculosis infection in a population.

b) Incidence of infection (Annual Infection Rate)

It is the percentage of population under study who will be newly infected by *Mycobacterium tuberculosis* among the non-infected of the preceding survey during the course of one year. It reflects the annual risk of being infected (or re-infected) in a given community. This parameter is also known as ‘tuberculin conversion’ index, and is considered one of the best indicators for evaluating the tuberculosis problem and its trend. The higher the rate, the greater the problem.

c) Prevalence of disease

It is the percentage of individuals whose sputum is positive for tubercle bacilli on microscopic examination. It is the best available practical index to estimate the number of infectious cases or “case load” in a community.

d) Incidence of new cases

It is the percentage of new tuberculosis cases confirmed by bacteriological examinations per 1,000 population occurring during one year. Although this index would not reflect the case load to be dealt with, it would reveal the trend of the problem in the community, including the impact of control measures.
e) **Prevalence of drug–resistant cases**

It is the prevalence of patient excreting tubercle bacilli resistant to anti-tuberculosis drugs. This index is directly related to chemotherapy.

f) **Mortality rate**

In the past, tuberculosis mortality rate i.e. the number of deaths from tuberculosis every year per 1,000 or 100,000 population was used as an index of the tuberculosis problem in a community. Since the introduction of an effective chemotherapy and preventive measures tuberculosis mortality rate has lost importance as an epidemiological index for measuring the magnitude of the tuberculosis problem.
**HIV AND TB CO-INFECTION**

Globally, the HIV epidemic is worsening the TB situation, increasing the number of TB cases and accelerating the spread of the disease.\textsuperscript{25} Tuberculosis and HIV infection together form a very grave public health hazard. WHO estimates that there are 2.5 million HIV seropositive persons in India. There are reports that prevalence of tuberculosis infection is higher in seropositive persons.\textsuperscript{26} The immune defects produced by HIV influence the natural history of tuberculosis infection. Thus the HIV pandemic has altered both the epidemiology of tuberculosis and the measures for approaches to its control.\textsuperscript{25}

It is also hypothesized that persons infected with the tubercle bacillus are at an increased risk of developing clinical disease if they become infected with HIV. Recently, WHO has given alarming estimates of HIV related tuberculosis is now recognized as one of the most common opportunistic disease among persons seropositive for HIV.\textsuperscript{26}

Tuberculosis prevalence and incidence are increasing in some parts of the world.\textsuperscript{27} There are about 16-20 million tuberculosis cases in the world and nearly 8 million cases are added each year. About 22 million people are currently suffering from HIV/AIDS. Nearly 85\% of HIV infections are now taking place in the developing world, where one third of population is already infected with *Mycobacterium tuberculosis*, exposing these people to a higher risk of developing tuberculosis.\textsuperscript{27,28}

The Joint United Nations Programme on HIV (UNAIDS) estimates that of the global total of 39.4 million people living with HIV 70.1\% are in sub-Saharan African and 16.1\% are in South East Asia. Unfortunately these are parts of the world where tuberculosis has been flourishing unhindered since ages, forming a deadly synergy of
HIV and tuberculosis. About one-third of those living with HIV world-wide are co-infected with *M. tuberculosis*. In 1990 it was estimated that HIV contributed 4% to the total tuberculosis cases i.e. (3,00,000) in the world and this estimate may rise to 14% i.e. 1.4 million cases per year by 2000 AD if both HIV and tuberculosis are not tackled effectively.\(^\text{29}\)

Estimated HIV prevalence in new TB cases as seen in 2008 is shown in figure 2. There were an estimated 11.1 million (range, 9.6-13.3 million) prevalence cases of TB in 2008 equivalent to 164 cases per 100,000 population. In that year, an estimated 1.3 million (range, 1.1-1.7 million) deaths, including 0.5 million (range, 0.45-0.62 million) deaths among women, occurred among HIV negative incident cases of TB. This is equivalent to 20 deaths per 100,000 populations. There were an estimated 0.5 million deaths among incident TB cases who were HIV positive; these deaths are classified HIV deaths in the 10\(^\text{th}\) revision of the international statistical classification of diseases (ICD-10). The number of TB deaths per 100,000 populations among HIV-negative people plus the estimated TB deaths among HIV-positive people equates to a best estimate of 28 deaths per 100,000 populations.\(^\text{20,28,29}\)
Fig. 2 Shows estimated HIV Prevalence in New TB Cases as seen in 2008

In India, about 5.1 million people are infected with HIV; about half of them are co-infected with *M. tuberculosis*, approximately 0.2 million of these co-infected persons will develop active tuberculosis each year in association with HIV infection. Persons co-infected with *M. tuberculosis* and HIV have 5% - 8% annual risk and 30% or greater lifetime risk of developing active tuberculosis.\textsuperscript{20,25,26,29}
DRUG RESISTANT TUBERCULOSIS

Tuberculosis was declared a “Global Emergency” by the World Health Organization (WHO) on World TB Day in 1993. This unprecedented declaration was prompted predominantly by two developments; the resurgence of TB in the West from the mid 80s, where the disease had been showing a steady decline from the beginning of the century and a number of outbreaks of multi drug resistant tuberculosis (MDR-TB) in many parts of the world, in the late 1980s and early 1990s.30

Drug- resistant tuberculosis is not a new phenomenon. It has been reported since the early days of introduction of anti-TB chemotherapy, but multi-drug resistant tuberculosis (MDR-TB) and more recently extensively drug-resistant tuberculosis (XDR-TB) has been areas of growing concern and are posing a threat to global efforts of tuberculosis control.31,32 Prevalence of drug resistant TB mirrors the functional state and efficacy of TB control programme and realistic attitude of the community towards implementation of such programs. Poor TB control generates MDR-TB and the misuse of second-line drugs generates XDR-TB. More than 400,000 cases of MDR-TB emerge every year as a result of poor management of drug sensitive as well as drug-resistant TB. Directly Observed Treatment Short-Course (DOTS) plus proposed by the World Health Organization highlighted the comprehensive management strategy to control MDR-TB.32,33

In 2006, XDR-TB was reported in all regions of the World and it has become a serious emerging threat to global public health especially in countries with a high prevalence of Human Immunodeficiency virus (HIV). XDR-TB has raised the possibility that the current drug susceptible TB will be replaced with a form of TB with severely restricted treatment options.5,32,33
• Definition of Drug Resistance

Drug resistance in Mycobacteria is defined as a decrease in sensitivity to a sufficient degree to be reasonably certain that the strain concerned is different from a sample of wild strains of human type that have never come in contact with the drugs.

Types of Drug Resistance

Epidemiologically drug resistance in tuberculosis is broadly classified in two categories.

1) Primary Drug Resistance

It is defined as drug resistance in a patient who has never received anti-tubercular treatment in the past or received for less than one month. It is caused by infection with organisms from another patient excreting drug resistant organisms. Primary drug resistance is said to be an indicator of tuberculosis control efforts in the past.

2) Acquired Drug Resistance

It is defined as resistance that develops in a patient during the course of treatment or who has received prior chemotherapy. The level of acquired resistance is a measure of on-going TB control measures. Recently, the terms “resistance in new cases” and “resistance in Previously treated cases” have been proposed by WHO & IUATLD for use because of the difficulty to confirm the validity of the patients past history of treatment.

When one is not sure whether the resistance is primary or acquired due to concealed history of previous treatment or unawareness of treatment taken before, it is known as
**Initial Drug Resistance.** Thus initial resistance is primary resistance plus some undisclosed acquired resistance.

Further, a drug resistance isolate can be categorized as single or multiple drug resistant.

- **Multi-Drug Resistant TB (MDR-TB)**

It is defined as resistance to at least two most potent anti-TB drugs viz Isoniazid (H) and Rifampicin (R) with or without resistance to other anti-TB drugs.

- **Extensively Drug Resistant TB (XDR-TB)**

It is defined as resistance to at least Isoniazid and Rifampicin (MDR-TB) as well as further resistance to any of the fluoroquinolones and any of the three second line injectable agent (Kanamycin, Amikacin or Capreomycin).

♀ **Anti Tubercular Drugs**\(^{35,36}\)

Although the tubercle bacilli was discovered over a century back (1882, Robert Koch) drugs have been available for not more than 70 years. In the 19\(^{th}\) century, bed rest and change in environment emerged as important forms of treatment.

Chemotherapy for tuberculosis became possible only after the discovery of streptomycin in 1944. Subsequently introduction of Isoniazid and Pyrazinamide in 1952 formed the basis of primary chemotherapy of TB in 1950s to 1960s. Discovery of Rifampicin in 1959 had a very important role in shortening the total duration of treatment of TB.

Fluoroquinolones, newer macrolides, congeners of rifampicin etc are added anti mycobacterial drugs available in the therapeutic armamentarium of TB.
According to their clinical utility, the anti-TB drugs can be divided into

1) First line drugs  2) Second line drugs

1) **First Line Drugs**

These drugs have the greatest level of efficacy with an acceptable degree of toxicity.

These include

1) Isoniazid

2) Rifampicin

3) Ethambutol

4) Streptomycin

5) Pyrazinamide

2) **Second Line Drugs**

These drugs have either low level of efficacy or high toxicity or both. These include

- Aminoglycosides (Kanamycin, Amikacin)
- Capreomycin
- Ethionamide
- Cycloserine
- Para aminosalicylic acid
- Thiacetazone
Mechanism of Drug Resistance\textsuperscript{30,33,34}

Drug resistance in \textit{M.tuberculosis} arises exclusively by random, single step or multistep spontaneous mutation at a low but predictable frequency in large bacterial population and not by adaptation after exposure to the drug. In the tubercle bacilli the sites of resistance are chromosomally located and are not plasmid borne.

Resistance of \textit{M.tuberculosis} to anti-tubercular drugs is man made amplification of natural phenomenon. \textit{M.tuberculosis} that has never been exposed to anti-tubercular drugs is almost never resistant, though natural resistance to specific drugs has been documented for \textit{M.bovis} (pyrazinamide).

During bacterial multiplication, resistance develops through spontaneous mutation and with a frequency that has been defined. Mutations resulting in resistance of \textit{M.tuberculosis} to rifampicin occur at a rate of $10^{10}$ per cell division and lead to an estimated resistance prevalence of 1 in $10^8$ bacilli in drug free environment. The rate for Isoniazid is approximately $10^7$ to $10^9$, resulting in resistance in 1 in $10^6$ bacilli. Bacillary populations greater than $10^7$ are common in lung cavities in infected patients thus resistant organisms or mutants evolve in the absence of antimicrobial exposure, but they are diluted within the majority of drug susceptible mycobacteria. The antimicrobials provide the selective pressure which favours a resistant cell which then multiplies to become predominant, especially in patients with a large load of bacilli like those with extensive cavitation disease.

Exposure to a single drug suppresses the growth of bacilli susceptible to that drug but permits the multiplication of drug resistant organisms hence monotherapy is not recommended in the treatment of TB. This phenomenon is called acquired resistance. Subsequent transmission of such bacilli to other persons may lead to disease which is
drug resistant from the outset, a phenomenon known as primary resistance. Every
drug active against *M. tuberculosis* is bound to select for resistance. Multiple drug
resistance due to spontaneously occurring mutations is virtually impossible, since
there is no single gene involved in such a process and mutations resulting in resistance
to the various different classes of drugs are genetically unlinked.

For example, likelihood of spontaneous mutations resulting in resistance to both
isoniazid and rifampicin is the product of individual probabilities i.e., 1 in $10^{14}$. This
is in fact one of the essential reasons for the use of multiple regimens in the treatment
of tuberculosis.
<table>
<thead>
<tr>
<th>Drugs</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid</td>
<td>It is a prodrug. Mycobacterial catalase-peroxidase converts Isoniazid into an active metabolite. Inhibits biosynthesis of mycolic acid-long branched lipids.</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Inhibits DNA-dependent RNA polymerase in bacterial cells by binding its beta subunit, thus preventing transcription to RNA and subsequent translation to proteins.</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>Bacteriostatic drug. Exact mechanism of action is still not known. Several hypothesis indicate more than one target for the drug. Inhibition of RNA metabolism, spermidine biosynthesis, phospholipids biosynthesis, biosynthesis of arabinogalactan are some of the proposed mechanisms.</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>Inhibits mycolic acid synthesis.</td>
</tr>
<tr>
<td>Kanamycin /</td>
<td>Inhibits protein synthesis by tightly binding to the conserved A site of 16 S rRNA in the 30S ribosomal subunit.</td>
</tr>
<tr>
<td>Capreomycin</td>
<td></td>
</tr>
<tr>
<td>Cycloserine</td>
<td>Inhibits reactions in which D-alanine is involved in bacterial cell wall synthesis.</td>
</tr>
<tr>
<td>Thiacetazone</td>
<td>Inhibits cyclopropane mycolic acid synthases (CMASs) as its cellular targets in mycobacteria.</td>
</tr>
<tr>
<td>Fluroquinolones</td>
<td>Exert their effects by trapping a DNA drug enzyme complex and specifically inhibiting ATP-dependent enzymes topoisomerase II (DNA gyrase) and topoisomerase IV.</td>
</tr>
</tbody>
</table>
**Causes of Drug Resistance**

Drugs resistance in TB has microbial, clinical and programmatic causes. From a microbiological perspective the resistance is caused by a genetic mutation that makes a drug ineffective against the mutant bacilli. An inadequate or poorly administered treatment regimen allows drug resistant mutants to become the dormant strain in a patient infected with TB. However it should be stressed that MDR-TB is a man made phenomenon poor treatment, poor drugs and poor adherence lead to the development of MDR-TB. Mismanagement of MDR-TB with erratic use of second-line drugs may lead to development of XDR-TB Development of drug resistance may result from

- **Inappropriate Treatment regimens**

  Inappropriate combination of drugs, choice of drugs, inadequate dosage, inadequate duration of treatment.

- **Program Related factors**

  Inadequate guidelines, Non- compliance with guidelines, inadequate training of health staff irregular drug supply. No monitoring of treatment.

- **Patient Factors**

  Non-adherence to the prescribed regimen, lack of information, malabsorption, socioeconomic status.
Different Bacterial Population of *Mycobacterium tuberculosis*

Mycobacteria present in a tuberculosis lesion have presence of following different populations.\(^{37-40}\)

- **Rapidly Growing Bacilli**
  The first population is the actively growing extracellular organisms in pulmonary cavities within liquefied caseous debris and on the cavity wall where there is abundant oxygen supply and a neutral PH. It is in this Population where drug that is most effective in this population is **Isoniazid** and the drugs which also contribute are **Rifampicin and Streptomycin**, where the latter is most active in an alkaline medium present in an extracellular environment.

- **Slowly Growing Bacilli**
  This population is usually present in an acid environment, inside the macrophages areas of inflammation, and in the early phase of the disease. **Pyrazinamide** is bactericidal against these intracellular organisms and works well in an acidic pH. It is the most effective drug on these bacilli, but **Rifampicin and Isoniazid** also contribute and have bactericidal activity against intracellular bacilli.

- **Semi- Dormant Bacilli**
  These are the bacilli which usually remain dormant but have short spells of metabolism. During dormancy phase, no drug is effective but during their spurts of metabolism **Rifampicin** is the drug which is most effective against these bacilli.

- **Dormant Bacilli**
  Mitchison D\(^{39}\) also postulated there to be a population D consisting of dormant, non replicating organisms that were not vulnerable to antimicrobial action. These bacilli
were held responsible for relapses, the assumption being that the microbes were reconstituted as pathogenic after medication were with drawn. However, in the updated model, this population has been deleted, leaving a three population model.

Thus, **Rifampicin** is the only drug that is bactericidal against most kinds of bacterial populations as **Isoniazid** too is also effective against many of these. It has been estimated that the population of slowly growing organisms in macrophages and caseous lesions is less than $10^5$, whereas in cavities the rapidly growing population numbers $10^7$-$10^9$ organisms.

- **Case Definitions**

  - **New Case:** A patient who has never had treatment for tuberculosis or has taken anti-tuberculosis drugs for less than one month.

  - **Relapse:** A patient declared cured of TB by a physician, but who reports back to the health service and is found to be bacteriologically positive.

  - **Treatment After Default:** A patient who received anti-tuberculosis treatment for one month or more from any source and who returns to treatment after having defaulted, i.e., not taken anti-TB drugs consecutively for two months or more.

  - **Treatment Failure:** A smear-positive patient who is smear-positive at 5 months or more after starting treatment. Failure also includes a patient who was initially smear-negative but who becomes smear-positive during treatment.

  - **Chronic:** A patient who remains smear-positive after completing a retreatment regimen.
MDR-TB: Global

A review by WHO of a series of 63 surveys of drug-resistant TB carried out between 1985 and 1994 led to the conclusion that the problem of drug resistance was global. The rate of MDR-TB was very low in most of the surveys ranging from 0-10.8% in the case of primary resistance and from 0-48% for acquired resistance. Multi-drug resistance was reported to range from 0.5-14.3% in surveys where there was no distinction between primary and acquired resistance. In most regions of the world, the rates of MDR-TB were very low, except in New York and Nepal where high rates of acquired type MDR-TB were reported. It is evident that prevalence of drug resistant TB varied considerably throughout the world. The reason for this variation in different surveys were the degree of selection of the patient studied, the degree of misuse of drugs, the quality of enquiry regarding previous treatment and the inadequate culture and drug susceptibility facilities in many parts of the world.

- Considering the limitation of previous studies, a WHO / IUATLD global project of drug resistance surveillance spread over 35 countries in five continents was carried out between 1994 and 1997. Median prevalence of primary and acquired multi-drug resistance was reported to be 1.4% (0-14.4%) and 13% (0-54.4%) respectively, particularly high prevalence of multi-drug resistance was found in the former Soviet Union, Asia, Argentina and the Dominican Republic. The WHO for the first time introduced the term MDR “hotspot” where high prevalence of MDR has been observed. The “hotspots” referred to the countries or regions where the combined prevalence of MDR-TB exceeded 5%.
• A second WHO / IUATLD Global Project on drug resistance surveillance in 58 countries geographical sites was carried out in 1996-1999.\textsuperscript{44,45} Median prevalence of primary and acquired multi-drug resistance was reported in 1% (0-14%) and 9% (0-48%) respectively. Most of the Previous “hotspots” of MDR-TB were confirmed again however new areas in Russia and China were added.

• A third WHO/ IUATLD global project on drug resistance surveillance in 77 Countries / geographical sites was carried out in 1999-2002\textsuperscript{46} representing 20% of the global total new smear positive TB cases. Median prevalence of primary and acquired multi drug resistance was reported in 1.1% (0-14.2%) and 7% (0-58.3%) respectively. There were ten counties regarded as hotspots with MDR-TB Prevalence viz. Ecuador (6.6%), Henan (7.8%), Latvia (9.3%), Lithuania (9.4%), Liaoning (10.4%), Estonia (12.2%), Uzbekistan (13.2%), Tomsk Oblast (13.7%), Israel (14.2%) and Kazakhstan (14.2%). Three countries China, India and the Russian Federation accounted for 62% of the estimated global burden.

• A fourth WHO/ IUATLD global project on drug resistance surveillance included Drug Susceptibility Test Results (DST) from 91,577 Patients from 93 settings in 81 countries and 2 special Administrative Regions (SARs) of China collected between 2002 and 2007, and representing 35% of the global total of notified new smear positive TB cases.\textsuperscript{47} It included data from 33 countries that have never been previously reported. New data were included from the high TB burden countries like India, China, Russian Federation, Indonesia, Ethiopia, Philippines, Vietnam, Thailand and Myanmar.
• The median prevalence of primary and acquired MDR-TB globally was 2.9% (2.2-3.6%) and 15.3% (9.6-21 1%) respectively. It is estimated that 489,139 MDR-TB cases emerged in 2006 globally and the global proportion of multi-drug resistance among all new and previously treated cases was 4.8%.

China and India carry approximately 50% of the global burden and Russia a further 7%.

MDR-TB: India

Development of drug resistance in India was noted since the beginning of the chemotherapeutic era. Indian Council & Medical Research (ICMR) conducted two surveys in 1965-67 to estimate the prevalence of drug resistance.\textsuperscript{48, 49} Since then several studies have been conducted in different parts of the country. Multi-drug resistance among new cases varied between 0-5%\textsuperscript{51-62} A study conducted by Tuberculosis Research Centre, Chennai in collaboration with the National Tuberculosis Institute Bangalore using WHO/IUATLD guideline between 1999-2002 including six districts in India showed that primary MDR-TB ranged from 0.7-2.8%\textsuperscript{58-62} According to the third global reporting WHO prevalence of MDR-TB among new cases in India (Wardha) was 0.5%.\textsuperscript{46}

The prevalence of acquired Multi-drug resistance rates also varied from 6-100%\textsuperscript{56, 60, 63-67} in the same time period. In a study conducted in Gujarat\textsuperscript{51} it was found that 95% of the rifampicin resistant strains were also resistant to isoniazid or streptomycin both.

The WHO/IUATLD Surveillance in India reported the combined prevalence of MDR-TB to be 13.3% in 1998; however this was done on a small sample of 2,240 people around Delhi city and therefore was not representative of the country.\textsuperscript{43}
The WHO/IUATLD global drug resistance surveillance carried out between 1969-1999 reported that the median prevalence of primary and acquired MDR-TB to be 3.4% and 25% respectively\(^4\). Zignol et al\(^68\) estimated the incidence of primary and acquired MDR-TB cases to be 2.4% and 14.7% respectively. The estimated incidence of MDR-TB among new and previously treated cases was 4.1% of all the TB cases in India.

The fourth global surveillance of WHO/IUATLD carried out in 2002-2007\(^47\) also reported new data from Gujarat giving the first reliable source of data with regard to MDR-TB among previously treated cases in India. Data from nine sites in India show that drug resistance among new cases is relatively low; however new data from Gujarat indicate that 17.2% MDR-TB among retreatment cases is higher than previously anticipated and it is estimated that 110,132 MDR-TB cases emerged in India in 2006, representing over 20% of the global burden. The estimated prevalence of MDR-TB among all TB cases was 4.9%.\(^13\) M/XDR-TB 2010 global report on surveillance and response estimated MDR-TB among new TB cases as 2.3% and in previously treated patients at 17.2%. It is estimated that 99,000 cases of MDR-TB emerged in India in 2008.

- **XDR-TB: Global**

Although limited data exist in the literature about XDR-TB and second-line drug resistance patients among MDR-TB patients; Centers for Disease Control and Prevention, USA. surveyed the Network of Supranational Reference Laboratories for \textit{M.tuberculosis} isolates that were resistant to second line anti-TB drugs during 2000-2004.\(^69\) As of January 2010, a cumulative total of 58 countries has confirmed at least one case of XDR-TB. \textbf{Around 5.4% of MDR-TB cases were found to have XDR-TB globally.}\(^70\)
❖ XDR-TB: India

Although isolated reports both published and unpublished indicate the existence of XDR-TB in the country, it is not possible as yet to estimate its magnitude and distribution from the available data. According to the data reported on XDR-TB from India, it varied from 1.5-11%\(^{71-74}\) of MDR-TB. M/XDR-TB 2010 global report on surveillance and response estimated the prevalence of XDR among MDR-TB patients as 3.2\%.\(^{70}\)

❖ THE MYCOBACTERIA

In the future it will not be difficult to decide what tuberculosis is and what is not….The demonstration of tubercle bacilli….will settle the question.

Robert Koch\(^{75}\)

- Taxonomy and Nomenclature\(^{76-78}\)

The genus *Mycobacterium* (Lehmann and Neumann, 1896) has been placed in the family ‘*Mycobacteriaceae*’ in the Section 16 ‘Mycobacteria’ in the Bergy’s manual of systematic Bacteriology, 9\(^{th}\) edition (Sneath et al 1986).

The genus *Mycobacterium* is currently the only genus in this family. The family *Mycobacteriaceae* along with other seven families of the order *Actinomycetales* was placed in the part 17: “*Actinomycetes* and related organisms” in the Bergey’s manual of Determinative Bacteriology, 8\(^{th}\) edition (Buchanan and Gibbons, 1974)

The recently published 9\(^{th}\) edition of the manual (Sneath et al; 1986) has been presented as various ‘sections’ based on a few readily determinable criteria. Each section bears a vernacular name. All accepted genera have been placed in what seems
the most appropriate section, although allocation of certain genera has presented difficulties for example *Corynebacterium* (section 15),

*Mycobacterium*(section 16), *Nocardia* and *Rhodococcus*(section 17) share many chemotaxonomic properties, which could justify their placement in one grouping. However, for determinative purposes, they have been arranged as noted above.

**Classification**

Robert Koch described the tubercle bacilli in 1882. Before the end of the 19th century, the bovine, avian, reptilian, piscine and saprophytic varieties of mycobacteria had also been described. Despite sporadic reports of isolation of many varieties of non-tuberculosis mycobacteria from clinical specimens, only *Mycobacterium tuberculosis* and *Mycobacterium bovis* were taken seriously as a cause of human disease while other isolates were given dismissive epithets such as “atypical”, “pseudotuberculosis”, and “tuberculoid” bacilli. As their classification was in chaos, they were dubbed ‘Anonymous Mycobacteria”. Interest in their role as human pathogens commenced in the 1950s with the description of two distinct diseases namely, “swimming pool granuloma” and “Buruli ulcer” caused by *Mycobacterium marinum* and *Mycobacterium ulcerans* respectively and the demonstration of their aetiological role in the tuberculosis like pulmonary disease.

The “atypical” mycobacteria isolated from clinical material have been referred to by many names such as “paratubercle”, “pseudotubercle”, “anonymous”, “opportunistic”, “nontuberculous”, “tuberculoid” and “Mycobacteria other than tubercle bacilli (MOTT). The most appropriate and least offensive name for these organisms seems to be Non- tuberculous Mycobacteria (NTM). Runyon classified
these Mycobacteria on the basis of type of colony, rate of growth, pigment production and biochemical reactions into four groups: 

1) Photochromogens

2) Scotochromogens

3) Non-Photochromogens

4) Rapid Growers

Currently there are more than 100 recognized or proposed species in the genus ‘Mycobacterium.’ These species produce a spectrum of infections in humans and animals ranging from localized lesions to disseminated disease. Although some species cause only human infections, others have been isolated from a wide variety of animals. Many species are also found in water and soil.

For the most part, mycobacteria can be divided into two major groups based on fundamental differences in epidemiology and association with disease; those belonging to the *Mycobacterium tuberculosis complex* and those referred to as *Non-tuberculous mycobacteria (NTM).*

*Mycobacterium Tuberculosis Complex*

- *M. tuberculosis*
- *M. bovis*
- *M. africanum*
- *M. microti*
Non Tuberculous Mycobacteria

- Slow growing nonphotochromogens
  - *M. avium complex*
  - *M. ulcerans*
  - *M. gastri*
  - *M. genavense*
  - *M. haemophilum*
  - *M. malmoense*
  - *M. shimoidei*
  - *M. simiae*
  - *M. xenopi*
  - *M. terrae complex*
  - *M. branderi*
  - *M. triplex*
  - *M. celatum*
  - *M. heidelbergense*
  - *M. conspicuum*

- Photochromogens
  - *M. kansasii*
  - *M. asiaticum*
  - *M. marinum*
  - *M. intermedium*
- **Scotochromogens**
  - *M. szulgai*
  - *M. scrofulaceum*
  - *M. gordonae*
  - *M. ulcerans*
  - *M. interjectum*
  - *M. cookii*
  - *M. hiberniae*
  - *M. lentiflavum*
  - *M. conspicuum*
  - *M. heckeshornense*
  - *M. tusciae*
  - *M. kubicae*
  - *M. bohemicum*

- **Rapid Growers**
  - *M. fortuitum*
  - *M. chelonae*
  - *M. abscessus*
  - *M. smegmatis*
  - *M. peregrinum*
  - *M. immunogenum*
  - *M. mucogenicum*
  - *M. wolinskyi*
M. goodii
M. septicum
M. alvei
M. novocastrense
M. mageritense
M. canariasense

- Rarely Pathogenic or Not Yet Associated with Infections

M. agri
M. thermoresistible
M. vaccae
M. phlei
M. rhodesiae
M. sphagni
M. murale
M. obuense
M. parafortuitum
M. flavescens
M. gadium
M. gilvum
M. fallax
M. hassiacum
M. brumae
M. chitae
M. chubuense

M. duvalii

- Noncultivable Mycobacteria

M. leprae

Characteristic Features

Mycobacteria are slender bacilli and sometimes exhibit filamentous and branching forms resembling fungal mycelium (from Greek, Myces meaning fungus). Hence they are so named meaning ‘fungus like bacterium.’

Members belonging to the genus ‘Mycobacterium’ show following characteristics

- The mycobacterium due to their lipid rich waxy cell wall are difficult to stain with commonly used basic aniline dyes such as gram stain, but once stained, they resist decolourisation with acid and alcohol. They are therefore called ‘Acid-alcohol fast bacilli’ (AFB).
- Most mycobacteria are slow-growers, the generation time being 2-24 hrs. These bacteria require 4-8 weeks of incubation to produce demonstrable colonies on the solid media.
- They contain mycolic acids which consist of 60-90 carbon atoms which are cleaved to C-22, C-26 fatty acid methyl esters by pyrolysis.
- They contain a high (61-71%) guanine and cytosine content in the DNA.

Mycobacterium tuberculosis complex

Originally, this group included the human and the bovine tubercle bacillus, but in 1970, the latter was given the separate specific name ‘Mycobacterium bovis’, the other species within this group are the vole tubercle bacillus of voles named
‘Mycobacterium microti’ and a heterogeneous group of strains isolated in the equatorial Africa and termed ‘Mycobacterium africanum’.

These species have been shown to be very closely related by the antigenic analysis, DNA hybridization studies and numerical taxonomy. All members of this group are obligate pathogens and have not been shown to replicate outside the animal or human body. They are strict mesophiles, showing little or no growth below 30°C and above 39°C. This temperature range of growth, together with lack of pigment, slow growth rate and a failure to grow on media containing p-nitro benzoic acid 500mg/l enables this group to be distinguished from other mycobacteria.

- **Mycobacterium tuberculosis**
  It grows well on Lowenstein-Jensen (LJ) medium (eugonic growth). It is aerobic, reduce nitrate to nitrite, usually produce large amounts of niacin and unless resistant to isoniazid, have strong heat resistant catalase activity.

- **Mycobacterium bovis**
  It grows poorly on Lowenstein–Jensen medium (dysgonic growth) but is enhanced by replacing glycerol by pyruvate. It is microaerophilic resistant to pyrazinamide, sensitive to TCH and does not produce niacin or reduce nitrate to nitrite.

- **Mycobacterium microti**
  Originally isolated from the vole, properties of *M. microti* are intermediate between *Mycobacterium tuberculosis* and *Mycobacterium bovis*.

- **Mycobacterium africanum**
  It is a heterogeneous group of strains, intermediate in properties to *M. tuberculosis* and *M. bovis*. Contrary to what its nomenclature might suggest the species is not
restricted to Africa and accounts for almost 1.5% cases of tuberculosis is South–East England.

**Mycobacterium Tuberculosis**\(^{75,81,82}\)

Robert Koch isolated *Mycobacterium tuberculosis* and proved its role in the causation of Tuberculosis by satisfying Koch’s postulates.

- **Koch’s postulates:**
  - The bacterium should be constantly associated with the lesions of the disease.
  - It should be possible to isolate the bacterium in pure culture from the lesions.
  - Inoculation of such pure culture into suitable laboratory animal should reproduce the lesions of the disease.
  - It should be possible to reisolate the bacterium in pure culture from the lesions produced in the experimental animals.

- **Habitat**
  According to habitat, the Mycobacterium species are grouped as below

1) **Obligate Mycobacteria**

Mycobacteria that multiples only inside the living host, e.g *M.tuberculosis, M.bovis, M.asiaticium, M.farcinogenes, M. haemopichum* etc.

2) **Facultative Mycobacteria**

The mycobacterial species can multiply outside the living host and can cause infection in animals or humans e.g *M aviun, M.chelonae, M. fortuitum, M.kanssii, M.marimum* etc.
3) **Saprophytic Mycobacteria**

Free living Mycobacterium that multiplies in soil, water, marshyland and estuaries without causing disease in humans and animals. e.g. *M. gastri*, *M. gordonae*, *M. terrain*, *M. triviate* etc.

4) **Opportunistic Mycobacteria**

The *Mycobacteria* normally present in soil, dead and decaying material and water and may occasionally cause infection in humans e.g *M. kansasii*, *M. scrofulaceum* etc.

- **Morphology :-**

  *M. tuberculosis* is slender, straight or slightly curved, rod shaped organisms, measuring 2-4um in length and 0.2-0.6 um in breadth, non-motile, non-sporing, weakly Gram+ve aerobic or microaerophilic occur singly, in pairs or in clumps. Branching may be sometimes evident. Filamentous or mycelium like growth may occur but it easily fragments into rods or coccoid elements.

- **Staining Reactions**

Mycobacteria are not readily stained by the Gram method as the high lipid content of the cell wall excludes the usual aniline dyes. Special staining procedures are therefore required to promote the uptake of a strong dye. Once the mycobacteria have been stained, they are not easily decolorized even with acid-alcohol. This resistance to decolourisation is called **acid-fastness**.

The property of acid-fastness is not absolute and may be partly or completely lost at some stage of growth by some proportion of the cells of some species of mycobacteria. In 1882, Robert Koch stained the tubercle bacilli with hot **alkaline**

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methylene blue as the primary stain and Vesuvin as the decolouriser and counterstain. Ehrlich discovered the ‘acid-fast’ property of the bacteria in 1882 and stained the bacilli with hot fuchsin in the presence of aniline oil as a mordant, destaining with a dilute mineral oil. In 1883, Ziehl changed the mordant to phenol and Neelsen, in 1884, combined the dye and mordant to form carbol fuchsin. Although pioneered by Ehrlich, the acid-fast staining technique is known as the “Ziehl-Neelsen method” and has remained essentially unchanged since 1884. Cold staining techniques by Kinyoun (1915) as well as the use of fluorescent dyes to visualize the Mycobacteria have also been developed.

- **Mycobacterial Envelope**

The cell envelope essentially distinguishes the Mycobacteria from other prokaryotes. Mycobacteria in general give a weakly positive response to the Gram stain but are phylogenetically more closely related to Gram-positive bacteria. However, similarity to Gram-ve bacteria includes the paucity of peptidoglycan and the fact the mycobacterial cell wall contains mesodeaminopimelic acid (DAP) and outer lipid bilayer.

Since *Mycobacterium tuberculosis* is one of the few species of bacteria able to survive inside the phagocytic cells of the host, it is likely that its envelope has special properties defending the bacteria against host microbicidal processes.

The envelope consists of two distinct parts: the plasma membrane and around it the cell wall. The plasma membrane provides osmotic protection and facilitates transport of ions and molecules. The cell wall provides mechanical support.
1. **Plasma Membrane**

Plasma membrane of mycobacteria appears as classic bilayers with two electron dense layers separated by a transparent layer in ultra thin sections. Mycobacterial membranes have same distinctive components, notably the lipopolysaccharides, lipoarabinomannan (LAM) and phosphatidylinositol mannosides.

2. **Cell Wall**

The electron microscopy revealed mycobacteria to possess a relatively thick cell wall. The cell wall is about 20 nm thick and appears to be separated from the cell membrane by a periplasmic space of 3 to 10 nm across. The cell wall is complex. It consists of lipids, proteins and polysaccharides. The lipids account for as much as 60% of the dry weight of the cell. The cell wall is made up of four distinct layers.

- **Peptidoglycan (Murein) Layer.**
  It is the innermost layer which maintains the shape and rigidity of the cell. It is composed of N-acetylglucosamine in β-1,4 linkage to N-glycolylmuramic acid. The murein layer is cross linked by tetrapeptide bridges containing L-alanine, D-glutamate, diaminopimelate and D-alanine. Some of the N-glycolylmuramic acid residues are linked by phosphodiester bonds to an overlying layer of branched chain, polysaccharide macromolecules called Arabinogalactans.

- **Arabinogalactan Layer.**
  It lies external to the peptidoglycan layer. The distal arabinose residues of this layer are linked to the overlying mycolic acid.

- **Mycolic Acid Layer**
  It is the principal constituent of the cell wall. Mycolic acids are large α - substituted β-hydroxy fatty acids, attached by ester bonds to the terminal arabinose units of
arabinogalactan. In *M. tuberculosis*, the unique mycolic acid 6,6’ – dimycolyltrehalose is known as **CORD FACTOR**. This molecule is associated with virulence of *M. tuberculosis* and has a wide range of biologic activities including cell membrane cytotoxicity, inhibition of granuloma formation, adjuvanticity, anti tumor activity and ability to activate the alternative complement pathway.

- **Mycosides**

  This is the outermost layer of ribbon like fibrils which extend into the matrix of the intermediate layer. Mycosides are superficially situated peptidoglycolipids which consist of fatty acids linked to an oligopeptide to which various sugar groups are attached. They are important in colonial morphology, serotyping by agglutination and phage typing. They have a protective role against phagocytosis.

  The mycobacterial cell wall is not only thicker than other bacterial cell wall but also uniquely lipophillic. The principal characteristics of the organisms like acid-fastness, aggregation of the cells, resistance to many bactericidal agents including lytic enzymes by invaded cells, impenetrability of some nutrients and even antibiotics arise from this external barrier only. If this wall is removed they are no more different, from the common gram negative organisms.

- **Nutritional Requirements**

  The mycobacteria vary markedly in their metabolic activities nutritional requirements and rate of growth. The basic nutritional requirements of mycobacteria are carbon, nitrogen, oxygen, phosphorus and sulphur together with metals especially iron and magnesium.
1. **Carbon Sources** :-

Mycobacteria are metabolically very versatile and various species of mycobacterium can grow on a wide range of carbon sources including carbohydrates, organic acid, hydrocarbons and even carbon-dioxide. Laboratory culture medium, traditionally contains glycerol as the carbon source because it is the only carbon source that can be utilized by all species of mycobacterium although it is not the optimal carbon source for many species. Pyruvate is a better carbon source for many species, various sugars such as glucose, fructose, sucrose, mannose, inositol and mannitol can also serve as preferred carbon sources. Oleic acid is a useful carbon source but is toxic when present in high concentration, this can be avoided by complexing the acid with albumin (Dubos and Middle brook 1947) from which it is slowly released.

2. **Nitrogen Sources**

Nitrogen source is essential for mycobacterial growth and is obtainable from inorganic sources including ammonium, nitrate and nitrite ions or organic nitrogenous compounds, including a wide range of amino acids, amides, amines, purines and pyrimidines. Ammonium ions afford one of the principal nitrogen sources.

3. **Iron**

This metal is essential for the growth of mycobacteria and requires two classes of compounds synthesized by the bacteria for its uptake, namely mycobactins and exochetins.

4. **Other Elements**

Mycobacteria require several inorganic elements for growth including sodium, potassium, magnesium, sulphur and phosphorous as trace elements. Magnesium is
essential for growth and plays an important role in the maintenance of ribosomal function.

- **Metabolic Pathway**

In general, metabolic activities of mycobacteria are similar to those of other bacteria and basic pathways for assimilation of nutrients, energy production, metabolism and biosynthesis of macromolecules are like those of most bacteria. Of course, mycobacteria do have several unique biochemical activities related to the synthesis of mycobacteria specific compound like mycolic acid or phenolic glycolipid. The reason mycobacteria grow very slowly (generation time of 3 hrs for rapid growers and 18-24hrs for slow growers) are not known. Possible explanations include

- Decreased diffusion or transport of required nutrients across the thick, hydrophobic cell wall.
- Differences in key respiratory enzymes.
- Lower rate of nucleic acid synthesis.
- High energy necessity for the production of thick cell wall lipids.
- Few copies of Ribosomal Ribonucleic acid (rRNA) gene in the mycobacterium cells.

- **TRANSMISSION**

Although tuberculosis can affect any organ of the body, the lung is virtually always the portal of entry. The bacilli are most commonly discharged into the atmosphere by aerosolization of pulmonary secretions by a diseased pulmonary patient in coughing, sneezing and speaking. Aerosol droplets are 2-10 μm in diameter, containing perhaps one to three tubercle bacilli. The surrounding moist material soon dries and thereafter the bacillus remain suspended in the air for long periods and can be inhaled by any
person in the vicinity. The small size of these infectious particles and their tendency not to settle enables them to reach the alveoli and to avoid the mucocilliary escalator. Droplet nuclei carrying tubercle bacilli are produced by patients with active pulmonary tuberculosis in proportion to the liquidity of the secretions and the number of bacilli excreted i.e. they are most numerous in persons with a productive cough and positive sputum smears. The number of organisms in the airborne aerosol also depends on the expulsive force of the cough and the presence of cavitations in the lungs.

Other routes of transmission of tubercle bacilli such as the skin, or through the placenta are uncommon and of no epidemiological significance. In the past, transmission of infection with *Mycobacterium bovis* through consumption of milk from infected cows was common, but this means has been brought under control in all developed countries by elimination of diseased cattle and pasteurization of milk and milk products. Transmission is still prevalent in developing countries due to consumption of unpasteurized milk, poorly heat treated meat and closer contact with infected animals.

However, infections can occur by way of inoculation when bacilli are introduced into or through the skin. Infection from this source is an occupational hazard among laboratory workers who must handle tuberculosis cultures and infected tissues. Fomites such as book, clothes, bedding and eating utensils are not involved in the spread of infection and need no special attention.

Tuberculosis is clearly an airborne disease due to droplet nuclei infection in the majority of patients. The probability of contact with a source case of *M. tuberculosis* infection, the intimacy and duration of contact, the degree of
infectiousness and the environment of the contact are all important determinants of transmission. Sputum smear-positive patients play the greatest role in the spread of infection. Patients with sputum smear negative and culture positive are much less infectious and those with culture negative pulmonary disease and extra-pulmonary tuberculosis are essentially non-infectious. Ability of the bacilli to cause infection in newly exposed contacts depends on the adequacy of innate antibacterial defences of the person. Crowding in poorly ventilated wounds is one of the most important factors in the transmission of tubercle bacilli, since it increases the intensity of contact with a case. Studies have shown that the infection rate among close contacts ranges from 25-50% even in the worst overcrowded and substandard conditions. In 90% of infected persons the organism becomes sequestered in dormant foci and cause no clinical disease. Of the remaining 10% of infected persons, 5% may develop early progressive disease and rest of the 5% may develop late recrudescent disease after several decades of infection. On the basis of epidemiologic data, it appears that exposure generally must be close and sustained, the environment heavily laden with droplet nuclei, and the prospective host unprotected by inborn defences, previously activated immune mechanisms or both if an infection sufficient to produce disease is to be established.

**Persons at Risk for Tuberculosis**

The following high-risk groups, designed by the Centers for Disease Control and Prevention (CDC) Advisory council for the Elimination of tuberculosis should be screened for tuberculosis.

1) Close contacts (those sharing the same household or other enclosed environment) of persons known or suspected to have tuberculosis.
2) Persons infected with HIV

3) Persons who infect illicit drugs or other locally identified high-risk substance users.

4) Persons who have medical risk factors known to increase the chance for disease if infection occurs.

5) Residents and employees of high-risk congregate settings (such as correctional institutions, nursing homes, mental institutions, other long-term residential facilities, and shelters for the homeless.)

6) Health care workers who serve high risk clients.

7) Foreign born persons, including children recently arrived (within 5 years) from countries that have a high incidence or prevalence of tuberculosis.

8) Some medically under served low-income populations.

9) High-risk racial or ethnic minority populations as defined locally.

10) Infants, children and adolescents exposed to adults in high-risk categories.

❖ IMMUNOPATHOGENESIS

Infection with tubercle bacilli evokes Cell Mediated Immunity in 2-8 weeks after infection. It is induced by recruiting and localizing cytokines, Henceforth, progression of disease depends upon the number of bacilli inhaled, native susceptibility and the immunologic response of the host. The deposited bacilli undergo free replication, followed by local accumulation of neutrophils and macrophages that signal initiation of a Delayed Type of Hypersensitivity (DTH) reaction. This is the main line of defence. Activated macrophages and epitheloid histiocytes congregate to form
Granulomas. Several epitheloid cells fuse to form the hallmark Langhans multinucleated giant cells. The organisms become sequestrated in the granuloma as dormant foci, which remain contained and active disease may not occur in the majority of infected persons. Cell-mediated immunity against *M. tuberculosis* evokes development of positive tuberculin skin test.

Droplet nuclei of tubercle bacilli when inhaled reach the alveoli. The alveolar macrophages attack them with the help of lysozyme and phagosymes. These cause phagocytosis and kill tubercle bacilli. However, natural host defense success depends on quantity and virulence of the organism. Thus when virulence and bacillary load are high, the macrophages are unable to control the infection and present the tubercle bacilli to the higher immune cells of the host i.e. the T-lymphocytes,. The CD4 + lymphocytes (helper T cells) and CD8 + lymphocytes (suppressor) cytotoxic T–cells get stimulated by recognition of the MTB antigen coupled with the major histocompatibility complex II (MHC-II) and MHC-I respectively. CD4+ T cells have a major role in the immunopathogenesis.

Th 1 and Th 2 cells are subsets of CD4 + T cells. Either Th 1 Th 2 gets stimulated depending upon the individual genetic make up and antigenic response. Th 1 secretes interleukins (IL-2, IL-12) and interferon gamma which stimulates macrophages. These activated macrophages secrete cytokines, and hydrolytic enzymes which kill bacilli and lead to the development of cell mediated immunity (CMI) However, if Th 2 is stimulated, IL 4 , IL 5, IL 10 and tumour necrosis factor alpha are secreted. These cause caseation necrosis and granuloma formation, which lead to cavities. This is what is known as delayed hypersensitivity (DTH) which has a detrimental effect.
CMI and DTH are two pathological events that are clinically important as CMI can develop due to natural MTB infection or may be induced by BCG vaccination.\textsuperscript{91}

Two to ten weeks are required after infection for the development of CMI. This concept of immunological memory is the basis of BCG vaccination. On the other hand delayed type of hypersensitivity causes caseation, necrosis and walling up of the lesion which sometimes leads to the temporary arrest of the disease which is a part of Koch’s phenomenon. But in most cases active TB can occur once the immunity of the host goes down i.e. activation of the Th 2 immunity due to the dormant bacilli.\textsuperscript{92}

When infection with the tubercle bacillus occurs for the first time, a focus of caseation located near the periphery of the lung is formed known as the Gohn’s focus. The involved draining lymphatics and lymph nodes together with the lung lesion is called the \textbf{Ghon’s complex or the primary complex}. This complex can develop into active disease or may heal by fibrosis, calcification or complete clearing of the lesion. This depends upon the individual’s immunity towards the infection.\textsuperscript{91}

During the formation of the primary complex, CMI is not strong enough to control the infection. The bacilli then spread to the sites of quiescent foci. If these foci remain active, it is known as progressive primary TB. After the first few years of primary infection when TB develops, it is known as post primary pulmonary TB. It is reactivation of quiescent foci. The upper lobe is a common site due to the higher oxygen concentration. This concept makes it clear that adult TB is due to reactivation of TB rather than acute inhalation or re-exposure to MTB.\textsuperscript{91}

The success of the bacillus in infecting a person and the success of the host in eliminating the infecting bacillus is a complex interplay of factors related to the host and those of the bacillus. While bacillary factors such as virulence is important,
numerous observations have been made on the host immunity that may determine the host-agent relationship and the pattern of disease in an individual.\textsuperscript{92}

- **Bacterial Factors:**
  The pathogenesis of any disease is related to several factors of both the infecting organisms and the infecting host. In case of mycobacteria, there is no way by which the organism can directly access the host cells. Hence, the bacilli must i) colonise the surface epithelium. ii) Enter the host cells. iii) Multiply in host tissues. iv) Overcome the host defence. v) Damage host tissues.\textsuperscript{91}

- **Virulence Factors**

  \textit{M.tuberculosis} does not produce any exotoxin and its capsule does not prevent phagocytosis. The damage caused by \textit{Mycobacterium tuberculosis} is therefore a combined effect of toxins and the dysfunction of the immune system. One of the several toxic factors is the lipid fraction isolated from petroleum ether extracts of virulent bacilli known as “\textbf{Cord Factor}”. The cord factor belongs to a family of trehalose 6, 6 dimycolates (TDM). It is known to be extremely toxic for mice due to its effect on the mitochondria.\textsuperscript{91}

  Mycobacteria are also known to inhibit fusion between phagosomes and lysosomes. This appears to be related to the exclusion of adenosine triphosphatase (ATPase) from phagosomal membranes, preventing acidification and arrest of the maturation of the phagosome.\textsuperscript{82,91}

  The capsule of the mycobacterium, especially by virtue of its lipid rich inner compartment provides resistance to host defences. Capsular components contain superoxide dismutase (SOD) and catalase peroxidase (KatG) and these may inactivate reactive oxygen derivatives (hydroxyl ions, hydrogen peroxide and super oxide) as well as nitric oxide produced by the phagocytic cells.\textsuperscript{93}
Capsular components such as the major sulphatide of the tubercle bacilli (SL-1) can inhibit the production of these reactive substances. *Mycobacterium tuberculosis* is also known to modulate immune response through phenolic glycolipids, arabinomannan and phosphatidylinositolmannosides (PIM). Basically, these inhibit lymphoproliferation and change the pattern of cytokine production.\textsuperscript{95}

**Host Factors:**

Factors related to the host form the second pillar along with bacterial factors on which the outcome of infection by mycobacterium rests.\textsuperscript{84,91}

**Age:** Infants and the elderly are vulnerable to tuberculosis. Infants are more vulnerable because the immune system has not attained its proficiency. In old age, a consequence of increased life expectancy, there are several reasons for increased vulnerability. Under-nutrition in the elderly is one of the factors. Further, a deficiency of the antioxidant, glutathione, has been reported among smokers and in old age. Tuberculin reactors passing through adolescence and young adulthood and children in the first five years of life are also at an increased risk.

**Nutritional factors:** Normal growth and development are essential factors that provide “resistance” to infection. Under-nutrition, unbalanced diet due to factors such as ignorance and financial compulsions are problems world wide. Second grade nutrients (fast food) and increased consumption of foods rich in energy but nutritionally sterile (soft drinks, popcorins, sweets, french fries etc) seem to account for an imbalance in those who can otherwise afford a balanced diet. The ill-effects of these nutritional factors may be compounded by factors that cause immune weakness, nicotine, recreational drugs, immunosuppression therapy, and exposure to stressful events.
LABORATORY DIAGNOSIS

“…………Absence of evidence is not Evidence of Absence…. ”

Carl Sagan.

Microbiology laboratory contributes to the diagnosis and management of tuberculosis in
1) Detection and isolation of mycobacteria.
2) Identification of the species of the isolate.
3) Antibiotic susceptibility testing of the isolate.

Tuberculosis ranks high among laboratory acquired infections. Therefore all mycobacterial procedures must be performed in a class II A or II B or Class III biological safety cabinet (BSC).  

- Specimen Collection and Transportation

The correct collection and transportation of samples to the laboratory is essential in ensuring that results are accurate and reliable. Collection of proper clinical specimens requires careful supervision to details by health care professionals. 

In general, all specimens must be collected in clean, sterile, wide-mouth, leak proof, screw tight disposable containers appropriately labeled with 1) patient name 2) hospital number 3) Date and time of collection. Specimens should be transported to the laboratory as early as possible along with complete requisition form and refrigerated if processing is delayed. Because mycobacterial disease may occur at almost any site in the body, a variety of clinical material may be submitted to the laboratory for examination. However, pulmonary tuberculosis being the most
common form of the disease, respiratory secretions is the most common specimen submitted for examination. 97

I) Pulmonary Specimens

Pulmonary specimens may be obtained by any of the following methods. 96, 99

- Spontaneously produced sputum
- Induced sputum
- Transtracheal aspiration
- Bronchio alveolar lavage
- Gastric lavage

1) Spontaneously produced Sputum

Spontaneously produced sputum is the specimen of choice. Early morning sputum obtained shortly after the patients awaken in the morning is considered to be the best specimen as mycobacteria are at their highest concentration. Twenty-four hour collections are now discouraged because the sample containing the highest concentration of mycobacteria will be proportionately diluted by subsequent low-yield samples and the chances for bacterial and fungal overgrowth during the prolonged collection process are significantly increased. 90, 98

Sputum is very frequently contaminated with oropharyngeal flora and the upper respiratory tract secretions, so it should be processed as soon after collection as possible to minimize the degree of overgrowth with contaminants. 96

Good sputum collections are dependent on proper patient education and a complete supervision of all the aspects of the sputum collection. Hence, collecting a good sputum sample requires that the patient be given clear instructions. 97
Three sputum samples, two spot specimens and one early morning sputum is required for the diagnosis of tuberculosis as per RNTCP guidelines.

The sample should be collected during the acute stage of the illness before the administration of antibiotics, as it can reduce the yield.

A highly purulent secretion should be collected as far as possible and the material should be expectorated into a sterile container without any contamination with saliva.

Aerosols containing *M. tuberculosis* may be formed when the patient coughs to produce a specimen. Thus the sputum sample is collected outside in the open air or away from other people and not in confined spaces such as a room, in the laboratory or toilets.

**The patient should be instructed to**

1) Rinse the mouth with water.

2) Stand facing the wall, away from wind.

3) Keep both hands on hips.

4) Take a deep breath, hold it momentarily and then cough deeply and vigorously.

5) Cover their mouth carefully while coughing and to discard tissue papers in an appropriate receptacle.

The sample should be sent to the laboratory within an hour of collection. If there is an anticipated delay in sending the same, the samples should be refrigerated to avoid contamination or overgrowth by normal commensals.
The irregular and intermittent release of mycobacteria into the bronchial lumen from mucosal ulcers or loculated cavities often results in a variable pattern of recovery from respiratory secretions. Thus a minimum of three sputum samples should be collected to maximize the chance of recovery of mycobacteria.\textsuperscript{90}

2) **Induced Sputum**\textsuperscript{90,97}

The saline induction procedure can best be done on ambulatory patients who are able to follow instructions. It is particularly important in those patients who cannot raise spontaneous sputum. This procedure should be performed only in an enclosed area with appropriate air flow and by operators wearing particulate respirators and taking all appropriate safety measure to avoid exposure.

**The patient should be instructed**

- Procedure is being performed to induce coughing to raise sputum with an aerosol solution of 10\% NaCl.

- Salt solution is irritating.

- The patient is instructed to inhale slowly and deeply through the mouth and to cough at will, vigorously and deeply, coughing and expectorating into a sterile collection tube.

- Procedure is discontinued if the patient fails to raise sputum after 10 minutes or feels any discomfort.

- 10 ml of sputum should be collected, if the patient continues to raise sputum, a second specimen should be collected.
Specimen must be labeled as ‘**Induced Sputum**’ so as not be mistaken with salivary specimen.

Delivered promptly to the laboratory and refrigerated if processing is delayed.

3) **Gastric Lavage**

Gastric lavage is used to collect sputum from patients who may have swallowed sputum during the night. The procedure is limited to senile, non ambulatory patient.

Gastric lavage reveals the organisms in 30 to 40% of the cases and the yield may be greater in infants with extensive disease.

❖ **Main Indication for the collection of Gastric Lavage**

- In infants and young children who tend to swallow the sputum instead of expectorating the material out.

- Unco-operative patients who do not expectorate adequate amount of sputum.

- Patients who cannot expectorate because of other disorders eg coma and neurological disorders.

- Patients with radiological evidence of tuberculosis but the sputum smear being negative.
Important Steps About the Collection

- The patient should be admitted before collection.
- The procedure should be performed early in the morning, when the patient has been fasting for the preceding eight hours, securing the specimen at this time would minimize the dilution of the bronchial secretions swallowed during the night by saliva.
- Inhalation of superheated nebulised saline prior to gastric lavage has been reported to increase the bacteriologic yield.
- Following insertion of nasogastric tube the stomach contents are aspirated. Then a small amount of sterile distilled water (not more than 50 to 70 ml) is instilled through the nasogastric tube and the aspirate is added to the first collection.
- The collection should be made in a sterile bottle or container.
- The sample should be transported and processed immediately to avoid killing the bacilli as *Mycobacterium tuberculosis* poorly tolerate the gastric acidity.
- In case of an anticipated delay in the transport of the sample, the acidity should be immediately neutralized by adding 5ml of 10 % Tri-sodium phosphate. A.Rattan, suggested to use 10 % sodium carbonate (added by dropper) to develop just pink color (pH7) indicated by phenol red, or with 40% anhydrous sodium phosphate to develop green color with indicator bromothymol blue, for immediate neutralization of specimen.
4) **Bronchoalveolar Lavage Fluid (BAL)**

Fibreoptic bronchoscopy has been extensively used to ascertain the diagnosis in patients who produce inadequate sputum and in those with sputum smear negative pulmonary tuberculosis. Various bronchoscopic specimens such as bronchoalveolar lavage fluid (BAL), bronchial washings, and transbronchial lung biopsy have been evaluated and found to be useful.\(^9\)

II) **Extrapulmonary Specimens**

- **Urine:**

  The first few milliliters of urine should be allowed to flush the external urethra. Thereafter, clean – voided total volume of the **first early morning urine specimen** is collected in a sterile container on three consecutive days and transported to the laboratory as early as possible. Twenty four hour urine specimens are undesirable because of excessive dilution, higher contamination and difficulty in concentrating.\(^8,9\)

- **Miscellaneous “Sterile” Specimens**

  Cerebrospinal fluid, synovial Fluid and other body fluids are considered to be usually sterile if collected with proper aseptic precautions. Such fluids need not be decontaminated before culture. Processing can commence with centrifugation for 30 minutes at 3600 g to concentrate the bacteria. The supernatant is decanted and the sediment is vortexed thoroughly before preparing the smear and inoculating media. **Sufficient quantity of specimens (at least 10ml)** is critical for isolation of acid-fast bacilli from body fluids as the bacilli are scanty. Low volume fluid samples can be
added directly to approximately 10 ml of 7H9 or 7H11 broth and incubated directly.\textsuperscript{80,90}

- **Swabs and Aspirate**

Aspirate is the best type of specimen to collect. Swab collection for mycobacteria is hardly ever practiced as there is virtually no indication for obtaining material in a swab. It is because the hydrophobic nature of the lipid-containing cell wall of the bacteria inhibits the transfer of the organism from the swab to the aqueous culture medium. However if swab is received for culture of mycobacteria, then the tip should be placed directly on the surface of the culture medium or into a tube containing approximately 5 ml of 7H9 broth and incubated for 4-8 weeks. *Mycobacteria*, if present may be found forming colonies in the fibers of the swab at the junction with the culture media.\textsuperscript{90}

- **Tissue Biopsy**

Tissue biopsy specimens of lymph nodes, liver etc, are aseptically collected in a vial containing normal saline and transported to the laboratory immediately. **Tissue in formalin should never be sent for culture.**\textsuperscript{80}

- **Blood Culture**

Several approaches may be used for the recovery of mycobacteria from blood cultures. Berlin and associates report on the use of biphasic system using modified 7H11 oleic acid albumin as the agar phase and brain-heart infusion as the broth phase.\textsuperscript{90}

The use of the **Lysis- Centrifugation blood culture system** (Isolator; Wampole Laboratories, Cranbury, NJ) has increased the yield and shortened the time of recovery of mycobacteria from blood cultures.
The lysis centrifugation tube contains an anticoagulant and a lysing agent to effect rupture of both erythrocytes and neutrophils. Thus, intracellular mycobacteria are released into the broth milieu further enriched by the lysis of the red blood cells. Each tube holds 5ml of blood, and cell lysis can be enhanced by gently inverting the tube several times immediately after adding the sample. Following centrifugation of the tube at 3000 g for 20 to 30 minutes, the eluate is discarded and 1.6 ml of sediment is divided into 0.2 ml aliquots for transfer to appropriate culture media.

- **Stool Specimens**

Acid-fast stain and/or culture of stool from patients with AIDS have been used to identify patients who may be at risk for developing disseminated disease. The clinical utility of this practice remains controversial.80,90

- Stool specimens are collected in a clean (not necessarily sterile) container with a tightly fitting lid, as for routine bacterial cultures.

- A direct smear is first prepared from a small quantity of the specimen & stained for acid-fast bacilli, using either the Ziehl–Neelsen or Kinyoun carbol fuchsin techniques or the rhodamine-auramine fluorescence method.

- If the smears are negative for acid-fast bacteria, the specimen is not processed further.

- If acid-fast bacilli are seen in the smear, 1g of faeces is suspend in 5ml of Middlebrook 7H9 broth and subjected to the NaOH digestion-decontamination as used for sputum specimens.
The genus *Mycobacterium* comprises the ‘Acid-fast bacilli,’ organisms that share the property of acid-fastness, i.e., resistance to decolourization by weak mineral acids after staining with one of the aryl methane dyes. The standard stain for demonstration of acid fastness is Ziehl-Neelsen (ZN) stain. Many modifications have been described but all basically use carbol fuchsin to stain the organisms and mineral acids to decolourize the background. The background is then counterstained with another dye such as malachite green or methylene blue to give red acid-fast bacilli against a green or blue background. Some methods also use alcohol for decolourization, this gives a cleaner slide but it is important to realize that not all mycobacteria are alcohol fast.  

Two types of acid-fast stains are commonly used:

1) Carbol fuchsins stains: a mixture of fuchsin with phenol (carbolic acid).
   a. Ziehl-Neelsen (hot stain)
   b. Kinyoun (cold stain)

2) Fluorochrome Stain: Auramine O, or Auramine-Rhodamine dyes.
### Table -4 Acid-Fast-Staining Procedure

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Procedure</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ZIEHL-NEELSEN PROCEDURE</strong></td>
<td><strong>KINYOUN COLD PROCEDURE</strong></td>
<td><strong>AURAMINE FLUOROCHROME PROCEDURE</strong></td>
</tr>
<tr>
<td><strong>Carbolfuchsin:</strong> Dissolve 3 g of basic fuchsin in 10 ml of 90-95% ethanol. Add 90 ml of 5% aqueous solution of phenol.</td>
<td><strong>Carbolfuchsin:</strong> Dissolve 4g of basic fuchsin in 20 ml of 90-95% ethanol and then add 100 ml of a 9% aqueous solution of phenol (9g of phenol dissolved in 100 ml of distilled water.</td>
<td><strong>Phenolauramine:</strong> Dissolve 0.1 g of auramine O in 10ml of 90-95% ethanol and then add to a solution of 3 g of phenol in 87 ml of distilled water. Store the stain in a brown bottle.</td>
</tr>
<tr>
<td><strong>Acid-alcohol:</strong> Add 3 ml of concentrated HCl slowly to 97 ml of 90-95% ethanol, in this order. Solution may get hot!</td>
<td><strong>Acid-alcohol:</strong> Add 3 ml of concentrated HCl slowly to 97 ml of 90-95% ethanol, in this order. Solution may get hot!</td>
<td><strong>Acid-alcohol:</strong> Add 0.5ml of concentrated HCl to 100ml of 79% alcohol.</td>
</tr>
<tr>
<td><strong>Methylene blue counterstain:</strong> Dissolve 0.3g of methylence blue chloride in 100 ml of distilled water.</td>
<td><strong>Methylenebluecounterstain:</strong> Dissolve 0.3g of methylence blue chloride in 100 ml of distilled water.</td>
<td><strong>Potassium Permanganate:</strong> Dissolve 0.5g potassium permanganate in 100 ml of distilled water.</td>
</tr>
</tbody>
</table>

Cover a heat- fixed, dried smear with a small rectangle (2×3 cm) of filter paper.

Apply 5-7 drops of carbolfuchsin stain to thoroughly moistened filter paper.

Heat the stain-covered slide to steaming, but do not allow to dry. Allow to stand for 5 mins.

Heating may be done by gas burner or over an electric staining rack.

Remove paper with forceps, rinse slide with water, and drain.

Decolorize with acid-alcohol until no more stain appears in the washing (2 min).

Counterstain with methylene blue (1-2min).

Rinse, drain, and air dry (1-2 min).

Examine with 100× oil-immersion objective.

Mycobacteria are stained red and the background light blue.

Cover a heat- fixed, dried smear with a small rectangle (2×3 cm) of filter paper.

Apply 5-7 drops of carbolfuchsin stain to thoroughly moistened filter paper. Allow to stand for 5 min.

Add more stain if paper dries. Do not steam!

Remove paper with forceps, rinse slide with water, and drain.

Decolorize with 3% acid-alcohol until no more stain appears in the washing (2 min).

Counterstain with methylene blue (1-2 min).

Rinse, drain, and air dry (1-2 min).

Examine with 100× oil-immersion objective. Mycobacteria are stained red and the background light blue.

Cover a heat- fixed, dried smear with carbol auramine and allow to stain for 15 min. Do not heat or cover within filter

Rinse with water and drain. Decolorize with acid-alcohol (2min).

Rinse with deionized water and drain.

Flood smear with potassium permanganate for at least 2 and not more than 4 min.

Rinse with tap water. Drain.

Examine with 253 objectives using a mercury vapor burner and BG-12 filter or a strong blue light. Mycobacteria are stained yellow-orange against a dark background.
The detection of acid-fast bacilli (AFB) in stained smears examined microscopically is the first bacteriologic evidence of the presence of mycobacteria in a clinical specimen. It is the easiest and quickest procedure that can be performed, and it provides the physicians with a preliminary confirmation of the diagnosis. Also, because it gives a quantitative estimation of the number of bacilli being excreted, the smear is of vital clinical and epidemiologic importance in assessing the patients' infectiousness. Smears may be prepared directly from clinical specimens or from concentrated preparations. 99, 100, 101

- **Microscopical Examination of Smears**

Careful smear examination is an essential part of today's tuberculosis control program. To attain excellence in microscopic examination, one should have a good microscope and a comfortable work area. Methods of reading smears vary from laboratory to laboratory. At CDC, no time limit has been set for examining a smear. Instead, a system has been adopted that ensures that a representative area of the smear is examined. To ensure that an area is covered only once, the smear should be searched in an orderly manner by making a series of three parallel sweeps the length of the smear or nine parallel sweeps the width of the smear. Each field should be searched thoroughly, with a rapid change to the next field. 100
• Reporting of Smear Results

In reading smears, the microscopist should provide the clinician with a rough estimate of number of AFB detected. Smears are graded as per RNTCP guidelines.

Table-5  RNTCP Grading of Smears

<table>
<thead>
<tr>
<th>Number of bacilli</th>
<th>Result</th>
<th>Grading</th>
<th>Number of Fields Examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 or more bacilli / field</td>
<td>Positive</td>
<td>3+</td>
<td>20</td>
</tr>
<tr>
<td>1-10 bacilli / field</td>
<td>Positive</td>
<td>2+</td>
<td>50</td>
</tr>
<tr>
<td>10-99 bacilli / 100 fields</td>
<td>Positive</td>
<td>1+</td>
<td>100</td>
</tr>
<tr>
<td>1-9 bacilli / 100 fields</td>
<td>Positive</td>
<td>Scanty</td>
<td>100</td>
</tr>
<tr>
<td>No bacilli seen in 100 fields</td>
<td>Negative</td>
<td>-</td>
<td>100</td>
</tr>
</tbody>
</table>

The major limitation of the Acid-fast staining is that a relatively large number of bacilli must be present to be seen microscopically. Acid-fast smears are generally negative where there are fewer than 10,000 bacilli / ml of sputum and many microscopic fields need to be examined to identify bacilli even when there are 10,000 to 50,000 bacilli / ml. This is a serious problem in patients without cavitary tuberculosis, who tend to have fewer bacilli in their sputum including HIV infected patients with tuberculosis in developing countries.\textsuperscript{96, 99, 101}

Several techniques have been described to improve the yield of smear positivity. Use of fluorochrome staining is more sensitive but less specific than acid-fast staining, as the fluorescent bacilli stand out brightly against the background. The smear is examined at lower magnification and therefore, more fields can be visualized in a short period but confirmation of positive results with Ziehl-Neelsen or Kinyoun
staining is essential, as false-positive fluorochrome results are not uncommon. Use of N-acetyl-L-cysteine concentration method, sodium hypochlorite (NaOCL) liquefaction chitin sedimentation and phenol ammonium sulfate sedimentation have been described to improve the sensitivity of direct microscopy.96, 99

**Culture**96,99,101

- Culture is much more sensitive than microscopy, being able to detect as few as 10 bacteria / ml of specimen.
- Growth of the organism is necessary for precise identification.
- Drug susceptibility testing requires culture or organisms.
- Genotyping of cultured organism may be useful to identify epidemiological links between patients or to detect laboratory cross contamination.
- In general, the sensitivity of the culture is 80-85%, with a specificity of approximately 98%
  - Before proceeding for culture it is necessary to carry out a homogenization, decontamination process by using chemicals.
  - Homogenization of the specimen particularly sputum is required to liquefy the mucus, thus releasing the bacilli trapped in the mucus.
  - Decontamination is done to kill the commensals present in the specimen.
  - Concentration by centrifugation at high speed is required to concentrate the bacilli in a small volume without inactivation.
Table -6 Agents Commonly Used for Decontamination and Concentration of Specimens

<table>
<thead>
<tr>
<th>AGENT</th>
<th>COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetylle-L-cysteine plus 2% NaOH</td>
<td>Mild decontamination solution with mucolytic agent NALC to free mycobacteria entrapped in mucus. Limit exposure to NaOH to 15 minutes.</td>
</tr>
<tr>
<td>Dithiothreitol plus 2% NaOH</td>
<td>Very effective mucolytic agent used with 2% NaOH. Trade name of dithiothreitol is Sputolysin. Reagent is more expensive than NALC. Limit exposure to NaOH to 15 minutes.</td>
</tr>
<tr>
<td>Trisodium phosphate, 13% plus benazalkonium chloride (Zephiran)</td>
<td>Preferred by laboratoies that cannot carefully control time ofexposure to decontamination solution. Zephiran should be neutralized with lecithin and not inoculated onto egg-based culture medium.</td>
</tr>
<tr>
<td>NaOH, 4%</td>
<td>Traditional decontamination and concentration solution. Time of exposure must be carefully controlled to no more than 15 minutes. NaOH, 4%, effects mucolytic action promote concentration by centrifugation.</td>
</tr>
<tr>
<td>Trisodium phosphate, 13% plus</td>
<td>Can be used for decontamination of specimens when exposure time can be completely controlled. Not as effective as TSP-Zephiran mixture.</td>
</tr>
<tr>
<td>Oxalic acid, 5%</td>
<td>Most useful in processing specimens that contain Pseudomonas aeruginosaas a contaminant.</td>
</tr>
<tr>
<td>Cetylpyridinium chloride, 1%, plus 2% NaCl</td>
<td>Effective as a decontamination solution for sputum specimens mailed from outpatient clinics. Tubercle bacilli have survived 8-day transit without significant loss.</td>
</tr>
</tbody>
</table>
Culture Media

Many different culture media have been devised for the growth of mycobacteria. They are egg based, agar based and liquid media.  

Table-7 Suggested media for cultivation of mycobacteria form clinical specimens

<table>
<thead>
<tr>
<th>SOLID MEDIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>I) Agar Based</td>
</tr>
<tr>
<td>Middlebrook 7H10</td>
</tr>
<tr>
<td>Middlebrook 7H11</td>
</tr>
<tr>
<td>II) Egg Based</td>
</tr>
<tr>
<td>Lowenstein –Jensen (L-J)</td>
</tr>
<tr>
<td>L-J Gruft</td>
</tr>
<tr>
<td>Petragnani Medium</td>
</tr>
<tr>
<td>American Thoracic Society Medium (ATS)</td>
</tr>
<tr>
<td>III) LIQUID MEDIA</td>
</tr>
<tr>
<td>BACTEC 12 B Medium</td>
</tr>
<tr>
<td>Middlebrook 7H9 broth</td>
</tr>
<tr>
<td>Septi- Chek AFB</td>
</tr>
<tr>
<td>Mycobacterial Growth Indicator tube</td>
</tr>
<tr>
<td>Commercially supplied broths for continuously monitoring systems for mycobacteria</td>
</tr>
</tbody>
</table>
Table-8: Mycobacterial Isolation Media and their composition

<table>
<thead>
<tr>
<th></th>
<th>MEDIUM</th>
<th>COMPONENTS</th>
<th>INHIBITORY AGENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lowenstein- Jensen medium</td>
<td>Coagulated whole eggs, defined salts, glycerol</td>
<td>Malachite green 0.025g / 100 ml</td>
</tr>
<tr>
<td>2</td>
<td>Lowenstein- Jensen medium with antibiotics</td>
<td>Coagulated whole eggs, defined salts, glycerol</td>
<td>Malachite green 0.025g / 100 ml Cycloheximide 400 µg / ml, Lincomylin 2 µg / ml Nalidixic acid 35 µg / ml</td>
</tr>
<tr>
<td>3</td>
<td>Gruft Modification of Lowenstein- Jensen Medium</td>
<td>Coagulated whole eggs, defined salts, glycerol, RNA 5 mg / 100 ml</td>
<td>Malachite green 0.025g / 100 ml Penicillin 50 U/ml Nalidixic acid 35 µg / ml</td>
</tr>
<tr>
<td>4</td>
<td>Petagnini Medium</td>
<td>Coagulated whole eggs, egg whole milk, potato, potato flour, glycerol</td>
<td>Malachite green 0.052g / 100 ml</td>
</tr>
<tr>
<td>5</td>
<td>American Thoracic Society Medium</td>
<td>Coagulated, fresh egg potato flour, glycerol</td>
<td>Malachite green 0.02g / 100 ml</td>
</tr>
<tr>
<td>6</td>
<td>Middle brook 7H10</td>
<td>Defined salts, Vitamins, cofactors oleic acid, albumin, catalase, glycerol dextrose</td>
<td>Malachite green 0.0025g / 100 ml</td>
</tr>
<tr>
<td>7</td>
<td>Middle brook 7H11</td>
<td>Defined Salts, Vitamins, cofactors oleic acid albumin, catalase, glycerol, 0.1% casein hydrolysate</td>
<td>Malachite green 0.0025g / 100 ml</td>
</tr>
</tbody>
</table>

Lowenstein-Jensen medium is most widely used in most clinical diagnostic laboratories for optimal recovery of mycobacteria, a minimum combination of solid and liquid medium is recommended. Cultures are incubated at $37^0$ C in the dark in an atmosphere of 5% to 10% carbon dioxide and high humidity. Cultures are examined
weekly for growth. Contaminated cultures are discarded and report as “contaminated, unable to detect presence of Mycobacteria.” Additional specimens are also requested. If available, sediment may be recultured after enhanced decontamination or by inoculating the sediment to a more selective medium. Growth appears after three weeks, sometimes delayed upto 8 weeks.80

❖ Rapid Manual And Automated Detection Systems

During the last two decades, several methods for achieving early growth 5-14 days as compared to 3-8 weeks with conventional method of M. tuberculosis have been developed. Some of the prominent methods are as follows101-116

- Radiometric BACTEC 460 TB

This is a radiometric automated system developed by Becton Dickinson. It consists of Middlebrook 7H9 broth, growth enhancers and small quantities of antimicrobial mixture of polymixin B, Amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA) to suppress growth of contaminants and radioactive $^{14}$C- labelled palmitic acid as the growth detector. Mycobacteria if present in the specimen will metabolize $^{14}$C labelled palmitic acid and produce $^{14}$CO2 which is measured by the instrument in the aspirated head gas and translated into a numerical value called the Growth Index (GI). Inclusion of NAP (beta nitro alpha acetylamine beta hydroxyl propiophenone) helps in distinguishing M. tuberculosis form other non-tuberculosis mycobacteria. This system has been widely used for drug susceptibility testing and is currently used as a comparative standard.
• BACTEC MGIT 960 System (BD Diagnostic)

It is a non-radiometric automated system with a capacity to incubate and continuously monitor 960 mycobacteria growth indicator tube (MGIT) every 60 minutes for increase in fluorescence. It uses modified Middlebrook 7H9 broth in which a supplement is added at the time of use. It has an indicator at the base of the tube that fluoresces under ultraviolet light. Oxygen quenches the fluorescence but, when oxygen in the medium is consumed, the indicator will fluoresce. A silicon film embedded with a ruthenium salt is present at the bottom of the tube as a fluorescence indicator. If viable mycobacteria are present in the tube, oxygen is consumed due to their metabolism, the quenching effect lowers accordingly, and the bottom of the tube fluoresces when exposed to UV light.

• MB / Bact Alert System (Bio Merieux)

It is a non-radiometric continuous monitoring system based on colorimetric detection of microbial generated CO₂. It consists of a bottle containing Middlebrook 7H9 broth with a colorimetric sensor. The bottles of the medium have CO₂ sensors at the bottom and are inoculated through a rubber septum by means of a syringe. As increasing amounts of CO₂ are produced by growing organisms, the sensor changes colour, which is detected by the instrument. The CO₂ sensor is impacted by a light whose reflected ray is monitored by a photodiode. If viable *Mycobacteria* are present in the bottle, the CO₂ produced by their metabolism causes a change in the colour of the sensor, from green to yellow, which alters the intensity of the reflected light ray. The instrument monitors the bottles at 10 minute intervals, alerts when they become positive and signals at the end of the incubation period. This has been reported to be useful for drug susceptibility testing of *M. tuberculosis*. 
- **Septi-chek AFB System (Roche)**

This is a biphasic medium system consisting of an enriched selective broth and a two-sided paddle with non-selective Middle-brook 7H11 agar on one side and two sections on the other side: one contains 7H11 agar with para nitro acetylamino β-hydroxy propiophenone (NAP) for differentiation of *M. tuberculosis* from non-tuberculous mycobacteria, the other section contain chocolate agar for detection of contaminants.

This method requires about 3 weeks of incubation. The unique advantage of this method is the simultaneous detection of *M. tuberculosis*, non-tuberculous mycobacteria (NTM), other respiratory pathogens and even contaminants.

- **Versa TREK system (TREK Diagnostics)**

This is a fully automated continuous monitoring system based on the detection of pressure changes within the headspace above the broth culture medium in a sealed bottle from the consumption of oxygen or production of gases by the metabolizing microorganisms. It uses a modified Middlebrook 7H9 medium to which the OADC enrichment is added. The pressure within each bottle is monitored by a manometer through a proper connector. Cultures presenting a decreased headspace pressure are reported as positive. If viable *Mycobacteria* are present in the bottle, the oxygen consumption due to their metabolism reduces the internal pressure.

A major disadvantage of many of the commercial liquid-based systems is high cost in terms of both instrumentation and reagents.
PHAGE BASED ASSAYS

Mycobacterial specific phages and reporter genes like luciferase have been successfully used for detection of growth and for assessing the drug susceptibility to anti-TB drugs. Indication of viability could be either emission of light from organism due to activation of luciferase gene or production of plaques on an indicator strain of mycobacteria.

Fast Plaque TB™ ASSAY (Biotec DIAGNOSTICS)

It is a novel, patent protected, phage amplification technology that has been developed for rapid detection and enumeration of *M. tuberculosis* from respiratory specimens. This method uses specific mycobacteriophages (viruses that infect *M. tuberculosis*) to detect the presence of viable TB bacilli in the clinical specimen. Mycobacteria are mixed with phages, which are allowed to adsorb and infect the cells. All unadsorbed extracellular phages are then inactivated using a phagicidal chemical (virucide), while the phages which have infected the viable TB bacilli remain protected and continue to replicate. After replication the progeny bacteriophages are released and detected by mixing with fast growing non-pathogenic helper cells (M. smegmatis) on an agar plate. The mycobacteriophages in turn infect, replicate and lyse these helper cells and lysis is detected as plaques (clear zones).

Microscopic Observation drug Susceptibility Assay (MODS)

MODS is a tissue culture plate based assay utilizing observation of Middlebrook 7H9 cultures with an inverted light microscope to detect the characteristic tangles of *M. tuberculosis* in liquid media. Drug-containing and drug free control wells allow concurrent drug susceptibility testing for rifampicin and isoniazid.
The Middlebrook 7H9 medium is supplemented with antimicrobial and nutritional supplements (PANTA and OADC) and sputum samples are previously decontaminated by the NALC-NAOH method. The plates are contained within zip lock polythene bags for safety and are read under the microscope daily (or on alternate days if preferred) from day five.
<table>
<thead>
<tr>
<th></th>
<th>OPTIMAL ISOLATION TEMPERATURE AND TIME FOR GROWTH</th>
<th>PIGMENTATION GROWTH IN</th>
<th>NIACIN TEST</th>
<th>NITRATE REDUCTION</th>
<th>TYPHOEUS RO Hydrolysis 10 DAYS</th>
<th>CATALASE</th>
<th>ARYLISULPHATASE 3 DAYS</th>
<th>UREASE</th>
<th>PYRORAZIMIDASE</th>
<th>IRON UPTAKE</th>
<th>GROWTH ON</th>
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<td></td>
<td>Light</td>
<td>Dark</td>
<td></td>
<td></td>
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<td>+</td>
<td>+</td>
<td>V</td>
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<td>-</td>
<td>-</td>
<td>+</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<td>V</td>
<td>-</td>
<td>-</td>
<td>&gt;45</td>
<td>V</td>
<td>+</td>
<td>-</td>
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<td>+</td>
<td>V</td>
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<td>+</td>
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<td>&gt;45</td>
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<td>+</td>
<td>-</td>
<td>+</td>
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</tr>
<tr>
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<td>-</td>
<td>y</td>
<td>&gt;45</td>
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<td>V</td>
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<td>+</td>
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<td>&gt;45</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Mycobacterium xenopi</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>&gt;45</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mycobacterium avium complex</td>
<td>37°C 10-21 d</td>
<td>Buff to pale yellow</td>
<td>-</td>
<td>-</td>
<td>&lt;45</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mycobacterium neoformans</td>
<td>30°C 14-21 d</td>
<td>Gray</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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</tr>
<tr>
<td>Mycobacterium malmoensei</td>
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<td>Buff</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>&lt;45</td>
<td>V</td>
<td>V</td>
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<td>Mycobacterium kansas 104</td>
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<td>-</td>
<td>+</td>
<td>&gt;45</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Mycobacterium melitensis</td>
<td>37°C 14-28 d</td>
<td>Buff</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>&gt;45</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>-</td>
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<tr>
<td>Mycobacterium abscessus</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>&gt;45</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>Mycobacterium smegmatis</td>
<td>37°C 3-5 d</td>
<td>Buff to yellow</td>
<td>-</td>
<td>+</td>
<td>&gt;45</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*: positive; -: negative; V: variable; blank: space, little or no data.
Phenotypic Characteristics for Identification of *M. tuberculosis* \(^9^0\)

- Strictly grows at 37\(^0\) C on Lowenstein Jensen or Middlebrook media.
- Fail to grow at 25\(^0\)C or 45\(^0\)C.
- Formation of non-pigmented, rough, tough and buff colonies.
- Slow Growth after 14 to 28 days of incubation at 37\(^0\) C.
- Niacin test positive (*M. simiae*, certain strains of *M. bovis*, occasional Strains of *M.marinum* and *M.chelonae* may also be niacin positive), therefore this characteristics must be used in conjunction with the other findings.
- Reduction of nitrates to nitrites.
- Ability to grow in the presence of Thiophene- 2 carboxylic acid Hydrazide (T2H).
- Lack of catalase activity.
- Selective inhibition of growth in L-J. medium containing paranitrobenzoic acid 0.5mg / ml and in broth culture media containing NAP.
The tuberculin test was introduced by Clemens Von Pirquet in 1907. It consists of introducing tuberculin, a protein of the tubercle bacillus, into the skin and observing whether or not an allergic reaction develops. A positive reaction to the test is generally accepted as evidence of past or present infection by *Mycobacterium tuberculosis*. The tuberculin test is based on the fact that the mycobacterial infection produces delayed hypersensitivity to certain products of the organisms in culture extracts called tuberculins.

This test is the only means of estimating the prevalence of TB infection in the population. Formerly old tuberculin (OT) was first prepared by Robert Koch in 1880 and wrongly promoted as a cure for TB. For preparing OT, tubercle bacilli are grown in a synthetic medium such as Sauton’s medium and are then killed with heat in a 100°C flowing steam cabinet. The remaining culture medium is concentrated to one tenth of its original volume over a steam bath. Injection of old tuberculin in patients with TB resulted in severe systemic upset, whereas non-TB individuals showed no or few symptoms. Fortunately it was recognized the tuberculin induced reactions in people who were infected with TB, the substance might prove a better diagnostic test than a therapeutic substance.

Over a period of years, refinements were made in the preparations of tuberculins, and in 1939 Siebert produced the reference lot of tuberculin called PPD-S (purified protein derivative) which has served as the international standard. PPD-S has been arbitrarily designated as containing 50,000 tuberculin units/mg. Other standard PPDs include PPD-RT23 prepared in Denmark for international use and British standard preparation known as PPD Weybridge. Tween 80 is added to PPD to prevent its
absorption to glass and plastics. One IU for PPD is defined as the biologic activity contained 0.000028 mg of PPD-S, consisting of 0.00002 mg of PPD plus 0.000008 mg of salts. Instead of expressing in IU, tuberculin unit (TU) is commonly used. One TU is defined as 0.00002 mg of PPD-S. PPD is distributed as a lypophilised powder. After evaluation of variety routes of administration of tuberculin including cutaneous, percutaneous, conjunctival and subcutaneous, the Mantoux test, an intracutaneous test developed by French physician Charles Mantoux gradually became the preferred method.

**Tuberculin Skin Tests**

There are three main tuberculin tests currently in use. These are single puncture test like **Mantoux test** and the multiple puncture tests like **Heaf test and Tine test**.

- **Mantoux Test**: It is the most widely used test for administration of tuberculin. This test is favoured when a more precise measurement of tuberculin sensitivity is required. The generally accepted and standard method of Mantoux test consists of injecting 0.1 ml PPD containing 1 TU (first strength), 5 TU (Intermediate strength) or 250 TU (Second Strength). 5 TU contains 0.1 ug/0.1 ml dose of PPD-S. In India, tuberculin test is routinely performed using either 1 TU or 5 TU. Higher strengths of tuberculin may be useful in patients with suspected tuberculosis where the test is negative with 1 TU. An injection of 0.1 ml of PPD-S is given intradermally in the volar surface of the forearm using a tuberculin syringe and a short bluntly bevelled 27 gauge needles. The result is read after 48 to 72 hours when redness and induration are seen. Since erythema is sometimes difficult to measure and is non-specific, it is disregarded while the induration is measured. The reading is taken in adequate lighting with the forearm partially fixed at the elbow. The diameter of induration is
assessed transversely to the long axis of the arm. The standard method is to measure the induration in its greater transverse diameter in mm using a ruler. An error of 2% in the measurement decreases the accuracy of the method by 25% and an error of 5% decreases the accuracy to 50%. Tuberculin skin reaction is usually read by measuring the induration by palpation. The margins of the induration are palpated by drawing the index finger lightly across the reaction. The measurement of induration by the pen method has been found to be an alternative. In this technique using a medium ball point pen, a line is drawn from a point 5-10 mm away from the margin of the skin induration towards its centre, until resistance is felt to further movement.

**Interpretation**

- An induration of **10 mm or above** is considered **positive** for general population in India.
- An induration of **< 5 mm** is is considered as **negative**.
- An induration **between 6-9 mm** is considered **doubtful**.
- An induration **>20 mm** considered to be a strong reaction, more chances of developing active tuberculosis than those with 10mm in duration.

**Causes of False Positive Tuberculin Test**

- Prior BCG Vaccination
- Wrong Technique
- Overdosage of Tuberculin
- Contaminated Tuberculin
- Atypical Mycobacterial Infection
Causes of False Negative Tuberculin Test

Factors Related to the Patients

- Infections (HIV, Measles, Mumps, influenza, typhoid, leprosy, malaria).
- Vaccination with measles, mumps, polio.
- Chronic Renal failure.
- Malnutrition.
- Malignancies- lymphoma and lymphatic leukemia.
- Sarcoidosis.
- Neonate or elderly.

Factors Related to Tuberculin Test

- Exposure of tuberculin to heat and light
- Improper dilution
- Contamination
- Inadequate dose
- Improper technique
- Inexperienced reader.

Heaf Test

The Heaf test is widely used in the UK and Ireland for mass screening for tuberculin sensitivity. Though Mantoux is the standard skin test, it is time consuming and requires skill to administer correctly. Heaf test is commonly performed in the field setting because of the speed and ease with which tuberculin can be administered. Tuberculin is introduced into the skin through tuberculin coated prongs and the skin reaction is usually recorded five to seven days later. The reaction is graded as 0-4.
Grade 0 – No Reaction

Grade I – One to four small raised erythematous dots.

Grade II- A raised ring of erythema with normal skin in the middle.

Grade III- A raised erythematous circle with the centre also filled with induration.

Grade IV- A raised erythematous circle with blister or ulcers.

A limitation of this test is that the amount of tuberculin introduced into the skin cannot be precisely controlled. The use of Heaf teat is restricted only when a large population with a low expected prevalence of tuberculosis infection is being studied.

➢ Tine Test

Tine test is relatively cheap and is a useful way of assessing PPD reactivity in the field setting. It consists of delivering tuberculin into the skin by puncture with a device having points either coated with dried tuberculin or by application through a film of tuberculin.

❖ IMMUNOLOGICAL DIAGNOSIS

There has been a long history of developing systems to diagnose tuberculosis based on the serological reaction, i.e. detection of a specific antibody. Currently, the development of such systems is very urgently needed due to the pressure for strengthening earlier diagnosis of diseases in the paucibacillary stage, pulmonary tuberculosis with negative sputum smears of adults, extrapulmonary tuberculosis, childhood tuberculosis and TB patients with HIV co-infection. In contrast to many cases of other acute bacterial and viral infections, there are several barriers to the successful applications of the serological reactions for diagnosing tuberculosis,
including the gap between active disease and latent infection, the wide profile of the disease from one with extensive cavitary lesion to an almost inactive minimal disease and distinction from NTM infection. These characteristics of tuberculosis comprise formidable factors against sensitivity and specificity of the expected diagnostics.\textsuperscript{118}

### Table-10: Types and Nature of Serodiagnostic Methods.\textsuperscript{118}

<table>
<thead>
<tr>
<th>Antigens</th>
<th>38 k Da, 16 kDa, 88k Da, MPT 51, Malate Synthase, CFP-10, Tb F6 polyprotein, antigen 85B, antigen A60, antigen 2,3-diacyltrehalose, 2,3,6-triacyltrehalose, cord factor, tuberculosphophatide, Lipoarabinomannan, Rv 3425</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td>Protein, Lipid, Polysaccharide and their complex.</td>
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<tr>
<td>Composition</td>
<td>Single antigen, Multiple antigens.</td>
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<td>Source</td>
<td>Native, recombinant</td>
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<tr>
<td>Ig Class</td>
<td>IgG, IgA</td>
</tr>
<tr>
<td>Assay</td>
<td>ELISA, Immunochromatography, Immunodor Rapid Test, Kaolin Agglutination Test.</td>
</tr>
</tbody>
</table>

**Interferon-gamma (INF-\(\gamma\)) assay (IGRAs)\textsuperscript{119-122}**

One of the most significant developments in the diagnostic armamentarium for TB in the last hundred years seen to be the assays based on IFN-gamma determination. The assays stem from the principle that T-cells of sensitized individuals produce IFN-gamma when they re-encounter the antigens of *M.tuberculosis*. Recent evaluations showed that IFN-gamma assays may have advantages over tuberculin skin testing. TSTs have been used worldwide for more than a century as an aid in diagnosing both latent TB infection (LTBI) and active tuberculosis. A positive TST
result is associated with an increased risk for current or future active tuberculosis. However, certain limitations are associated with the use of TSTs. A valid TST requires proper administration by the Mantoux method with intradermal injection of 0.1mL of tuberculin-purified protein derivative (PPD) into the volar surface of the forearm. In addition, patients must return to a health-care provider for test reading, and inaccuracies and bias exist in reading the test. Also, false-positive TSTs can result from contact with nontuberculous mycobacteria or vaccination with Bacille Calmette-Guerin (BCG), because the TST test material (PPD) contains antigens that are also in BCG and certain nontuberculous mycobacteria.

In 2001, QuantiFERON Test (QFT) became the first IGRA approved by FDA as aid for diagnosing *M. tuberculosis* infection. This test used an enzyme-linked immunosorbent assay (ELISA) to measure the amount of IFN-γ released in response to PPD compared with controls. However, QFT specificity was less than that of TST despite the use of *M. avium* antigen as a control for nontuberculous mycobacterial sensitization and saline as a negative control (19). QFT has not been available commercially since 2005. To improve specificity, new IGRA were developed.

These IGRA assess response to synthetic overlapping peptides that represent specific *M. tuberculosis* proteins, such as early secretory antigenic target-6 (ESAT-6) and culture filtrate protein 10 (CFP-10). These proteins are present in all *M. tuberculosis* and they stimulate measurable release of IFN-γ in most infected persons, but they are absent from BCG vaccine strains and from most nontuberculous mycobacteria.
Thus, as test antigens, these proteins offer improved test specificity compared with PPD. However, ESAT-6 and CFP-10 are present in *M. kansasii*, *M. szulgai*, and *M. marinum*, and sensitization to these organisms might contribute to the release of IFN-γ in response to these antigens and cause false-positive IGRA results. Because ESAT-6 and CFP-10 are recognized by fewer T lymphocytes and stimulate less IFN-γ release compared with PPD, a more sensitive ELISA than was used for QFT is required to measure IFN-γ concentrations and responses to ESAT 6 and CFP-10.

In 2005, the QuantiFERON-TB Gold test (QFT-G) (Cellestis Limited, Carnegie, Victoria, Australia) became the second IGRA approved by FDA as an aid for diagnosing *M. tuberculosis* infection. It assesses the immunologic responsiveness of tested patients to ESAT-6 and CFP-10. For QFT-G, separate aliquots of fresh whole blood are incubated with controls and with two separate mixtures of peptides, one representing ESAT-6 and the other representing CFP-10. The amount of IFN-γ released in response to ESAT-6 or CFP-10 (i.e., the ESAT-6 Response or the CFP-10 Response) is calculated as the difference in IFN-γ concentration in plasma from blood stimulated with antigen minus the IFN-γ concentration in plasma from blood incubated with saline (i.e., Nil). For QFT-G, the TB Response is the higher of the ESAT-6 Response or the CFP-10 Response. A stipulation for FDA approval was inclusion of interpretation criteria that addressed the potential for false-positive results accompanying high Nil values (i.e., >0.7 IU/ml). For IGRA to measure IFN-γ response accurately, a fresh blood specimen that contains viable white blood cells is needed. This requirement limited the use of early IGRA to facilities in which trained laboratorians could begin testing blood within a few hours of its collection.
The **QuantiFERON-TB Gold In-Tube test (QFT-GIT)** (Cellestis Limited, **Carnegie, Victoria, Australia**) was developed to address this limitation. In October 2007, QFT-GIT became the third IGRA approved by FDA as an aid for diagnosing *M. tuberculosis* infection. Control materials and antigens for QFT-GIT are contained in special tubes used to collect blood for the test, thus allowing more direct testing of fresh blood. One tube contains test antigens that consist of a single mixture of 14 peptides representing the entire amino acid sequences of ESAT-6 and CFP-10 and part of the sequence of TB7.7. The two accompanying tubes serve as negative and positive controls: the negative-control tube contains heparin alone, and the positive-control tube contains heparin, dextrose, and phytohemagglutinin. Blood (1 ml) is collected into each of the three tubes, mixed with the reagents already in the tubes, and incubated for 16--24 hours. Plasma is separated, and the IFN-γ concentration in the plasma is determined using the same sensitive ELISA used for QFT-G. To interpret QFT-GIT as approved by the FDA, the TB Response is calculated as the difference in IFN-γ concentration in plasma from blood stimulated with antigen (i.e., the single cocktail of peptides representing ESAT-6, CFP-10, and TB7.7) minus the IFN-γ concentration in plasma from blood incubated without antigen (i.e., Nil). In July 2008, T-Spot became the fourth IGRA to be approved by FDA. For this test, peripheral blood mononuclear cells (PBMCs) are incubated with control materials and two mixtures of peptides, one representing the entire amino acid sequence of ESAT-6 and the other representing the entire amino acid sequence of CFP-10. The test uses an enzyme-linked immunospot assay (ELISpot) to detect increases in the number of cells that secrete IFN-γ (represented as spots in each test well) after stimulation with antigen as compared to the media control (Nil).
T-Spot TB (ELISPOT)

The ELISPOT assay for diagnosis of *M. tuberculosis* infection is based on the rapid detection of T cells specific for *M. tuberculosis* antigens. IFN-gamma released from these cells can be detected by the extremely sensitive ELISPOT. Each such T cell gives rise to a dark spot and the readout is the number of spots. The T cells enumerated by the ELISPOT assay are effector cells that have recently encountered antigen in vivo and can rapidly release IFN-gamma when re-exposed to the antigen. In contrast, the long life memory T cells, which persist long after clearance of the pathogen are relatively quiescent and less likely to release IFN-gamma during the short period of exposure to antigen in the ex vivo ELISPOT assay.

❖ MOLECULAR METHODS

Molecular amplification techniques are mainly indicated for detection of organisms that require prolonged incubation and also that cannot be grown in vitro or the current culture techniques are too insensitive. Several molecular methods have been developed during the last decade for the direct detection and identification of *M. tuberculosis* in clinical specimens. These methods are able to potentially reduce the diagnostic turn around time from weeks to days, which acquire a greater relevance under special circumstances.\textsuperscript{123}

The basic principle of any molecular diagnostic test is the detection of specific nucleic acid sequence by hybridization to a probe, a complementary sequence and followed by the detection of the hybrid. DNA and RNA fragments have been proposed for the amplification target in *M. tuberculosis* and the most frequently used targets are 1S6110, a repetitive element, 16S ribosomal DNA (r DNA) and 16 S ribosomal RNA (r RNA).\textsuperscript{123,124}
Nucleic Acid Probes

Nucleic acid probes were the first nucleic acid-based technology to be used routinely in the clinical microbiology laboratory for the identification of mycobacteria in positive cultures. These probes which were initially radio labeled, but are now nonisotopic were pioneered by Gen-Probe (San-Diego, CA). The accuracy, sensitivity and specificity of these probes is very high when used to identify mycobacteria in culture.\textsuperscript{90}

1) DNA Probes

Based on information about specific gene sequence well defined oligonucleotide probes for identification of various clinically relevant mycobacteria have been developed.\textsuperscript{126,127}

2) Ribosomal r RNA based Probes

In recent years, ribosomal RNA gene region has been extensively explored for designing systems for ribosomal DNA finger printing and for development of probes as well as gene amplification assays for various mycobacterial species. These probes target r RNA, ribosomal DNA, spacer and flanking sequence r RNA targeting probes are 10-100 fold more sensitive than DNA targeting and may be used to confirm the diagnosis directly in the clinical specimens, the lowest detection limit is around 100 organisms.\textsuperscript{126,128}

3) PCR Methods

A variety of PCR methods have been developed for detection of specific sequence of \textit{M. tuberculosis} and other mycobacteria. These PCR assays may target either DNA or RNA and these could be based on conventional DNA based PCR, nested PCR and RT-PCR. Targets include insertion and repetitive elements various protein encoding genes, ribosomal r RNA etc.
The PCR allows sequencing of DNA present in only a few copies of mycobacteria to be amplified in vitro such that the amount of amplified DNA can be visualized and identified. If appropriate sequences specific for *M. tuberculosis* selected, 10-1000 organisms can be readily identified. The PCR methodology is rapid; results are available within a day of DNA extraction from the sample. The most common target used in the PCR is IS6110. This sequence is specific for *M. tuberculosis* complex and is present up to 20 times in the genome, thus offering multiple targets for amplification.\textsuperscript{124-128}

\begin{itemize}
  \item \textbf{Commercial Direct Amplification Tests (CDAT)\textsuperscript{123,129,130,131}}
  
  Many commercial direct amplification tests are available. These include

  \begin{itemize}
    \item COBAS AMPLICOR Mycobacterium system (Roche Molecular System, Branchburg, NJ) which uses the 16 S r RNA gene.
    
    \item Amplified *M. tuberculosis* Direct (AMTD) assay (Gene Probe, Inc, San Diego, Calif) in which 16 S r RNA is the target amplified and which is identified with an acridinium ester- labeled *M. tb* complex specific DNA Probe.
    
    \item LCx MTB Assay (Abbott LCx Probe system by Abbott Laboratories, Abbott Park, III) which uses a Lgase Chain reaction method, wherein detection is performed by micro particle enzyme immunoassay with the LCx fluorimetric analyzer.
    
    \item BD Probe Tec energy transfer system DTB (Becton Dickinson Biosciences, Microbiology Products, Sparks, Md.), in which the large sequences of IS6110 and 16S r RNA gene are both co-amplified. Detection is subsequently made by the increase in fluorescence polarization.
  \end{itemize}
\end{itemize}
• INNO- LIPA Rif. TB Assay (Innogenetics Ghenet, Belgium) for the detection of both *M. tuberculosis* and its resistance to rifampicin e.g the INNO-LipA RiF, TB assay.

• Genotype MTBDR Assay (Hain Life science, Nehren, Germany). This assay allows the rapid and specific detection of *M. tuberculosis* and the most frequent mutations leading to isoniazid and rifampicin resistance in clinical *M. tuberculosis* isolates. Both the PCR technology and the reverse hybridization technique used for the test have been proven to be robust and reproducible and results are easy to interpret.

• In diagnostic Mycobacteriology, the introduction of PCR or CDAT was considered as an exciting new milestone. The probabilities of confirming a diagnosis of tuberculosis in paucibacillary situations are higher with PCR than with the routine diagnostic procedures such as sputum smear microscopy, followed by chest X-rays. The advantage of the test is that it can be performed from a single specimen and yields results within a day. This makes the diagnostic process shorter and more patient friendly, and in turn it may reduce transmission of infection. However, the disadvantage of PCR is that it may also detect non-viable tubercle bacilli and PCR negative data must be considered more carefully because of false negatives due to the presence of inhibitors of PCR in some samples. These techniques however, should be performed always in conjunction with microscopy and culture, and the results should be always interpreted with the patient’s clinical data.¹²⁴
DRUG SUSCEPTIBILITY TESTING

With the emergence of drug resistance, determination of drug susceptibility testing is important for starting appropriate treatment. The principle of *M. tuberculosis* drug testing established more than three decades ago by Canetti et al. and has evolved overtime.

Drug susceptibility testing is one of the most difficult procedures to standardize for the Mycobacteriology laboratory.

- **Direct and Indirect Susceptibility Tests**

Susceptibility testing may be performed by either the direct or the indirect method. The direct method uses as inoculum a smear positive concentrate of the specimen containing more than 50 acid-fast bacilli per 100 oil immersion fields. The specimen may be diluted based on smear positivity. The direct test provides early results (3 weeks) and the inoculum are true representatives of the bacterial population. But the direct method is less standardized and contamination may occur.

For the indirect test, the inoculum is the primary culture isolate. In this test, the preparation of uniform inoculum is easier, but it is essential to avoid selecting a predominant resistant or susceptible population of cells in subculture.

- **Conventional Phenotypic Methods**

Conventional susceptibility testing essentially determines whether an isolate is resistant to an agent by evaluating growth or metabolic activity in the presence of the drug.
There are three standard methods using solid media.

1) **Absolute Concentration Method.**

2) **Resistance Ratio Method**

3) **Proportion Method.**

1) **Absolute Concentration Method**

This method uses a standardized inoculum grown on drug free media and media containing graded concentrations of the drug to be tested. Several concentrations of each drug are tested and resistance is expressed in terms of the lowest concentration of the drug that inhibit the growth i.e Minimal inhibitory concentration (MIC) (Defined as 20 colonies or more at the end of four weeks.)

2) **Resistance Ratio Method**

It compares the resistance of test strains of tubercle bacilli with that of a standard strain (H_{37}Rv), Parallel sets of media, containing two fold dilutions of the drug are inoculated with a standard inoculum prepared from both the test and standard strains of the tubercle bacilli. Resistance is expressed as the ratio of the minimal inhibitory concentration (MIC) of the test strain divided by the MIC of the standard strain in the same set.

If the ratio of the MICs for the test strain to the standard strain is less than or equal to two, the isolate is susceptible. If the ratio is more than or equal to eight, the isolate is resistant.
3) Proportion Method

Originally described by Middlebrook and Corn, the proportion method was later refined by Canetti et al\textsuperscript{132} at the Pasteur Institute in Paris. This technique is most widely used for drug susceptibility testing throughout the world and currently the method of choice. This method enables the precise quantitation of the proportion of mutants resistant to a given drug. Dilution of the inoculum is made so that growth on control media and drug containing media results in the production of a number of colonies that can be counted. The inoculum is inoculated on to both drug-free and drug containing media. The ratio of the number of colonies growing on the drug containing medium to the number of colonies on drug free medium indicates the proportion of drug resistant bacilli. From these bacterial colony counts, the proportion of mutants resistant to the drug concentration tested can be determined and expressed as a percentage of the total number of viable colony forming units in the population.

Criteria of resistance: Any strain with 1% bacilli resistant to any of the four drugs is classified as resistant to that drug. There are two variants of this method. The simplified variant requires the testing of only one drug concentration and is widely used in most public health settings. The standard variant uses several drug concentrations and is mainly used in research laboratories.

- Automated Methods

The proportion method has been modified to be performed in broth culture used in radiometric BACTEC 460. This method is quicker than the agar proportion method but has the drawback of using radioisotopes. Non-radioactive liquid-based culture systems have also been adapted to perform susceptibility testing, such as BACTEC MGIT 960 systems, the MB/BacT system and the Versa TREK. Many studies have
now been published on the application of the MGIT system for the rapid detection of resistance to first and second-line anti tuberculosis drugs.\textsuperscript{133, 134}

- Recently Developed Phenotypic Methods

1) Phage- Based Assay

The phage based assay relies on the ability of \textit{M.tuberculosis} to support the growth of an infecting mycobacteriophage. Mycobacteriophages have been used both to detect viable organisms in specimen and to perform rapid susceptibility testing to Rifampicin. There are currently two formats of phage-based assays that have been described for the rapid (48-72 hrs) detection of drug resistance in \textit{M.tuberculosis}.\textsuperscript{135}

Fast Plaque-TB Rif. Biotec Laboratories

Presence of PLAQUES indicates phages and hence viable bacilli after exposure to Rifampicin considering resistant. While absence of plaques indicate no phages, hence no viable bacilli after exposure to Rifampicin, considering sensitive\textsuperscript{135}

Luciferase Reporter Phages

It is based on the efficient production of a light signal by viable mycobacteria infected with specific reporter phages expressing the firefly luciferase gene. Light production is dependent on phage infection, expression of the luciferase gene and the level of cellular adenosine triphosphate (ATP). Signals can be detected within minutes after the infection. \textit{M.tuberculosis} isolates Susceptible to INH or RIF result in extinction of light production, while drug- resistant strains continue to produce light.\textsuperscript{136}
2) **Colorimetric methods**

A relatively new method of rapid susceptibility testing uses colorimetric method to indicate growth. A colored indicator is added to the medium after exposure of *M.tuberculosis* to the antimycobacterial drug (INH and RMP). The presence of viable mycobacteria is detected by a change in color of the indicator. The assays are usually performed in microtiter plates with a range of concentration of the drugs, and in this way an MIC can be derived. The indicators used are Tetrazolium salts (XTT and MTT), Alamar blue and resazurin.

The turnaround time of these tests is usually 7-14 days which again compares very favourably with current methods. The pooled sensitivities for the detection of RMP and INH resistance were both 98% while the pooled specificities were 99% and 98% respectively. 137,138

3) **Nitrate Reductase Assay**

The nitrate reductase assay (NRA) is a quite simple technique based on the capacity of *M.tuberculosis* to reduce nitrate to nitrite, which is detected by adding a chemical reagent to the culture medium. *M.tuberculosis* is cultivated on L-J medium in the presence of an antibiotic and is ability to reduce nitrate is measured after 10 days of incubation. Resistant strains will reduce the nitrate which is revealed by a pink red color in the medium, while susceptible strains will lose this capacity as they are inhibited by the antibiotic. 139,140,141
4) Microscopic observation drug susceptibility (MODS)

Microscopic Observation Drug Susceptibility (MODS) has been described for the early detection of *M. tuberculosis* growth in liquid medium, allowing a more timely diagnosis and drug susceptibility testing. The method is based on the observation of the characteristic cord formation of *M. tuberculosis* visualized microscopically in liquid medium with the use of an inverted microscope (Caviedes, 2000). The presence of growth in the control well but not in the drug containing wells indicates susceptibility to that drug. The accuracy of the susceptibility tests has been equally impressive, with most studies showing excellent correlation between the MODS result and that of a reference method (agar proportion or BACTEC 460).142-144

5) E-test (AB BIO DISK)

The E test is an antibiotic-impregnated strip in which the test antibiotic is adsorbed to produce a concentration gradient from the top of the strip to the bottom, through at least 15 doubling dilutions. The diffusing antibiotic provides a continuous minimal inhibitory concentration (MIC) gradient in the agar, which can be interpreted by reading the meniscus of growth inhibition against a calibration scale printed on the strip.145
GENOTYPIC METHODS

As with the molecular identification systems, a number of in-house and commercial assays for detecting resistance mutations in \textit{M. tuberculosis} have been described. Most work has been done on detecting resistance to INH and RMP partly because the resistance mutations are well described and partly because these two drugs form the cornerstone of therapy of TB.\textsuperscript{101}

**Commercial Assays\textsuperscript{101, 146, 147}**

Two commercial reverse hybridization assays have been released for the detection of RMP and INH resistance.

INNO-Lipa Rif TB assay (Innogenetics Ghent, Belgium)

Genotype MTB DR assay (Hain Lifesciences Nehren, Germany)

- The INNO-LipA Rif assay was introduced several years ago and is based on reverse hybridization of amplified DNA from cultured strains or clinical samples to 10 probes, covering the core region of the \textit{rpoB} genes, of \textit{M. tuberculosis} immobilized on a nitrocellulose strip. This assay only detects resistance to RMP.

- The Geno Type MTB DR is based on the DNA strip technology and permits the molecular genetic identification of the \textit{M. tuberculosis complex} and its resistance to Rifampicin and/or Isoniazid from cultured strains or smear-positive clinical specimens. It is based on the detection of the most common mutations in the \textit{rpoB} gene for Rifampicin resistance and \textit{Kat G} gene for high level isoniazid resistance and \textit{inh A} gene for low level isoniazid resistance.
Microarrays

Microarrays, also known as biochips or DNA chips, have been proposed as genotypic methods for detecting drug resistance in *M. tuberculosis*. They are based on the hybridization of DNA obtained from clinical samples to oligonucleotides immobilized on a solid support such as miniaturized glass slides. They have been tested to detect resistance to INH and RIF.¹⁴⁸