"STUDIES ON DRUG RESISTANCE PATTERNS AND THEIR MECHANISMS IN *MYCOBACTERIUM TUBERCULOSIS*"

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BHARATI VIDYAPEETH UNIVERSITY, PUNE

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THE FACULTY OF SCIENCE

BY

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CERTIFICATE

This is to certify that the work incorporated in the thesis entitled **"Studies on drug resistance patterns and their mechanisms in *Mycobacterium tuberculosis*"** for the degree of **'Doctor of philosophy' (Ph.D.)** in the subject of **Microbiology** under the F**aculty of Science** has been carried out by Miss PatilSeemaDattatraya in the Department of Microbiology at BharatiVidyapeeth Deemed University, Medical college, Pune and Rajiv Gandhi Institute of Information Technology and Biotechnology, Pune during the period from 27/07/2011 to 31/12/2014 under the guidance of Dr. M.G. Bodhankar.

Place: Pune

Date:

CERTIFICATION OF GUIDE

This is to certify that the work incorporated in the thesis entitled **"Studies on drug resistance patterns and their mechanisms in *Mycobacterium tuberculosis*"** submitted by Miss Patil Seema Dattatraya for the degree of '**Doctor of Philosophy'(Ph.D.)**in the subject of **Microbiology** under the **Faculty of Science** has been carried out in the Department of Microbiology, Bharati vidyapeeth Deemed University Medical college, Pune and Rajiv Gandhi Institute of Information Technology and Biotechnology, Pune during the period from 27/07/2011 to 31/12/2014 under my direct supervision/guidance.

Place: Pune

Date:

Dr. M. G. Bodhankar

(Research Guide)

DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled "**Studies on drug resistance patterns and their mechanisms in *Mycobacterium tuberculosis***" submitted by me to the Bharati Vidyapeeth Deemed University, Pune for the degree of '**Doctor of Philosophy' (Ph.D.)** in **Microbiology** under the **Faculty of Science** is original piece of work carried out by me under the supervision of Dr.M.G.Bodhankar. I further declare that it has not been submitted to this or any other university or institution for the award of any degree or diploma.

I also confirm that all the material which I have borrowed from other sources and incorporated in this thesis is duly acknowledged. If any material is not duly acknowledged and found incorporated in thesis, it is entirely my responsibility. I am fully aware of the implications of any such act which might have been committed by me advertently or inadvertently.

Place: Pune

Date: (Miss Seema Dattatraya Patil)

Dedicated to

Our beloved Vice-Chancellor

Honorable *Dr. Shivajirao Kadam*

Bharati Vidyapeeth Deemed University, Pune.

Whose blessings & constant encouragement

have always been with me throught my endeavour

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Place: Pune

Date: (Miss Seema Dattatraya Patil)

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ABBREVIATIONS AND ACRONYMS

Abbreviation	Full form
AFB	Acid Fast Bacilli
AM	Amikacin
Amx/Clv	Amoxicillin/Clavulanate
Arg	Arginine
ARI	Annual Risk of TB in India
ART	Anti Retroviral Treatment
ACSM	Advocacy, Communication& Social Mobilization
ATT	Anti Tuberculosis Therapy
BD	Becton Dickinson
BMRC	British Medical Research Council
Cat I, II, III	Category I,II,III
Category IV	Regimen for MDR TB
Category V	Regimen for XDR TB
CC	Conjugate Control
Cfx	Ciprofloxacin
CM	Capreomycin
Cfz	Clofazimine
Clr	Clarithromycin
CP	Continuation Phase
CPC	Cetyl Pyridinium Chloride
CNS	Central Nervous System
Cs	Cycloserine
Cys	Cysteine
CTD	Central TB Division
DANIDA	Danish International Development Agency
DFID	Department for International Development

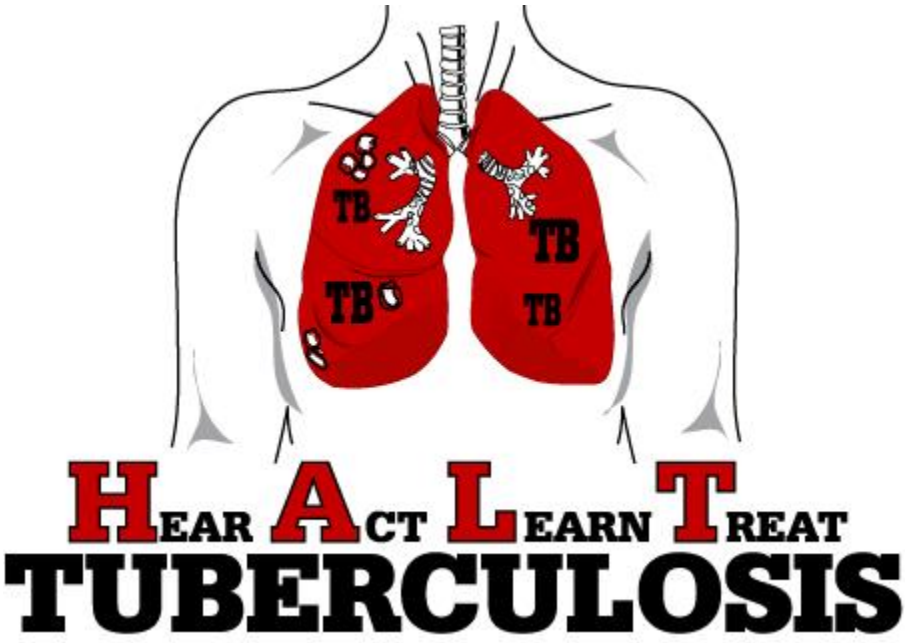
DNA	Deoxy-ribo nucleic acid
DMC	Designated Microscopy Centre
DOT	Directly Observed Treatment
DRS	Drug Resistance Surveillance
DR-TB	Drug resistant Tuberculosis
DST	Drug Sensitivity Testing
DTC	District TB Centre
DTH	Delayed Type hypersensitivity
DTO	District TB Officer
E	Ethambutol
EQA	External Quality assessment
Eto	Ethionamide
FQ	Fluoroquinolone
GDF	Global Drug Facility
GFFATM	Global Fund to Fight AIDS, TB and Malaria
Gfx	Gatifloxacin
GLC	Green Light Committee
GoI	Government of India
GMSD	Government Medical Store Depot
H	Isoniazid
HAART	Highly Active Anti-Retroviral Therapy
HCW	Health Care Worker
His	Histidine
HIV	Human Immunodeficiency Virus
HRD	Human Resource Development
ICMR	Indian Council of Medical Research
IFN- γ	Interferon gamma
IP	Intensive Phase
Ile	Isoleucine
IMA	Indian Medical Association

Ipm/Cln	Imipenem/Cilastatin
IRL	Intermediate Reference Laboratory
IUATLD	International Union Against TB and Lung Diseases
Km	Kanamycin
LAM	Lipoarabinomannan
Leu	Leucine
Lfx	Levofloxacin
LJ	Lowenstein-Jensen
LJ with NAP	Lowenstein-Jensen with Nalidixic acid and Penicillin
LRS	Lala Ram Syrup TB Institute, Delhi
LPA	Line Probe Assay
Lzd	Linezolid
MDR-TB	Multidrug-Resistant Tuberculosis
Mfx	Moxifloxacin
MGIT	Mycobacterium Growth Inhibitory Testing
MIC	Minimum Inhibitory Concentration
MO-TC	Medical Officer- TB Control
MUT	Mutation
NaCl	Sodium Chloride
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydrogen (reduced)
NIRT	National Institute for Research in Tuberculosis
NGO	Non- Governmental Organization
NRL	National Reference Laboratory
NRHM	National Rural Health Mission
NRTI	Nucleoside Reverse Transcriptase Inhibitors
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitors
NSP	New sputum positive
NTI	National TB Institute, Bangalore
NTM	Non-Tuberculous Mycobacteria

NTP	National Tuberculosis Program
PANTA	Pilymyxin B, Amphotericin B, Nalidixicacid, Trimethoprim, Azlocillin
PHI	Peripheral Health Institution
PAS	p-amino salicylic acid
PCR	Polymerase Chain Reaction
Pto	Proethionamide
PTB	Pulmonary Tuberculosis
OADC	Oleic acid, Albumin, Dextrose and Catalase
Ofx	Ofloxacin
QRDR	Quinolone resistance determining region
PNB	p-nitrobenzoic acid
R	Rifampicin
RIF	Rifampicin
RNTCP	Revised National TB Control Prgramme
S	Streptomycin
SCID	Severe Combined Immunodeficiency
SIADH	Syndrome of Inappropriate Antidiuretic Hormone
Ser	Serine
SEAR	South East Asian region
STR	Streptomycin
SD	Standard Diagnostics, Seoul, South Africa
SNRL	Supra-National Reference Laboratory
SOP	Standard Operating Procedures
SSCP	Single strand confirmation polymorphism
STR	Standardized Treatment Regimen
TB	Tuberculosis
TDR-TB	Totally Drug-Resistant Tuberculosis
Thz	Thioacetazone
TLR2	Toll-like Receptor 2
TRC	TB Research Centre, Chennai

Trd	Terizadone
UNICEF	United Nations International Children Emergency Fund
Val	Valine
VM	Viomycin
WHO	World Health Organization
WT	Wild type
XDR-TB	Extensively Drug Resistant Tuberculosis
XXDR-TB	Extremely Drug-Resistant Tuberculosis
Z	Pyrazinamide
ZN	Ziehl-Neelsen

DRUG RESISTANT TUBERCULOSIS



INTRODUCTION

INTRODUCTION

March 24th, 2015 marked 133th anniversary of Robert Koch's discovery of *Mycobacterium tuberculosis* (MTB). More than half a century has passed since the discovery of first anti-tubercular drug but the end of White plague is still not in sight. Tuberculosis (TB) had been on decline in West to an extent that people had started thinking in terms of its elimination. However, in 1993 increasing reports of Multi-drug Resistant Tuberculosis (MDR-TB) were noted from USA [(90)] and other parts of the world [(136)] and WHO declared TB as a Global Emergency.

In spite of newer modalities for diagnosis and treatment of TB, unfortunately, millions of people are still suffering and dying from this disease. TB is one of the top three infectious killing diseases in the world: HIV/AIDS kills about 3 million people each year, TB kills 2 million while malaria kills 1 million [(247)]. Even though tubercle bacillus was identified nearly 133 years ago, a definitive understanding of pathogenesis of this disease is still deficient. Although, it can affect people of any age, individuals with weakened immune systems, e.g. with HIV infection, are at increased risk. Since the immune system in healthy people walls off the causative bacteria, TB infection in healthy people is often asymptomatic. This bacterium lives and multiplies in the macrophages, thus avoiding the natural defense system in the patient's serum. Infection with TB can result in two stages: asymptomatic latent tuberculosis infection (LTBI) or tuberculosis disease. If left untreated, the mortality rate with this disease is over 50% [(247)].

Global Scenario

According to WHO, TB is a worldwide pandemic and MDR-TB has reached in all corners of the world while 100 countries had reported Extensively Drug Resistant TB (XDR-TB) by the end of 2013. Among the 15 countries with the highest estimated TB incidence rates, 13 are in Africa and half of all new cases are in six Asian countries, viz., Bangladesh, India, China, Indonesia, Myanmar and Thailand [(314)].

TB is contagious and airborne. It is a disease of poverty affecting mostly young adults in their most productive years. Vast majority of TB deaths are in the developing world. 1.5

million People died from TB (including 5, 10,000 women) in 2013, including 1.1 million (13%) people with HIV. About 75% of these cases were in the African region. Globally in 2013, an estimated 4, 80,000 people developed MDR-TB and there were an estimated 2, 10,000 deaths from MDR-TB. Most TB cases and deaths occur among men, but TB remains among the top three killers of women worldwide. Half of the HIV positive people who died from TB in 2013 were women (1, 80,000). Of the estimated 8.6 million new TB cases worldwide in 2013, 2.9 million were women. There were an estimated 5, 50,000 TB cases among children (under 15 years of age) and 80,000 TB deaths among HIV negative children in 2013. The majority of cases worldwide in 2013 were in the South-East Asia and Western Pacific Regions (56%). A further one quarter was in the African Region while India and China alone accounted for 24% and 11% of total cases, respectively [313].

According to WHO report-2014, globally 3.5% of new and 20.5% of previously treated TB cases was estimated to have had MDR-TB in 2013 and on an average; an estimated 9.0% of patients with MDR-TB had XDR-TB [313].

A WHO fact sheet dated March 2010 [309] on TB stated that every second, someone in the world get newly infected with TB bacilli and one in every 10 of these newly infected people will become sick or infectious later in life. Since concurrent infection with HIV weakens the immune system, people with co-infection of HIV and TB are much more likely to develop TB. It is a leading cause of death among HIV-positive people. In Africa, HIV is the single most important factor contributing to the increase in the incidence of TB since 1990. The same fact sheet [309] stated that in 2008, globally speaking, there were 9.37 million new cases of TB with the African region and the South East Asian Region (SEAR) having a share of 30% and 34% respectively.

The global community woke up to this disease when in 1993, WHO declared TB as a global emergency. It was estimated that by 2004, the world as a whole would have achieved the Millennium Development Goal (MDG) of halting and reversing the incidence to half of its 1990's prevalence and mortality rate. Now the revised time limit to achieve that MDG is by 2015 [293].

Robert Heinrich Herman Koch.

Invented causative agent of Tuberculosis (*Mycobacterium tuberculosis*)

Nobel awards: Nobel Prize in Medicine (12th December, 1905) for his investigations and discoveries in relation to tuberculosis [39].

To control the TB, Directly Observed Treatment Short course (DOTS) is a universally accepted program. It is an internationally recognized strategy for delivering the basics of TB case finding and cure. For patients it is not simply a clinical approach, but it is a management strategy for public health systems, including political commitment, case-detection through quality-assured bacteriology, short-course chemotherapy, ensuring patient adherence to treatment, adequate drug supply and sound reporting and recording systems [(293)].

Indian Scenario

In India, TB has been mentioned in the *Vedas* and the old *Ayurvedic* scriptures. Historically speaking, fight against TB in India can be broadly classified into three periods: early period, before the discoveries of X-ray and chemotherapy; post-independence period, during which nationwide TB control programs were initiated and implemented; and the current period, during which the ongoing WHO-assisted TB control program is in place.

Early period of TB control:

It was marked with non-availability of any chemotherapeutic agents, absence of diagnostic X-ray facilities and lack of any TB control program and it lasted around middle of the 20th century. During this period, as no drug treatments with combinations of drugs were available/ effective against TB, a sanatorium movement originated in Europe and quickly spread worldwide. Popular rationale for sanatoria was that are regimen of rest, good nutrition, open fresh air and high altitude offered the best chance that the sufferer's immune system would "wall off" pockets of pulmonary tuberculosis (PTB) infection. In 1863, for the treatment of tuberculosis, Hermann Brehmer opened the world's first sanatorium named *Brehmerschen Helanstalt for Lungenkranke* in the city of Gorbersdorf (Soko-owsko), Silesia (now Poland) [(167)].

In India, the first open air sanatorium for treatment and isolation of TB patients was founded in 1906 in Tiluania, near Ajmer city of Rajasthan, followed by the first TB dispensary in Mumbai in 1917 [(257)]. By 1925, chest radiology started playing diagnostic role in detecting deep-seated areas of TB consolidation. By 1945, the capacity of this

apparatus was enhanced to embody the Mass Miniature Radiography (MMR) version. The first genuine success against TB was in immunizing against tuberculosis, developed from attenuated bovine strain of tuberculosis by Albert Calmette and Camille Guerin in 1906 was BCG (Bacillus of Calmette and Guerin) vaccine. It was first used on human in France on July 18, 1921. In 1948, with support from WHO and UNICEF, a BCG vaccine production center in Gundy, Madras (now Chennai), was set up. In 1951, India started a mass BCG campaign to control TB. This was the first nationwide campaign against TB [(216)] and for the first time in the history of India, message of health and prevention of disease was taken to the remotest parts of the country.

Post-independence initial nationwide TB control programs:

This period can be conveniently subdivided into two phases as:

a) *District TB program-*

In 1961, the Indian government and Anantpur district in Andhra Pradesh state was the first model district TB center (DTC) prepared District Tuberculosis Program. This program was aimed at integration of TB control schemes with the existing government health services to reduce the TB problem in the community as economically as possible [(2)]. Shortly after establishing the Anantpur DTC, it became evident that although case finding could be done at any place without difficulty, the major problem in the fight against TB was that of keeping the patients on continuous treatment until cure was achieved [(114)]. Using this district TB center model, in 1962, the Indian government launched the National TB Control Program (NTCP).

b) *Era of short-course chemotherapy-*

It is reported that in the middle of 20th century, around the time India gained independence in 1947, effective drugs against TB started becoming available (Streptomycin: 1944, Para-amino salicylic acid: 1946, Thioacetazone: 1950, Isoniazid: 1952 and Rifampicin: 1966) [(249)]. In 1956, under the auspices of the Indian Council of Medical Research (ICMR), the government of Chennai state, the WHO and the British Medical Research Council (BMRC), the Indian government established the Tuberculosis

Research Center (TRC) in Chennai. This center provided information on the mass domiciliary application of chemotherapy in the treatment of pulmonary TB. In 1959, National Tuberculosis Institute (NTI) was established at Bangalore to evolve, through research, a practicable TB program that could be applied in all parts of the country by training medical and paramedical workers to efficiently apply proven methods in rural and urban areas [196].

Chemotherapy for TB underwent revolutionary changes in the seventies owing to the availability of two well- tolerated and highly effective drugs, Rifampicin and Pyrazinamide. These drugs allowed short-course chemotherapy (SSC) and made it possible to simplify treatment and reduce its duration. Discovery of Rifampicin in 1967 is considered one of the greatest achievements in the history of development of anti-TB drugs. Since its discovery, no new drug has been discovered yet which is as efficacious as Rifampicin against TB. However, the emergence of MDR-TB and extensively drug resistant tuberculosis (XDR-TB) has spurred interest in the development of novel drugs. For the effective treatment outcome, there is a dire need of new drugs with a different mechanism of action that can tackle both drug sensitive as well as drug-resistant strains. Bedaquiline is one such new drug with unique mechanism of action [101]. Food and drug Administration has approved Bedaquiline and Delamanid for the treatment of MDR-TB under specific conditions in December 2012 [313].

Current WHO-assisted ongoing TB control program:

In 1992, Government of India, WHO and the Swedish International Development Agency (SIDA), reviewed the national program and concluded that it suffered from managerial weaknesses, inadequate funding, over-reliance on X-ray, nonstandard treatment regimens, low rates of treatment compliance and completion and lack of systematic information on treatment outcomes [287]. In1993, WHO declared TB to be a global emergency, devised the DOTS strategy and recommended all countries to adopt this strategy. This strategy was built on five pillars viz., political commitment and continued funding for TB control programs, diagnosis by sputum smear examinations, uninterrupted supply of high-quality anti-TB drugs, drug intake under direct observation and accurate reporting and recording of all registered cases.

World Bank acknowledged that the DOTS strategy was the most economical health intervention and agreed to provide credit assistance for the NTCP, initially for the coverage of a population of 271 million persons, which was later revised to cover a population of 730 million persons. Presently, other bilateral and multilateral agencies, Danish International Development Agency (DANIDA), Department for International Development (DFID), US Agency for International Development (USAID), Global Fund to Fight HIV/AIDS, Tuberculosis and Malaria (GFFATM), Global Drug Facility (GDF) and WHO are providing invaluable support to the program. The Global Fund to fight HIV/AIDS, Tuberculosis and Malaria is the single biggest source of external funding for TB control [(262)].

To give new thrust and to revitalize the NTCP, with assistance from the mentioned international agencies, in 1997, the Revised National TB Control Program (RNTCP) was launched [(290)]. It formulated and adopted the internationally recommended DOTS strategy as the most systematic and cost-effective approach to revitalize the TB control program in India. Today, India's DOTS program is the fastest- expanding and the largest program in the world in terms of patients initiated on treatment; and the second largest, in terms of population coverage [(132)].

HIV and TB:

TB and HIV form a lethal combination, each speeding the others progress. Someone who is HIV positive and infected with TB bacilli is 100 times more likely to develop progressive TB than HIV negative person infected with TB bacilli. TB is the leading cause of death among people who are HIV positive. In Africa, HIV is the single most important factor contributing to the increase of TB since 1990 [(305)] [(301)].

TB is a leading cause of death in people with HIV infection, accounting for 1.1 million people who develop TB in 2013; it is the commonest HIV-associated opportunistic disease in the world [(313)]. It accelerates HIV disease progression, increasing infectivity and reducing HIV treatment efficacy [(49)] [(74)].

In 2013, of the total TB patients tested for HIV 13% were found to be HIV positive [(313)]. The interaction between HIV and TB in persons co-infected with HIV and TB is

bidirectional and synergistic. The level of immunosuppression determines clinical presentation of the resulting disease [145] [245]. Pulmonary involvement occurs in about 75% of all HIV/TB- infected patients [60] [4]. It was observed that the most frequent extra-pulmonary form of TB in HIV-positive people is with involvement of the lymph nodes, with cervical region being the commonest [15]. Co-infection of HIV and TB also results in more rapid development of MDR-TB [68] [128].

A national policy to coordinate common activities for HIV/AIDS and TB has been formulated by the National AIDS Control Organization and the Central TB Division. TB and TB/HIV interactions are reciprocally included in the national policies of both programs [188]. Among the 1.5 million TB cases reported under the national program in 2008, an estimated 73,720 cases were HIV-infected. Implementation of the revised "*national framework of joint TB/HIV collaborative activities*" began in early 2008 and interventions now cover the entire country. An intensified TB/HIV package initiated in 2008[196], is now being implemented in the entire country.

Current guidelines of National AIDS Control Organization, 2007 (NACO) recommended that irrespective of HIV status of the patient; TB requires a minimum of 6 months of treatment- with 4 drugs (including Rifampicin) in the intensive phase and 2 drugs in the continuation phase [188]. Treatment consists of Isoniazid (INH), Rifampicin (RIF), Ethambutol (EMB) and Pyrazinamide (PYZ) for two months followed by INH and RIF for 4 months, given either daily or intermittently. It further classifies the currently available antiretroviral (ART) agents as: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors, chemokine receptor antagonists and entry (fusion) inhibitors. A reduction by 80% in incident TB in HAART (Highly Active Antiretroviral Therapy) - treated patients (treated with a combination of at least 3ART drugs) was demonstrated as compared to ART- naive HIV- infected persons in Brazil [174]. Regimens recommended for use in India for HIV/TB patients are a combination of two NRTIs with Efavirenz or less commonly, Nevirapine. The NRTI combinations used commonly are Zidovudine with Lamivudine; Stavudine with Lamivudine; and rarely "Abacavir with Lamivudine" or "Didanosine with Lamivudine" [188].

A commonly observed side effect of HAART is immune reconstitution inflammatory syndrome (IRIS), which is defined as transient worsening of existing symptoms, signs or radiographic manifestation or transient appearance of new symptoms, signs or radiographic manifestation after initiation of HAART. Tuberculosis is the most frequent pathogen associated with IRIS, of which, lymph node enlargement is the commonest manifestation. In one study, the incidence of IRIS in TB alone was 2%; with HIV co-infection, it was 7% and in those started on HAART, it was 36% [187].

Current challenges:

Even today in India, two deaths occur every 3 minutes from TB. Major challenges to control TB in India include poor primary health-care infrastructure in rural areas of many states; unregulated private health care leading to widespread irrational use of first-line and second-line anti-TB drugs; spreading HIV infection; poverty; lack of political will and above all ineffective administration[272]. A collaborative effort is in the progress between NTCP and National Rural Health Mission (NRHM), which is a reform initiative of which the goal is to improve primary health care in rural areas. In addition to this, NTCP has established several initiatives in coordination with the private sector and the Indian Medical Association (IMA) to improve TB care.

Surprisingly, in India, people are still under the impression that TB is a disease of poor people, mostly those living in slums. The rich and affluent persons need to know that their cooks/ servants/ drivers can be asymptomatic carriers of this deadly disease, right in their mansions, and hence they can potentially be infected with TB even without stepping into these slums. The consumption of unpasteurized milk or dairy products made from raw milk is another potential source of TB for humans, as there is ample evidence that bovine TB (*Mycobacterium bovis*) is transmitted to humans [213,251].

Drug Resistant Tuberculosis:

Mismanagement of TB paves the way to drug resistant TB like Multi Drug Resistant Tuberculosis (MDR-TB; 1990), Extensively Drug Resistant Tuberculosis (XDR-TB; 2006) and Extremely Drug Resistant Tuberculosis (XXDR-TB) / Totally Drug Resistant Tuberculosis (TDR-TB; 2007) [170].

Multi Drug Resistant Tuberculosis (MDR-TB) is defined as tuberculosis, which is caused by the strains of *Mycobacterium tuberculosis* that are resistant at least to Isoniazid (INH) and Rifampicin (RIF), the most powerful first line anti tuberculosis drugs.

Extensive Drug Resistant Tuberculosis (XDR-TB) is defined as disease caused by strains of *Mycobacterium tuberculosis* that are resistant to not only INH and RIF (i.e., MDR-TB) but also to any fluoroquinolone and any of second-line anti-TB injectable drugs like amikacin, kanamycin or capreomycin. These forms of TB do not respond to the standard six-month treatment with first –line anti-TB drugs and can take up to two years or more to treat with drugs that are less potent, more toxic and much more expensive [(61)].

Extremely or Totally drug resistant Tuberculosis (XXDR-TB/TDR-TB) is defined as tuberculosis caused by strains of *Mycobacterium tuberculosis* that are resistant to all the existing anti-tuberculosis drugs. All these drug resistant forms of TB are the emerging threats to the success of anti-TB programs.

Drug resistance may be broadly classified as primary and acquired. Drug resistance in a patient who has never received anti-TB treatment previously is termed as primary resistance. Acquired resistance is that which occurs because of specific previous treatment. WHO and the IUATLD (International Union Against Tuberculosis and Lung Diseases) have now replaced the term *primary resistance* with the term *drug resistance among new cases*; and *acquired resistance*, with *drug resistance among previously treated cases* [(289) (291)]. The emergence of drug resistance in TB patients is mostly a result of deteriorating TB control programs. Factors related to the development of drug resistance includes: 1) inadequate or inefficient administration of effective treatment; 2) poor case holding; use of substandard drugs; 3) inadequate or irregular drug supply; 4) ignorance of health care workers in the treatment and control of TB; 5) interruption of chemotherapy due to side effects; 6) non-adherence of patients to the prescribed regimens; 7) availability of anti-TB drugs without prescription; 8) illiteracy; 9) low socioeconomic status of patients; 10) massive bacillary load; 11) laboratory delays in identification and susceptibility testing of *M. tuberculosis* isolates and 12) lack of the use of uniform laboratory methodology and quality control measures [(202)].

As per WHO guidelines, the current protocol for the treatment of MDR-TB recommends standardized treatment regimen for empirical treatment in patients who have previously received only first-line TB drugs[61), (299)]. The standardized regimen includes mixture of essential drugs- Streptomycin, Pyrazinamide, Ethambutol and Thioacetazone; and second-line drugs- Aminoglycosides (Amikacin, Kanamycin, and Capreomycin), Thioamides (Ethambutol, Prothionamide), Fluoroquinolones (Ofloxacin, Ciprofloxacin), Cycloserine/Terizidone and Para-aminosalicylic acid. Surgery should be considered for a patient with bacilli resistant or probably resistant to all except two or three relatively weak drugs. Unfortunately, many such patients will have too extensive disease and/or too poor lung function for surgery to be possible. If the patient has a large localized cavity with little other disease, reasonably lung function and only two or three (weak) drugs available, surgery should be seriously considered.

According to WHO estimates, India has the world's largest tuberculosis epidemic. WHO statistics for 2013 giving an estimated incidence figure of 25% cases of TB for India out of a global incidence of cases. It is estimated that about 40% of the Indian population is infected with TB bacteria, the vast majority of which have latent rather than active TB. Most of the statistics for India is from the government Revised National Tuberculosis Control Program (RNTCP) which was started in 1997. Tuberculosis is the biggest health issue as all the existing forms of drug-resistant strains of *Mycobacterium tuberculosis*- MDR, XDR and TDR are prevalent in the country. TDR-TB was reported in Jan, 2012 by Dr Udwadia Z.F. from Hinduja hospital, in Mumbai (Maharashtra) [(274)]. In 2012, India declared TB to be a notifiable disease- meaning that with immediate effect all private doctors, caretakers and clinics treating TB patients had to report every case of TB to the government [(258)]. According to WHO-2014 report, out of an estimated 300 000 cases of MDR-TB, more than half of these were detected in India, China and the Russian Federation. The estimated percentage of TB cases with MDR-TB is 2.2 (range: 1.9-2.6) and 15 (range: 11-19) in new and retreatment cases in 2013 [(313)]. The prevalence of XDR-TB has been reported from India, which varies between 2-4% to as high as 33.3% among HIV infected persons suffering from MDR-TB [(64)].

Maharashtra's current burden of drug-susceptible TB is 1,37,320 cases and there are 4,397 MDR-TB cases apart from the 115 XDR-TB cases, which are more difficult to treat [193]. It is reported that in last 3 years the state has recorded 165 XDR-TB cases. Out of these, 115 were recorded in 2013. Barring nine cases, 123 are from Mumbai, 25 from Navi Mumbai and 8 from Pune [116].

According to RNTCP-Drug Surveillance (DRS) report (2012) in Gujarat, Maharashtra and Andhra Pradesh, MDR-TB prevalence is low i.e. less than 3% among new cases and 12-17% in re-treatment cases. Since available data from India cover only a small portion of this vast country, there is need for continuous surveying of drug resistance by a network of investigators in different regions of the country, by employing a common protocol, with an emphasis on quality control, which will serve as a useful parameter in the evaluation of current and past chemotherapy programs.

A study using computer simulated epidemiological model predicted that natural history of a TB epidemic will last over one to several centuries , rising from very low levels to epidemic proportions , then slowly declining to endemic levels [26, 288].

Downward trend in incidence of TB was accelerated following introduction of Anti-tuberculosis therapy (ATT) in late 1940s and early 1950s. Ironically, worldwide emergence and spread of MDR-TB is one of the saddest chapters in the history of disease control, underscoring the foregone conclusion that new drugs alone cannot provide enduring solutions to problems of infectious disease, if substantial long-term commitment to public health is lacking. Drug resistance is largely man-made and is a consequence of suboptimal regimens and treatment interruptions [292]. While TB is 100% curable, MDR-TB is difficult to treat.

Resistance to antimicrobial agents is an innate characteristic of *M. tuberculosis*. It is related to genetic mutations that occur naturally in large populations of microorganisms. These mutations are thought to be associated with loss of fitness so that, in the wild state, where specific antimicrobial agents have never been used, this resistance has no clinical significance. Clinically significant drug resistance constantly has its origins in the incorrect use of antimicrobial agents and is in this sense a 'man-made' phenomenon [36].

After MDR-TB cases rose in 1990s, WHO and IUALTD established a Global project on Anti-tubercular Drug Resistance Surveillance. One of the goals was to assess extent of drug resistance through setting of Supra National Reference Laboratories (SRLS). In 2000, Stop-TB Partnership Green Light Committee (GLC) was created to provide access to preferentially priced Second-line drugs to combat rise of MDR-TB [292, 107].

Traditional laboratory methods of drug sensitivity testing takes a minimum of 10-12 weeks, which is too long a period for sputum positive case especially MDR-TB cases both from the patient's treatment and community point of view. Agar proportion method on Lowenstein-Jensen and Middlebrook 7H10 or 7H11 are considered gold standard but this takes 10-12 weeks. Automated Radiometric BACTEC 460 and Fluorescence based (MGIT) methods are available but they too take at least 3 weeks [52]. Luciferase phage reporter system in which bacteria are infected with a phage carrying the luciferase enzyme has been reported to give good results in a few days but the system is expensive and not standardized in most laboratories [234].

Rifampicin resistance is a surrogate marker for detection of potential resistance; Line Probe Assay offers a promising route to rapid identification of INH and RIF resistance [298].

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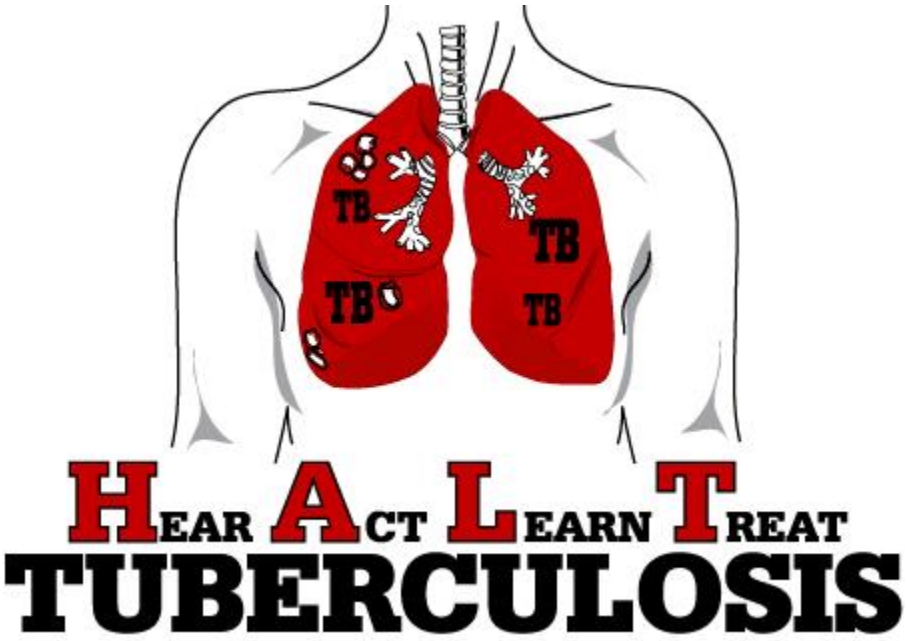
DRUG RESISTANT TUBRECULOSIS



OBJECTIVE

AIMS AND OBJECTIVES

AIMS:

To detect drug resistance patterns and their mechanisms in the isolates of *Mycobacterium tuberculosis*.

OBJECTIVES:

1. To screen the TB patients in the Bharati Hospital, Pune.

2. To isolate and identify *Mycobacterium tuberculosis* from pulmonary TB patients.

3. To detect drug resistance pattern of *Mycobacterium tuberculosis* isolates by proportion method as MDR/XDR-TB strains.

4. To study mechanisms of drug resistance of isolates of MDR strains by GenoType MTBDR *plus* assay.

5. To detect the presence of co-existing HIV infection and Drug resistance pattern in HIV patients with TB.

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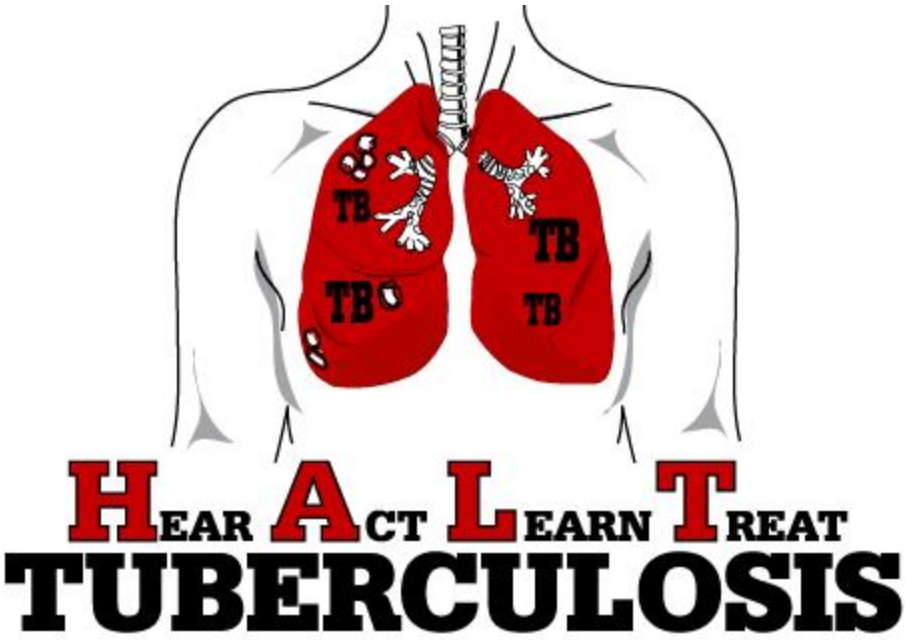
DRUG RESISTANT TUBERCULOSIS



REVIEW OF LITERATURE

REVIEW OF LITERATURE

Historical Aspects:

Tuberculosis (TB) is a disease of antiquity. TB is also referred as Pthisis [110, 204], Scrofula [11], Pott's disease [16], White Plague [154] etc. in ancient medical literature. It is estimated to be 15,000-20,000 years old. Before 19th century, human bones of Neolithic age showed presence of the bacteria *Mycobacterium tuberculosis* [30, 31]. TB became a major problem when industrial revolution led to crowded living conditions and favoured the spread.

In ancient Indian history, it is known as Rajyakshama [325], in Latin it is Consumptione [55]. It was also known as "romantic disease" [28, 92].

Chronological Historical developments in tuberculosis are:

1. 3000 and 2400 BC – First Evidence of TB was reported in Egyptian mummies.
2. 2700 BC – Emperor Shemnong for the first time mentioned the evidence of TB in Chinese literature.
3. 1550BC – Eberspapyrus reported the Pulmonary and cervical involvement in TB.
4. 1500 BC – In Rigveda TB was reported as Yakshma and Balsa in Athrvaveda.
5. 600BC –
   i. Methods of treatment with breast milk were reported in Sushrutha Samhita.
   ii. An advice to TB affected people to go to higher altitudes was reported in Yajurveda.
6. 460 BC – Hippocrates reported TB as Phthisis (a Greek word, to describe the ravages of tuberculosis; from the root phthoe).
7. 17th century –
   1. TB was reported as White Plague (name due to the white color of tubercules).
   2. Hippocrates wrote a Book on spread of TB.

3. Hippocrates has reported the first description of clinical features and it was believed that the disease could be cured due to "Divine Right of Sovereigns".

8. 1663 –
   1. The Book of Prayer contained the Royal Touch Ceremony for cure of TB.
   2. For the first time the theory of infectivity of the disease through contact was proposed by Fracstoro.
9. 1768 – Robert Whytt reported the first clinical description of Tubercular Meningitis.
10. 1779 –
    1. Percivall Pott described the Vertebral TB.
    2. William Stark described the Pathogenesis of TB.
    3. J. L. Schonlein Proposed the word "Tuberculosis" (Latin, tuberculum -small swelling on/within the organ)
    4. Rene Laennec – Died of TB at age of 45
11. 1840s – A Sanatorium movement for treatment of TB in Europe and US.
12. 1880S – TB was notifiable disease in Britain and spitting in Public was stopped.
13. 1882- 24th March – Robert Koch demonstrated *Mycobacterium tuberculosis* as a causative agent of TB.
14. 1890 – Robert Koch developed "Tuberculin" test.
15. Beginning of 20th century TB in UK was a major health problem.
16. 1902 – First International conference on TB proposed the "Cross of Larraine" as the International symbol of fight against TB.
17. 1906 – Albert Calmette and Camille Guerin – developed BCG vaccine against TB.
18. 1908 – Charles Montoux – Introduced Intradermal test for diagnosis of TB.
19. 1944 – Streptomycin was discovered – Beginning of the modern

	era of TB.
20. 1952 –	Discovery of Isoniazid – First oral Mycobactericidal drug.
21. 1957 –	Discovery of effective anti-TB drug, Rifampicin – reduced the recovery time.[195]
22. 1980s –	1. Emergence of Drug Resistant MDR-TB strains and rise of TB cases globally. 2. Franz Ziehl and Friedrich Neelsen – Introduced ZN staining for *Mycobacteria*
23. 1998 –	Genetic sequencing of *Mycobacterium tuberculosis* by Tohru Miyoshi-Akiyama, Kazunori Matsumura, Hiroki Iwai, Kelji Funatogawa and teruo Kirikae from Japan.
24. 2006 –	First case of Extensively Drug Resistant TB (XDR-TB) in South Africa.
25. 2008 –	First case of Extremely Drug Resistant TB (XXDR-TB) in Italy.
26. 2012 –	First report of totally drug resistant TB (TDR-TB) in India [274].

General Characteristics of *M. tuberculosis*:

Morphology:

Mycobacterium tuberculosis are non-motile, non-spore forming, weakly Gram positive, aerobic or microaerophilic, straight or slightly curved rod shaped bacteria [212]. Some have coccobacillary, branched, filamentous growth in the form of serpentine cords. Sensitivity of different staining methods is influenced by concentration of bacilli in sputum.

Presence of the "Beaded appearance" in some stained cells may be due to [141]-

(a) Mechanical damage to cell wall during smear preparation
(b) Irregular uptake of dye during staining

(c) Centers of respiratory enzymatic activity mimicking mesosomes in other bacteria
(d) Age of the culture

However, the most accepted one is effective anti-tubercular treatment. Beaded bacilli indicate good progress and strongly stained bacilli indicates poor prognosis.

Mycobacteria typically measure 0.5µm x 3µm classified as acid-fast bacilli and has a unique cell wall structure crucial to their survival. *Mycobacteria* have complex outer envelope made up of high lipid content, contributing to hydrophobicity of *Mycobacteria*, tendency to form clumps and cords, resistance to lysis procedures and ability to survive for longer periods. Innermost layer is plasma membrane containing proteins, phosphatidylinositol, mannosides and lipoarabinomannan (LAM) followed by peptidoglycan layer determining shape of cell.

Mycolic acids are a defining characteristic of members of genus *Mycobacterium* [(253)]. Each species of *Mycobacterium* synthesizes unique set of mycolic acids, which is exploited for identifying *Mycobacteria* and resulting pattern compared with a library of reference patterns to identify the species. Mycolic acid induces acid fastness and its degree is directly proportional to concentration of mycolic acid in cell wall. Higher the concentration of mycolic acid, higher will be the acid fastness.

Lehman and Neuman gave the name "Mycobacterium" to this bacterium in 1896 because of the mould like pellicles produced by these bacteria when grown in liquid media [(250)].

Classification of Mycobacteria:

According to the Bergey's Manual of Determinative Bacteriology, 8th edition, *Mycobacteria* are classified in to XVII division as-

Higher order taxa:

Domain: Bacteria; **Phylum:** Actinobacteria; **Class:** Actinobacteria; **Order:** Actinomycetales; **Family**: Mycobacteriaceae; **Genus: Mycobacterium**.

Species:

1. *Mycobacterium tuberculosis* complex -*M. tuberculosis, M. bovis, M. africanum, M. ulcerans, M. canetti, M. caprae, M. microti, M. pinnipedii.*
2. Non-tuberculous *Mycobacteria/ Mycobacteria* other than tuberculosis (MOTT)/Atypical *Mycobacteria* – in 1950, on the basis of growth rate and pigment production, Runyon grouped these in to four groups as-[141]

   (a) Photocromogenes – produce non-pigmented colonies when grown in the dark and pigmented colonies only after exposure to the light and re-incubation.

   Ex. *M. kansasii, M. marinum, M. simiae, M. genavense*, *M. asiaticum*

   (b) Scotocromogenes – produce deep yellow to orange colonies when grown in the presence of the dark.

   Ex. *M. scrofulaceum, M. szulgai, M. xenopi, M. celatum, M.gordonae, M. flavescens*

   (c) Non-photochromogenes – *M. avium intracellulare* complex, *M. paratuberculosis*, *M. terrae, M. trivial, M. shimoidae*

   (d) Rapid growers – *M. fortuitum, M. chelonae, M. abscessus*, *M .thermoresistible*

3. Saprophytic *Mycobacteria – M. gordonae, M. asiaticum, M. terrae, M. gastri, M. paratuberculosis.*

4. Noncultivable – *M. leprae*

Staining reaction of Mycobacteria:

Mycobacteria are difficult to stain[69]. The high lipid content in their cell wall renders them impermeable to the dyes used in Gram stain. Penetration is facilitated by phenol and heating acts as mordants. It can be stained by using strong dyes like those that concentrated carbol fuchsin mixed with phenol and facilitating the dye penetration by

gentle heating. This staining was first introduced by Ziehl and Neelsen and is commonly known as ZN staining. Over the years, this conventional heat method of ZN staining has been modified and is now being replaced by the 'cold method' of acid fast bacteria (AFB) staining. There are two other methods widely used in different laboratories viz. Kinuyon and Gabbett's modifications [141, 112]. By these staining methods, *M. tuberculosis* appears as pink colored bacilli and all other nonacid fast structures appear blue in colour (Figure 1 and 2). Morphologically AFB may appear as deeply stained, short, straight, or slightly curved bacilli. Sometimes, it may take up long filamentous form; this is due to the presence of 'cord factor' in the cell wall of such strains. This cord factor is considered as **'virulence factor'**. Some *M. tuberculosis* strains may exhibit a beaded appearance. Hok in1962 further increased the concentration of Basic fuchsin and combined steps of decolorization and counterstaining for staining of AFB [45].

In 1966, Rao *et al.* used chloroform (instead of ethanol) as solvent and reported that this modification was as efficient as ZN method. These cold staining methods are particularly useful in field survey where heating poses a problem [225].

In fluorescent staining technique, Auramine O or Auramine Rhodamine stains are used. When stained with Auramine O alone, bacteria appear yellow on a green background (Fig no.4), while with a mixture of Auramine and Rhodamine, they appear more golden coloured against dark background (Fig no.3). Fluorescent staining is more sensitive than acid fast staining. [99]

Cultural characteristic of *Mycobacterium tuberculosis*:

M. tuberculosis is nutritionally fastidious which requires special media containing serum, coagulated egg proteins for its growth. Robert Koch grew *Mycobacterium tuberculosis* for the first time using heat coagulated bovine serum [124].

In 1930, Lowenstein modified the Dorset egg medium. In 1946 Copper and Cohn suggested use of malachite green for suppression of contaminants. Jensen further (1955) modified it by using glycerol as carbon source instead of starch. This modification called as Lowenstein-Jensen medium, is today the popular medium for cultivation of tubercle bacilli [124]. First agar based medium, containing bovine serum and oleic acid was

described by Middlebrook and Duboes in 1947 [317]. Middlebrook 7H10 and 7H11 are widely used agar based media today.

Soutan (1912) modified liquid media for the cultivation of *Mycobacterium tuberculosis*. Later Middlebrook *et al.* formulated Middllebrook 7H12 broth, liquid medium with ^{14}C labeled palmitic acid as substrate. [53] This medium has revolutionized the isolation of tubercle bacilli.

Culture of tubercle bacilli is important in many ways. It not only provides a more sensitive and specific means of diagnosis of disease, especially smear negative cases, but also provides pure growth for identification of the organism by various phenotypic and genotypic tests. The pure growth is also helpful for performing Drug Susceptibility Testing (DST) indirectly.

An ideal culture medium for isolation of tubercle bacilli:

(a) should give early and luxuriant growth from small inoculums

(b) should enable preliminary separation of species based on pigment production and colony morphology.

(c) should be able to suppress the growth of contaminants effectively.

(d) should be user friendly- easy to prepare, should have longer shelf-life and

(e) should be economical.

However, such an ideal medium is at present not available as all contemporarily used media have some or the other inherent drawbacks.

Different media used for cultivation of *M. tuberculosis*:

I. Solid Media:
   (a) Egg based
      - Lowenstein- Jensen medium
      - Gruft modification of L.J. medium
      - American thoracic society (ATS) medium
   (b) Agar based
      - Middlebrook 7H10 medium
      - Middlebrook 7H10 selective medium
      - Middlebrook 7H11 medium
      - Middlebrook 7H11 selective medium

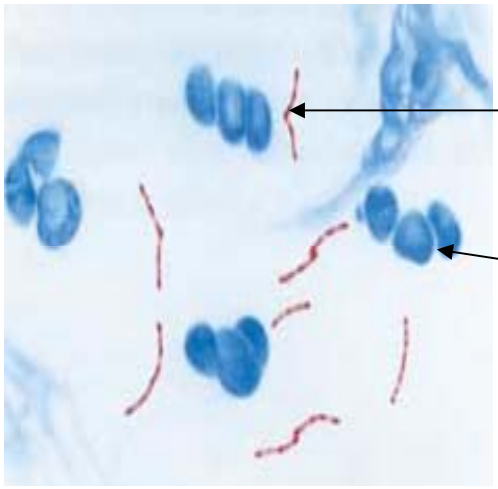


Figure 1: ZN Staining of AFB- *M. tuberculosis*

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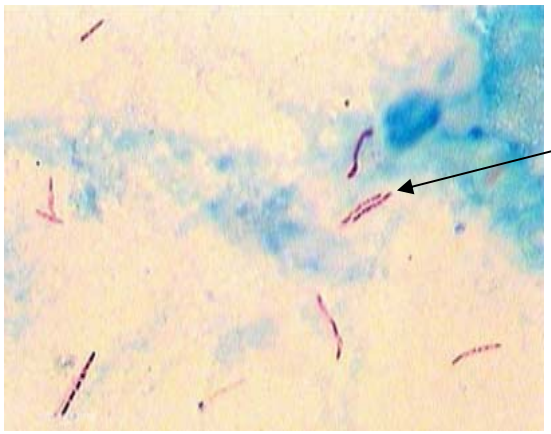

Figure 2: Cold acid-fast staining of *M. tuberculosis*

Source: acid fast staining in Tuberculosis – slide share. Available on www.slideshare.net/doctorrao/acid-fast-staining-in-tuberculosis

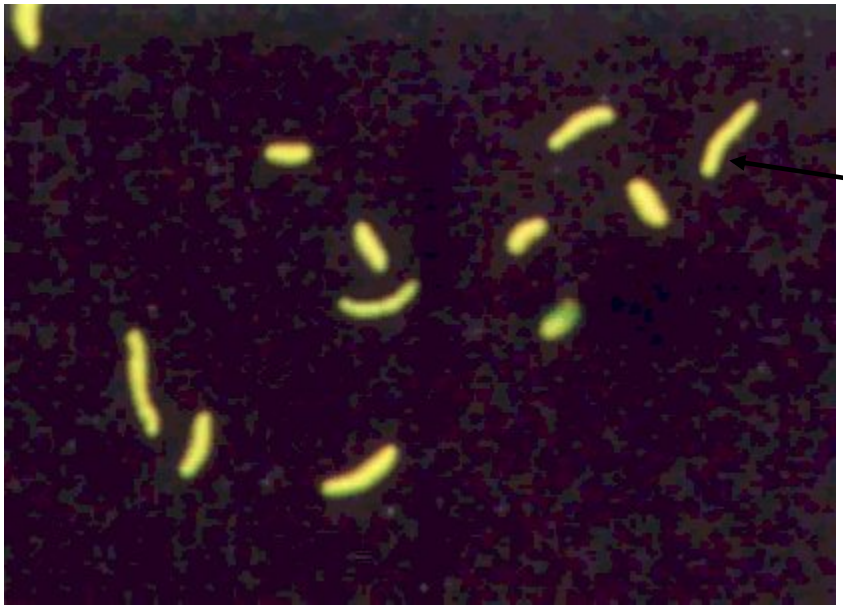

Figure 3: Staining of *M .tuberculosis* by mixture of Auramine and Rhodamine:

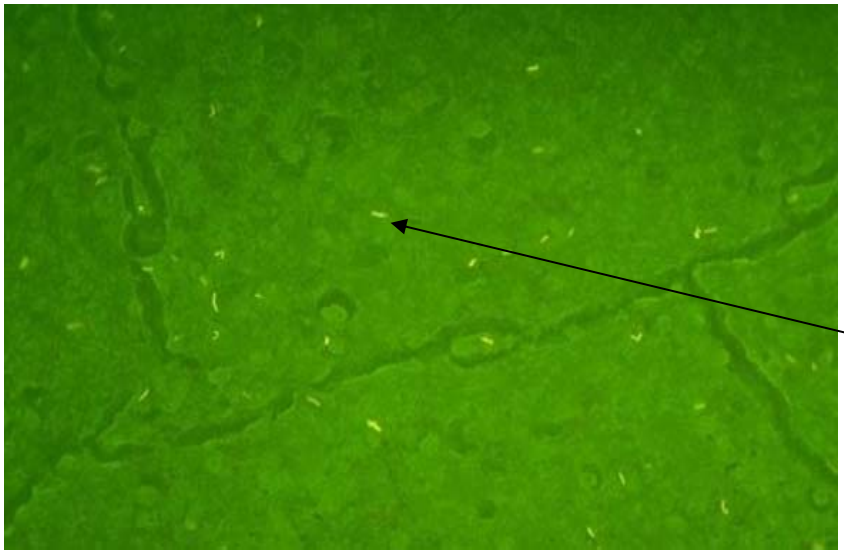

Figure 4: Staining of *M. tuberculosis* by Auramine alone:

Source (figure 3 and figure 4): acid fast staining in Tuberculosis – slide share. Available on www.slideshare.net/doctorrao/acid-fast-staining-in-tuberculosis.

II. Liquid Media:

(a) Radiometric

- Middlebrook 7H12 medium (BACTEC460TB)

(b) Non-Radiometric

- Middlebrook 7H9 broth
- Septi Check AFB system
- Mycobacterial Growth Indicator Tube(MGIT)
- MB-Redox tube

(c) Continuous Growth Monitoring Systems:

- ESP Culture System- II
- BACTEC 9000MB
- BACTEC MGIT 960TB
- MB/BacT

The more commonly used culture media and commercial systems are:

1) Lowenstein Jensen medium-

This is the most commonly used medium for cultivation of tubercle bacilli. It consists of coagulated whole egg, defined salts, glycerol, asparagine etc. Addition of malachite green in concentration of 0.0025gm% makes it somewhat selective [141].Many modifications have been described. Gruft modification, which in addition to previously described ingredients contains RNA (5mg %), Antibiotics like Penicillin (50ug/ml) and Nalidixic acid (35mg/ml) is more selective than original L.J. medium. Petran and Vera have suggested the use of Cycloheximide (400mg/ml), Lincomycin (2mg/ml) and Nalidixic acid (35mg/ml) which gives both antibacterial and antifungal coverage to medium [207].(Figure 5)

The reported rate of isolation of tubercle bacilli on L.J. medium ranges from 70-85%[235,121].It is affected by type and quality of specimen, time lag between collection of specimen and its inoculation on the medium, concentration of malachite green, use of antitubercular therapy by the patient and number of slants used. The rates are also affected by AFB smear results.

Lowenstein Jensen Medium –

Selective. Always in screw capped bottle. Bluish Green.
Contains – Egg protein – Solidifying agent
Mineral salts – Mg sulphate, Mg citrate
Asparagine
Malachite Green – Selective agent
Sterilized by - Inspissation

Figure 5: Lowenstein Jensen Medium

Stager *et al.* have shown that L.J. medium was substantially inferior to BACTEC in recovery of organism, but only marginally in comparison to Middlebrook 7H11 medium if 2 L.J. slopes are inoculated [252].But Wilson *et al.*, in their study on L.J. and Middlebrook 7H11 medium have reported recovery of 40% and 81% respectively. [318]

The average time taken to detect growth on L.J. medium in smear positive is 18-22 days and 28-31 days in smear negative cases [52]. The rate of contamination on L.J. medium varies from 0% to 9% [23].Because of these drawbacks, L.J. medium is gradually phased out from routine use in Mycobacteriology Laboratories in developed countries.

2) Middlebrook 7H10 and 7H11 media:

Both these media are prepared from basal medium of defined salts, vitamins, co-factors, oleic acid, albumen, catalase, glycerol, dextrose. Middlebrook 7H11 in addition contains 0.1% casein hydrolysate which improves recovery of INH resistant strains [97, 119].Middlebrook 7H10 is made selective for growth of *M. tuberculosis* by adding antibiotics like cycloheximide (360µg/ml), lincomycin (2µg/ml) and nalidixic acid (20µg/ml). Stager *et al.* have shown that there is not much difference in recovery rates of Middlebrook7H11 medium in comparison to L.J. medium (79% and 76% respectively).

Both these media show high contamination because they contain malachite green in 10 times smaller quantities than L.J. media [141].

3) BACTEC 460TB:

This technique utilizes ^{14}C labeled palmitic acid in Middlebrook 7H12 liquid medium. *Mycobacterium* if present utilizes these radio-labeled substances for metabolism and release $^{14}CO_2$ which is detected by BACTEC system and reported in terms of growth index. The bottles which yield growth index of more than 10 are considered positive. [119]

Choong Park *et. al*., in their study comparing BACTEC system with L.J. medium, observed that BACTEC system detected 93% cases while L.J. medium could detect 82%

of cases. BACTEC required an average of 7 days for detecting MTB while L.J. media required 18 days. Contamination rate for BACTEC and L.J. were 6.2% and 9.1% respectively. [52]

Thus, BACTEC system in comparison to L.J. medium is more sensitive method, takes less time and contamination rate is minimum. Despite these advantages, it has certain limitations, such as inability to observe colony morphology, high cost, use of radio-active materials, use of needles and cross contamination. [119, 191]

4) Septi-check AFB system:

This system consists of a capped tube containing Middlebrook 7H9 broth with enriching substances and antibiotics. The solid phase is in the form of a paddle. One side of the paddle is covered with Middlebrook 7H11 agar. The reverse side is divided into two halves, one containing L.J. media with Nalidixic acid, Amphotericin B, Polymyxin B (NAP) and other contains chocolate agar. This arrangement enables the system to detect *M. tuberculosis*, Nontuberculous *Mycobacteria* and contamination simultaneously. Overall isolation rate (80-95%), time for detection (20 days) is better than L.J. medium [111, 268] with low contamination rate [117].

5) Mycobacterial Growth Indicator Tube (MGIT):

It contains Middlebrook 7H9 broth along with nutritional supplement, antibiotic cocktail and a fluorescent compound which is embedded in silicon sensor. Oxygen diminishes the fluorescent output of the sensor. As the actively growing and respiring *Mycobacteria* consume the dissolved oxygen, the sensor glows indicating *Mycobacterial* growth. The fluorescence is observed by UV light of wavelength 365 nm. This system has better recovery rate, shorter time (7.2 days) than L.J. medium but less than that of BACTEC 460 method [50, 152, 38].

6) Microscopic observation of broth culture:

This simple technique is based on the fact that morphology of *M. tuberculosis* in liquid culture is characteristic and recognizable, consisting of tangles or cords of the organism.

When broth culture is viewed with inverted microscope, characteristic *Mycobacterial* growth can be detected long before it becomes visible to unaided eye. A 24 well plate containing Middlebrook 7H9 broth with oleic acid, albumin, dextrose and catalase (OADC) supplement and polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin (PANTA) antibiotic cocktail is inoculated with decontaminated sputum sample. The plates are examined daily from days 5 to 15, on alternate days from days 16to 25 and twice weekly from days 26 to 40, under inverted light microscope at 40x magnification.

A Peruvian study has shown that, this technique can detect 94% of cases, mean time required for culture positivity in pre and post treatment cases were 8.2 and 10 days respectively. This technique is relatively cheaper, rapid, compares well with standard methods of culture and hence is especially useful in disease endemic and resource poor countries. [65]

7) Continuous Monitoring System:

a. MGIT 960 TB:

It is a fully automated system for the growth and detection of *Mycobacteria* with a capacity to incubate and continuously monitor 960 Mycobacterial growth indicator tubes (MGIT tubes) every 60 minutes for increase in fluorescence. Growth detection is based upon the metabolic utilization of oxygen by *Mycobacteria*. Yield obtained by MGIT 960 is slightly lesser than radiometric method but significantly more than L.J. medium. This system could detect growth 1.5 days and 12 days earlier than BACTEC 460 and L.J. medium respectively [267].

b. MB-BacT method:

This method relies on continuous colorimetric CO_2 detective device to indicate Mycobacterial growth in a closed system. A solid sensor at the base of each vial contains the colorimetric indicator, which changes from green to yellow when $CO_2$is produced in the vial. Each compartment of the instrument, where vials are incubated, contains a rechlectometer and a detection unit. The measured values are transmitted every 10

minutes to a computer which indicates vials with Mycobacterial growth based on sophisticated algorithm.

A Swiss study comparing this method with BACTEC 460 and L.J. medium revealed that MBBacT could recover *M. tuberculosis* in 86.3% cases, BACTEC in 91.8% and L.J. in 79.5% cases. Time taken for culture positivity was 17.5 days, 14.3 days and 24.3 days respectively. Rates of contamination on MBBacT and BACTEC were 9% and2.7% respectively. Despite its inferior performance in comparison to BACTEC, researchers recommend it on the grounds of lesser work load and minimized exposure to laboratory workers. [206]

c. BACTEC 9000 MB system:

It is fluorescence based continuously monitoring and detection system which uses a modified Middlebrook 7H9 broth, growth supplement, and antibiotic cocktail. This system is more efficient in recovering *M. tuberculosis* than L.J. but less so in comparison to radiometric method. Mean recovery time in smear positive cases was 12.2 days and 9.3 days with BACTEC 9000 MB and BACTEC 460 respectively. Rate of contamination was significantly higher with BACTEC 9000 MB i.e. 6.8% in comparison to 1.6% in BACTEC 460 system. Despite these drawbacks authors recommended this system as an alternative to radiometric method due to comparable rate and total elimination of problems associated with radiometric method. [93]

d. ESP II culture system:

This fully automated monitoring system is based on the pressure changes within the headspace above the broth culture medium in a sealed bottle i.e. either gas production or gas consumption due to Mycobacterial growth. It has better recovery rate, shorter recovery time (18 days) than L.J. and BACTEC. [78]

Identification of *Mycobacterium tuberculosis* complex:

Identification of *Mycobacterium* species is done by

(a) Rate of growth: Growth appearing in < 1week is considered as rapid grower. *M. tuberculosis* takes 3-4 weeks to grow.

(b) Pigment production: *M. tuberculosis* colony is buff colored. Some atypical *Mycobacteria* produce pigment.

(c) Colony appearance: *M. tuberculosis* shows rough colony appearance.

(d) Biochemical tests (Table-1):

The most important and commonly used biochemical tests for the identification of *M. tuberculosis* are Niacin test; Nitrate reduction test and Catalase test (68°c) [141].

Table-1

Common Biochemical Properties of *M. tuberculosis* and other *Mycobacterium spp*. [141]

Mycobacterium species	**Niacin test**	**Nitrate reduction test**	**Catalase Ph-7.0, 68°C**	**Arylsulphatase Test(3 days)**	**Urease**	**Pyrazinamidase**	**MA**
M.tuberculosis	+	+	-	-	+	+	-
M.africanum	V	V	-	-	V	-	-
M.bovis	V	-	-	-	+	-	-
M.ulcerans	-	-	+	-	V	-	-
M. kansasii	-	+	+	-	+	+	-
M. marinum	V	-	-	V	+	+	-
M. scrofulaceum	-	-	+	-	+	V	-
M.gordonae	-	-	+	-	-	V	-
M.avium complex	-	-	-	-	-	+	V
M. fortuitum	-	+	+	+	+	+	+
M. chelonei	V	-	+	+	+	+	-
M. abscessus	-	-	+	+	+	+	+
M. smegmatis	-	+	+	-	-	-	-
M. szulgai	-	+	+	-	+	-	-

Cell wall structure of *Mycobacterium tuberculosis*: (Figure 6)

The cell wall structure of *Mycobacterium tuberculosis* deserves special attention because it is unique among prokaryotes and it is a major determinant of virulence for the bacterium. *Mycobacterium tuberculosis* has a tough cell wall that prevents passage of nutrients into and excreted from the cell, therefore giving it the characteristic of slow growth rate. The cell wall of the pathogen looks like a Gram-positive cell wall. The cell wall complex contains a polypeptide layer, a **peptidoglycan layer**, but otherwise it is composed of free complex lipids. Over 60% of the mycobacterial cell wall is lipid. The cell wall contains lipid complexes including acyl glycolipids and other complex such as free lipids and sulfolipids. There are porins in the membrane to facilitate transport. Beneath the cell wall, there are layers of arabinogalactan and peptidoglycan that lie just above the plasma membrane [(264)].The well-developed cell wall contains a considerable amount of fatty acid, mycolic acid, covalently attached to the underlying peptidoglycan-bound polysaccharide arabinogalactan, providing an extraordinary lipid barrier. This barrier is responsible for many of the medically challenging physiological characteristics of *M. tuberculosis*, including resistance to antibiotics and host defense mechanisms. The peptidoglycan polymer confers cell wall rigidity and also contributes to the permeability barrier. Another important component of the cell wall is lipoarabinomannan; a carbohydrate structural antigen on the surface is immunogenic and facilitates the survival of mycobacteria within macrophages [(146,123)].The lipid fraction of *Mycobacterium tuberculosis* cell wall consists of 3 major components, as mycolic acids, cord factor and wax-D.

Mycolic acids: These are unique α-branched lipids found in cell walls *Mycobacterium tuberculosis*. They make up 50% of the dry weight of the mycobacterial cell envelope. Mycolic acids are strong hydrophobic molecules that form a lipid shell around the organism and affect permeability properties at the cell surface. Mycolic acids are thought to be significant determinant of virulence in *Mycobacterium tuberculosis*. Probably, they prevent attack of the mycobacteria by cationic proteins, lysozyme and oxygen radicals in the phagocytic granule. They also protect extracellular mycobacteria from complement deposition in the serum. The *Mycobacterium tuberculosis* cell wall contains 3 classes of mycolic acids: alpha-, keto- and methoxymycolates.

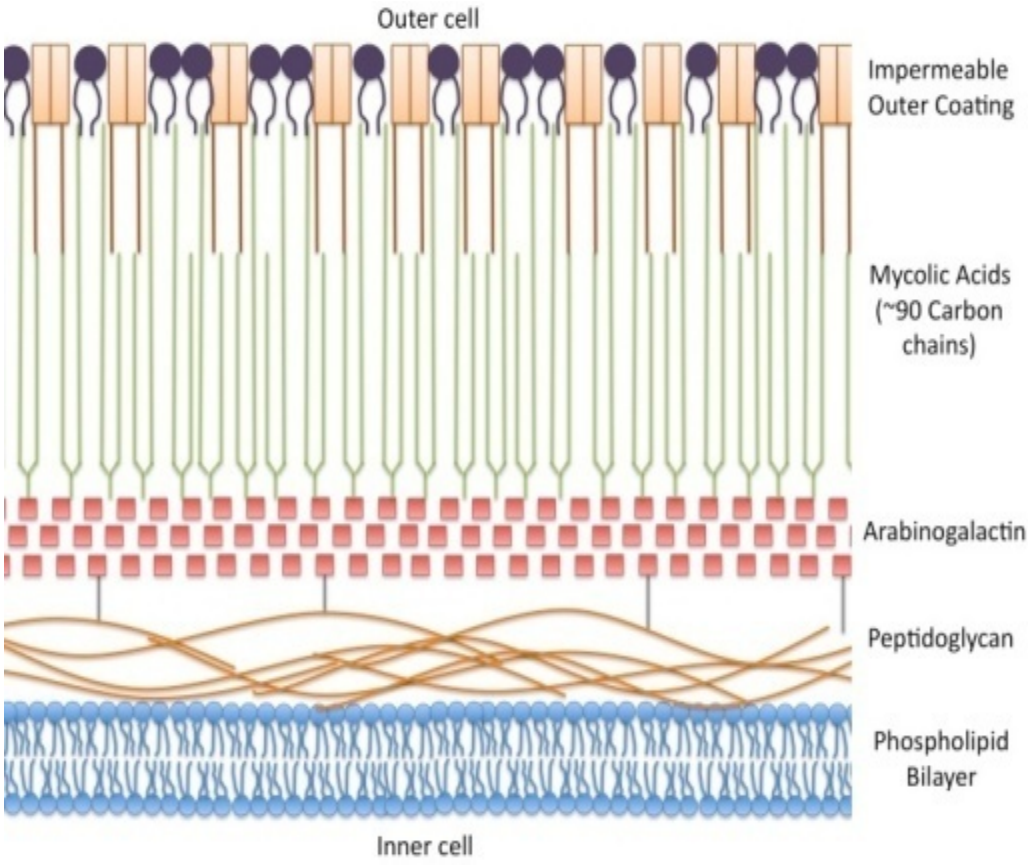

Figure 6: Cell wall structure of *Mycobacterium tuberculosis*

Source: Smith J P- Yale J Bio Med (2011). Department of Epidemiology of Microbial Diseases and Global Health, Yale School of Public health, new haven, Connection 06510, USA. jonathan.p.smith@yale.edu

Cord factor: It is responsible for the serpentine cording. Cord factor is toxic to mammalian cells and is also an inhibitor of polymorpho-nuclear cell migration. Cord factor is most abundantly produced in virulent strains of *Mycobacterium tuberculosis*.

Wax-D: It is the major component of **complete Freund's adjuvant** (CFA) in the cell envelope.

The high concentration of lipids in the cell wall of *Mycobacterium tuberculosis* has been associated with some properties as-

1. impermeability to stains and dyes.
2. resistance to many antibiotics and resistance to killing by acidic and alkaline compounds.
3. resistance to osmotic lysis via complement deposition and
4. resistance to lethal oxidations and survival inside of macrophages.

Genome structure of *Mycobacterium tuberculosis*:

Mycobacterium tuberculosis has circular chromosomes of about 4,411,529 nucleotides long (Figure 7). The G+C content is about 65.6% [(190)]. Several regions showing higher than average G+C content correspond to sequences belonging to a large gene family that includes the polymorphic G+C-rich sequences (PGRSs). The genome of *M. tuberculosis* was studied by using the strain *M. tuberculosis* H37Rv and it was published in 1998. The genome contains about 4000 genes. 40% of these genes have had their function characterized, with possible function postulated for another the 44%. Six pseudogenes are also present within the genome of *Mycobacterium tuberculosis*. Genes that code for lipid metabolism are a very important part of the bacterial genome and 8% of the genome is involved in this activity [(58)]. 250 genes of the genome are involved in fatty acid metabolism, with 39 of these involved in the polyketide metabolism generating the waxy coat. The different species of the *M. tuberculosis* complex show 95-100% DNA relatedness based on studies of DNA homology and the sequence of the 16SrRNA gene is exactly the same for all the species [(14)]. There are fifty genes coding for functional RNA molecules. These molecules are the three species produced by the unique ribosomal RNA operon, the 10Sa RNA involved in degradation of proteins encoded by abnormal messenger RNA, the RNA component of RNase P and 45 transfer RNAs. The genes

encoding tRNAs that recognize 43 of the 61 possible sense codons are distributed throughout the genome.

Insertion sequences: sixteen copies of the promiscuous insertion sequence IS6110 and six copies of the more stable element IS1081 reside within the genome of H37Rv [(208)].One copy of IS1081 is truncated. Most of the insertion sequences in *M. tuberculosis* H37Rv appear to have inserted in intergenic or non-coding regions, often near transfer RNA genes. Plasmids in *Mycobacterium tuberculosis* are important in transferring virulence because genes on the plasmids are more easily transferred than genes located on the chromosome. One such 18kb plasmid in the *Mycobacterium tuberculosis* H37Rv strain was proven to conduct gene transfers.

Genes encoding proteins: 3,924 open reading frames were identified in the genome, accounting for ~91% of the potential coding capacity. A few of these genes appear to have in-frame stop codons or frame-shift mutations and may either use frame shifting during translation or correspond to pseudogenes. Examination of the amino-acid composition of the *Mycobacterium tuberculosis* proteome shows the significant presence of amino-acids Ala, Gly, Pro, Arg and Trp, which are all encoded by G+C rich codons and a comparative reduction in the amino-acids encoded by A+T rich codons such as Asn, Ile, Lys, Phe and Tyr. There is presence of two groups of proteins rich in Asn or Gly that belongs to new families, PE and PPE. About 10%, the PE/PPE families that encode acidic, glycin-rich proteins take up the coding capacity. These proteins have a conserved N-terminal motif, deletion of which impairs growth in the macrophages and granulomas. The names PE and PPE derive from the motifs Pro-Glu (PE) and Pro-Pro-Glu (PPE), whose genes are clustered and are often based on multiple copies of the polymorphic repetitive sequences referred to as PGRSs and major polymorphic tandem repeats (MPTRs), respectively.

Metabolism of *Mycobacterium tuberculosis*:

The *Mycobacterium tuberculosis* genome encodes about 190 transcriptional regulators, including 13 sigma factors, 11 two-component system and more than 140 transcription regulators. Several regulators have been found to respond to environmental distress, such

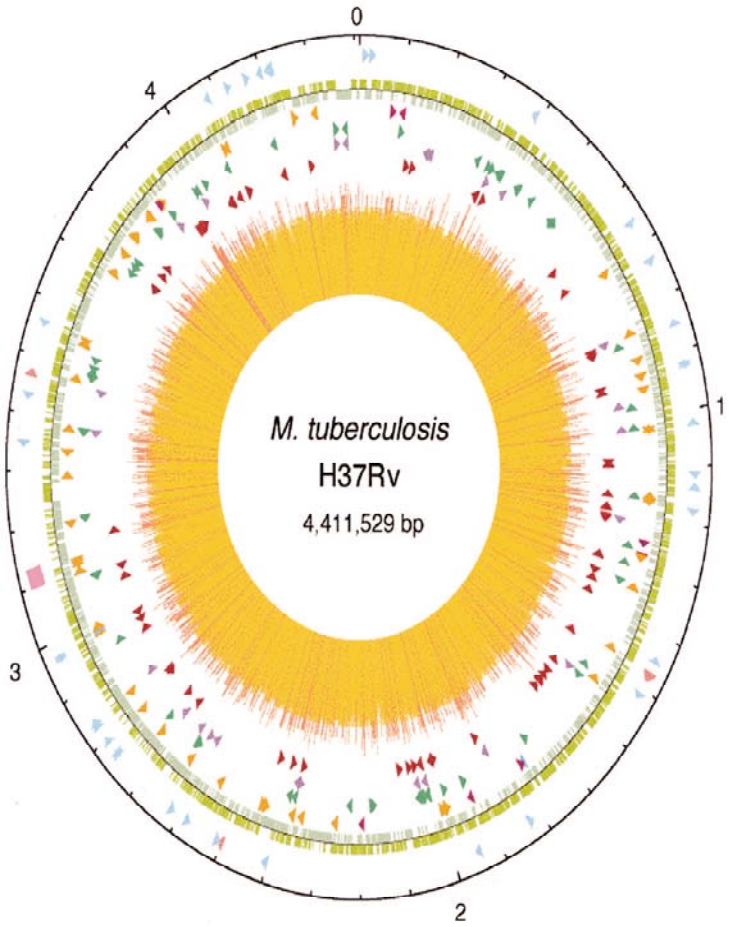

Figure 7: Circular map of the chromosome of *Mycobacterium tuberculosis* H37Rv.

The outer circle shows the scale in Mb, with o representing the origin of replication. The first ring from the exterior denotes the positions of stable RNA genes (tRNAs are blue, others are pink)and the direct repeat region (pink cubes); the second ring inwards shows the coding sequences by strand (clockwise, dark green; anticlockwise, light green); the third ring depicts repetitive DNA (insertion sequences, orange; 13E12 REP family, dark pink; prophage, blue ; the fourth ring shows the positions of the PPE family members (green); the fifth ring shows the PE family members (purple, excluding PGRS); and the sixth ring shows the positions of the PGRS sequences (dark red). The histogram (centre) represents G+C content, with <65% G+C in yellow, and >65% G+C in red.

Source: S.T. Cole *et al.* Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature (1998), 393; 537-544.

as extreme cold or heat, iron starvation, and oxidative stress [321]. To survive in these harsh conditions for a prolonged period in the host, *Mycobacterium tuberculosis* had learned to adapt to the environment by allowing or inhibiting transcription according its surroundings [200].

Metabolic pathways:

Mycobacterium tuberculosis has the potential to synthesize all the essential amino acids, vitamins enzymes and co-factors, although some of the pathways involved may differ from those found in other bacteria. *Mycobacterium tuberculosis* can metabolize a variety of carbohydrates, hydrocarbons, alcohols, ketones and carboxylic acids [286,226]. In addition to many functions involved in lipid metabolism, the enzymes necessary for glycolysis, the pentose phosphate pathway the tricarboxylic acid and glycoxylate cycles are also present. A large number (~200) of oxidoreductases, oxygenases, dehydrogenases and many oxygenases containing cytochrome P450, that are similar to fungal proteins involved in sterol degradation are present. Under aerobic growth conditions, ATP will be generated by oxidative phosphorylation from electron transport chains involving a ubiquinone cytochrome-B reductase complex and cytochrome-C oxidase. Components of several anaerobic phosphorylative electron transport chains are also present, including genes for nitrate reductase, fumarate reductase and possibly nitrite reductase. Two genes encoding haemoglobin-like proteins, which may protect against oxidative stress or be involved in oxygen capture, are also present. The ability of *Mycobacterium tuberculosis* to adapt its metabolism to environmental change is significant as it not only has to compete with the lung for oxygen but also adapt to the microaerophilic/ anaerobic environment at the heart of the burgeoning granuloma.

Cholesterol catabolism:

It has been studied extensively because of its possible therapeutic applications in tuberculosis infections. It has been shown that TB infections require cholesterol for virulence *in vivo*, because *Mycobacterium tuberculosis* utilizes cholesterol as an electron source during infection [200, 34]. As a result, therapies targeting this catabolic dependency may be developed to treat TB, once it is better understood.

The cholesterol catabolic pathway in *Mycobacterium tuberculosis* is regulated by KstR1and KstR2 proteins, which regulate gene expression. These proteins function by binding to the operator and repressing transcription of its regulon, which represses cholesterol catabolism genes, in the absence of cholesterol [276, 130].

The uptake of cholesterol uses the Mce4 transport system on the *Mycobacterium tuberculosis* cell wall and is regulated by KstR [200, 136, 48]. This Mce4 is an ATP- binding cassette transport system consisting of more than 8 proteins.

Cholesterol is first oxidized to cholestenone with the dehydrogenase, using the reduction of NAD^+ to NADH as the oxidizing agent. The catabolism of cholesterol is then split into two parts: the alkyl side chain degradation which eventually feeds into the TCA cycle and the ring cleavage of the steroid body. This process yields one molecule of acetyl-CoA and 2 molecules of propanyl-CoA, which can then generate energy via TCA cycle or be used for anabolism.

Role of cholesterol in tuberculosis infections:

Mycobacterium tuberculosis infect host and persist inside phagosomes, where there are limited nutrients. The unique ability of this pathogen to utilize cholesterol, which is a component of human cell membranes, plays a role in its persistence [200]. Furthermore, because the cholesterol catabolism pathway requires a large number of oxygenases, it is no surprise that *Mycobacterium tuberculosis* infects the lungs where oxygen concentrations are highest [280].

Therapeutic application:

As *M.tuberculosis* requires cholesterol to persist during an infection, inhibiting the Mce4 transporter can be a novel therapeutic option. Understanding the mechanism of the Mce4 transport system can provide insight into possible anti-mycobacterial targets. If the KstR proteins are prohibited from unbinding its operator and de-repressing its regulon, *Mycobacterium tuberculosis*'s cholesterol catabolism genes would not be expressed. As a result, the ability for *Mycobacterium tuberculosis* to persist inside macrophages would be lost and thus would be faster to treat.

Antigens of *Mycobacterium tuberculosis*:

M.tuberculosis has a complex antigenic structure, which includes Tuberculin antigens, Lipoarabinomannan, 38kD, 16kD, Ag85A, Ag85B, MPT63, MPT64, MTB39, MTB48, MTb81, MTC28, Rv1009, ESAT6, CFP10-ESAT6 and katG [(62)].

1. Tuberculin antigens:

Robert Koch studied antigens present in filtrates of cultured *M. tuberculosis*. During a search, as a remedy for TB, Koch described in 1891 tuberculin reactivity known as "Koch Phenomenon". He induced this in a guinea pig by injecting intramuscularly either killed tubercle bacilli or heat concentrated filtrate of medium in which bacilli had been grown termed as **"Old Tuberculin"** into an animal which was previously exposed to *M. tuberculosis*. To his surprise, the animal showed an exaggerated reaction on the skin which ulcerated after 48hrs. This reaction was named as "Koch's phenomenon". The protein was a crude extract which contained other protein extracts from the medium which induced nonspecific dermal reactions in uninfected people also. This problem was overcome by Seibert in 1934 by purifying the reagent known as "Purified Protein Derivative" (PPD) which is now used in Tuberculin Skin Test (TST).

Tuberculin Skin Test (TST): (Montoux test)

It is an intradermal Delayed Hypersensitivity reaction performed to elicit dermal response in a person previously exposed to *M. tuberculosis* and/ or its antigenic extracts. The principle of the test is based on Koch's phenomenon of delayed hypersensitivity mediated by T lymphocytes. It is a test carried out to detect exposure to *M. tuberculosis*, BCG or to any of the tubercular proteins. A positive tuberculin test (Figure 8 and Table 2) indicates the presence of a tubercular focus in the body which may be either active, healing or has healed. It is an ideal adjunct for detection of ARI (annual risk of infection) in the population and also to diagnose pulmonary tuberculosis in children with a positive radiological finding. The test is also used to assess effectiveness of control measures and to indicate those requiring BCG vaccination/ preventive therapy. Weak or Negative reactions do not exclude TB diagnosis. Some are intrinsically poor reactors and in others reactivity is depressed due to advanced disease, old age, malnutrition and

immunosuppression (HIV).This test is not 100% sensitive/specific and positive reaction does not necessarily signify presence of disease [(94)].

Procedure: 0.1ml of PPD is injected intracutaneously into the forearm. After 48-72 hours, erythema with induration is seen at the site of injection. Diameter of induration is measured and then interpreted as to whether patient has been infected with *M. tuberculosis*. An induration of more than 5mm is positive (Figure 8). A false positive result may be seen in immunosuppressed persons including HIV infected patients. In such individuals, the dosage may be increased up to 100 units of PPD.

Quantiferon TB Gold (QTF-G):

It is a FDA approved Enzyme Linked Immunosorbent Assay, which measures a component of Cell Mediated Immunity (CMI) to *Mycobacterium tuberculosis* as a means to diagnose Latent TB infection and TB disease. When heparinised whole blood is incubated overnight with mixtures of synthetic peptides, early secretary antigen target 6 (ESAT-6) and culture filtrate protein target 10 (CFP-10) simulating two proteins in *Mycobacterium tuberculosis*, Interferon gamma (IFN-γ) is released from sensitized lymphocytes. This IFN-γ is quantified to assess responses to multiple antigens. Advantages are: it does not require more than one patient visit, less subjective to reader bias and error and unaffected by prior BCG vaccination[(166)].Centers for Disease Control and Prevention (CDC) have given guidelines for using this assay in all circumstances in which tuberculin skin test is used and provides specific cautions while interpreting negative results from selected populations[(89)].

ELISPOT assay:

Enzyme linked immunospot assay also detects T cell responses to *Mycobacterium tuberculosis* antigens ESAT-6, CFP-10 and TB-7.7. This has 89% agreement with TST and correlated more closely with exposure to index case than TST. Whether QTF-G or ELISPOT blood tests are more specific or sensitive than TST in latent/active TB is still unclear. [(157)]

Figure 8: Tuberculin Test

Source: Image Courtesy of Centers for Disease Control and Prevention

Table 2-

Interpretation of Tuberculin Test

Diameter of Induration	**Considered positive for**
≥5mm	Persons at high risk for tuberculosis: - Patients with chronic diseases (eg. Infection with HIV) - Persons with recent exposure to tuberculosis - Patients with findings on radiographs suggestive of tuberculosis. - Employees of hospitals and long-term facilities.
≥10mm	Persons at risk for tuberculosis: - Injectable drug users. - Persons in close living conditions. - Persons born in countries with high prevalence of tuberculosis.
≥15mm	Persons who do not belong to either of the other groups.

2. Lipoarabinomannan (LAM):

It is structurally and functionally related to "O" antigenic lipopolysaccharides of other bacteria. Biological activities of LAM include strong seropositivity, inhibition of interferon gamma mediated activation of macrophages, induction of cytokines production and release by macrophages, scavenging reactive oxygen intermediates and suppression of T cell proliferation[29].

3. Other antigens:

Seventeen *Mycobacterium tuberculosis* antigens (38kD,16kD, Ag85A, Ag85B,MPT32, MPT63, MPT64, Mtb39, MTB48, Mtb81,MTC28,Rv1009, ESAT6, CFP10,CFP10-ESAT6,katG and ManLAM) were prepared by cloning, expression, and purification from *E.coli*. [320]

1) **38kD:** Used in serodiagnosis of pulmonary tuberculosis by ELISA.
2) **16kD:** Helps in differentiation of *Mycobacterial* species.
3) **Ag85A:** Play a significant role in the formation of lipid storage bodies and thus also in the establishment and maintenance of a persistent tuberculosis infection.
4) **Ag85B:** Secreted protein Ag85B-ESAT6 of *Mycobacterium tuberculosis* was successfully fused and expressed in *E.coli* DH5 alpha. It may become a new type of vaccine against tuberculosis.
5) **MPT32:** Glycosylated and non-glycosylated forms of the MPT-32 glycoprotein of *M. tuberculosis* induce different DTH responses.
6) **MPT63:** Involved in cell-host interactions to facilitate endocytosis/phagocytosis. Also a target site for drugs.
7) **MPT64:** Evaluation of this antigen as a possible component of subunit vaccine for *M. tuberculosis*.
8) **Mtb 39:** It is an immunodominant protein antigen of *M. tuberculosis*. It belongs to the "proline-proline-glutamic acid" family proteins. There are various types of Mtb-39 proteins called as Mtb39a-e. Despite their good specificity, these purified antigens are weakly immunogenic when injected alone, but they becomes strong immunogenic when combined with other antigens like Mtb32 and ESAT-6 to form hybrid proteins called as Mtb72F and H-1.

9) **MTB48:** Inclusion of recombinant MTB48 in prototype serodiagnostic test increases assay sensitivity for *M. tuberculosis* infection when it is combined with other known immunodominant antigens, such as the 38 kDa antigen.
10) **Mtb81:** Useful in serodiagnosis of TB.
11) **MTC28:** Specific for *M. tuberculosis* complex.
12) **Rv1009:** Promote proliferation of *Mycobacteria* and may be useful for culture of *Mycobacteria* presented in clinical samples.
13) **ESAT-6:** It is a 6kDa early secretory antigenic target of *Mycobacterium tuberculosis*. It is a secretory protein and T cell antigen. It induces highly potent T-cell responses and production of gamma interferon (INF-γ), which plays a critical role in protective cell-mediated immunity against tuberculosis. It is used in TB diagnosis by the whole blood interferon γ test known as Quantiferon-TB Gold, in conjunction with CFP-10 and TB7.7. ESAT-6 directly binds to the TLR2 receptor and inhibits downstream signal transduction.
14) **CFP-10:** It is also known as ESAT-6- like protein esxB or secreted antigenic protein MTSA-10, which is encoded by exsB gene. It forms a 1:1 heterodimeric complex with ESAT-6. Both genes are expressed from the RDI region of the bacterial genome and play a key role in the virulence of the infection.
15) **CFP-10/ESAT-6:** This complex is associated with the pahtogenecity of *M. tuberculosis*. In the mycobacterial cell, these two proteins are interdependent on each other for stability. CPF-10/ESAT-6 has a hydrophobic nature as well as high content of α- helical structures formed by the individual proteins, which lie anti-parallel to each other and forms a four-helix bundle. Its long flexible arm projecting off the four-helix bundle, formed by the seven amino-acid C-terminal of CFP-10, is essential for binding and attaching to the surface of host white blood cells; such as macrophages and monocytes.
16) **KatG:** These genes encode a dual function catalase-peroxidase enzyme which protects the cell against excess hydrogen peroxide and therefore, contributes to its survival in macrophages.
17) **ManLAM:** It is a mannosylated lipoarabinomannan, characterized by the presence of mannosyl caps on the terminal D-arabinan. It is an anti-inflammatory molecule that

inhibits production of TNF-α and IL-12 production by human dendritic cells and human macrophages in vitro and to modulate *M. tuberculosis*- induced macrophage apoptosis via binding to host macrophage mannose receptors. This is particularly important in deactivating host macrophages to allow the bacteria to survive and multiply

Soluble urokinase plasminogen activator receptor (SUPAR) levels are elevated in TB patients and associated with mortality [(79)]. Soluble antigens are antigens found in supernatant following high-speed centrifugation of a lysate of *Mycobacteria*, which removes cell wall fragments and other particulate matter. These antigens include most cytoplasmic, secreted proteins and soluble carbohydrates. Insoluble antigens are in pellet following high speed centrifugation of lysate and include cell wall components; membrane bound proteins, high molecular weight carbohydrates, lipids, mycolic acids, phenolic glycolipids etc.

Virulence mechanisms of *Mycobacterium tuberculosis*:

The virulence of *Mycobacterium tuberculosis* is extraordinarily complicated and multifaceted. Although the organism apparently does not produce any toxins, it possesses a hugh repertoire of structural and physiological properties that have been recognized for their contribution to mycobacterial virulence and to pathology of tuberculosis.

Some general properties of *Mycobacterium tuberculosis* that contribute to its virulence are [(265)]:

1) **Special mechanisms for cell entry**- The tubercle bacilli can bind directly to mannose receptors on macrophages via the cell wall-associated mannosylated glycolipid, lipoarabinomannan (LAM) or indirectly via certain complement receptors or Fc receptors.
2) **Intracellular growth**- *Mycobacterium tuberculosis* can grow intracellularly. This is an effective means of evading the immune system. In particular, antibodies and complement are ineffective. Once *Mycobacterium tuberculosis* is phagocytosed, it can inhibit phagosome-lysosome fusion by secretion of a protein that modifies the phagosome membrane. It may remain in the phagosome or escape from the

phagosome, in either case, finding a protected environment for growth in the macrophage.

3) **Detoxification of oxygen radicals**- *Mycobacterium tuberculosis* interferes with the toxic effects of reactive oxygen intermediates produced in the process of phagocytosis by three mechanisms:-
   i) Compounds including glycolipids, sulpfatids and LAM down regulate the oxidative cytotoxic mechanism.
   ii) Macrophage uptake via complement receptors may bypass the activation of a respiratory burst.
   iii) The oxidative burst may be counteracted by production of catalase and superoxide dismutase enzymes.
4) **Antigen 85 complex**- This complex is composed of a group of proteins secreted by *Mycobacterium tuberculosis* that are known to bind fibronectin. These proteins may aid in walling off the bacteria from the immune system and may facilitate tubercle formation.
5) **Slow generation time**-Because of *Mycobacterium tuberculosis*'s slow generation time, the immune system may not readily recognize the bacteria or may be triggered sufficiently to eliminate them.
6) **High lipid content in cell wall**- This accounts for impermeability and resistance to antimicrobial agents, resistance to killing by acidic and alkaline compounds in both the intracellular and extracellular environment, and resistance to osmotic lysis via complement deposition and attack by lysozyme.
7) **Cord factor- (trehalose 6, 6' dimycolate) –**It is a glycolipid found in the cell walls of mycobacteria, which causes the cells to grow in serpentine cords. It is primarily associated with virulent strains of *Mycobacterium tuberculosis.* It is known to be toxic to mammalian cells and to be an inhibitor of Polymorpho-nuclear cells migration. Its exact role in *Mycobacterium tuberculosis* virulence is unclear, although it has been shown to induce granulomatous reactions identical to those seen in TB.

   Of approximately 4000 genes in the *Mycobacterium tuberculosis* genome, 525 are involved in cell wall and "cell processes", 188 genes encode regulatory proteinsand91are involved in "virulence, detoxification and adaptation". Over 200

genes are identified as encoding enzymes for the metabolism of fatty acids [265]. This large number of *Mycobacterium tuberculosis* enzymes that putatively use fatty acids may be related to the ability of the pathogen to grow in the tissues of the infected host, where fatty acids may be the major carbon source. These genes as well as those from other classes may be directly or indirectly involved in virulence. Some of these and their products or activities are-

a) **19-kDa protein**: It is a secreted antigenic protein that is immunologically recognized by T cells and sera from TB patients. When *Mycobacterium tuberculosis* enters macrophages and other phagocytic cells, this surface-exposed glyco-lipoprotein is thought to cause host signaling events as it interacts with its receptor, Toll-like receptor-2 (TLR2). It can activate human neutrophils.
b) **Glutamine synthase**: It plays an important role in nitrogen metabolism. It is also involved in the synthesis of poly-L-glutamate-glutamine cell wall component found in pathogenic mycobacteria.
c) **Erp** – It is a surface located protein secreted by *Mycobacterium tuberculosis*. Its function in virulence is unknown.
d) **Mas-** It encodes mycocerosic acid synthase, an enzyme that catalyzes the synthesis of long-chain, methylated branched fatty acid, mycocerosic acid, which found in pathogenic mycobacteria.
e) **FbpA –** Mycobacteria have three mycolyl-transferase enzymes, encoded by 3 genes, *fbpA, fbpB* and *fbpC* that transfer long-chain mycolic acids to trehalose derivatives. The proteins can also bind the cell matrix protein fibronectin. These proteins are known as the antigen 85A, 85B and 85C complex (Antigen 85 complex). These proteins produce a formidable immunologic response, which led to the creation of a new live vaccine that was made by introducing the *Mycobacterium tuberculosis fbpB* gene into *M. bovis* BCG [56].
f) **OmpA –**It is a porin-like protein that can form pores in liposomes, a general property of porin family proteins. An OmpA expression is induced by low pH as well as by engulfment into macrophages.
g) **HbhA –**It is a heparin-binding hemagglutin protein which is localized on the surface of virulent mycobacteria. It is important for *Mycobacterium tuberculosis*

interaction with pneumocytes and this interaction may play a role in extrapulmonary dissemination.

h) **LAM-**It is a complex glycolipid that contains repeating arabinose- mannose disaccharide subunits. It is a major component of the *Mycobacterium tuberculosis* cell wall. It is known to be immunomodulator analogous to the 19-kDa protein. It down regulate host immune responses to *Mycobacterium tuberculosis* infection to protect the bacterium from potentially lethal mechanisms like the respiratory burst.

i) **MbtB –**It is an operon, consisting of mbtA to mbtJ genes, which encodes enzymes whose function is to synthesize mycobactin and carboxymycobactin, the major siderophores produced by *Mycobacterium tuberculosis*. The operon is a part of a regulon which is repressed in high iron conditions. MbtB is an enzyme that catalyzes an essential step in mycobactin synthesis.

   Like many other bacterial pathogens, during infection, *Mycobacterium tuberculosis* requires an iron acquisition system consisting of **siderophores**, to obtain iron from host iron-containing proteins such as transferring and lactoferrin. Thus, the iron availability, made possible through the activities of siderophores, promotes the growth and virulence of the *Mycobacterium tuberculosis.*

j) **Oxidative stress proteins** – Most aerobic organisms have enzymes that degrade peroxides and superoxide, which are normal byproducts of aerobic respiration but also are toxic oxygen radicals. These enzymes, generally superoxide dismutases, catalases and peroxidases, are also important for the response to various external oxidative stresses. Since phagocytic cells produce oxygen radicals during respiratory burst to kill invading bacteria, it is not surprising that these enzymes may contribute to *Mycobacterium tuberculosis* virulence. Enzymes found in *Mycobacterium tuberculosis* which combat oxygen radicals include **AhpC**, an alkyl hydroperoxide reductase that detoxifies organic hydroxyperoxides and **SodA** and **SodC**, two species of superoxide dismutase that degrade superoxides, which are normal by-products of aerobic respiration and are also produced by the phagocytic respiratory burst.

k) **Nitrate reductase** –*Mycobacterium tuberculosis* was originally thought to be an obgligate aerobe, but there are numerous experimental indicators that the bacterium can grow in microaerophilic environments, especially during the later stages of infection, e.g., in lung granulomas. Wild type *Mycobacterium tuberculosis* has been shown to possess an inducible nitrate reductase which allows respiration using NO_3 as a final electron acceptor. If anaerobic or microaerophillic growth is an important feature of *Mycobacterium tuberculosis* physiology during infection, the existence of nitrate reductase could be a significant factor in sustaining growth under these conditions.

l) **Adherence** – The specific bacterial adhesions involved in the complex interaction between *Mycobacterium tuberculosis* and the human hosts are largely unknown. This includes the heparin-binding hemagglutin (**HbhA)**, a fibronectin-binding protein and a polymorphic acidic, glycine-rich protein, called **PE-PGRS**. HbhA is a surface-exposed protein that is involved in binding *Mycobacterium tuberculosis* to epithelial cells but not to phagocytes. It could be involved in extrapulmonary spread after the initial long-term colonization of the host. Fibronectin- binding proteins (**FbpA)**, first identified as the α-antigen (**Antigen 85complex**), can bind to the extracellular matrix protein fibronectin in vitro. This property may represent a mechanism of tissue colonization. This property may represent a mechanism of tissue colonization.

It has been shown recently that *Mycobacterium tuberculosis* produces **pili** during human infection, which could be involved in initial colonization of the host [(7)]. On the basis of electron microscopic evidence, *Mycobacterium tuberculosis* produces a dense fibrillar meshwork composed of thin coiled, aggregated fibers resembling pili that extend many microns away from the bacterial surface. These structures have been named *Mycobacterium tuberculosis* pili or **MTP**. Additionally, biochemical and genetic data demonstrate that *Mycobacterium tuberculosis* produces pili, whose pilin subunit is encoded by *Rv3312A* gene. The serum of TB Patients with active TB has been shown to contain IgG antibodies to MTP, which suggests that the structures are produced in vivo during human infection. It is

suggested that these MTP act as adhesion and may be an important host colonization factor of *Mycobacterium tuberculosis*.

Major virulence factors in *Mycobacterium tuberculosis* [158]:

1) Cell wall: Antigen85, LAM, MmaA4,PDIM, PcaA
2) Cellular metabolism: FadD33, Isocitrate lyase, LipF, Nitrate reductase, PanC/PanD
3) Heat-shockprotein: HsX
4) Iron uptake: Mycobactin
5) Magnesium uptake: MgtC
6) Regulation: DevRS, HspR, IdeR, MprAB, PhoP, SigA, SigE, SigF, SigH
7) Secreted proteins: ESAT-6/CFP-10
8) Secretion system:ESX-5
9) Stress protein: AhpC, KatG, SodA, SodA, SodC
10) Unclassified: Erp, HbhA, PE/PE-PGRS

Pathogenesis and immunology of tuberculosis:

Virulence of *M. tuberculosis* and effectiveness of host defense mechanism decide clinical and histological features of TB. Immune reactions in TB are:

a) principally cell mediated depending on macrophages activation and granuloma formation and
b) exhausted macrophages and other cells, in which tubercle bacilli are replicating, are recognized and destroyed(apoptosis)

Tubercle bacilli entering tissues are taken up by macrophages. Entry of *M. tuberculosis* into human monocytes is controlled by a phagocytic mechanism mediated by complement receptor and macrophage mannose receptors. Heparin or fibronectin binding proteins on bacterial surface facilitates binding to epithelial cells or macrophages.

Once inside macrophage, the intracellular *Mycobacteria* use various survival strategies including

a) prevention of oxidative burst in phagocytizing cells and inhibition of phagosome-lysosome fusions
b) resistance to lysosomal enzymes and reactive oxygen intermediates by using mycosides, LAM and secreting superoxide dismutase.
c) escape from phagosome into cytoplasm.

If bacilli are not destroyed, they replicate and kill the infected cell or may be transported within phagocytes to regional lymph nodes, where they are engulfed by antigen presenting cells (APC). Some bacilli are transported further leading to extra pulmonary forms.

Epitopes for *Mycobacteria* within phagosomes inside APC are presented on cell surface by MHC class II molecules to CD4+ helper T cells, which undergo activation and clonal proliferation. These then produce a range of cytokines, which activates macrophages. If tubercle bacilli proliferate within APC and escape from phagosomes, epitopes will be presented to CD8+ T cells by MHC class I molecules.CD8+ contains cytotoxic T cells that lyses these bacillus [(94)].

Macrophage Activation and Granuloma Formation:

Activated macrophages aggregate to form characteristic lesion of TB called "Granuloma". These macrophages resemble epithelial cells, hence termed as Epitheloid cells. Some of these fuse to form multinucleate "Giant cells".

Entire granuloma is more capable of destroying *Mycobacterium tuberculosis* than isolated macrophages; palisading macrophages consume oxygen diffusing into granuloma, hence center undergoes necrosis producing material similar to cottage cheese termed as "Caseation". This anoxic center is unfavorable to TB bacilli and many die. These defense mechanisms render disease quiescent in 95% of primarily infected patients but some survive in latent/persisted state. In a normal healthy person, the necrotic area heals leaving a coin lesion.

Genetic factors in Mycobacterial immunity:

Not all those exposed to tubercle bacilli develop overt (active) TB. Therefore, some genetically determined innate resistance to disease is suspected. An allel, "beg" encodes a protein termed Natural resistance associated macrophage protein (NRAMP) in mouse and its homolog NRAMP1 is present in humans. Polymorphism in gene encoding this protein will affect susceptibility to smear positive Pulmonary TB. Natural mutations in genes for IFNγ receptor and Interlukin 12 receptor increases susceptibility to Mycobacterial infections [(94)].

The Disease:

Predisposing factors for TB include [(8)]:

1) Close contact with large populations of people, i.e. schools, nursing homes, dormitories, prisons, etc.
2) Poor nutrition,
3) IV drug use,
4) Alcoholism and
5) HIV infection is the major predisposing factor for *Mycobacterium tuberculosis* infection, 10% of all HIV-positive individuals harbor *Mycobacterium tuberculosis*, which is 400-times the rate, associated with the general public. Only 3-4% of infected individuals will develop active disease upon initial infection and 5-10% within one year. These percentages are much higher if the individual is HIV positive.

   Disease progression depends on:

   i) Strain of *Mycobacterium tuberculosis*
   ii) Prior exposure
   iii) Vaccination
   iv) Infectious dose and
   v) Immune status of the host

M. tuberculosis enters the body by inhalation of infective droplets. Smear positive patients contain at least 5000 bacilli in 1 ml of sputum and are the principal source of infection. Not all patients infected by tubercle bacillus develop overt disease [(159)]. Only 10% of non immunocompromised infected people develop active TB, 5% within first 2 years following infection and 5% during their lifetime. Disease ratio in HIV positive people is higher. Interval between initial infection and overt disease varies from few weeks to several decades.

Initial lesion is usually in lung from which the organisms reach various organs via lymphocytes and bloodstream. Hematogenous spread occurs in Primary TB with implantation of bacilli in many organs. In some people, especially children, these foci progresses to serious fatal disease involving menings, kidney, bones and pleurae. Foci developing in endothelium of major blood vessels rupture giving rise to widespread small granulomata, termed as "Miliary TB" [(94)].

Primary TB:

Disease occurring in person who is never exposed to a tubercle bacillus is termed as "primary TB". Infection results in focus of disease (Ghon focus) at sites of implantation of bacillus, usually lung. This focus together with enlarged, infected regional lymph nodes is termed as "Primary Complex". Most primary complexes resolve spontaneously, only a few TB bacilli enter persistence/latency. Such infected but healthy people are not invariably tuberculin positive.

Stages of the disease [(265)]:

Stage 1:

Tuberculosis begins when droplet nuclei reach the alveoli (Figure 9). When a person inhales air that contains droplets, most of the larger droplets become lodged in the upper respiratory tract (the nose and throat), where infection is unlikely to develop. However, the smaller droplet nuclei may reach the small air sacs of the lung (the alveoli), where infection begins (Figure 10).

Stage 2:

It begins 7-21 days after initial infection, *Mycobacterium tuberculosis* multiplies virtually unrestricted within unactivated macrophages until the macrophages burst. Other macrophages begin to extravasate from peripheral blood. These macrophages also phagocytose the organism; however, they are also unactivated and hence cannot destroy the bacteria.

Stage 3:

At this stage, lymphocytes begin to infiltrate. The lymphocytes, specifically T-cells, recognize processed and presented *Mycobacterium tuberculosis* antigen in context of MHC molecules. This results in T-cell activation and the liberation of cytokines including γ- interferon (IFN). The liberation of IFN causes in the activation of macrophages. These activated macrophages are now capable of destroying *Mycobacterium tuberculosis*.

It is at this stage that the individual becomes tuberculin-positive. This is the result of the host developing a vigorous cell mediated immune (CMI) response. A CMI response must be mounted to control *Mycobacterium tuberculosis* infection. An antibody-mediated immune response (AMI) will not aid in the control of tuberculosis infection because the organism is intracellular and if extracellular, it is resistant to killing by complement due to the high lipid concentration in its cell wall.

Although a CMI response is necessary to control a *Mycobacterium tuberculosis* infection, it is also responsible for the pathology associated with tuberculosis. Activated macrophages may release lytic enzymes and reactive intermediates that facilitate the development of immune pathology. Activated macrophages and T-cells also secrete cytokines that may also play a role in the development of immune pathology, including interleukin 1 (IL-1), tumor necrosis factor (TNF) and γ-IFN.

It is also at this stage that **tubercle formation** begins. The center of the tubercle is characterized by "caseation necrosis", meaning it takes on a semi-solid or "cheesy" consistency (Figure 11). *Mycobacterium tuberculosis* cannot multiply within these tubercles because of the low pH and anoxic environment. *Mycobacterium tuberculosis* can however, persist within these tubercles for extended periods.

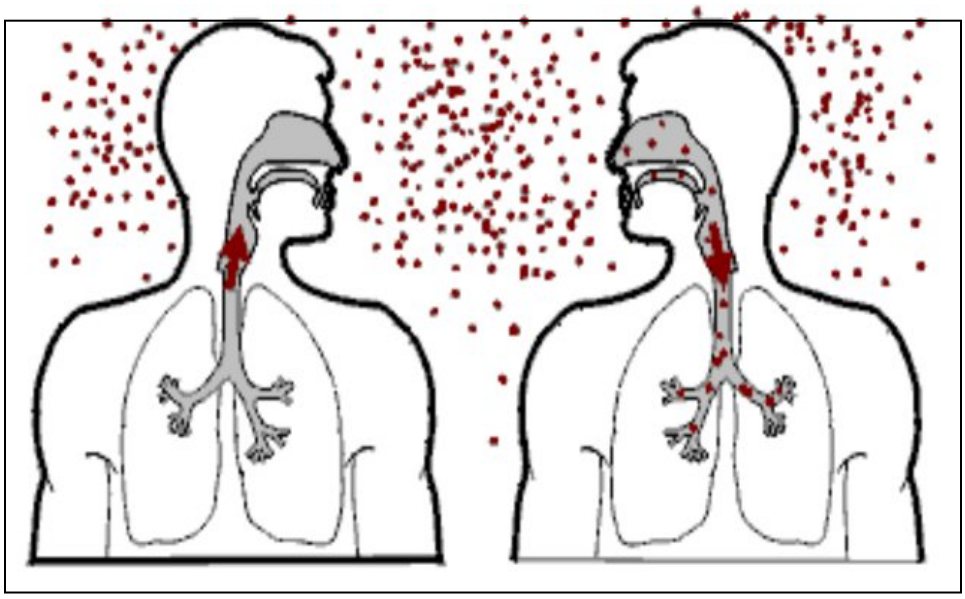

Figure 9: Tuberculosis - Air Borne Infection

Source: Transmission of Tuberculosis; East African Community, HEALTH

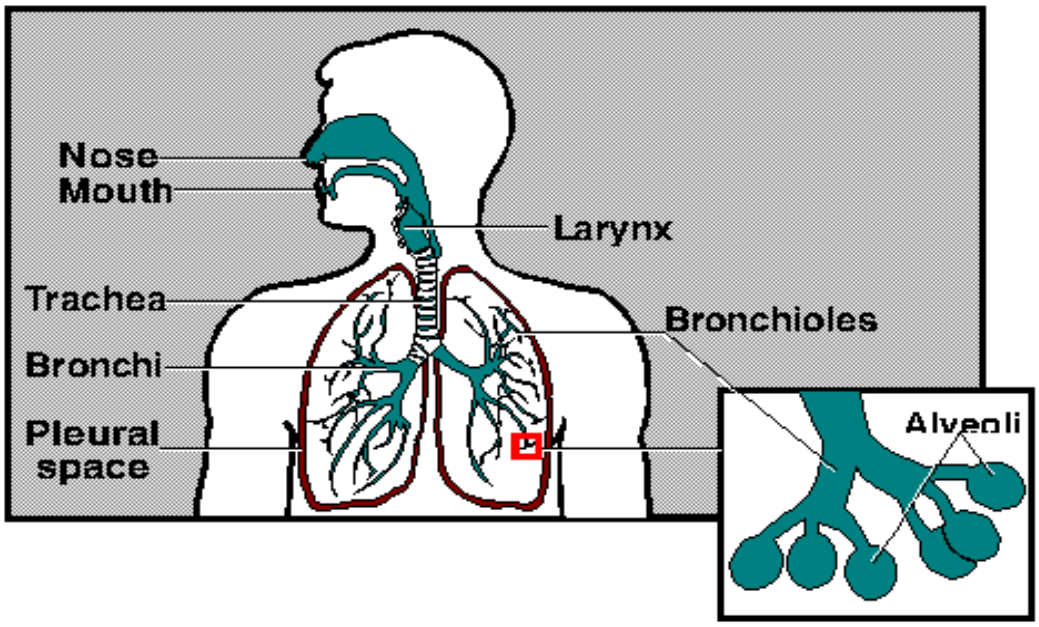

Figure10: Setteling of *M. tuberculosis* in Lungs Alveoli

Source: Textbook of bacteriology.net.

Stage 4: Although many activated macrophages can be found surrounding the tubercles, many other macrophages present remain unactivated or poorly activated (Figure 12). *Mycobacterium tuberculosis* uses these macrophages to replicate and hence, the tubercle grows.

The growing tubercle may invade a bronchus. If this happens, *Mycobacterium tuberculosis* infection can spread to other parts of the lung. Similarly, the tubercle may invade an artery or other blood supply line. The hematogenous spread of *Mycobacterium tuberculosis* may result in extrapulmonary tuberculosis otherwise known as **miliary tuberculosis**. The name "miliary" is derived from the fact that meta-statisizing tubercles are about the same size as a millet seed, a grain commonly grown in Africa.

The secondary lesions caused by miliary TB can occur at almost any anatomical location, but usually involve the genitourinary system, bones, joints, lymph nodes and peritoneum. These lesions are of two types:

1. **Exudative lesions** result from the accumulation of polymorpho-nuclear leucocytes around *Mycobacterium tuberculosis* where the bacteria replicate with virtually no resistance. This situation gives rise to the formation of a "soft tubercle".
2. **Productive or granulomatous lesions** occur when the host becomes hypersensitive to tuberculoproteins. This situation gives rise to the formation of a "hard tubercle".

Stage 5: For unknown reasons, the caseous centers of the tubercles liquefy. This liquid is very conducive to *Mycobacterium tuberculosis* growth and the organism begins to rapidly multiply extracellularly. After time, the large antigen load causes the walls of nearby bronchi to become necrotic and rupture. This results in cavity formation. This also allows spilling into other airways and rapidly spreading to other parts of the lung.

As stated earlier, only a very small percentage of *Mycobacterium tuberculosis* infections result in disease and even a smaller percentage of the infections progress to an advanced stage. Usually the host will begin to control the infection at some point. When the primary lesion heals, it becomes fibrous and calcifies. When this happens, the lesion is referred to as the **Ghon complex**. Depending on the size and severity, the Ghon complex may never subside. Typically, the Ghon complex is readily visible in chest X-ray.

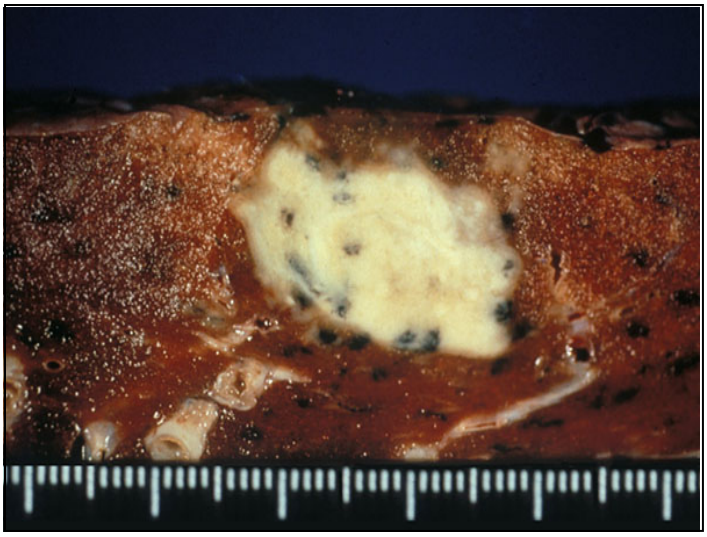

Figure 11: Caseaion and necrosis showing cheesy liquefaction in lung

Source:PathologTuberculosis.library.med.utah.edu/webPath/TUTORIAL/MTB/MTB.

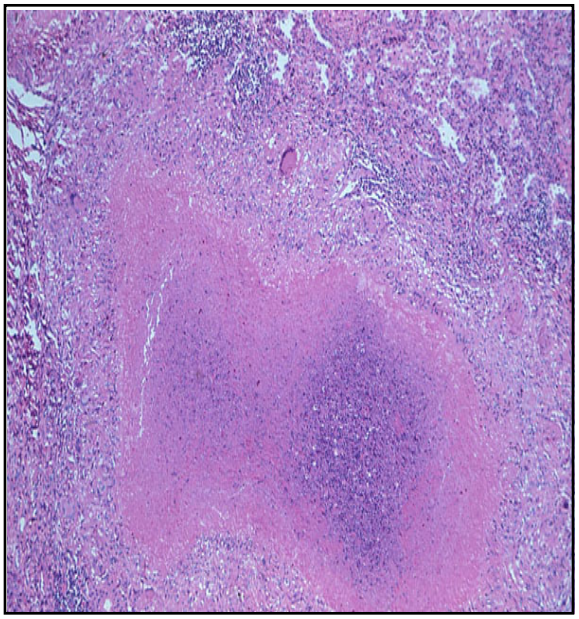

Figure 12: Microscopic examination of Classical tubercular lesion- Granulloma with typical Langharhan's giant cells, epittheloid cells, lymphocytes and fibrosis**.**

Source: Lawrencobroxmeyer.wordpress.com

Small metastatic foci containing low numbers of *Mycobacterium tuberculosis* may also calcify. However, in many cases these foci will contain viable organisms. These foci are referred to **Simon foci**. The Simon foci are also visible in chest X-ray and are often the site of disease reactivation.

Post-Primary TB (Secondary TB):

It develops in previously infected people either because of endogenous reactivation of latent disease or of exogenous re-infection. It usually occurs within 5 years after primary infection. Characteristic feature is extensive tissue necrosis. Tuberculomas may develop and conditions within them do not favour bacterial growth. Necrotic tissue is softened and liquefied by macrophages derived proteases and if lesion erodes into bronchus, liquefied contents are discharged and cavity is formed which is an ideal environment (well oxygenated) for bacterial replication.

Bacilli escaping cavities may be expectorated, infecting other people. Spread to distant organs is uncommon, due to obliteration of draining lymphatics and capillaries by tissue necrosis and deposition of scar tissue. Localization of disease in post primary TB depends on immune reactivity. Cavitation is absent in immunocompromised people and dissemination, atypical forms of disease occurs frequently [(94)].

Secondary TB results from massive hematogenous spread of tubercle bacilli. In children, it is often the consequence of recent primary infection, but in adults it may be either due to recent infection or reactivation of old disseminated foci. The unit lesion is usually yellowish granuloma (Figure 13) 1-2 mm in diameter that resembles the millet seed. Miliary pattern on chest radiography is the hallmark of miliary tuberculosis (Figure 14).

Clinical features:

Clinically tuberculosis is broadly classified as:-

1. Pulmonary Tuberculosis and

2. Extrapulmonary Tuberculosis

1) Pulmonary tuberculosis can manifest as - (Table 3)

a) Primary pulmonary TB

b) Progressive pulmonary TB

c) Secondary pulmonary TB

d) Tubercular pleural effusion

Table 3-

Differences in the Stages of tuberculosis

Early Infection	Early Primary progressive(active)	Late Primary progressive(active)	Latent
• Immune system fights infection. • Infection generally proceeds without signs or symptoms. • Patients may have fever, paratracheal lymphadenopathy or dyspnoea. • Infection may be subclinical and may not advance to active disease.	• Immune system does not control initial infection. • Inflammation of tissues ensues • Patients often have nonspecific signs or symptoms (e.g. Fatigue, weight loss, fever). • Nonproductive cough develops. • Diagnosis can be difficult: findings on chest radiographs may be negative for mycobacteria.	• Cough becomes productive. • More signs and symptoms as disease progresses. • Patients experience progressive weight loss, rales and anemia. • Findings on chest radiograph are normal • Diagnosis is via cultures of sputum	• Mycobacteria persist in the body. • No signs or symptoms. • Patients do not feel sick. • Granulomatous lesions calcify and become fibrotic, become apparent on chest radiographs. • Infection can reappear when immunosupperessi -on occurs.

Source: Tuberculosis: Pathophysiology, clinical features and diagnosis. Critical Care Nurse, April2009; 29(2):34-43.

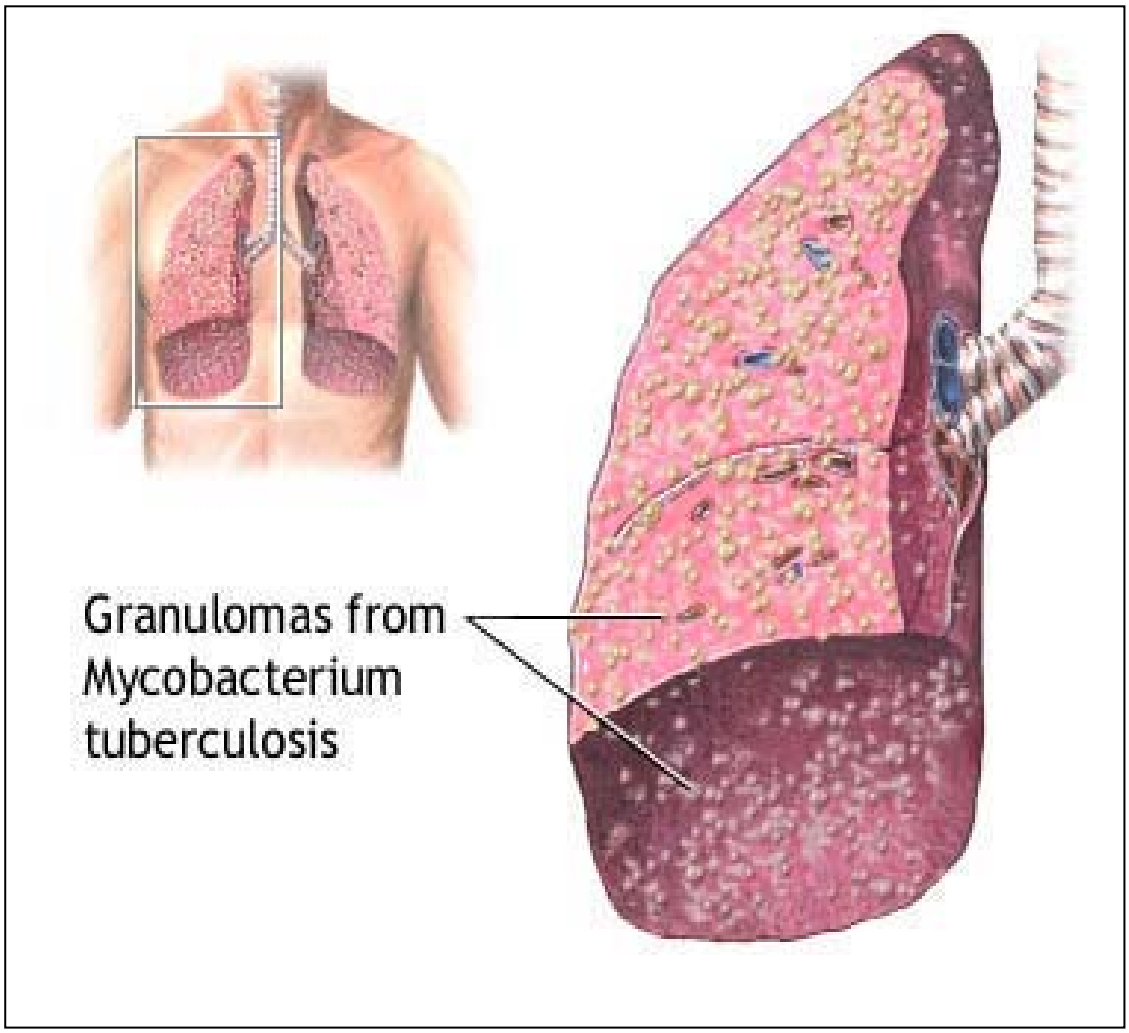

Figure 13: Granulomas from *Mycobacterium tuberculosis* in lungs.

Source: Schiffman G (15TH January, 2009). "Tuberculosis symptoms". eMedicine Health.

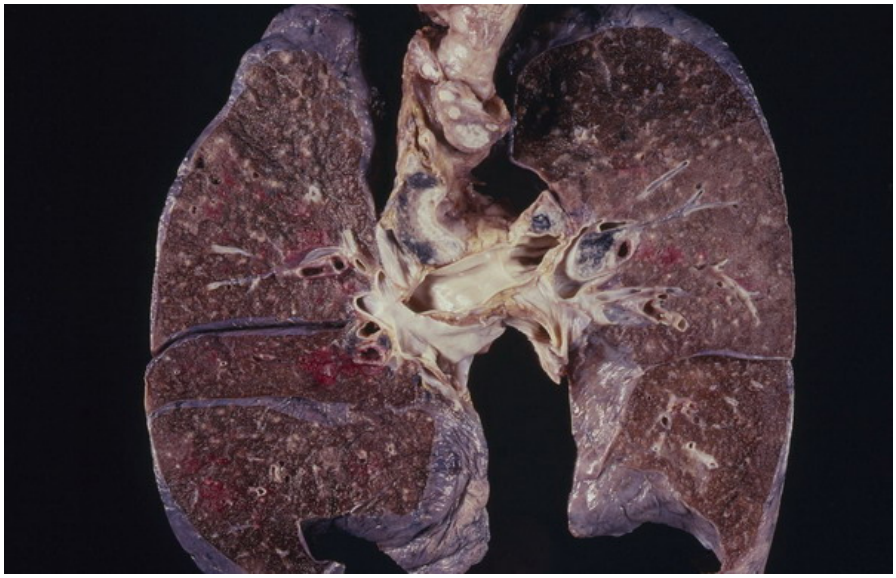

Figure 14: Miliary tuberculosis in lungs

Source: Tuberculosis-Lung-Gross. www.flicker.com.

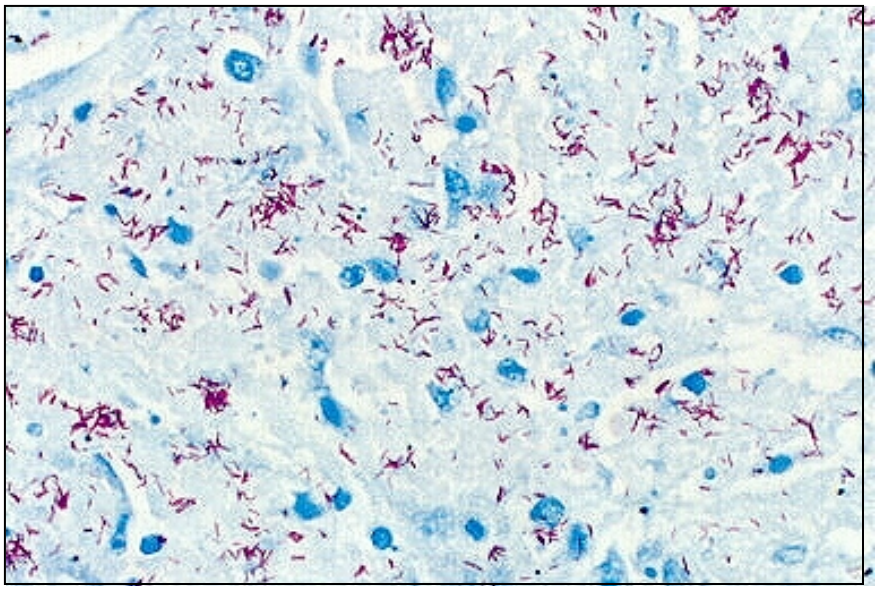

Figure 15: ZN staining showing presence of acid-fast *Mycobacterium tuberculosis* in histo-pathological section of granuloma tissue.

Source: Gallery of histological sections prepared by Roy Ellis, IMVS Divisions of Pathology. The Queen Elizabeth Hospital, Woodville Road, Woodville, South Australia.

2) Extra pulmonary TB

TB affects every organ/tissue in human body. Depending on the affected organ/tissue, extra pulmonary tuberculosis can manifest as

a) Tubercular meningitis
b) Tubercular Lymphadenitis
c) Tubercular Ascitis
d) Intestinal Tuberculosis
e) Endometrial Tuberculosis
f) Genital Tuberculosis
g) Tuberculosis of Spine (Pott's disease)
h) Tuberculosis of Hip and other joints
i) Tuberculosis of bones
j) Renal tuberculosis
k) Tuberculosis of pericardium
l) Tubercular peritonitis etc.

Pathophysiology of Tuberculosis:

Once inhaled, the infectious droplets settle throughout the air-ways. The majority of the bacilli are trapped in the upper parts of the airways where the mucus-secreting goblet cells exist. The mucus produced catches foreign substances and the cilia on the surface of the cells constantly beat the mucus and its entrapped particles upward for removal [(91)].This system provides the body with an initial physical defense that prevents infection in most persons exposed to tuberculosis [(122)].

Bacteria in droplets that bypass the mucobacillary system and reach the alveoli are quickly surrounded and engulfed by alveolar macrophages [(9, 91)], the most abundant immune effector cells present in alveolar spaces [(138)]. These macrophages, the next line of host defense, are part of the innate immune system and provide an opportunity for the body to destroy the invading mycobacteria and prevent infection [(279)]. Macrophages are readily available phagocytic cells that combat many pathogens without requiring previous exposure to the pathogens. Several mechanisms and macrophage receptors are involved

in the uptake of the mycobacteria [279]. The mycobacterial lipoarabinomanan is a key ligand for a macrophage receptor [194]. The complement system also plays a role in the phagocytosis of the bacteria [148]. The complement protein C3 binds to the cell wall and enhances recognition of the mycobacteria by macrophages. Opsonization by C3 is rapid, even in the air spaces of a host with no previous exposure to *M. tuberculosis* [84]. The subsequent phagocytosis by macrophages initiate a cascade of events that results in either successful control of the infection followed by latent tuberculosis or progression to active disease, called primary progressive tuberculosis [91]. The outcome is essentially determined by the quality of the host defenses and the balance that occurs between host defenses and the invading mycobacteria [279,103].

After being ingested by macrophages, the mycobacteria continue to multiply slowly [91], with bacterial cell division occurring every 25to 32 hours [212, 9]. Regardless of whether the infection becomes controlled or progresses, initial development involves production of proteolytic enzymes and cytokines by macrophages in an attempt to degrade the bacteria [279,194]. Released cytokines attract T lymphocytes to the site the cells. Macrophages then present mycobacterial antigens on their surface to the T-cells [279]. This initial immune process continues for 2-12 weeks; the microorganisms continue to grow until they reach sufficient numbers to fully elicit the cell-mediated immune response, which can be detected by a skin test [212, 91,279].

For persons with intact cell-mediated immunity, the next defensive step is formation of granulomas around the organisms [238]. These nodular-type lesions formed from an accumulation of activated T lymphocytes and macrophages, creates a microenvironment that limits replication and spread of the mycobacteria [279,194].This environment destroys macrophages and produces early solid necrosis at the center of the lesion; however, the bacilli are able to survive [70]. In fact, *M. tuberculosis* can change their phenotypic expression, such as protein regulation, to enhance survival [148]. By 2-3 weeks, the necrotic environment resembles soft cheese, often referred to caseous necrosis and is characterized by low oxygen levels, low pH, and limited nutrients. This condition restricts further growth and establishes latency. Lesions in persons with an adequate immune system generally undergo fibrosis and calcification, successfully controlling the infection so that the bacilli are contained in the dormant, healed lesions [70]. Lesions in

persons with less effective immune systems progress to primary progressive tuberculosis [212, 191, 148, 70](Figure 16).

For less immunocompetant persons, granuloma formation is initiated yet ultimately is unsuccessful in containing the bacilli. The necrotic tissue undergoes liquefaction and the fibrous wall loses structural integrity. The semi-liquid necrotic material can then drain into a bronchus or nearby blood vessel, leaving an air-filled cavity at the original site. In infected patients, droplets can be coughed from the bronchus and infect other persons. If discharged into a vessel, occurrence of extrapulmonary tuberculosis is likely. Bacilli can also drain into the lymphatic system and collect in the tracheobronchial lymph nodes of the affected lung, where the organisms can form new caseous granulomas [70].

Clinical manifestations of Tuberculosis:

- **Early symptoms** of active pulmonary tuberculosis can include-
    1. Cough with expectoration for more than 3 weeks, not responding to routine antibiotic treatment.
    2. Rapid excessive weight loss, loss of appetite and night sweats.
    3. Low grade fever of long duration, with evening rise in body temperature.
- The infection can progress to more serious with **typical symptoms** of TB, which includes-
    1) Chest pain and cough with blood stained sputum.
    2) A pulmonary shadow in X-ray and an increased ESR as supportive investigation findings.
    3) The gold standard of TB diagnosis demonstrates *Mycobacterium tuberculosis* in the clinical sample.
    4) Additional symptoms pertaining to the organ/tissue involved.

Blood picture in Tuberculosis:

In Tuberculosis a full blood count is never diagnostic but normocytic anaemia and lyphopenia are common while neutrophilia is rarely found. Iron deficiency anaemia may develop with isoniazid treatment. Urea and electrolytes are usually normal, although hypocalcemia and hypoatremia are possible in tuberculous meningoencephalitis due to SIADH. In advanced disease, hypoalbuminemia, hyperproteinemia and hyperglobunemia may be present. Erythrocyte sedimentation rate is usually raised [86].

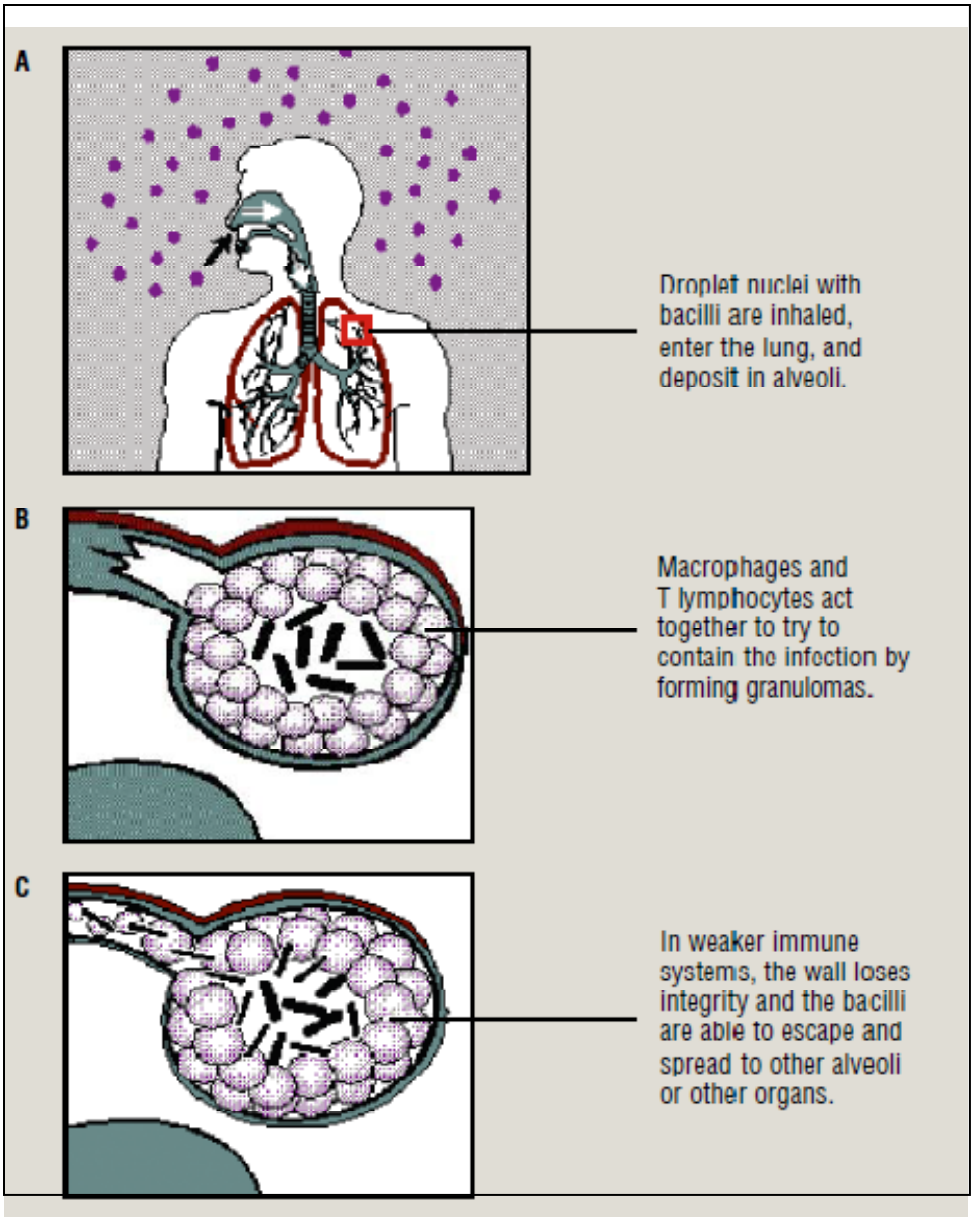

Figure16: Pathophysoilogy of tuberculosis: A. Inhalation of bacilli. B. Containment in a granuloma. C. Breakdown of granuloma in less immunocompetent individuals

Source: Centers for disease control and preventation, Geneva.

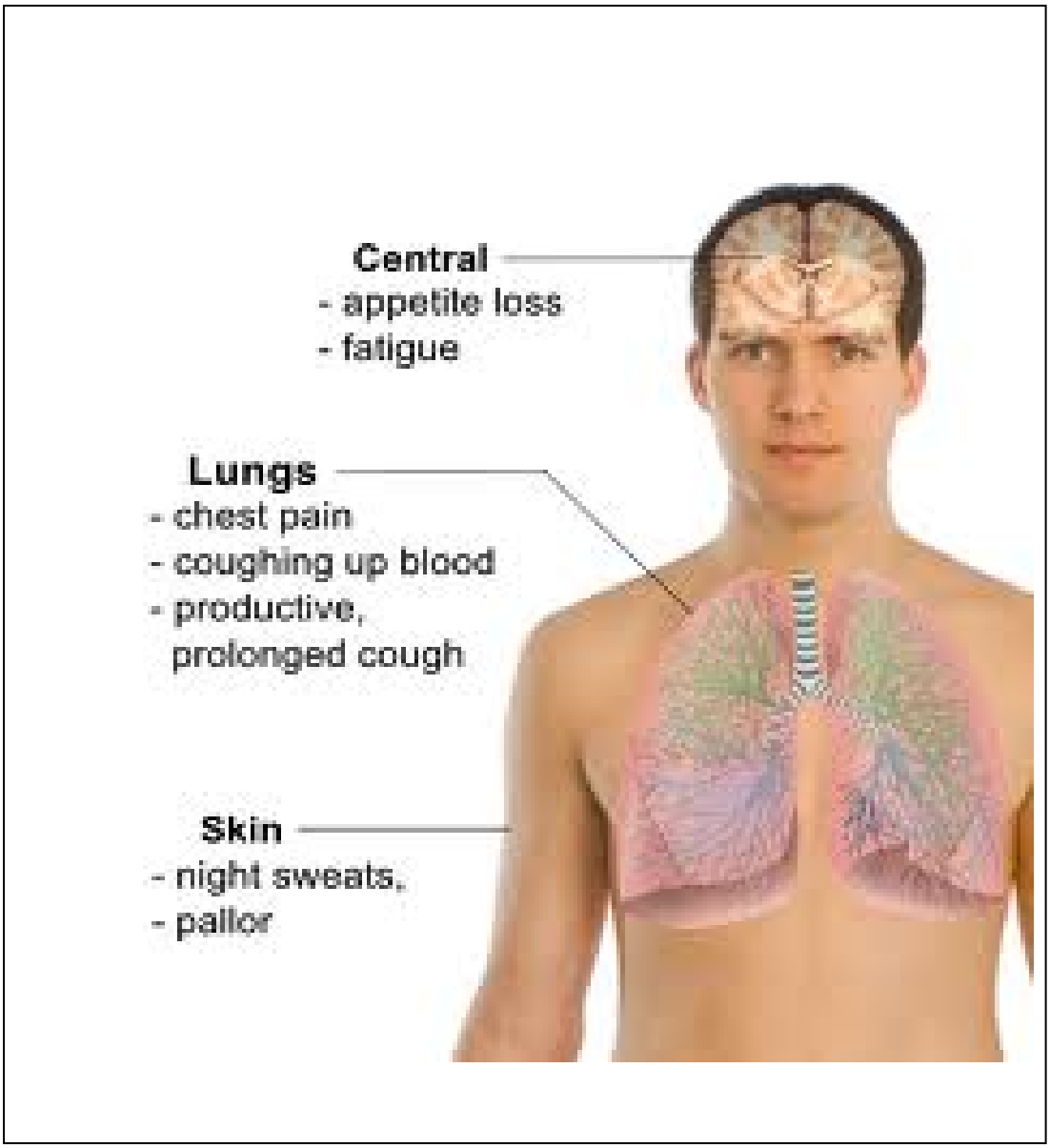

Figure17: Main Symptoms of pulmonary tuberculosis

Source: Schiffman G (15th January 2009). "Tuberculosis symptoms" e Medicine Health.

High percentages of immature polymorphonuclear leukocytes in the circulating blood are evidence of excessive loss of polymorphonuclear leukocytes from the blood stream and an attempt on the part of the bone marrow to make good the loss. In pulmonary tuberculosis, the demand for leukocytes indicates necrosis and extension of the lesion [(63)].Since *M. tuberculosis* is an intracellular pathogen, the serum components may not get access and may not play any protective role [(221)].

Epidemiology of Tuberculosis:

According to WHO, more than 1/3rd of world's human population has been infected by *Mycobacterium tuberculosis*. Every year, more than 8 million new cases occur and more than 2 million die. Major burden of global TB is borne by economically poor countries. One in seven adult deaths and one in 4 preventable adults are due to TB, even though it is among the most cost effective of all adult disease to treat. It is the major cause of death of children, killing at least 5, 00,000annually. In 1993, WHO took the unprecedented step of declaring TB as "Global emergency". In 2007, there were an estimated 13.7 million chronic active cases [(302)], while 8.8 million new cases and 1.45 million deaths, mostly in developing countries were reported in 2010[(261)]. 0.35 Million of these deaths occur in those co-infected with HIV [(310)]. Tuberculosis is the second most common cause of death from infectious disease, after HIV [(71)].

The absolute number of tuberculosis cases has been decreasing since 2005 and new cases since 2002 [(261)]. China has achieved particularly dramatic progress with an 80% decline in its TB mortality rate [(100)].The distribution of tuberculosis is not uniform across the globe. About 80% of the population in many Asian and African countries test positive in tuberculin tests while only 5-10% U.S. population [(144)].

In 2007, the country with the highest estimated incidence rate of TB was Swaziland with 1200 cases per 100,000 people. India had the largest total incidence, with an estimated 2.0 million new cases [(302)]. In developed countries, tuberculosis is less common and is mainly an urban disease. In the United Kingdom, the national average was 15 per

100,000 in 2007andthe highest incidence rates in Western Europe were 30 per 100,000 in Portugal and Spain. In United States, the overall tuberculosis case rate was 4 per 100,000 persons in 2007 [303].

The incidence of TB varies with age. In Africa, tuberculosis primarily affects adolescents and young adults [294]. However, in countries where tuberculosis has gone from high to low incidence, such as the United States, TB is mainly a disease of older people or of the immunocompromised [144,44].

Tuberculosis incidence is seasonal, with peaks occurring every spring/summer [72,163,203, 142]. The reasons for this are unclear but may be related to Vitamin D deficiency during the winter [142,139].

In 2012, twenty-two high burden countries accounted for 80% of world's cases. These countries are mainly in Sub-Saharan Africa, East Asia, Eastern Mediterranean, Europe and South America.

Calculations of incidence of TB on the basis of case finding and notification are very unreliable. Determinations of "Annual Infection Rate" or "Annual Risk of Infection" define better the epidemiological situation of TB. Infection for this purpose is defined as conversion to tuberculin positivity and annual risk may be calculated from results of tuberculin test surveys of children and young adults, provided BCG vaccination is not taken. Annual infection rate is employed to get indirect estimates of number of infectious TB cases in community [94].

Tuberculosis in India:

Tuberculosis is one of the major public health problems in India. According to WHO, India has the world's largest tuberculosis epidemic. According to WHO-2011, India is the highest TB burden country with an estimated incidence figure of 2.2 million cases of TB out of a global incidence of 8.7 million cases [259]. Tuberculosis is the biggest health issue that lies around India but what makes is worse is the newly and recently discovered global phenomenon of Totally Drug-Resistant Tuberculosis (TDR-TB) [273].

Tuberculosis in India is rich in its high rates because of the pollution dispersed throughout the country. Pollution causes many effects in the air. Since TB can be gained through air; the chances of TB remain high and in a consistent movement going uphill for India. Another major cause for the growth of TB in India is- it is a developing country and its economy is still developing. TB raises high in India because of the majority of people not being able to afford the treatment drugs prescribed to diagnosed patients. It is reported that at present, only the 1.5 million patients already under the Indian government's care get free treatments for regular TB. That leaves patients already under the India's growing private sector to buy drugs for themselves and most struggle to do that [(10)]. Consequently, high priced treatment drugs and the struggles of "poor patients" also brawl through the poor treatment they receive in response to acquiring TB. It is estimated that just 16% of patients with drug-resistant TB are receiving appropriate treatment [(239)]. In India, the particular type of TB infections are majority resistant to regular antibiotic treatment (MDR-TB, XDR-TB, TDR-TB), therefore, not one or two medications will help, rather a combination of different medications must be taken for over a course of 18-24 months, depending on how deep the infection[(183,115)]. Since the 1960, two drugs- Isoniazid and Rifampicin – have been the standard TB treatment [(239)]. In addition to antibiotics, a vaccine is available to limit the spread of bacteria after TB infection. The vaccine generally used in countries or communities where the risk of TB infection is greater than 1% each year [(271)], thus the country of India; whose TB infection rate is at a peak (world's third highest TB infected country), and is consistently growing, and giving 20% of the world's diagnosed patients a home [(274)]. At present the anti TB treatment offered in public and private sector in India is not satisfactory and needs to be improved [(175)].

The Indian government's Revised National TB Control Programme (RNTCP) started in India during 1997. The program uses the WHO recommended Directly Observed Treatment Short Course (DOTS) strategy to develop ideas and data on TB treatment. This group's initial objective is to achieve and maintain a TB treatment success rate of at least 85% in India among new patients [(275)]. In 2010, the RNTCP made a major policy decision that it would change focus and adopt the concept of Universal Access to diagnosis and TB treatment for all TB patients. By doing so, they extend out a helping hand to all

people diagnosed with TB, and in addition, provide better quality services and improve on therapy for these patients. It is also suggested that patients with TDR-TB only be treated "within the confines of government sanctioned DOTS-Plus programs to prevent the emergence of this untreatable form of tuberculosis" [(274)].

Impact of HIV pandemic:

Coexistence of HIV infection and TB is one of the most serious threats to human health since Black Death and called "The Cursed Duet" [(54)]. People infected with tubercle bacilli have about 10% chance of developing TB during remainder of their lives [(104)]. But HIV positive person who is already infected by *Mycobacterium tuberculosis* has 5-15% chance of developing overt disease annually or up to 50% during remaining short lifespan [(77)].

Impact of HIV/AIDS pandemic on global toll of TB has been devastating. According to WHO in 2001, there were 37.2 million adults and 2.7 million children infected by HIV worldwide and 4.3 million adults and 0.8 million children newly infected during that year and 3 million died because of AIDS. Tuberculosis was the immediate cause of 15% of all adult AIDS deaths. This suggest that TB is the leading cause of HIV related mortality and morbidity and HIV is most important factor fueling a TB epidemic in high HIV prevalence populations[(189)].

Although treatment induces bacteriological cure, patient's future will be shortened. Reason is not fully understood, one reason is that immune responses in TB and in other infections induce cytokines that enhances HIV replication leading to AIDS with shortening of life. Other reason is that INFγ and other immunological mediators released in TB lead to transactivation of HIV provirus and subsequent replication. One more reason is TB causes CD4+ T cell lymphoma that synergizes with that induced by HIV [(197)]. Whatever may be the cause, the consequence, worsening HIV prognosis.

Epidemiology of Drug Resistance in Tuberculosis:

A selection of resistant mutants leads to resistance in tuberculosis, as there is no plasmid in *Mycobacterium tuberculosis*. Standard anti-tuberculosis treatment (ATT) regimens use 3-4 drugs because probability of finding a mutant resistant to two drugs is 10^{-10} to 10^{-16} [(127)].

Types of Drug Resistance are:

(a) **Intrinsic resistance:** It refers to the innate ability of a bacterium to resists the activity of a particular antimicrobial agent through its inherent structural or functional characteristics. Intrinsic drug resistance in *Mycobacterium tuberculosis* has been attributed to its unique cell wall properties, including the presence of mycoloc acids, which are high-molecular weight α-alkyl, β-hydroxy fatty acids covalently attached to arabinogalactan and which constitute very hydrophobic barrier responsible for resistance to certain antibiotics [126]. In addition, *Mycobacterium tuberculosis* posses β-lactamase enzymes, which confer intrinsic resistance to β-lactam antibiotics, while efflux mechanisms appear to play an important role in resistance to antibiotics such as tetracycline and the aminoglycosides [140].

(b) **Acquired resistance:** It results by selection of mutants in patients receiving inadequate therapy. It is mainly caused by spontaneous mutation in chromosomal genes and selective growth of such drug-resistant mutants may be promoted during suboptimal drug therapy [137].

Factors responsible for acquired resistance are:

(i) Factors related to patient/clinician.
- Poor compliance of patient to therapy.
- Misdiagnosis.
- Poor quality of drugs.
- Poor therapy schemes.

(ii) Factors related to healthcare centers
- Dysfunction of healthcare centers.
- Difficult access to healthcare centers.
- Lack of drug supply.

Globally 10% of *Mycobacterium tuberculosis* strains exhibit resistance to at least one anti-tubercular drug. Rifampicin (RIF) resistance is much more serious, as most strains resistant to this drug are also resistant to Isoniazid (INH). Therefore, Rifampicin (RIF) resistance is a surrogate marker for detection of potential resistance.

Multidrug resistance (MDR):

High rates of MDR-TB were observed in early 1990's in different countries and were associated with the dismantling of public health programs. The highest rates were in the

former USSR, the Baltic States, Argentina, India and China and were associated with the poor or failing of national Tuberculosis Control Programs [(94)].

According to WHO-2013, globally MDR-TB occurs in several "Hot spots". Countries with a very high incidence include Argentina, Dominican Republic, Estonia, Latvia, and Russia, Asian countries like India, Thailand and Bangladesh. WHO has established 22 supranational Reference Laboratories to strengthen local laboratories and to validate data of drug resistance collected all over the world.

Extensively Drug Resistant Tuberculosis:

The term XDR-TB appears to have been used for the first time in March 2006. A report was published jointly by USCDC and WHO, describing strains of *M. tuberculosis*, referred to as XDR-TB that were resistant not only to INH and RFM but they were also resistant to at least three of six classes of second line anti-TB drugs. In 2008, 963 cases of XDR-TB were reported to WHO globally from 33 countries compared with 772 cases from 28 countries in 2007. According to WHO-2013, globally XDR-TB occurs in 92 countries.

Extreme/Total Drug resistance (XXDR/TDR):

These are the strains of *Mycobacterium tuberculosis* which shows resistance to all available anti-tuberculosis drugs. Within a year of the first reports of extensively drug-resistant tuberculosis (XDR-TB) in 2006, two patients with strains having resistance to all first and second-line anti-TB drugs which were reported from Italy [(69)].In 2009, 15 TB patients in Iran the not responding to all the existent anti-TB drugs tested were reported [(305)]. New terms like "extremely drug resistant" (XXDR-TB) and "Totally drug-resistant TB" (TDT-TB) were used to define such drug resistance patterns. In December 2011, clinicians in Mumbai (India), described four patients with "TDR-TB" [(199)].

Cases of **MDR-TB** have been reported in every country surveyed [(202)]. MDR-TB most commonly develops in the course of TB treatment [(203)] and is most commonly due to inappropriate treatment given by doctors or failing to complete the treatment by patients. As MDR-TB is an air-borne infection, persons with active disease, can transmit the pathogens through the secretions of various respiratory activities like coughing, sneezing

etc. [202]. TB strains are often less fit and less transmissible and outbreaks occur more readily in people with weakened immune systems (e.g. patients with HIV) [204, 205,206,207, 208]. Outbreaks among non immunocompromised healthy people do occur [209], but less common [203].

According to WHO, 2014 report, 3.5% of new tuberculosis cases and 20.5% of previously treated for tuberculosis cases have MDR-TB. WHO estimates that there were about 0.5 million new MDR-TB cases in the world in 2013. About 60% of these cases occurred in Brazil, China, India, the Russian Federation and South Africa alone [210]. In 2013, the Mexico- United States border was noted to be "a very hot region for drug resistant TB", though the number of cases remained small [211].

Laboratory Diagnosis of Pulmonary TB:

Detection of *M. tuberculosis* in clinical samples varies greatly in terms of turnaround time cost and complexity. Ultimately, the 'holy grail' diagnostic for TB must fulfill all technical specifications for a good point-of-care test, screen for drug resistance concurrently and be adaptable to the various health system levels and to the countries with diverse economic status and TB burden. **Figure 18** depicts the steps in the routine diagnosis of TB.

Laboratory and Diagnostic studies of Tuberculosis:

One of the barriers in TB control is the delay in the diagnosis of the disease. Accurate and rapid diagnosis is the key component in the prevention of transmission. Although Ziehl-Neelsen (ZN) stain smear microscopy is most commonly employed for early detection, it is rather insensitive and fails to detect large number of cases. [98]

Active TB may be considered as a possible diagnosis when findings on a chest radiograph of a patient being evaluated for respiratory symptoms are abnormal, as occurs in most patients with pulmonary TB. The radiographs may show the characteristic findings of infiltrates with cavitation in the upper and middle lobes of the lungs (Figure 16). However, in specific patients, such as the elderly and patients with advanced infection by human immunodeficiency virus, may not have these typical findings. Compared with other patients, both groups have the classic cavitation less often and may have lower-lobe infiltrates as a prominent finding [9]. Although abnormal findings on a chest radiograph may suggest tuberculosis, they are not diagnostic for the disease [115].

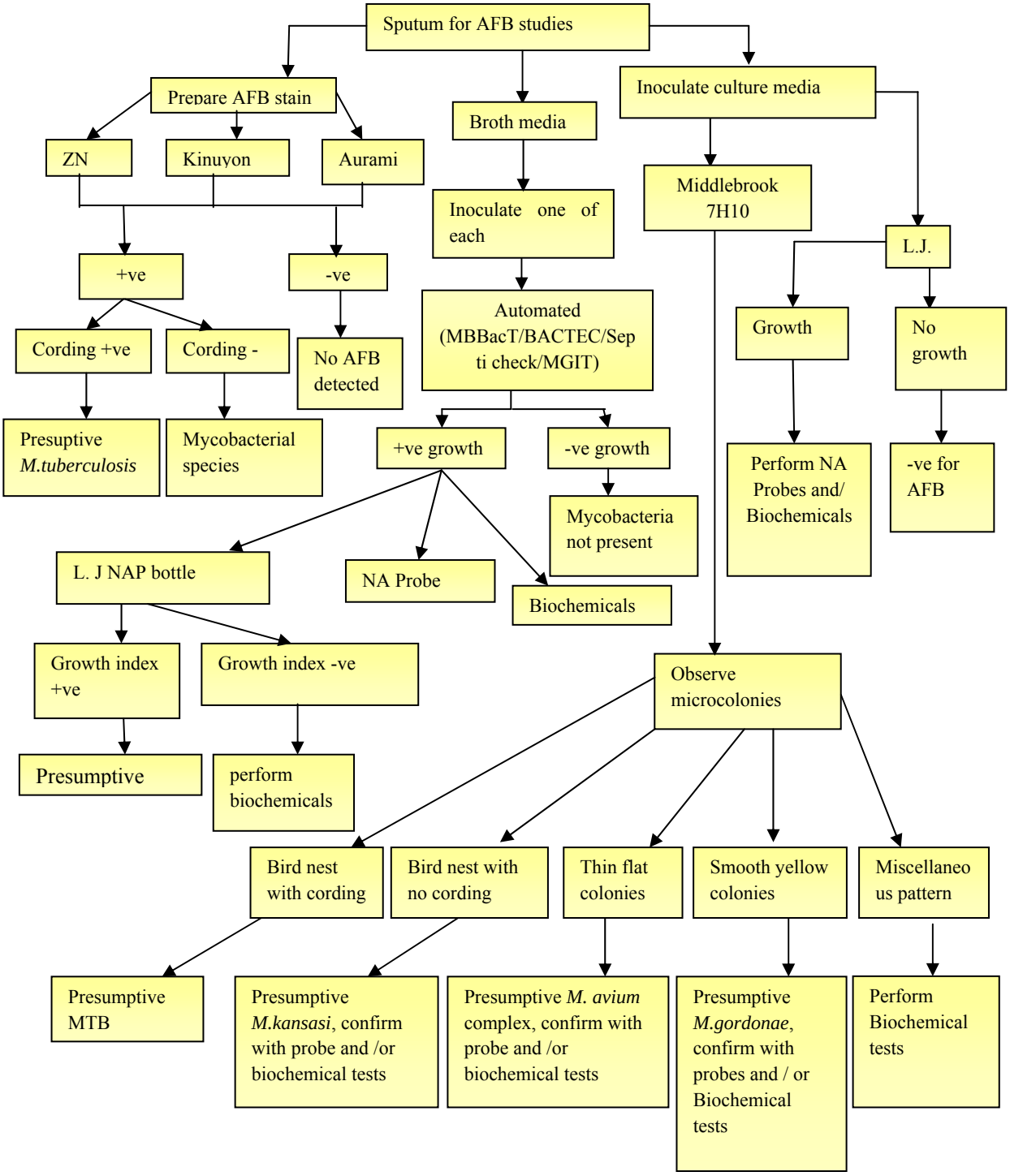

Figure 18: Algorithm for diagnosis of tuberculosis

Source: Laboratory approach to the recovery and identification of Mycobacteria from chapter 19- Mycobacteria, Page number -1071. Koneman's color Atlas and textbook of diagnostic Microbiology- 6th edi.

Traditionally, the first laboratory test used to detect active tuberculosis in a patient with abnormal findings on chest radiographs is examination of a sputum smear for the presence of acid-fast bacilli (Table 4). Also, because the bacilli have entered the sputum, the patient is infectious to others. According to the Centers for Disease Control and Prevention, two sputum specimens should be used for detection of pulmonary tuberculosis, with specimens collected in the morning on consecutive days.

For the test sputum smear is stained by ZN staining technique to demonstrate *Mycobacterium tuberculosis*. The test is not specific for tuberculosis, because other mycobacteria also give the same results. Results of sputum smears should be available within 24 hours of the specimen collection.

The definitive diagnosis of tuberculosis requires the identification of *Mycobacterium tuberculosis* in a culture of a diagnostic specimen. The most frequent sample used from a patient with a persistent and productive cough is sputum. As *Mycobacterium tuberculosis* is a slow growing organism, 3-6 weeks may be required for detectable growth on solid media. After medications are started, the effectiveness of the therapy is assessed by obtaining sputum samples for smears. Once again two sputum smears negative for *Mycobacterium tuberculosis* are necessary for the discontinuation of respiratory isolation. Culture conversion is an important objective evaluation of response to treatment [(115)].

Unfortunately, not all patients with tuberculosis can be detected by culture of sputum specimens, a situation that can lead to delayed or missed diagnosis. Additionally many critically ill patients have trouble in producing the sputum. For these patients, inhalation of an aerosole of normal saline can be used to induce sputum for collection. However, if sputum specimens still inadequate, bronchoscopy with bronchial washings or bronchoalveolar lavage (BAL) can provide sputum for diagnosis. It is collected by inserting a fiberoptic bronchoscope into the lungs. In patients with involvement of intrathoracic lymph nodes, specimens are collected by inserting a 19-guage flexible histology needle through a bronchoscopy tube.

Newer diagnostic techniques for faster detection of *M. tuberculosis* include nucleic acid (DNA and RNA) amplification tests by PCR.

Diagnosis of Latent TB (LTB) is carried out by Tuberculin skin test and recently introduced QuantiFERON-TB Gold test.

Serological diagnosis of Tuberculosis [113]:

Most of the serological tests which are used in diagnosis of TB have low turn around time, high negative predictive value and are useful as screening tests. The limitation of these tests is low sensitivity in smear negative patients, HIV positive cases and in disease endemic countries with a high infection rate. The tests are also expensive, require trained personnel and often have difficulty in distinguishing between *M. tuberculosis* and NTM. In these tests either mycobacterial antigen can be detected in clinical specimen of pateint or antibodies to mycobacterial antigens in sera of patients are detected.

Tests based on detection of mycobacterial antigen [51]:

1. Capture ELISA: is a quantitative test to detect LAM in urine specimen. Another semi-quantitative dipstick method is used for the detection of LAM in both pulmonary and extra-pulmonary specimens.
2. Detection of LAM in sputum: this test is based on the capture antibody derived from murine source (murine monoclonal antibody against LAM). The rabbit antiserum against *M. tuberculosis* is used as a source of detector of the antibody.
3. Antigen detection in body fluids: free mycobacterial antigen at a concentration of 3-20ng/ml can be detected in body fluids such as CSF and pleural fluid. In this method the antigens are detected by using polyclonal antibodies raised against crude mycobacterial antigens except for antigen 5 and LAM. Other most common antigens that can be detected by this method are Ag A60, 45/47kDa, Kpp90, 30kDa, P32, cord factor (trehalose dimycolate).the methods used for antigen detection are: the sandwich ELISA, inhibition ELISA, latex agglutination and reverse passive agglutination tests.

Table 4

Various Diagnostic tests (with time span)for identifying Tuberculosis:

Variable	Sputum smear	Sputum culture	PCR	Tuberculin test	QuantiFERON-TB test	Chest radio-graphy
Purpose of the test	Detect AFB	Identify *M. tuberculosis*	Identify *M. tuberculosis*	Detects exposure to *M. tuberculosis*	Measure immune reactivity to *M.tuberculosis*	Visualize lobar infiltrates with cavitation
Time required	< 24 hours	3-6 weeks with solid media	6-8 hours	48-72 hours	12-24 hours	minutes

Source: Tuberculosis: Pathophysiology, Clinical features and Diagnosis by Nancy A, Knechel RN. MSN, ACNP; April 2009, 29(2).

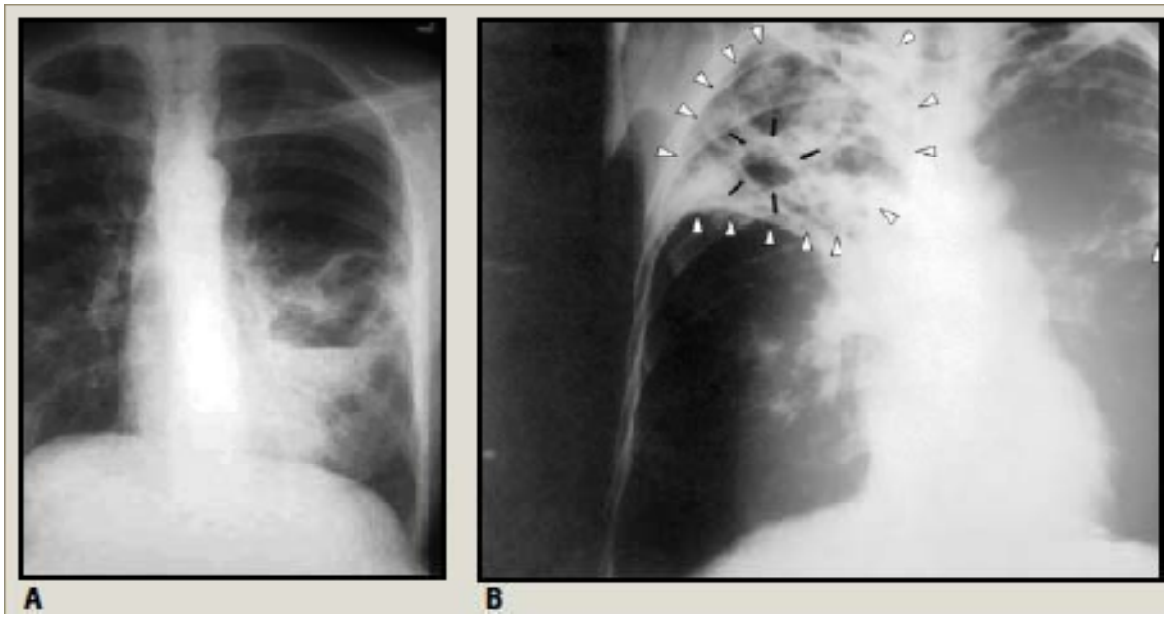

Figure 19: Chest radiograph in pulmonary tuberculosis: A. Infiltrates in left lung, B. Bilateral advanced pulmonary tuberculosis and cavitation in apical area of right lung.

Source: CDC, Public Health Image Library, Medical tests. PD US HHS CDC.

Tests based on detection of antibodies (indirect approach) [266]**:**

1. *TB STAT-PAK* test: It is an immunochromatographic test based on the detection of antibodies with a capacity to differentiate between active or dormant TB infection in whole blood, plasma or serum.
2. *Enzyme immune assay* for the detection of anti-mycobacterial superoxide dismutase antibody: superoxide dismutase is an important secretory protein of *M. tuberculosis* and has been evaluated for the serodiagnosis of tuberculosis.
3. *Insta test TB:* It is a rapid in vitro assay for the detection of antibody in active TB disease using whole blood or serum. The test employs an antibody binding protein conjugated to a colloidal gold particle and a unique combination of TB antigens immobilized on the membrane.

   Some of the other commercially available antibody tests for pulmonary TB are: MycoDot (Dot-blot), Detect-TB (ELISA), Pathozyme Myco (ELISA), Pathozyme TB (ELISA), Antigen A460 (ELISA), ICT diagnostics (membrane based) test.

Drug Susceptibility Testing (DST) for *Mycobacterium tuberculosis*:

Generally DST of *M. tuberculosis* is carried out in following instances:

- For relapse/retreatment cases of tuberculosis.
- To change drug regimen when drug resistance is suspected.
- Undertaking drug resistance surveillance studies in a region/country [229].

DST is one of the most difficult procedures to perform and standardize in Mycobacteriology laboratory. Proficiency in susceptibility tests demands an understanding of:

- Origin of drug resistance.
- Variation in stability of drugs subjected to different conditions of filtration, heat or storage.
- Alteration in activity of certain drugs when incorporated into different kinds of media.
- Type of susceptibility test performance.

- Reading and reporting of test results.
- Criteria of resistance.

There are different methods of Drug Susceptibility Testing (DST) for *Mycobacterium tuberculosis*:-

Indirect 1% proportion is the 'gold standard' DST method for *Mycobacterium tuberculosis*. This method was developed during 1960s and is still used in many laboratories, especially in developing countries, because it is inexpensive and easily accessible. [162] Because of long turnaround time (weeks to months) associated with such conventional DST methods, several new approaches have been developed for faster detection of drug-resistant TB [47]. These methods can be divided into two categories: culture-based or phenotypic methods and nucleic acid-based or genotypic methods.

1. **Phenotypic methods:**
   a. Conventional L.J. method.
   b. Automated methods.

2. **Genotypic methods:**
   a. Gene probe
   b. Cobas amplicor PCR
   c. Reverse Hybridization tests

DST for first-line anti-TB drugs is most accurate for Rifampicin (RIF) and Isoniazid (INH) and less reliable and reproducible for Streptomycin, Ethambutol (EMB) and Pyrazinamide (PZA). RIF resistance is a valid and reliable indicator of MDR-TB. [306] Automated liquid culture systems and molecular line probe assays are recommended by WHO as the current 'gold standard' for first-line DST [244, 46,269,143]. Second-line DST is complex and expensive. Liquid culture DST for aminoglycosides, polypeptides and fluroquinolones has been shown to have relatively good reliability and reproducibility for diagnosis of XDR-TB; however, DST for other second-line drugs (ethonamide, prothionamide, cycloserine, terizidone, para-aminosalicylic acid, clofazimine, amoxicillin-clavulanate, claritromycin, linezolid) is not recommended [306]. Automated

liquid culture systems are currently recommended by the WHO as the 'gold standard' for second-line DST [306,149,161].

Phenotypic Drug Susceptibility Testing (DST):

Phenotypic DST methods are accurate and inexpensive but are disadvantaged by relying on *Mycobacterium tuberculosis*, rendering them time consuming [271]. These methods require perfect standardization and result reading is subjective. Phenotypic DST methods are performed on solid or liquid media as direct or indirect tests [219]. Direct methods are those used directly on patient samples where a set of drug- containing and drug-free media is inoculated directly with a patient specimen. Indirect DST involves inoculation of drug-containing media with a pure culture grown from the original patient specimen [306].

There are 3 methods of conventional DST:

1. Absolute concentration method
2. Proportion control method and
3. Resistance ratio method

Other culture based DST methods are Microscopic Observation Drug susceptibility (MODS) [173,60,20,59], Colorimetric Redox Indicator (CRI) [1, 83] methods and the Nitrate Reductase Assay (NRA)[277, 106, 284].These methods have received WHO approval [73].Such methods have similar accuracy to commercial liquid culture systems and could be implemented in high-burden, low-income settings with minimum cost; however, these tests require extensive operator training, standardization and quality assurance before implementation [285].

Commercial liquid culture Drug Susceptibility Testing:

Commercial automated methods use liquid culture for DST; these methods are more sensitive than conventional and yield results rapidly. However, these are expensive, require elaborate set up and trained labor [180].

BACTEC MGIT 960:

It is a most widely used automated DST system. Both automated and manual versions are available. This is an indirect qualitative method for detection of resistance to Streptomycin, INH; RIF and EMB [180].The test takes 4-13 days to give a result. It is done by using a critical concentration of the drugs which is slightly lesser than in proportion method of DST-Streptomycin (1µg/ml), INH (0.1µg/ml), RIF (1µg/ml) and EMB (5µg/ml). It can also be used for testing Pyrazinamide by comparing growth in a medium without drug (growth control) with growth on medium with drug. Continuous analysis of fluorescence by the BACTEC MGIT 960 instrument in the drug-containing tube compared with the fluorescence of the growth control tube is used to determine susceptibility results. This method has been demonstrated to be equivalent to the proportion method standard and has been endorsed by the WHO [21,296,209,198].

MBBacT:

MBBacT is non-radiometric antimicrobial susceptibility system for testing *Mycobacterium tuberculosis* isolates cultured from clinical samples. In this system primary culture bottle growing *Mycobacterium tuberculosis* is used to inoculate drug-containing bottles and a drug-free control. DST sets are entered into the instrument and continuously monitored until a positive or negative result is obtained. An organism is determined to be susceptible when the antibiotic-containing bottle shows a positive detection time greater than drug-free control. In contrast, when the antibiotic containing bottle shows a detection time shorter than the drug-free control, the tested organism is determined to be resistant [209].

The rifampicin, isoniazid, streptomycin and ethambutol susceptibility test results were obtained in 6 days with the MBBacT [236].The absence of any false-susceptibility or false-resistance results with MBBacT indicates the excellent ability of the system for rapid testing of the susceptibility of *Mycobacterium tuberculosis* [33,237].

It demonstrates that (i) the MBBacT system is a reliable method for testing the susceptibility of *Mycobacterium tuberculosis*; (ii) the overall agreement of results is excellent for the three major anti-tuberculosis drugs, isoniazid, rifampicin and

pyrazinamide (iii) additional studies are required in order to improve ethambutol and streptomycin testing results, particularly at the high concentration; (iv) an undiluted growth control should be used, especially for testing pyrizinamide and (v) the MBBacT turnaround time for SIRE and PYZ testing is as fast that of the radiometric method [22,210].

BacT/ALERT MB (BioMereux Inc., Durham, North Carolina, USA) -This is also a non-radiometric automated system employed for culture and DST of *Mycobacterium tuberculosis*. Principle and applications are similar to BACTEC system.

Versa TREK system (Trek Diagnostic Systems, West Lake, Ohio, USA) is another automated system available for culture and DST of *Mycobacterium tuberculosis* [209,199].

Microscopic Observation Drug Susceptibility (MODS):-

A MODS is a liquid culture-based test used for culture and DST on sputum samples directly. It can assess INH and RIF's susceptibility either directly on sputum samples or indirectly on *Mycobacterium tuberculosis* isolates. Characteristic microscopic cording appearance of *Mycobacterium tuberculosis* in liquid media indicates growth. Cultures containing supplemented Middlebrook 7H9 medium are microscopically examined for microcolonies which can be detected in a median of 7 days. INH and RIF can be incorporated in the testing process to enable MDR-TB detection [59].Drug-free and drug - containing media are inoculated with specimens from patients or *Mycobacterium tuberculosis* isolates and cultures are microscopically examined. Growth of *Mycobacterium tuberculosis* in drug-free media indicates a positive culture, whereas growth of *Mycobacterium tuberculosis* in both drug-free and drug-containing media indicates resistance [173, 60, 59].

Meta-analysis of direct and combined (direct and indirect) testing of MODS performed by the WHO indicates that the method is 98% sensitive and 99% specific for the detection of RIF resistance [308]. High sensitivity and specificity are retained in direct MODS testing. The capability to perform MODS-DST directly on sputum samples in a short time and the low cost of reagents are advantages of this technique [59] but the WHO only endorse its implementation at reference laboratory level [308].

Colorimetric Redox Indicator Methods (CRI):

The principle of CRI method is reduction of a colored indicator, which is added to culture medium after cultured *Mycobacterium tuberculosis*, has been exposed to test antibiotic[199].Drug resistance is detected by a change in colour of the indicator, which is directly proportional to number of viable *Mycobacteria* remaining in the medium after exposure to the antibiotic. Different indicators have been evaluated giving comparable results in agreement with the proportion method. Among the different growth indicators used are the tetrazolium salts XTT, MTT, and the redox indicators Alamar blue and resazurin [160].

Data analysis performed by WHO showed that CRI methods are highly sensitive and specific for detection of RIF and INH resistance (98 and 97% sensitive respectively; 99 and 98 % specific respectively) and these methods were endorsed by the WHO in 2010[61]. CRI methods are indirect tests performed on *Mycobacterium tuberculosis* isolates; therefore, turnaround time to results is not faster than conventional phenotypic DST [308, 160].

Nitrate Reduction Assay (NRA):

The NRA (Greiss Nitrite test) – It is a simple test based on the capacity of *Mycobacterium tuberculosis* to reduce nitrate to nitrite. By incorporating 1mg/ml potassium nitrate (KNO_3) in Lowenstein-Jensen medium, reduction of nitrate can be detected using Greiss reagent, which produces a colored reaction [162, 53]. In presence of antibiotic at the critical concentration, development of a red-pink colour in the medium indicates resistance, but susceptible strains lose the capacity to reduce nitrate in presence of antibiotic, thus produce no colour. This test is also an indicator of growth even before seeing the macroscopically [162].The turnaround time is less than conventional method. WHO recommends that it can be used as a direct test on clinical samples for resistance detection [308]. It can also be used as an indirect test to test the cultures. Data on combined (direct and indirect) use showed that the NRA is 97% sensitive and 100% specific for RIF resistance detection and 97% sensitive and 99% specific for INH resistance detection[308]. Diagnosis accuracy data for direct testing alone does not differ

significantly. Reagents for NRA are nonproprietary and relatively inexpensive, but indirect testing using NRA is not faster than conventional phenotypic DST using solid media [162, 308].

Traditional laboratory methods of drug sensitivity testing takes a minimum of 10-12 weeks, which is too long a period for sputum positive case especially MDR-TB cases both from the patient's treatment and community point of view [228]. Agar proportion method on Lowenstein-Jensen and Middlebrook 7H10 or 7H11 are considered to be gold standard but this takes 10-12 weeks. Automated Radiometric BACTEC 460 and Fluorescence based (MGIT) methods are available but they too take at least 3 weeks [52]. Luciferase phage reporter system in which bacteria are infected with a phage carrying the luciferase enzyme has been reported to give good results in a few days but the system is expensive and not standardized in most laboratories [234].

Other new and existing phenotypic DST methods are Slide Culture technique, Mycobacteriophage method, Fast plaque assay, Luciferase reporter phage method, E-test, TK medium and Thin layer agar method. These are not approved by WHO.

Genotypic Drug Susceptibility Testing (Genotypic DST):

There are many molecular methods for detection of *M. tuberculosis* complex in clinical samples. This includes Cobas Taqman MTB PCR test (Roche) [134], Artus MTB PCR (Qiagen) [73], amplified *Mycobacterium tuberculosis* direct (AMTD, Gen-probe, Sandigo) [192]. Some of these tests are also capable of simultaneous detection of drug resistance in *Mycobacterium tuberculosis* complex. These tests are nucleic acid amplification based tests (NAAT) and includes hybridization assay [17, 25, 6, 171, 35, 95]. By molecular analysis, most common mutations have been detected and genotypic methods target these mutations and identify the resistance pattern [240,120]. Easily detectable resistance pattern is that of RIF and is suitable for genotypic DST because 95% of RIF mutations are located in the 81 bp region of *rpoB* gene which is known as Rifampicin Resistance Determining Region (RRDR) [25].

Line Probe Assays (LPA):

LPA is a rapid, reliable and sensitive test method for detection of resistance to RIF and INH. The major advantage is that it can be done on samples, which are smear-positive for AFB. Thus this test helps in early diagnosis of possible MDR-TB case. This method

involves two steps, amplification of target DNA using specific probes, followed by reverse hybridization forming specific bands [80]. Absence of wild type probes or appearance of mutation bands indicate resistance. Two commercial LPA's are in use:

1. INNO-LiPA Rif.TB test (Innogenetics NV, Gent, Belgium)
2. MTBDR *Plus* test (Hain Lifescience GmbH, Nehren, Germany).

Resistance is indicated by detection of the most common single nucleotide polymorphism (SNP). LPAs are very specific for detection of first line drug resistance in smear positive cases [198, 35, 150, and 248,247,246]. LPAs have a high sensitivity (≥97%) and specificity (≥99%) for detection of RIF resistance alone or in combination with INH (sensitivity≥90%; specificity≥99%), on isolates of *Mycobacterium tuberculosis* and on smear-positive sputum specimens. Conventional culture is avoided and this helps in initiating correct treatment [220].

Hain Life Sciences have released the new GenoType MTBDR*sl* test in 2009. This is designed to detect second line drug resistance and in turn XDR-TB cases. The drugs tested include fluoroquinolones, ethambutol, aminoglycosides and cyclic peptides and this can be used in combination with the MTBDR*plus* test to identify XDR-TB [133, 32, 109].

Hain test protocol (LPA):

The Genotype MTBDR*plus* test is based on DNA strip technology and permits the molecular genetics identification of *Mycobacterium tuberculosis* complex and its resistance to RIF and/or INH from cultivated samples and smear positive pulmonary samples-direct patient material.

- The identification of RIF resistance is enabled by the detection of most significant mutation in the *rpoB* gene (coding for the β subunit of RNA polymerase).
- For testing of high level of INH resistance, the *katG* gene (coding for the catalse-peroxidase) is examined.
- For testing of low level INH resistance, the promoter region of *inhA* gene (coding for NADH enoyl ACP reductase) is examined.

Genotype MTBDR*sl* is based on the DNA-strip technology [131, 109] and permits the simultaneous molecular identification of the *Mycobacterium tuberculosis* complex and its resistance to second-line anti-tuberculosis drugs:

- Fluroquinolones like ofloxacin and moxifloxacin- by the detection of the most common mutations in the *gyrA* gene.
- The injectable antibiotics (viomycin, kanamycin, amikacin and capreomycin) - by detection of the most common mutations in the *rrs* gene.
- The first line drug Ethambutol- by detection of the most common mutations in the *embB* gene from smear positive pulmonary clinical specimens or cultivated samples.

The whole procedure is divided into 3 steps: DNA extraction from cultured material (culture bottle/liquid medium) or direct clinical specimen (pulmonary smear positive decontaminated), a multiplex amplification of the resistance determining region of the gene under question is performed using biotinylated primers. Following amplification, labeled PCR products are hybridized with specific oligonucleotide probes immobilized on a strip. Captured labeled hybrids are detected by colorimetric development, enabling detection of the presence of *Mycobacterium tuberculosis* complex, as well as the presence of wild-type and mutation probes for resistance. If a mutation is present in one of the target regions, the amplicon will not hybridize with the relevant probe.

Mutations are therefore detected by lack of binding to wild-type probes, as well as by binding to specific probes for the most commonly occurring mutations. The post-hybridization reaction leads to the development of coloured bands on the strip at the site of probe binding.

Gene Xpert *Mycobacterium tuberculosis*/Rifampicin Assays:

One of the most promising new point-of-care (POC) diagnostic technologies to be developed in recent years is the GeneXpert (Cepheid, Sunnyvale, California, USA). The Gene Xpert machine is a fully automated closed system that performs both sample preparation and real-time PCR, producing results in less than 2 hours. The Xpert MTB/RIF test, designed for the Gene Xpert system, is capable of detecting the *Mycobacterium tuberculosis* complex (MTBC) while simultaneously detecting RIF resistance (targeting the RRDR of the *rpoB* gene). Analytical sensitivity and specificity is 100% according to a recent study using RIF resistant and sensitive *Mycobacterium tuberculosis* isolates and non-tuberculous bacteria, fungi and viruses [(25)].

The assay is designed for direct genotypic DST from unprocessed sputum or sediment from a concentrated specimen. Sample reagent is poured into the sample tube, incubated for 15 minutes, pippeted into the Xpert cartridge and inserted into the GeneXpert machine for processing. Highly trained staffs are not required to run the machine or interpret the results [79]. The Xpert MTB/RIF test has the potential to be used in the moderately equipped laboratories; however, it is unlikely to be used as a POC diagnostic test in the most peripheral settings, like rural health centers, due to poor infrastructure and limited resources [50]. Variables that may affect the tests overall performance are HIV prevalence, strain diversity, prevalence of specific drug resistance-conferring mutations, patient-related diagnostic delays and default rates [281].

Cobas-R TaqMan –R MTB Test [57]:

It is a Real-time PCR test for qualitative detection of *Mycobacterium tuberculosis* Complex DNA.

This test uses real-time polymerase chain reaction (RT-PCR) nucleic acid amplification and hydrolysis probes for the detection of *Mycobacterium tuberculosis* in liquefied, decontaminated human respiratory specimens, including expectorated and induced sputum and bronchial lavages (BAL).

Features and benefits:

- Ease of use with high throughput.
- Up to 48 tests per run.
- Results in only 2.5 hours after sample preparation.
- Overall inductivity of 99% when evaluated with all species of *Mycobacteria* found in the *Mycobacterium tuberculosis* complex, reduced contamination with Roche's AmpErase Enzyme.
- Runs on the COBAS-R TaqMan-R Analyzer.
- Automated amplification and detection in a closed system.
- Enhanced quality control in the laboratory.

Serodiagnosis of Tuberculosis:

Serology and serological tests have very little role in the diagnosis of tuberculosis.

Serodiagnosis based on Antigen Detection: *Mycobacterium tuberculosis* has been known to secrete more than 33 different proteins. One of these is found predominantly in

Mycobacterium tuberculosis complex. Recently, SD Bioline, South Africa has developed a simple, rapid immunochromatography assay using mouse monoclonal anti-MPT64 for rapid discrimination between the *Mycobacterium tuberculosis* complex and MOTT bacilli. This test is useful in rapid identification of MTBC [195, 147].

Treatment of Tuberculosis:

Therapeutically tuberculosis treatment has two objectives:

(a) Interrupt tuberculosis transmission by rendering patients noninfectious and

(b) Prevent morbidity and death by curing patients with tuberculosis.

Classes of anti-TB drugs [231]: The anti-TB drugs can be grouped (Table 5) based on efficacy, experience of use and drug class.

Table 5

Grouping of anti-TB drugs

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

Source: WHO, Treatment of Tuberculosis guidelines; 4th edi. (WHO, 2010).

First- line drugs for the treatment of tuberculosis:

1. Isoniazid (H).
2. Rifampicin (R).
3. Pyrazinamide (Z).
4. Ethambutol (E).

These agents are recommended on the basis of their bactericidal activity, sterilizing activity, low rate of induction of drug resistance.

Second- line drugs:

1. Injectible aminoglycosides- Streptomycin (S),Kanamycin (Km),Amikacin (Am),
2. Injectible polypeptide – Capreomycin (Cm), Viomycin (Vm)
3. Oral agents – Etionamide (Eto), Cycloserine (Cs), para-aminosalicylic acid (PAS)
4. Fluoroquinolones – Levofloxacine (Lvx), Ciprofloxacin (Cfx), Ofloxacin (Ofx) Gatifloxacin (Gfx), Moxifloxacin (Mfx)
5. Unproven efficacy – Clofuzinamine (Cfz), Amoxicillin/Clavulanic acid (Amx/Clv), Linezolid (Lzd), Thioacetazone (Thz), Imipenem/Cilastatin (Ipm/Cln), high- dose Isoniazid (high-dose H), Clarithromycin (Clr).

These second-line drugs have a lower degree of efficacy and a higher degree of intolerability and toxicity.

All the first-line anti-tuberculosis drugs names have a standard three-letter and a single-letter abbreviation. Drug regimens are abbreviated in a standardized manner. First-line drugs (Group-1) are recommended in a four-drug combination for the treatment of drug-susceptible tuberculosis. Second-line anti-tuberculosis drugs (Groups- 2, 3 and 4) are reserved for drug-resistant tuberculosis. Third-line anti-tuberculosis drugs (Group 5) have unclear efficacy.

Third –line drugs:

Other drugs that may be useful, but are not on the WHO list of second-line drugs:

1. Rifabutin
2. Macrolides- e.g. clarithromycin
3. Linezolid
4. Thioacetazone

5. Thioridazine
6. Arginine
7. Vitamine D
8. R207910

These drugs may be considered "third-line drugs" and are listed here either because they are not very effective (e.g.clarithromycin) or because their efficacy has not been proven (e.g. linezolid, R207910). Rifabutin is effective, but is not included on the WHO list because for most developing countries, it is impractically expensive [13].

New tuberculosis drugs:

Several new drugs e.g. Nitroimidizoles, PA-824, OPC-67683, TM207, SQ109, Phenothiazines, Oxazolidinones etc. have emerged recently as potential candidates for the treatment of tuberculosis. In most cases, there mechanism of action is distinct from that of the classical anti-TB drugs.

Bedaquiline is one such new drug with a unique mechanism of action that can tackle both drug sensitive as well as drug resistant strains. It targets adenosine triphosphate synthase, an enzyme necessary for generation of energy for the *Mycobacterium tuberculosis.* Food and Drug Administration has approved Bedaquiline and Delamanid for MDR-TB in December 2012[101].

Treatment failure and Relapse in Tuberculosis:

It is suspected when a patient's sputum cultures remain positive after 5 months of standard treatment. These cultures have to be tested for susceptibility to first and second-line drugs. If the patient's clinical condition is deteriorating, add at least two and preferably three drugs that have never been used and to which the bacilli are likely to be susceptible [164, 165]. The mycobacterial strains infecting patients who experience a relapse after apparently successful treatment are less likely to have acquired drug resistance. It is prudent to begin the treatment of all patients who have relapsed with all four first-line drugs plus streptomycin, pending the results of susceptibility testing.

Drug-Resistant Tuberculosis:

Strains of *Mycobacterium tuberculosis* resistant to individual drugs arise by spontaneous point mutations in the **Mycobacterial** genome. Because there is no cross-resistance among the commonly used drugs, the probability that a strain will be resistant to two

drugs is the product of the probabilities of resistance to each drug and thus is low. The development of drug-resistant tuberculosis is invariably the result of monotherapy.

Drug-resistant tuberculosis is classified as: [186]

a. **Primary drug resistance:** It is defined as presence of drug resistance to one or more anti-TB drugs in a patient who has never received prior anti-TB chemotherapy.
b. **Acquired resistance:** It is defined as resistance to one or more anti-TB drugs, which arises during the course of treatment, usually as a result of non-adherence to the recommended regimen or faulty prescribing. This is also referred as a secondary resistance.
c. **Initial resistance:** It is defined as the presence of drug resistance to one or more anti-TB drugs in a new tuberculosis patient who presents to the treatment centre.
   This category includes those patients with primary resistance as well as patients with undiagnosed acquired resistance.
d. **Multidrug resistance (MDR):** It refers to resistance to more than one anti-TB drug. It can occur both as primary and secondary resistance and is often associated with HIV infection as well as with chronic patient. It is defined as resistance to at least both INH and RIF.
e. **Extensively drug resistant (XDR):** It refers to resistance to INH, RIF and any Fluoroquinolone and at least one of the three injectable drugs: capreomycin, kanamycin and amikacin.
f. **Extremely drug resistant (XXDR) / Totally drug resistant (TDR):** It refers to resistance to all anti-TB drugs.
g. **Transient drug resistance:** It is a temporary drug resistance which occurs just before sputum conversion in a patient who is responding to therapy. This normally occurs in the form of few resistant colonies which usually do not multiply and does not warrant any change in the treatment.

Drug resistant tuberculosis can be prevented by adherence to the principles of sound therapy [40,185]:-

1. Inclusion of at least two bactericidal drugs to which the organism is susceptible.
2. Use of FDC products.
3. Verification that patients complete the prescribed course.

Mode of Action and Mechanism of Drug Resistance in Tuberculosis :[140, 242, 322]

Aside from natural resistance mechanisms, *Mycobacterium tuberculosis* has acquired resistance mechanisms to anti-tuberculosis drugs by spontaneous mutations in chromosomal genes. The rate at which resistance emerges differs for all the anti-tuberculosis agents, it being highest for ethambutol (EMB) and lower for rifampicin (RIF) and quinolones. Mutations in the genome of *Mycobacterium tuberculosis* that can confer resistance to anti-tuberculosis drugs occur spontaneously with an estimated frequency of 3.5×10^{-6} for INH and 3.1×10^{-8} for RIF.

Various anti-tuberculosis drugs with their mode of action and mechanism of drug resistance:-

1) Isoniazide (INH):

It enters the cell as a prodrug that is activated by a catalase-peroxidase encoded by *katG*. The peroxidase activity of the enzyme is necessary to activate INH to a toxic substance in the bacterial cell. A lack of mycolic acid synthesis eventually results in loss of cellular integrity and the bacteria die. Middlebrook *et al.* initially demonstrated that a loss of catalase activity results in INH resistance mutations. Mutations are found between codons 138 and 328 with the most commonly observed gene mutation being at codon 315of the *katG* gene. The Ser315Thr substitution is estimated to occur in 30-60% of INH resistant isolates. The *katG* 463 (CGG-CTG) (Arg-Leu) amino acid substitutions is the most common polymorphism found in the *katG* gene and are not associated with INH resistance.

One of the targets for activated INH is the protein encoded by the *inhA* locus. InhA is an enoyl-acyl carrier protein (ACP) reductase which is proposed to be the primary target for resistance to INH and ETH. ETH, a second line drug, is a structural analogue of INH that is also thought to inhibit mycolic acid biosynthesis and several studies have suggested that low-level INH resistance is correlated with resistance to ETH. Activated INH binds to the InhA NADH complex to form a ternary complex that results in inhibition of mycolic acid biosynthesis. Six point mutations associated with INH resistance within the structural *inhA* gene have been identified (Ile16 Thr, Ile21Thr, Ile21Val, Ile47Thr, Val78Ala and Ile95Pro). These mutations are associated with INH resistance but not

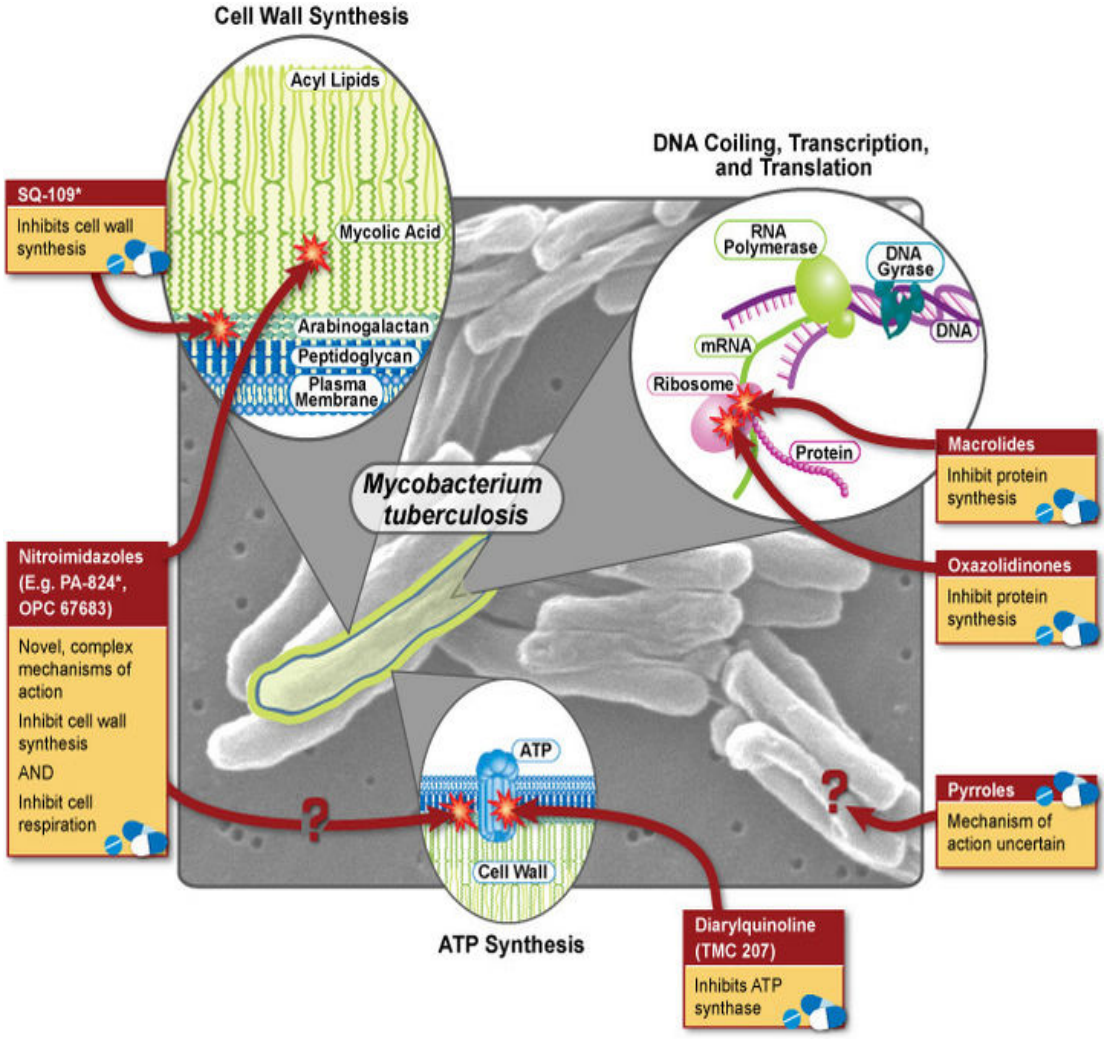

Figure 20: Mechanism of action of anti-tuberculosis drugs.

Source: CDC, Originally from National Institute of Health, PD US NIH, Tuberculosis.

frequently reported in clinical isolates. *InhA* promoter mutations are more frequently seen and are present at positions -24(GT), -16(A-G), or -8(T-G/A) and -15(C-T). These promotor mutations result in over expression of *inhA* leading to low level INH resistance.

2) Rifampicin (RIF):

It interferes with transcription by the DNA-dependent RNA polymerase. RNA polymerase is composed of four different subunits (α, β, β'and σ) encoded by *rpoA, rpoB, rpoC* and *rpoD* genes respectively. RIF binds to the β-subunit hindering transcription and thereby killing the organism. Extensive studies on the rpoB gene in RIF resistant isolates of *Mycobacterium tuberculosis* identified a variety of mutations and short deletions in the gene. A total of 69 single nucleotide changes; 3 insertions, 16 deletion and 18 multiplenucleotide changes have been reported. Changes in the codons Ser531 and His526 have been documented in more than 70% of the RIF-resistant isolates.

3) Streptomycin (STR)

It interacts with the 16S rRNA and S12 ribosomal protein (*rrs* and *rpsl*) including ribosomal changes, which cause misreading of the mRNA and inhibition of protein synthesis.

Point mutations in STR resistant isolates have been reported in *rrs* and *rpsl* genes in 65-67% of STR resistant isolates. In the *rrs* gene a C-T transition at positions 491,512 and516 and a A-C/T transversion at position 513 were observed in the highly conserved 530 loop. The 530 loop region is part of the aminoacyl-tRNA binding site and is involved in the decoding process. The C-T transition at codon 491 is not responsible for resistance to STR as it occurs in both STR resistant and susceptible strains but is strongly associated with the global spread of *Mycobacterium tuberculosis* with a Western Cape F11 genotype. Other mutations in the 915 region [903(C-A/G) and 904 (A-G)] have also been reported to have an association with STR resistance. Mutations in the *rpsl* gene at codon 43 (AAG-AGG/ACG) (Lys-Arg/Thr) and codon 88 (AAGAGG/CAG) (Lys-Arg/Gln) are associated with STR resistance. MIC analysis of STR resistant isolates indicates that amino acid replacements in the *rpsl* genes correlates with a high level of resistance, whereas mutations in the *rrs* gene correlate with an intermediate level of resistance. In

addition, it has been suggested that low levels of STR resistance are also associated with altered cell permeability or rare mutations which lie outside of the *rrs* and *rpsl* genes.

4) Kanamycin and Amikacin:

These are aminoglycosides which inhibit protein synthesis and thus cannot be used against dormant *Mycobacterium tuberculosis*. Aminoglycosides bind to bacterial ribosomes and disturb the elongation of the peptide chain in the bacteria. Mutations in the *rrs* gene encoding for 16S rRNA are nucleotide changes at positions 1401, 1402 and 1484 of the *rrs* gene have been found to be specifically associated with resistance.

5) Ethambutol (EMB):

It inhibits an arabinosyl transferase (*embB*) involved in cell wall biosynthesis. Telenti ***et. al***. identified three genes, designated *emb*CAB, that encode homologous arabinosyl transferase enzymes involved in EMB resistance. Various studies have identified five mutations in codon 306 [(ATG-GTG), (ATG-CTG), (ATG-ATA), (ATG-ATC) and (ATG-ATT)] which results in 3 different amino acid substitutions (Val, Leu, Ile) in EMB-resistant strains.

6) Pyrazinamide (PZA):

It is an important first-line drug used along with INH and RIF. It plays a unique role in shortening tuberculosis treatment from the previous 9-12 months to 6 months because it kills a population of bacilli present in acidic pH environment in the lesions that is not killed by other drugs. It is a pro-drug converted to pyrazinoic acid (POA), by the enzyme pyrazinamidase. The anti-tuberculosis activity of PZA is associated with the disruption of proton motive force required for essential membrane transport functions by POA at acidic pH. The resistant strains have a diverse nucleotide changes scattered throught the *pncA* gene. Mutations in the *pncA* gene correlate well with phenotypic resistance to PZA. However, PZA resistant strains without *pncA* mutations are also observed suggesting that another mechanism may be involved in conferring PZA resistance in these strains. In addition, not all mutations (e.g. Thr114Met) are associated with PZA resistance.

7) The fluoroquinolones (FQ):

It target and inactivate DNA gyrase, a type II DNA topoisomerase. DNA gyrase is encoded by *gyrA* and *gyrB* and introduces negative supercoils in closed circular DNA molecules. The quinolone resistance-determining region (QRDR) is a conserved region in the *gyrA* (320bp) and *gyrB* (375bp) genes which is the point of interaction of FQ and gyrase. Missense mutations in codon 90, 91and 94 of *gyrA* are associated with resistance to FQs. A polymorphism at *gyrA* codon 95 is not associated with FQ resistance and is used with the *katG*463 polymorphism, to classify *Mycobacterium tuberculosis* into three phylogenetic groups.

8) Ethionamide:

It is a synthetic compound structurally related to INH, is a pro-drug, requiring activation by the monooxygenase EthA. Similar to INH, ethionamide inhibits mycolic acid synthesis by binding the ACP reductase InhA. High level ethionamide resistance is associated with mutations in *ethA* or *inhA* genes. Other potential mechanisms of resistance are associated with deletion in *mshA* genes of *Mycobacterium tuberculosis* which results in defective mycothiol biosynthesis and resistance to ethionamide, likely due to defective activation of drug.

9) Capreomycin and Viomycin:

Capreomycin is a polypeptide antibiotic. Like streptomycin and kanamycin, it inhibits protein synthesis through modification of ribosomal structures at the 16S rRNA. Resistance to capreomycin and kanamycin is associated with mutations in the *rrs* gene encoding 16S rRNA. Mutations in the gene *tlyA* encoding a 2'-O-methyltranfarese of 16S rRNA and 23S rRNA have been implicated in resistance to capreomycin and viomycin.

10) Cycloserin (DCS):

It is a d-alanine analog, which interrupts peptidoglycan synthesis by inhibiting the enzymes d-alanine racemase (AlrA) and d-alanine: d-alanine ligase (Ddl). Over expression of AlrA and Ddl may result in resistance to DCS.

11) Para-aminosalicylic acid (PAS):

It inhibits folic acid biosynthesis and uptake of iron. Mutations in the *thyA* gene encoding the enzyme thimidylate synthesis of the folate biosynthesis pathway have been identified in PAS-resistant *Mycobacterium tuberculosis* clinical isolates, suggesting that PAS may act as a folate antagonist.

12) Linezolid (Lzd):

Due to its ability to penetrate macrophages, it is active against intracellular bacilli exerting its activity by binding to the ribosomal 50S subunits and thus inhibiting an early step in protein synthesis. It is most commonly used to treat drug resistant tuberculosis. Resistance to linezolid is rarely reported in clinical specimens and it is found to be associated with mutations at G2061T and G2576T in the 23S rRNA gene.

HIV-ASSOCIATED TUBERCULOSIS:

Impact of co-infection with HIV and *Mycobacterium tuberculosis*: -

The HIV pandemic, which is largely uncontrolled in many parts of the world, has contributed significantly to the spread of tuberculosis [(141)]. HIV infection and tuberculosis are synergistic diseases on the every level, from the molecular to the epidemiologic. For instance, the presence of HIV promotes the intracellular replication of the *Mycobacterium tuberculosis* and thereby promotes the transmission of *Mycobacterium tuberculosis* to others [(223,214)]. In many parts of the world, TB is the leading cause of death in persons infected with HIV. It has been estimated by the WHO that a third of the 36.1 million persons who are infected with HIV also have tuberculosis. The most powerful risk factor the reactivation of latent tuberculosis is co-infection with HIV.

Sputum smear microscopy remains the standard diagnostic test but is woefully inadequate in HIV, children and in drug-resistant tuberculosis [(222, 85)]. Xpert MTB/RIF test provides sensitive detection of TB and RIF resistance in less than 2 hours. This technology has been endorsed by the WHO in December 2010, essentially in high MDR-TB settings and high HIV prevalence settings. Global TB diagnostics is expanding and

availability of several newer tools is being scaled up, driven to a large extent by both drug resistance and HIV-TB co-infection [235].

In general, the standard treatment regimens are equally efficacious in HIV-negative and HIV- positive patients.

Three important considerations are relevant to tuberculosis treatment in HIV-infected patients:

- Increased frequency of paradoxical reactions.
- Drug interactions between antiretroviral therapy (ART) and Rifampicin.
- Development of Rifampicin monoresistance with widely spaced intermittent treatment.

The presentation of MDR-TB in the HIV-infected patient does not differ from that of drug-sensitive tuberculosis in the HIV-infected patient. However the diagnosis of tuberculosis in HIV-positive persons can be more difficult and may be confused with other pulmonary or systemic infections. As the HIV disease progresses and the individual become more immunocompromised, the clinical presentation is proportionately more likely to be extra-pulmonary or smear-negative than in HIV-uninfected tuberculosis patients. This can result in misdiagnosis or delays in diagnosis and in turn, higher morbidity and mortality. With the nation wide scale up of intensifies Tuberculosis Package, it is expected that more and more numbers of tuberculosis patients have known HIV status and if found to be HIV-positive, they must be linked to ART Centers and provided Co-trimoxazole preventive therapy (CPT).

The treatment of HIV-positive individual with MDR-TB is the same as for HIV negative patients. However treatment is more difficult and adverse events are more common. Deaths during treatment, partly due to other HIV-related diseases, are more frequent in HIV-infected patients, particularly in the advanced stages of immunodeficiency. Due to the increased frequency of adverse drug events, rigorous monitoring in this particular group of patients is required in order to ensure adherence to treatment, early identification and treatment of adverse events and reduce default.

Prevention of tuberculosis by Prophylactic methods [311]:-

1. **General Prophylactic Methods**:
    1. Avoid overcrowding.
    2. Avoid addictive drugs.
    3. Improve nutritional deficiency.
    4. Early detection and isolation and initiation of correct treatment.
2. **Specific Prophylactic Methods:**

a) **Chemoprophylaxis**: -

Treatment of selected persons (family members of contacts and children) with Latent Tuberculosis Infection (LTBI) aims at preventing active disease. This intervention was formerly called chemoprophylaxis. In most cases, candidates for treatment of LTBI are identified by the Tuberculin Skin Test (TST) of persons in defined high-risk groups. Isoniazid is administered at a daily dose of 5mg/kg (up to 300mg/d) for 9 months. Six months period of treatment has been recommended for HIV-negative adults. When supervised treatment is desirable and feasible, Isoniazid may be given at a dose of 15 mg/kg (up to 900 mg) twice weekly. An alternative regimen for adults is 4 months of daily Rifampicin.

Rifampicin regimen should be considered for persons who are likely to have been infected with an isoniazid-resistant strain. Isoniazid should not be given to persons with active liver disease. All persons at increased risk of hepatotoxicity should undergo baseline and then monthly assessment of liver function. Patients should be seen and questioned monthly during therapy about adverse reactions and should be given no more than 1 month's supply of drug at each visit. It may be more difficult to ensure compliance when treating persons with latent infection than when treating those with active tuberculosis.

Best way to prevent tuberculosis is to diagnose and isolate infectious cases rapidly and administer appropriate treatment until patients are rendered noninfectious and the disease is cured. Additional strategies include BCG vaccination and treatment of persons with latent tuberculosis infection who are at risk of developing active disease.

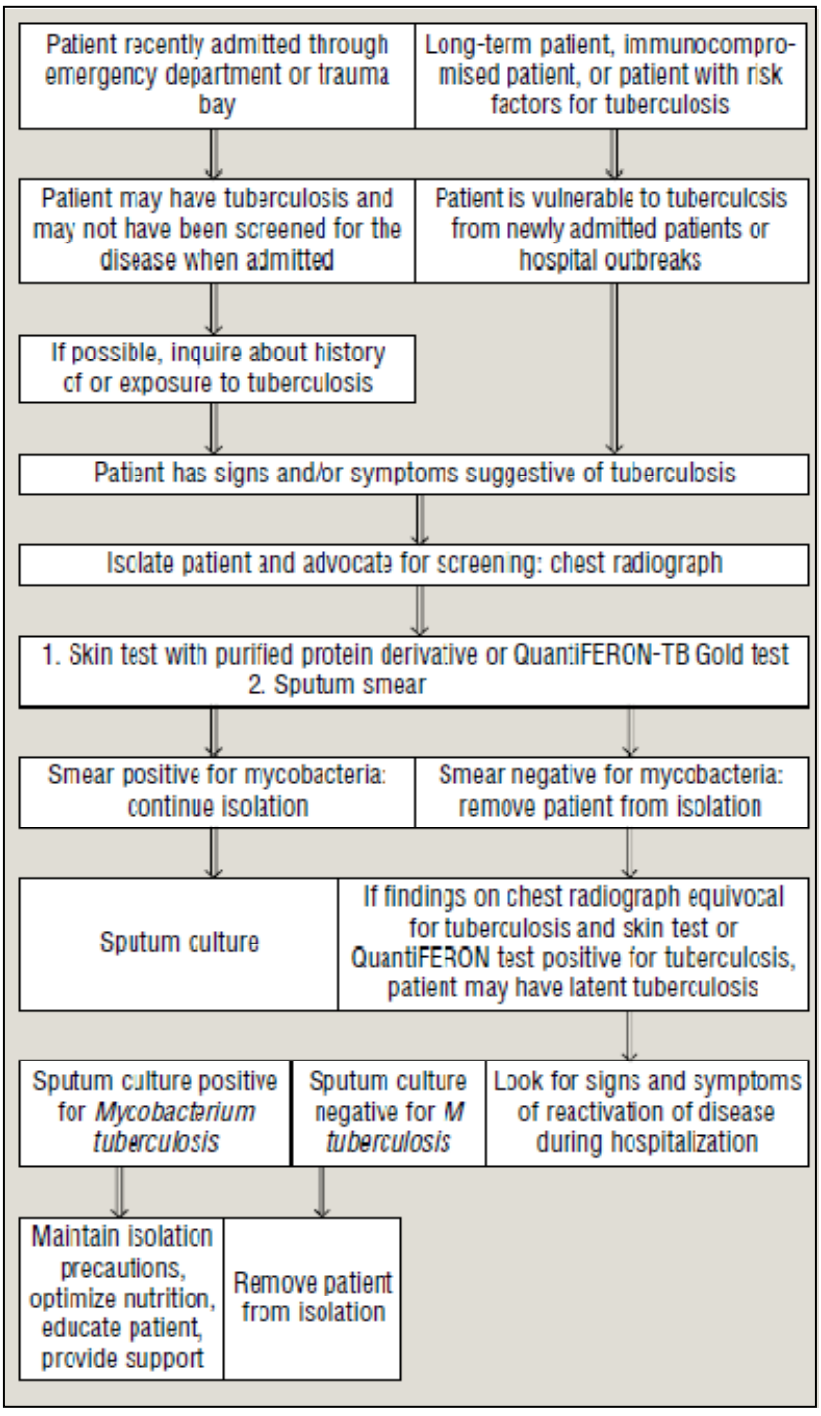

Figure 21: General plan-of-care algorithm for a suspected Tuberculosis patient.

Source: Center for disease control and prevention, Geneva

BCG VACCINATION:

Bacillus Calmette-Guerin (BCG) is a live attenuated vaccine against tuberculosis. It is prepared from bovine strain of tuberculosis bacilli. This *Mycobacterium bovis* is grown in glycerol broth and sub-culturing every 3rdweek over a period of 12 years. Experimental evidences have proved that by this method the *Mycobacterium bovis* lose its virulence but remain viable.

Schedule for BCG vaccination: 1. Dosage: 0.1ml

2. Time: soon after birth
3. Site: Deltoid
4. Route of administration: Intramuscular (I.M.)
5. Protection/Immunity: 10-12 yrs after vaccination.

The local tissue response begins 2-3 weeks after vaccination, with scar formation and healing within 3 months.

Albert Calmette, a French physician and bacteriologist, and his colleague, Camille Guerin, a veterinarian, developed this BCG vaccine at the Pasteur institute, France in 1908, and it was first used in humans in 1921[(87)]. India and Pakistan introduced BCG mass immunization in 1948, the first countries outside Europe to do so [(155)].

Adverse effects:

BCG immunization generally causes some pain and scaring at the site of injection. The main adverse effects are keloids- large, raised scars. The insertion of deltoid is most frequently used because the local complication rate is smallest when that site is used. Nonetheless, the buttock is an alternative site of administration because it provides better cosmetic outcomes.

BCG vaccine should be given intradermally. If given subcutaneously, it may induce local infection and spread to the regional lymph nodes, causing either suppurative or nonsuppurative lymphadenitis. If suppuration occurs, it may need needle aspiration. For non-resolving suppuration, surgical excision is required, but not incision. Uncommonly, breast and gluteal abscesses can occur due to haematogenous and lymphangiomatous spread. Regional bone infection (BCG osteomyelitis or osteitis) and disseminated BCG

infection are rare complications of BCG vaccination, but potentially life threatening. Systemic anti-tuberculous therapy may be helpful in severe complications [102].

If BCG is accidentally given to an immunocompromised patient (e.g., an infant with SCID), it may cause disseminated or life-threatening infection. The documented incidence of this happening is less than one per million immunizations given [43]. In 2007, the WHO stopped recommending BCG for infants with HIV, even if there is a high risk of exposure to TB [295], because of the risk of disseminated BCG infection (which is approximately 400 per 100,000) [270, 156].

PRINCIPLES OF TUBERCULOSIS CONTROL:

The highest priority in any tuberculosis control program is the prompt detection of cases and the provision of short-course chemotherapy to all tuberculosis patients under proper case-management conditions, including directly observed therapy. In addition, in low prevalence countries with adequate resources, screening of high-risk groups is needed. Measures to limit such transmission include: -

1. Isolation of persons with suspected tuberculosis until they are proven to be noninfectious.
2. Proper ventilation in rooms of patients with infectious tuberculosis.
3. Use of ultraviolet irradiation in areas of tuberculosis transmission.
4. Periodic screening of personnel who may come into contact with known or unsuspected cases of tuberculosis.

Revised National Tuberculosis Control Program (RNTCP):-

It is the state-run Tuberculosis control initiative of the Government of India which incorporates the principles of Directly Observed Treatment Strategy (DOTS)-the global TB control strategy of the World Health Organization (WHO). The program provides free of cost, quality Anti-tubercular drugs across the country through numerous **primary health centers** and the growing numbers of the private-sector DOTS- providers.

History:

India has an on-going National Tuberculosis Program (NTP) since 1962 which could diagnose only 30% of estimated tuberculosis patients and only 30% of these were treated successfully. Based on the findings and recommendations of the review in 1992, the

Government of India (GOI) evolved a revised strategy and launched the Revised National Tuberculosis Control Program (RNTCP) in the country. This RNTCP began as a national program in 1997.

By the end of 2000, 30% of the country's population was covered under RNTCP. By December 2005, around 97% (about 1080 million) of the population had been covered and by24 March of 2006, the entire country was covered under DOTS covering 1114 million people.

Objectives of RNTCP:

1. To achieve and maintain cure rate of at least 85% among New Sputum Positive (NSP) patients.
2. To achieve and maintain case detection of at least 70% of the estimated NSP cases in the community.

Monitoring, Supervision and Evaluation:

All states in India are currently implementing the 'Supervision and Monitoring strategy' - detailing guidelines, tools and indicator for monitoring the performance from the peripheral health institution (PHI) level to the national level. The quality program implementation is ensured by frequent internal and external evaluations. The program is focusing on the reduction in the default rates among all new and retreatment cases and is undertaking steps for the same.

Current focus:

RNTCP has completed 19 years of its implementation with 4 years of full nation-wide coverage. Since its inception the program, over 56 million patients have been successfully treated and an estimated 22 million lives saved through the use of DOTS and the Stop TB Strategy recommended by WHO [315]. RNTCP has definitely made strong strides towards achieving the Millennium Development Goals of relating to the prevalence and mortality due to tuberculosis by 2015 as compared to the 1990 levels. The

ultimate goal of the program remains a “TB-free India”, and clearly it is a long journey towards this goal. RNTCP is now moving beyond the objectives of 70% case detection rate and 85% cure rate in new smear positive patients, so that all TB patients have access to good quality diagnostic and treatment services. Further, the program has initiated actions to address the challenges of drug resistant- tuberculosis and TB-HIV co-infection. Key focus of the program is to prevent the emergence of drug resistance by provision of quality DOT services.

By the end of 2013, a total of 66 laboratories were accredited by the Government of India’s RNTCP to undertake quality-assured culture and DST for the program, of which 51 laboratories(including eight from the private sector) undertake DST for first-line drugs and five laboratories (including one from the private sector) undertake second-line DST. In addition, 41 laboratories (including four from private sector) implemented line probe assay for diagnosis of MDR-TB cases. In 2012, rapid DST through Xpert MTB/RIF was introduced in 32 sites, some for field demonstration and some for decentralization of DST in MDR-TB suspects. In 2013, an additional 40 Xpert sites were initiated in the government sector.

Following the banning of commercial serology for tuberculosis diagnosis in 2012, in March 2013 the Initiative for Promoting Affordable, Quality TB Tests (IPAQT) was launched.

SOPs for second-line DST, guidelines for certification of laboratories for second-line DST and policy guidance documents on how to use Xpert MTB/RIF under the program have been developed in 2013.

Since September 2012, all 35 states have been providing MDR-TB diagnostic and treatment services. In 2012, 14,143 laboratory-confirmed MDR-TB and 131 XDR-TB cases were started on second-line standard treatment. In addition, treatment enrolment is showing a significant increase: in the first semester of 2013, another 10273 MDR-TB and 152 XDR-TB cases were started on second-line standard treatment.

The incident HIV-positive TB cases in 2012 were estimated to be 130000. National surveillance has shown that the distribution of HIV among TB patients is highly heterogenous and is closely correlated with the distribution of HIV infection.

In 2012, the RNTCP involved over 2325 NGOs and 13997 private practitioners; 150 corporate hospitals and 312 medical colleges are implementing RNTCP.

The RNTCP has developed a case-based web-based patient tracking and data management system for all forms of TB (NIKSHAY) that are being scaled up nationwide.

The Mumbai program has developed an innovative urban tuberculosis control model. MDR-TB in Mumbai was brought into sharp focus in January 2012 with the reports of allegedly incurable, totally drug-resistant TB (TDR-TB). The "Mumbai mission for TB control" plan, released on 22nd March 2013, formulated a blueprint to ensure universal access to tuberculosis.

The DOTS plus (extended DOTS program including treatment of MDR-TB) services which were initiated in 2007 in Gujarat and Maharashtra have now been scaled up to the states of Andhra Pradesh, Delhi, Haryana, Kerala, West Bengal, Tamil Nadu, Rajasthan, Daman-Diu, Orissa and Jharkhand. In 2012, all states in India initiated DOTS- plus services.

The major challenge faced by the program is TB-HIV co-infection. To meet this challenge, RNTCP and National AIDS Control Program (NACP) have jointly drafted 'The National TB-HIV framework', which articulates the policy of TB/HIV collaboration in the country. Coordination mechanism have been established at the National, State and District levels for regular monitoring and joint review of the collaborative activities. As per the frame work, all clients attending HIV care settings are to be screened for tuberculosis and all TB patients are to be offered HIV counseling and testing. This leads to early diagnosis of HIV- infected TB patients, who then are linked to RNTCP and NACP for treatment of TB and HIV care and support including antiretroviral therapy (ART) and co-trimaxozole preventive therapy (CPT). The TB/HIV collaborative activities have been hailed as successful by the joint monitoring mission conducted in 2009.

To improve access to triable and other marginalized groups the program has implemented a triable action plan by providing additional TB units and Direct Microscopy Centre (DMCs) in triable/difficult areas, peripheral health worker for urban areas, compensation for transportation of patient and attendant in triable areas.

Achievements of RNTCP make us really proud. But because of irrational and unsupervised use of first and second line anti-TB drugs for the treatment of TB patients threatens the progress made by the program and needs to be actively discouraged. At the time it is very essential that quality DOTS services are able to reach such each and every TB patient in the country.

Today India's tuberculosis program needs to be up-date itself with the international tuberculosis guidelines as well as provide an optimal anti-tuberculosis treatment to the patients enrolled under it or it will land up being another factor in the genes of drug resistant tuberculosis [176].

Vision and Targets for RNTCP (2012-17):

The vision of the Government of India is for a "TB-free India" with reduction of the burden of the disease until it is no longer a major public health problem. To achieve this vision, the program has now adopted the new approach of Universal Access for quality diagnosis and treatment for all TB patients in the community. This entails sustaining the achievements of the program to date and extending the reach and quality of services to all persons diagnosed with tuberculosis.

By end 2015, the program aims to achieve the following:

a. Early detection and treatment of at least 90% of estimated tuberculosis cases in the community, including HIV associated TB.
b. Initial screening of all re-treatment smear-positive tuberculosis patients for drug-resistant TB and provision of treatment services for MDR-TB patients.
c. Offer of HIV Counseling and testing for all tuberculosis patients and linking HIV-infected TB patients to HIV care and support.
d. Successful treatment of at least 90% of all new tuberculosis patients and at least 85% of all previously-treated TB patients.
e. Extend RNTCP services to patients diagnosed and treated in the private sector.

Directly Observed Treatment Short Course (DOTS):-

Components of DOTS are:-

- Political and administrative commitment.
- Case detection by Sputum Smear Microscopy.
- Uninterrupted supply of good quality anti-TB drugs.
- Standardized treatment regimens with directly observed treatment for at least first two months.
- Systematic monitoring and accountability.

Diagnosis:-

It is made primarily based on Sputum Smear Examination using ZN staining and X-ray plays only a secondary role. Two sputum samples are collected over two consecutive days as spot and early morning specimens from Chest Symptomatic (Cough for two weeks or more) to arrive at the diagnosis. Use of two samples ensures that the diagnostic procedure has a high specificity and (>99%) sensitivity.

Treatment Regimen:

The program has now revised its categorization of patients from the earlier 3 categories (CatI, CatII and CatIII) to two categories (New and Previously treated cases) based on the recommendations of experts and endorsement by National Task Force for Medical colleges.

RNTCP previous treatment regimens (Table 6):

Treatment regimens followed vary according to the type of patient (whether the patient is a new case of tuberculosis or one who has been treated for tuberculosis previously), severity of illness and response to treatment [(230)]

Table 6

TB treatment regimens before 2011

Treatment Groups	Type of treatment	Intensive Phase(IP)	Continuation Phase(CP)
Category I	New sputum smear-positive. Seriously ill sputum smear-negative. Seriously ill extra-pulmonary.	$2H_3R_3Z_3E_3$	$4H_3R_3$
Category II	Sputum smear-positive relapse. Sputum smear-positive failure. Sputum smear-positive treatment after default.	$2H_3R_3Z_3E_3S_3$ + $1H_3R_3Z_3E_3$	$5H_3R_3E_3$
Category III	New sputum smear-negative, not seriously ill. New extra-pulmonary, not seriously ill.	$2H_3R_3Z_3$	$4H_3R_3$

RNTCP Change in Treatment Categories (Table 7):

For the purpose of treatment, tuberculosis patients are classified into 2 groups, namely "New" or "Previously treated", based on the history of previous treatment [(230)].

Table 7

Revised TB treatment categories (since 2011)

Treatment Groups	Type of patient	Intensive Phase(IP)	Continuation Phase(CP)
New (CatI)	New sputum smear positive New sputum smear negative New Extra-pulmonary New others	$2H_3R_3Z_3E_3$	$4H_3R_3$
Previously treated (CatII)	Smear-positive relapse Smear-positive failure Smear-positive treatment after default Others	$2H_3Z_3E_3S_3$/ $1H_3R_3Z_3E_3$	$5H_3R_3E_3$

Regimen for MDR-TB (Table 8 and 9) [231]:

This regimen comprises of 6 drugs- Kanamycin, Levomycin, Ethionamide, Pyrazinamide, Ethambutol and Cycloserine during 6-9 months of the Intensive phase and 4 drugs- Levofloxacin, Ethionamide, Ethambutol and Cycloserine during the 18 months of the Continuation phase.

RNTCP Regimen for MDR-TB: 6(9) Km Lvx Eto Cs Z E / 18 Lvx Eto Cs E

[Reserve/Substitute drugs: PAS, Mfx, Cm]

Special adjustments to the standard Regimen for MDR-TB are as follows:

- In case of intolerance to Kanamycin, then Capreomycin (or PAS if injectable agent not feasible) is the available substituting drug.
- In case of intolerance leading to discontinuation of other oral second-line drug, p-aminosalicylic acid (PAS) is the available substitute drug.
- Baseline Kanamycin mono-resistance should lead to substitution of Kanamycin with Capreomycin.
- Baseline Ofloxacin mono-resistance should lead to substitution of Levofloxacin with the combination of Mofloxacin and PAS.
- Baseline Ofloxacin and Kanamycin resistance (XDR-TB) should lead to detection of outcome, referral to Drug-Resistant TB (DR-TB) Centre for pre-treatment evaluation for regimen for XDR-TB.

All drugs should be given in a single daily dosage under directly observed treatment (DOT) by a DOT Provider. All patients will receive drugs under direct observation on 6 days of the week. On Sunday, the oral drugs will be administered unsupervised whereas injection of Kanamycin will be omitted. If intolerance occurs to the drugs, Ethionamide, Cycloserine and PAS may be split into two dosages and the morning dose administered under DOT. The evening dose will be self-administered. The empty blister packs of the self-administered dosages will be checked the next morning during DOT. Pyridoxine should be administered to all patients on regimen for MDR-TB.

Table 8

Regimen for MDR-TB dosage and weight band recommendations

Sr.no.	Drugs	16-25 kgs	26-45 kgs	46-70 kgs
1.	Kanamycin	500 mg	500 mg	750 mg
2.	Levofloxacin	250 mg	750mg	1000mg
3.	Ethionamide	375 mg	500 mg	750 mg
4.	Ethambutol	400 mg	800 mg	1200 mg
5.	Pyrazinamide	500 mg	1250 mg	1500 mg
6.	Cycloserine	250 mg	500 mg	750 mg
7.	Pyridoxine	50 mg	100 mg	100 mg
	Na-PAS (80% Weight/vol)[2]	5 mg	10mg	12 mg
	Moxifloxacin (Mfx)	200 mg	400 mg	400 mg
	Capreomycin (Cm)	500 mg	750 mg	1000 mg

If a patient gains 5 Kgs or more in weight during treatment and crosses the weight-band range, the DR-TB Centre committee may consider moving the patient to the higher weight-band drug dosages. Similarly if a patient loses 5 kgs or more in weight during treatment and crosses the weight band the DR-TB Centre committee may consider moving the patient to the lower weight band. The new higher/lower dosages are provided whenever the patient is due the next supply of drugs in the normal course of treatment and not as soon as change of weight is noted.

Large majority of the patients will fall into one of the above weight bands. However, there have been reports of some cases weighing less than 16 kg and more than 70 kg who may require some alteration in the dosage of the drugs in the MDR-TB regimen as follows:

Table 9

Dosage of regimen for MDR-TB for pediatric group <16 kg

Drug	**Daily-dose- mg/kg body weight**
Kanamycin/Capreomycin	15-20 mg/kg
Levofloxacin/ Moxifloxacin	7.5-10mg/ kg
Ethionamide	15-20 mg/ kg
cycloserine	15-20 mg/ kg
Ethambutol	25 mg/ kg
pyrazinamide	30-40 mg/ kg
Na- PAS	150 mg/ kg

The dosages for higher weight patients include the use of additional dosages of some second-line drugs for MDR-TB cases in patients weighing >70kg taking the dosage to Kanamycin/Capreomycin (1gm), Cycloserine (1 gm), Ethambutol (1.6gm) and Pyrazinamide (2gm). Other drug dosages are well within the maximum permissible dosage for each drug as per the WHO guidelines.

Treatment duration for Regimen for MDR-TB:-

The treatment is given in two phases, the Intensive phase (IP) and Continuation phase (CP). The total duration of treatment for regimen for MDR-TB is 24-27 months, depending on the IP duration. IP should be given for at least six months. After 6 months of treatment, the patient will be reviewed and the treatment changed to CP if the 4^{th} or 5^{th} month culture result in solid or liquid culture is negative respectively. If the 4^{th} or 5^{th} month culture result remains positive, the treatment is extended by one month. Extension of IP beyond 1 month will be decided on the results of sputum culture of 5^{th} or $6^{th}$and 6^{th} or 7^{th} months. If the results of the 4^{th} month culture are still awaited after 6 months of treatment, the IP is extended until the result is available, with further treatment being decided according to the culture result. The IP can be extended up to a maximum of 3 months after which the patient will be initiated on the CP irrespective of the culture result. The recommended duration for CP is 18 months.

Regimen for XDR-TB (Table 10):-

All XDR-TB patients should also be subject to a repeat full pre-treatment evaluation, but also including consultation by a thoracic surgeon for consideration of surgery. Identification must be done for the site (tertiary centers) with such surgical facilities.
MDR-TB patients diagnosed as XDR-TB would be given an outcome of "Switched to regimen for XDR-TB". The decision and initiation of regimen for XDR-TB is to be taken by the concerned DR-TB Centre Committee. Drugs will be centrally procured.

The **Intensive Phase** (6-12 months) will consist of 7 drugs – Capreomycin (Cm), Para-amino salicylic acid (PAS), Moxifloxacin (Mfx), High dose-INH, Clofazimine (Cfz), Linezolid (Lzd) and Amoxyclave (Amx/Clv).
The **Continuation Phase** (18 months) will consist of 6 drugs – Para-amino salicylic acid (PAS), Moxifloxacin (Mfx), High dose-INH, Clofazimine (Cfz), Linezolid (Lzd) and Amoxyclave (Amx/Clv).

RNTCP Regimen for XDR-TB: 6-12 Cm, PAS, Mfx, High dose-H, Lzd, Amx/Clv/ 18PAS, Mfx, High dose-H, Cfz, Lzd, Amx/Clv

[Reserve/Substitute drugs: Clrithromycin, Thiacetazone]

The dosage of the drugs would vary as per the weight of the patient (≤45 kg or >45kg). All drugs are to be given on a daily basis. Injections of Capreomycin will be given for 6 days/week (not on Sundays). All morning dosages are to be supervised by the DOT provider except Sundays. After taking DOT for morning dosages on Saturday, next day medicines would be given to the patient to be taken at home on Sunday. Empty blisters of medicines taken unsupervised in evening and on Sunday are to be collected by DOT Provider.

Table 10

Regimen for XDR-TB dosage and weight band recommendations

Drugs	Dosages/day	
	≤45 Kgs	>45 Kgs
Inj. Capreomycin (Cm)	750 mg	1000 mg
Para-amino salicylic acid (PAS)	10 mg	12 mg
Moxifloxacin (Mfx)	400 mg	400 mg
High dose-INH (High dose-H)	600 mg	900 mg
Clofazimine (Cfz)	200 mg	200 mg
Linezolid (Lzd)	600 mg	600 mg
Amoxyclav (Amx/Clv)	875/125 mg BD	875/125 mg BD
Pyridoxine (Pyz)	100 mg	100 mg
Reserve/Substitute drugs:		
Clarithromycin (Clr)	500 mg BD	500 mg BD
Thiacetazone (Thz)#	150 mg	150 mg

depending on availability, not to given to HIV positive cases

Technical specifications of drugs for treatment of XDR-TB under RNTCP (meant for local purchase of drugs for XDR-TB patients when centrally-procured supplies are not available) are available at the program website www.tbindia.nic.in.

The reserve/substitute drugs would be used in the following conditions:

- In case the patient was on PAS, PAS will be replaced with one of the reserve drugs in the regimen for XDR-TB.
- If the patient is unable to tolerate one or more of the drugs.
- If the patient is found to be resistant to Capreomycin.

Duration of Regimen for XDR-TB:

The Regimen for XDR-TB would be of 24-30 months duration, with 6-12 months Intensive Phase (IP) and 18 months Continuation Phase (CP). The change from IP to CP

will be done only after achievement of culture conversion i.e. 2 consecutive negative cultures taken at least one month apart. In case of delay in culture conversion, the IP can be extended from 6 months up to a maximum of 12 months. In case of extension, the DR-TB Centre Committee, which will be responsible for initiating and monitoring the regimen for XDR-TB, can decide on administrating Capreomycin injection intermittently (3 times/week) for the months 7 to 12.

Pre-treatment evaluation:

The patient should be hospitalized for pre-treatment evaluation and treatment initiation. Pretreatment evaluation should include a thorough clinical evaluation by a physician, chest radiograph and relevant haematological and biochemical tests detailed below. Since the drugs used for the treatment of MDR-TB are known to produce adverse effects, a proper pre-treatment evaluation is essential to identify patients who are at increased risk of developing such adverse effects. A thorough clinical examination should be done during the pre-treatment evaluation. It includes:

1. Detailed history (including screening for mental illness, drug/alcohol abuse etc.).
2. Weight.
3. Height.
4. Complete Blood Count with platelets count.
5. Blood sugar to screen for Diabetes Mellitus.
6. Liver Function Tests.
7. Blood Urea and Serum Creatinine to assess the Kidney function.
8. TSH levels to assess the thyroid function.
9. Urine examination- Routine and Microscopic.
10. Pregnancy test (for all women in the child bearing age group).
11. Chest X- Ray.

All MDR-TB cases will be offered referral for HIV counseling and testing at the nearest centre if the HIV status is not known or the HIV test is found negative with results more than 6 months old. TSH levels alone are usually sufficient to assess the thyroid function of the patient.

In case of pre-treatment evaluation for XDR-TB, an ECG, serum electrolytes and surgical evaluation should be added to the pre-treatment evaluation.

Patients should receive counseling on

1) The nature and duration of treatment.
2) Need for regular treatment.
3) Possible side effects of these drugs.
4) The consequences of irregular treatment or pre-mature cessation of treatment.

It is advisable to involve close family members during the counseling, since family support is an essential component in the management. Patient should be advised to report any side effects experienced by them.

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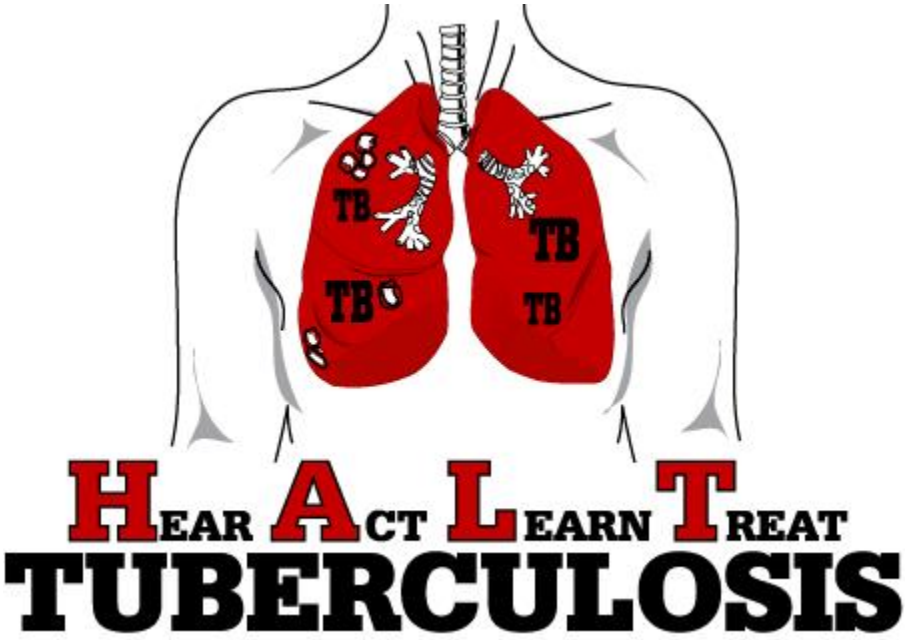
DRUG RESISTANT TUBERCULOSIS



MATERIALS andMETHODS

MATERIALS AND METHODS

1. Source of data:

The patients at Bharati hospital; Pune, with clinically suspected Tuberculosis forms the source of data for the study.

2. Sample collection:

Early morning expectorated sputum samples were collected in wide mouthed disposable sterile containers on three successive days. The samples were stained to confirm the presence of *Mycobacterium tuberculosis* and detection of its resistance pattern to first and second-line anti-tuberculosis drugs [96, 184,153].

2.1. Microscopic examination to detect *M. tuberculosis* - Acid fast bacilli (AFB):

The smears were screened for AFB by Ziehl-Neelsen staining technique and positive smears were graded as per revised national tuberculosis control program (RNTCP) guidelines [229].

2.1. Ziehl-Neelsen staining method:

2.1.1. Principle:

Use of heated strong carbol fuchsin enhances its penetration in the thick and waxy coated cell wall of *M. tuberculosis*. Mycolic acids and waxes form a complex with the basic dye, which resists washing out by acid decolorization [112].

2.1.2. Method:

1. Sputum smear was heat fixed and flooded wih strong carbol fuchsin and gently heated for 1min. by flaming from below the gas burner. Care was taken not to boil/drying of the stain.
2. Stain was allowed to remain on slides for an additional 4-5 minutes without heat and rinsed with deionized water.
3. Decolorization was done with 20% Sulphuric acid for 2 mins- till the entire red colour is removed.
4. Smear was rinsed with deionized water and flooded with methylene blue reagent for 1 minute.
5. Smear was rinsed with deionized water, air dried and examined under oil immersion objective (100x) for presence of AFB.

Results:

Mycobacterium tuberculosis (AFB) appeared as bright pink, straight/ slightly curved rods, at times having beaded appearance against blue background containing non-acid fast materials like pus cells and epithelial cells (Figure 1).

2.1.3. Grading of the sputum smear:

Smears were graded according to the number of AFB as:-

Table 11:

ZN smear evaluation and AFB report:-

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

2.2. Homogenization and Decontamination:

Majority of sputum specimens submitted to laboratory are contaminated by more rapidly growing normal flora. These overgrow before tubercle bacilli start to grow. Therefore specimens were submitted to harsh digestion and decontamination procedure that liquefies organic debris and eliminates unwanted normal flora [(229)].

All currently available digesting/decontaminating agents are to some extent toxic to tubercle bacilli. Therefore, to ensure the survival of maximum number of bacilli in specimen, decontamination procedure was precisely followed. As a general rule, contamination rate of 2-3% is acceptable in laboratories that process fresh specimens. A laboratory which experiences no contamination is probably using a method that kills too many of tubercle bacilli [(229)].

When culturing tubercle bacilli, 3 important preliminary steps ensured were:

(a) Specimens must be homogenized to free bacilli from mucus, cells/tissue in which they may be embedded. The milder the homogenization process, the better the recovery of tubercle bacilli.

(b) Decontamination should not diminish viability of tubercle bacilli.

(c) Success depends on

- Appropriate concentration of decontamination solution.
- Length of exposure time to these agents.
- Centrifugation speed and time used to sediment the tubercle bacilli.
- Temperature builds up in specimen during centrifugation.

By keeping in mind these points, a simple and widely used Petroff's method was selected for homogenization and decontamination of sputum samples. In this method *Mycobacterium tuberculosis* will not get killed and thus can be cultured.

2.2.1 Petroff's method:

5ml of sputum was transferred to centrifuge tube and double the volume of sterile 4% NaOH solution was added aseptically. Cap was tightened and incubated at 37°c for 30 minutes. Then 15ml sterile distilled water was added, centrifuged at 3000g for 15 minutes and the supernatant was discarded slowly into container with 5% phenol solution. Again equal quantity of sterile distilled water was added, centrifuged at 3000g for 15 minutes, supernatant was discarded and sediment was used for the isolation of *M. tuberculosis* [229].

2.3 Isolation of *M. tuberculosis* by using Lowenstein Jensen's (LJ) media:

2.3.1 Inoculation method:

From the sediment, one loopful was inoculated on the surface of slope of LJ media and incubated at 37°c for 48-72 hours.

2.3.2. Examination schedule:

Inoculated LJ media was examined after 48-72 hours to detect gross contaminants. Thereafter, culture was examined weekly once for 8 weeks on a specified day of week. Contaminated cultures were discarded.

2.3.3. Reading of cultures:

Typical colonies of *M. tuberculosis* (Figure 5) were rough, crumbly, waxy, non-pigmented (buff coloured) and slow growers i.e. appearing 2-3 weeks after inoculation.

If colonies have doubtful morphology, acid fastness was confirmed by Ziehl- Neelsen (ZN) staining. A very small amount of growth was removed from culture using a loop and gently rubbed into 1 drop of sterile saline on a slide. At this point, the ease with which the organisms emulsify in liquid was noted. Tubercle bacilli do not form smooth suspensions, unlike other bacteria. If no AFB was seen in the smear, it is reported as contaminant. If AFB was seen in the smear, it is considered as the *Mycobacterium* species [229]. Identification of *M. tuberculosis* was done by performing specific biochemical tests.

2.4. Identification of *Mycobacterium tuberculosis*:

Positive growth on LJ is speciated by several biochemical tests.

For conventional identification the tests used were:-

2.4.1. Niacin test:-

Procedure:-

1. 1.0 ml of sterile distilled water was added to the culture, (if growth is confluent; dig into the medium) to facilitate extraction of the niacin. Tube was placed in slanted position so as to cover the medium with water for 15 minutes. Alternatively, the culture tubes may be autoclaved at 121°C for 20 minutes and the water of condensation tested for Niacin.
2. 0.5 ml of the extract from the culture tube was pippeted out and placed in another tube. 0.5 ml (equal volume) of 4% aniline in ethyl alcohol was added in to it. This mixture should be colourless.
3. Finally 0.5 ml 10% (aqueous) cyanogen bromide (volume equal to that of the extract) was added to it.

Expected Result:

Immediate development of a yellow colour is indicative of a positive result (Figure 22). There is no colour change, if niacin has not been formed.

2.4.2. Nitrate reduction test:-

Procedure:

1. 3-4 drops sterile distilled water was added into tube and emulsified one loopful of colonies in the water.
2. After addition of 2 ml of the $NaNo_3$ substrate to it, shake gently and placed in 37°C water bath for 2 hours.
3. One drop of 1:2 dilution of concentrated HCL was added to it and gentle shake to mix it.
4. Finally 2 drops of 0.1 % aqueous n-1-naphthylethylenediamine dihydrochloride solution was added to it.

Expected Result:

Development of red colour indicates a positive result (Figure 23). Grade colour ranges from pink to deep red as 1+ to 5+ reactions.

2.4.3. Catalase activity, semi-quantitative test:-

Procedure:

1. Surface of L.J. medium, specially prepared as a butt by inspissations of the tube in upright position was inoculated with 0.1ml or loopful of a 7 day-old liquid culture and incubated with **cap loose at 37°C** for 2 weeks.
2. 1.0 ml of Twin-peroxide mixture made just before use (equal parts of 30% H_2O_2 and 10% Tween 80 in Distilled water) was added to it and the tube was kept in a upright position at room temperature for 5 minutes.
3. The height of the column of bubbles above the surface of the medium(Figure 24)was measured in millimeters and results were recorded as follows:
   Weak positive- Height of column <45mm
   Strong positive- Height of column >45
   Negative – No bubbling.

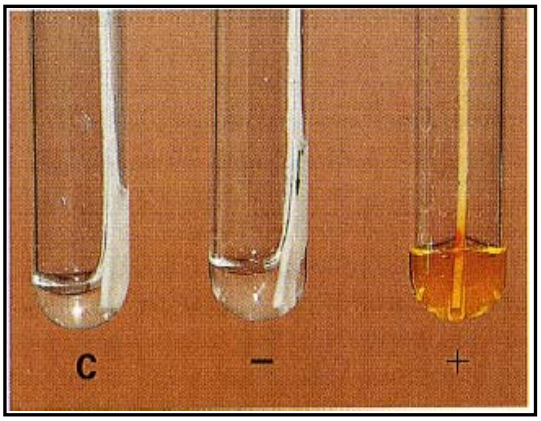


Figure 22: Niacin test

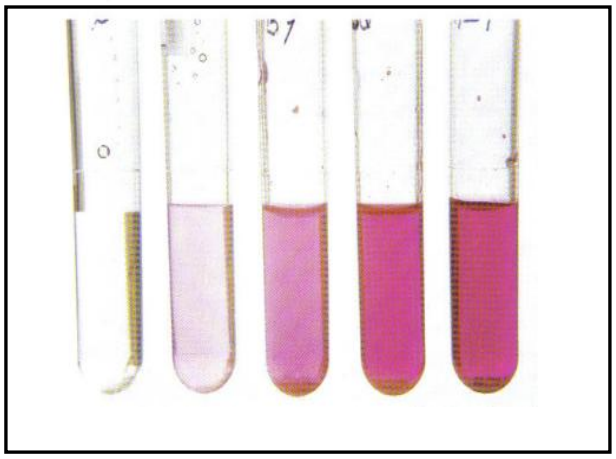


Figure 23: Nitrate reduction test

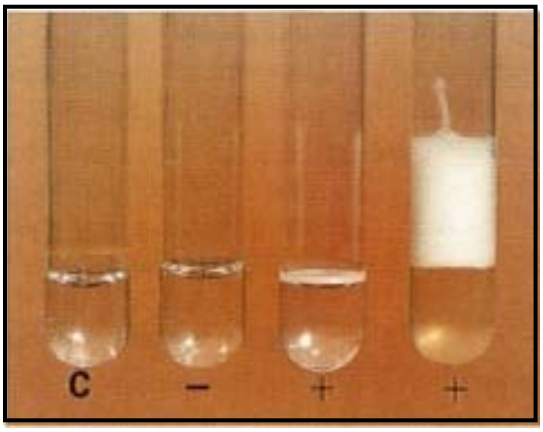


Figure 24: Catalase test

2.5. Recording and Reporting of Growth on LJ media (Table 12):

If laboratory findings are to be useful, they must be communicated in ways that make sense to different authorities. Culture procedures for TB bacteriology are notoriously time consuming, often taking 8-9 weeks to complete. For this reason, interim reports should be issued. The following schedule is recommended.

- If cultures are contaminated, a report should be sent out immediately
- At 8 weeks, a final report should be issued for culture negative specimens
- If cultures are positive and growth has been identified as *M. tuberculosis*, a report should be sent out immediately to referral authority.
- If colony count is <20/faint growth in 1 or 2 weeks, incubation is continued up to 4thweek to obtain a colony count of at least >50 colonies/loopful of growth.
- If growth is still insufficient at the end of 4th week, a subculture should be done on fresh LJ medium by touching all colonies. [229]

Table 12

Report of growth of *M. tuberculosis* on L.J. media

Reading of Primary culture and for DST	**Report**
No growth	Negative
1-100 colonies	Positive (+)
>100 discrete colonies	Positive(++)
Confluent growth / innumerable colonies	Positive(+++)

The reasons for increased negative results observed in laboratory may be: - [229]

- Delays between sputum collection and processing.
- Quality of sputum specimen.
- Increased temperature of incubation of cultures.
- Increased concentration of malachite green used for LJ preparation.
- Increased time and temperature of inspissations during media preparation.
- Centrifugation speed is below 3000g during sedimentation and shorter times for centrifugation.

2.6. Drug Susceptibility Test (DST)[229]:

As a guide for treatment of the disease, DST is carried out. One of the objectives of this study is to detect resistance pattern of *M. tuberculosis* isolates i.e. whether the isolate is MDR/ XDR strain, we have performed the DST of all biochemically identified isolates of the *M. tuberculosis* strains by "Proportion method".

2.6.1. Drug sensitivity testing by Proportion method:

For the detection of MDR strains of isolates, 1st line drugs were used while for the detection of XDR strains, 2nd line drugs (Table 13) were used. For this Hi- media kits of 1st and 2nd line anti-tuberculosis drug containing LJ media were used.

Table 13

Concentration of 1st and 2nd- line anti-tuberculosis drugs used for DST by Proportion method

1st line anti-tuberculosis drugs	Concentration (µg/ml)	2nd line anti-tuberculosis drugs	Concentration (µg/ml)
Isonoazide	0.2	Kanamycin	30
Ethanoamide	2	Amikacin	700
Pyrazinamide	200	Ethionamide	20
Rifampicin	40	D-cycloserine	30
Streptomycin	4	Clarithromycin	8
		Ciprofloxacin	125
		P-amino salicylic acid	25
		Rifabutin	0.5

Proportion method [229]:

Principle: In this method, the number of colonies from a standard inoculums growing on a medium containing drug is compared with the number of colonies from same sized inoculums on a medium without drug. When more than 1% of the mycobacteria grow in the presence of drug, it is regarded as a resistant strain.

Method:-

1. Preparation of inoculums:

1. A loopful of growth was taken aseptically from L.J. medium & inoculated into 1.0ml of sterile distilled water in a screw capped bottle.
2. Homogenization of the mixture was carried out for 10 minutes on a vortex mixture &kept for 10 minutes before opening the bottles.
3. Opacity of suspension was adjusted to match McFarland 0.5 standard with saline giving approximately 1.5 x10^8 CFU/ml. and then diluted to 1:10000.

2. Method of inoculation:

1. The kit and slants were equilibrated to room temperature & labelled it appropriately.
2. 100µl inoculums were added on L.J. medium slants (by using calibrated wire-loop).
3. All the slants were incubated at 37°C for 2-4 weeks and examined for the growth intermittently every week up to 6-8 weeks (Figure 25 and 26).

The standard strain of *M. tuberculosis* H37 RV was tested with both 1st and2nd line DST as internal quality control.

Interpretation of the results:

1. The isolate is termed either Resistant or sensitive or intermediate.
2. Evaluation can be done as follows:
   1. The colony characteristics & count on the control medium.
   2. Results obtained for respective dilutions calculated as the % of resistance.

Formula to be used to calculate the percentage of resistant cells:

$$\frac{\text{No. of colonies on the Drug medium}}{\text{No. of colonies on the control medium}} \times 100 = \% \text{ Resistance.}$$

Accordingly, results are interpreted as: 1) Resistant: when >1%

2) Sensitive: when <1%

3) Intermediate: when =1%

Figure 25: DST of isolates *M. tuberculosis* against first line anti-TB drugs

Figure 26: DST of MDR-TB isolates of *M. tuberculosis* against second line anti-TB drugs

2.7 Molecular methods for detection of drug resistance by line probe assay (LPA)[96]:-

This molecular method was used to detect the mutation pattern in the genes of *M. tuberculosis* which are responsible for the resistance of the specific anti-tuberculosis drugs. The protocol was used to perform LPA involves 3 steps:

(1) DNA Extraction :

DNA was extracted using *Mycobacterium* colonies grown on L.J. medium.

Quick protocol that yields DNA suitable for amplification was used.

1 loopful of growth from L.J. was suspended in 300µl of sterile distilled water (SDW)

↓

Pellet of bacteria was prepared by spinning it for 15 minutes at 10000 x g

↓

Supernatant was discarded and bacterial pellet was suspended in 100-300 µl of molecular grade water

↓

It was incubated for 30 minutes at 95°c in a water bath.

↓

It was spinned for 5 minutes at full speed and 5µl of the supernatant from it was directly used for PCR.

(In case, DNA solution is to be stored for an extended period, transfer supernatant to a new tube and store at -20°c).

(2) Amplification of DNA:

Amplification of extracted DNA was carried out by PCR method.

For this, preparation of the amplification mixture (45µl/sample) was carried out in a DNA-free room. First pre-tube mixture (master-mix) was prepared by adding ingredients.

Master Mix for PCR:

- 35µl (primer nucleotide mix) PNM
- 5µl 10x polymerase incubation buffer
- 2 µl $MgCl_2$ solution
- 0.2µl of thermostable DNA polymerase
- 2.8µl SDW
- 5µl extracted DNA solution (20-100 ng DNA) was added to make a final volume of 50µl.

For a negative control, 5µl of water was added instead of DNA solution. A master mix containing all reagents except for DNA solution was prepared and mixed well. It was aliquoted (45µl) in each of the prepared PCR tubes.

Amplification profile for PCR (Table 14):

Table 14

Amplification cycles used for the PCR were as:

Sr.no.	**Duration and Temperature**	**Number of cycles of amplification**
1.	5 min 95°c	1 cycle
2.	30sec 95°c 2 min 58°c	10 cycles
3.	25 sec 95°c 40 sec 53°c 40sec 70°c	20 cycles
4.	8min 70°c	1 cycle

Amplification products can be stored at +4 to -20°c. For checking the amplification reaction, 5µl of each sample may be directly applied to a 2% agarose gel without the addition of loading buffer. The amplicons have a length of approximately 63bp (Amplification Control), 115 bp (*M. tuberculosis* complex), 166 bp (*rpoB* gene), 120 bp (*katG* gene), and 110 bp (*inhA* gene) respectively.

(3) Hybridization :

The hybridization includes a chemical denaturation of the amplification products, hybridization of a single-stranded, biotin-labled amplicons to the membrane-bound strips coated with specific probes, stringent washing, addition of a streptavidin/alkaline phosphatase (AP) conjugate and an AP mediated staining reaction.

Steps carried out for the hybridization and development of assay were:-

A) Preparations for hybridization:

1. Pre-warming of shaking water bath / Twincubator to 45°c.was carried out.
2. HYB (Hybridization Buffer) and STR (Stringent Wash Solution) was prewarmed to 37-45°c before use.
3. Remaining all reagents was prewarmed to 37°c with the exception of conjugate concentrate (CON-C) and substrate concentrate (SUB-C) to room temperature.
4. Using a suitable tube, CON-C and SUB-C was diluted as 1:100 with the respective buffer (CON-C with CON-D, SUB-C with SUB-D).
5. Dilution of CON-C was carried out just before use.
6. Diluted SUB-C is stable for 4 weeks if stored at room temperature and protected from light.
7. According to the number of samples, membrane strips necessary for the running assay were taken out form the kit and the sample number was marked on the left side of the blue/red/green line on it.
8. These strips were then placed at bottom of different lanes in assay tray

B) Protocol for Hybridization:

20µl of Denaturation solution (DEN) was added to the bottom corner of each lane of the tray.

↓

20µl of amplified sample was added to the each DEN solution, mixed well and incubated at room temperature for 5 minutes and strips were taken out with the help of tweezers.

↓

1ml of pre-warmed HYB buffer solution was added to each lane and shake gently until the solution has a homogenous color.

↓

The corresponding strip was placed in the respective lane. Cover the strips completely by the HYB solution with coated side face upward.

↓

The tray was then placed in Twincubator and incubated at 45°c for 30 minutes.

↓

HYB buffer was completely aspirated from lanes of the tray.

↓

1ml of STR (stringent) solution was added on to each strip and incubated for 15 min at 45°c in Twincubator.

↓

STR solution was completely removed by aspiration and all remaining fluid was removed by turning the tray upside down and gently striking it on an absorbent paper.

↓

Each strip was washed once with 1ml of Rinse solution (RIN) for 1 minute on Twincubator

↓

1ml of diluted Conjugate was added to each strip and incubated for 30 minutes on Twincubator at room temperature.

↓

Conjugate solution was completely removed and each strip was washed twice for 1 minute with 1ml of RIN and then washed once for 1minute with 1ml of distilled water on Twincubator.

↓

1ml of diluted substrate was added to each strip and incubated for 6 minutes at room temperature. Tray was covered with aluminium foil to protect from light without shaking.

↓

Reaction was stopped by briefly rinsing twice with SDW.

↓

Using tweezers, strips were removed from the tray and dried between 2 layers of absorbent paper

Evaluation and Interpretation of the assay:

Developed strips were pasted to evaluation sheet in the designated fields by aligning the bands CC and AC with the respective lines on the sheet. The resistance status was determined and noted in the respective column; as a help for interpretation. Each strip has a total of 27 reaction zones.

Conjugate Control (CC):

A line must develop in this zone, documenting the efficiency of conjugate binding and substrate reaction.

Amplification Control (AC):

When the test is performed correctly, a control amplicon will bind to the Amplification control zone. If this band is developed, mistakes during extraction and amplification set-up and the carry-over of amplification inhibitors can be excluded. In case of a positive test result, the signal of AC zone can be weak or even vanish. This might be due to competition reactions during amplification. In this case, however the amplification reaction was performed correctly and the test need not be repeated. A weak or missing AC band in case of a negative test result indicates mistakes during amplification set-up or carry-over of amplification inhibitors. In this case, the test is not valid and the respective sample has to be repeated.

***M. tuberculosis* complex (TUB):**

This zone hybridizes with amplicons generated from all members of the *Mycobacterium tuberculosis* complex. If the TUB zone is negative, the tested bacterium does not belong to the *M. tuberculosis* complex and cannot be evaluated by this test system.

Locus controls (*rpoB, katG, and inhA*):

The locus control zones detect a gene region specific for the respective locus and must always stain positive.

Wild type probes:

These comprise the most important resistance areas of the respective genes. When all wild type probes of a gene appear positive, there is no detectable mutation within the examined regions. Hence, the strain tested is sensitive for the respective antibiotics.

In case of a mutation, the respective amplicon cannot bind to the corresponding wild type probe. The absence of a signal for at least one of the wild type probes, hence, indicates resistance of the tested strain to the respective antibiotics.

Only those bands whose intensities are about or stronger than that of the Amplification Control (AC) zone are to be considered. Each pattern deviating from the wild type pattern indicates resistance of the tested strain. The banding pattern obtained with the *rpo-B* probes allows drawing a conclusion about a rifampicin resistance of the strain tested, with the *kat-G* probes about a high-level isoniazide resistance and with the *inhA* probes, a conclusion about a low-level isoniazide resistance of the strain tested, respectively.

Mutation probes:

Only those bands whose intensities are about as strong as or stronger than that of the Amplification Control zone are to be considered. Each pattern deviating from the wild type pattern indicates resistance of the tested strain.

Limitations:

- The available data about extra-pulmonary smear-positive direct material is not sufficient to draw a conclusion about their applicability.
- As with any DNA-based assay, this test only screens the nucleic acid sequence and not the amino acid sequence. Therefore, it is possible that mutations that do not cause an amino acid exchange (silent mutations) will still produce the absence of one of the wild type probes.
- Newest data indicate that in spite of a L533P mutation, the respective *M. tuberculosis* strain may still be RMP sensitive. Hence, if the band WT8 is absent and the *rpoB* MUT3 probe does not develop, result of the phenotypic resistance determination should be considered.

- In recent years additional mutations within the tested *rpoB* gene region causing rifampicin resistance have been published. As these mutations are very rare, they were not accessible for validation purposes of this test system.
- Detects those resistances of the *M. tuberculosis* complex that have their origins in *rpoB*, *katG, inhA* regions examined in the assay. Resistances originating from mutations of other genes or gene regions as well as other rifampicin and isoniazid resistance mechanisms will not be detected by this test.
- The presence of multiple bacterial species in the sample to be analyzed might hamper the interpretation of the test.
- Theoretically, a resistance can exist in spite of a wild type pattern. If, at investigation, the sample contains a strain that has developed only a partial resistance which is not covered by the mutation probes, the wild type pattern will appear.
- If the sample contains more than one *M. tuberculosis* strain and one of these harbors mutations which is not covered by the mutation probes, the wild type pattern will appear. As with other diagnostic assays, the results of this test may only be interpreted in combination with additional laboratory and clinical data available to the responsible physician.
- It must be ensured that the template DNA is efficiently amplified during the amplification reaction. The test only works within the limits of the genomic regions of the probes from where it was chosen.
- Use of this assay is limited to qualified personnel well trained in the test procedure and familiar with molecular biological methods.

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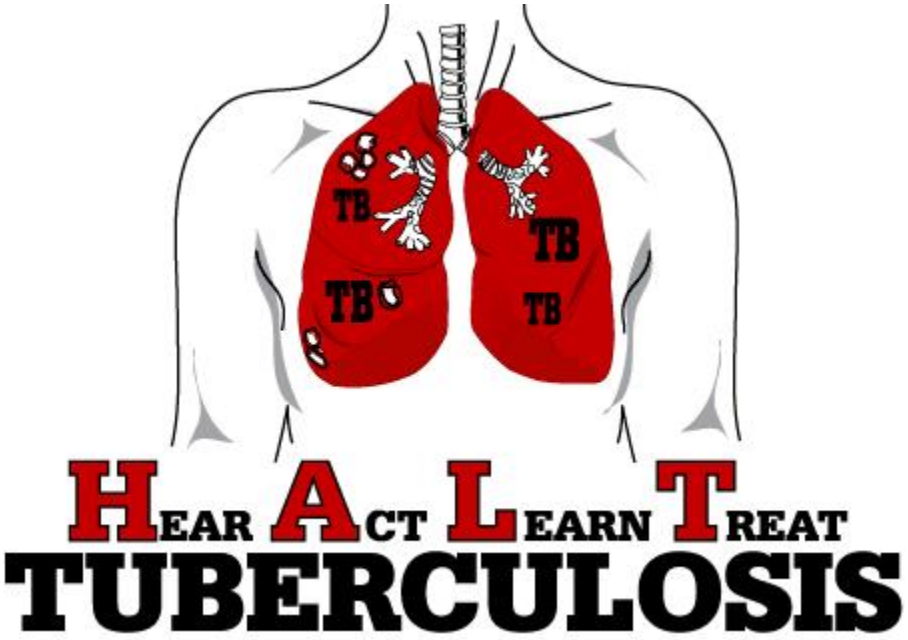
DRUG RESISTANT TUBERCULOSIS



RESULTS

RESULTS

In the present study, total numbers of sputum samples from clinically suspected pulmonary tuberculosis (PTB) cases screened for the presence of *Mycobacterium tuberculosis* by Ziehl-Neelsen staining were 1186. Out of that 130 were smear positive PTB cases. Out of these 130 smear positive sputum samples, only 123 were successfully grown on Lowenstein-Jensen media which were used for studying their pattern of drug resistance by proportion method. Out of these 123 culture positive *M. tuberculosis* isolates, 10 were MDR-TB strains showing resistance to first-line anti-tuberculosis drugs and out of these 10, only 2 were XDR-TB strains showing resistance to second-line anti-tuberculosis drugs.

All these 10 drug resistant *M. tuberculosis* isolates were used to study the mechanisms of drug resistance (to detect the mutations in the respective genes) by MTBDR*plus* assay. With this assay the commonest mutations in *rpoB* gene showing resistance to rifampicin and mutations in the *katG* gene as well as in the *inhA* genes showing resistance to isoniazid were observed.

Data Analysis:-

1. Age-Gender wise distribution of TB cases:

Majority of patients (24.4%) were found in the age groups of 21-30 years followed by age group of51-60 (17.7%). The least number was seen in 0-10 year age group.78 males and 45 females form the study group. Highest number of male was in age group of 21-30 years and highest number of females was in age group 11-30 years. (Figure 26)

(In calculation of percentage, values are rounded off to one place of decimal)

Male to female ratio is 1.7:1(Figure 28).

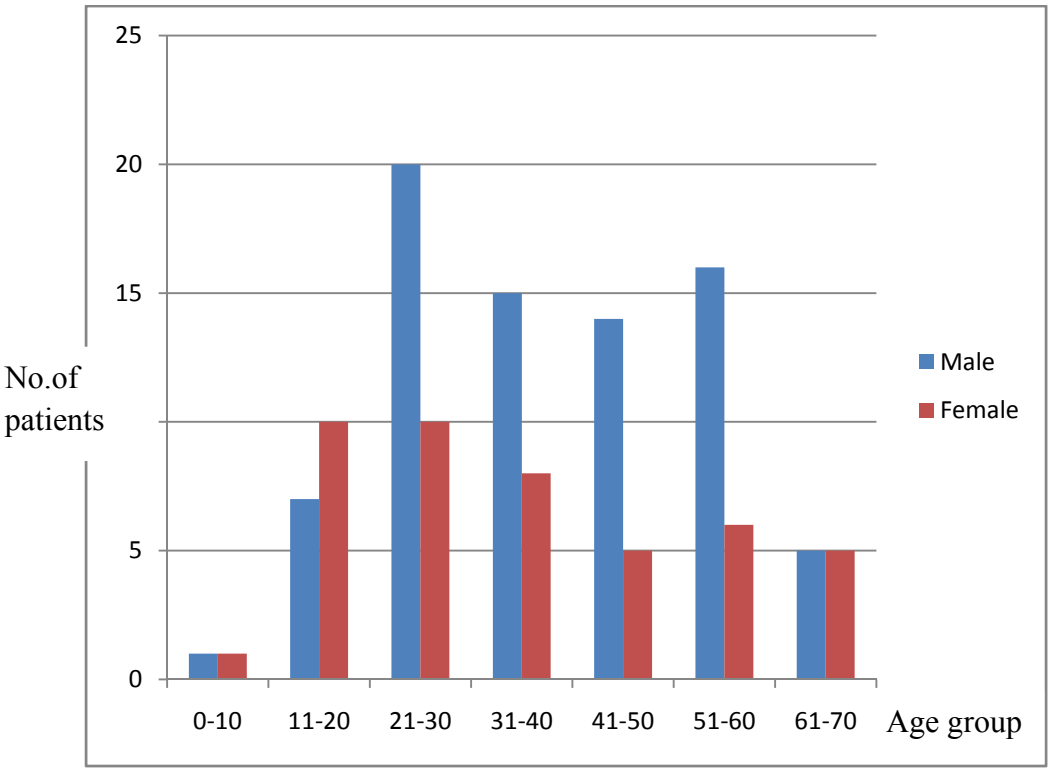


Figure 27: Bar-chart showing Age and Gender wise Distribution of TB-cases

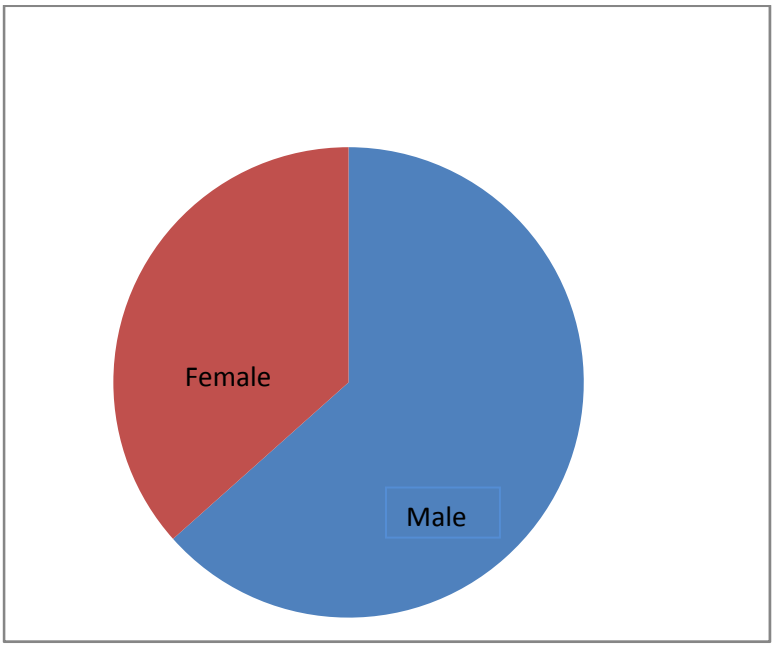


Figure 28: Pie chart showing distribution of Male and female TB cases (1.7:1).

2. AFB Smear positivity rate:

Out of 1186 clinically suspected cases, sputum smear for AFB was positive in 130 samples (10.96%) (Table 15 and Figure 29).

Table 15

AFB Smear positivity

ZN-smear	No. of cases	Percentage (%)
Positive	130	10.96%
Negative	1056	89.04%
Total	1186	100%

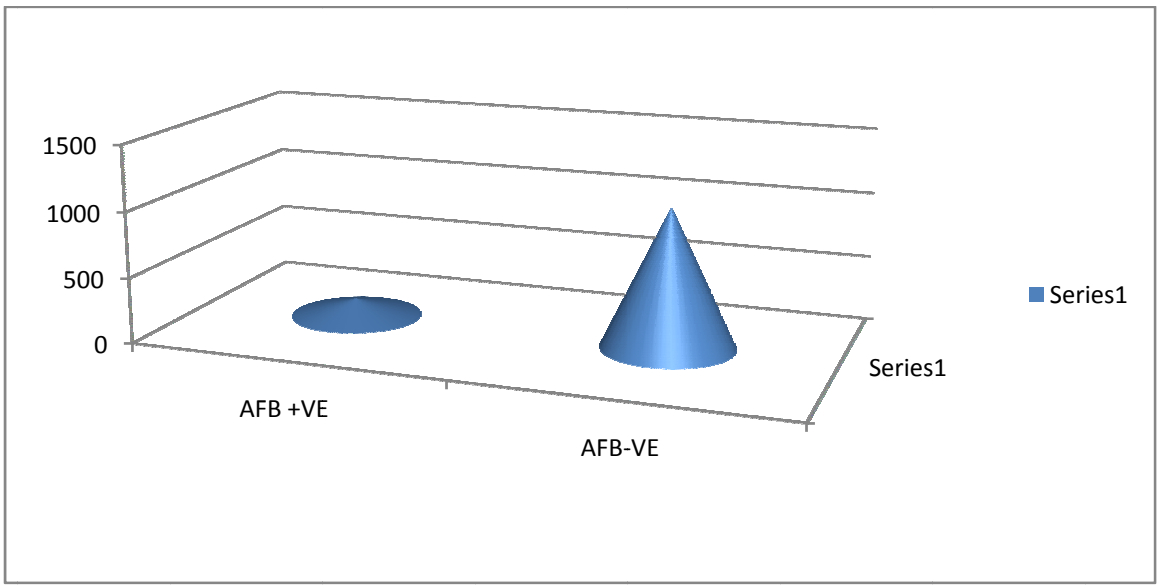


Figure 29: 3D Chart of Results of Demonstration of AFB by ZN- Staining

3. Culture positivity rate:

Out of 130 cases, 123 cases could be tracked during this study period. The smear positive samples were cultured on L.J. medium. The sensitivity of L.J. in comparison with smear is 94.61%. Comparison of results of Microscopy versus Culture on L.J. is shown in Table-16 and Figure 30.

Table 16

Result of Cultures of *M. tuberculosis* isolates on L.J.

AFB smear positive	L.J. positive	L.J. negative	Total
130	123 (94.61%)	07 (5.38%)	130 (100%)

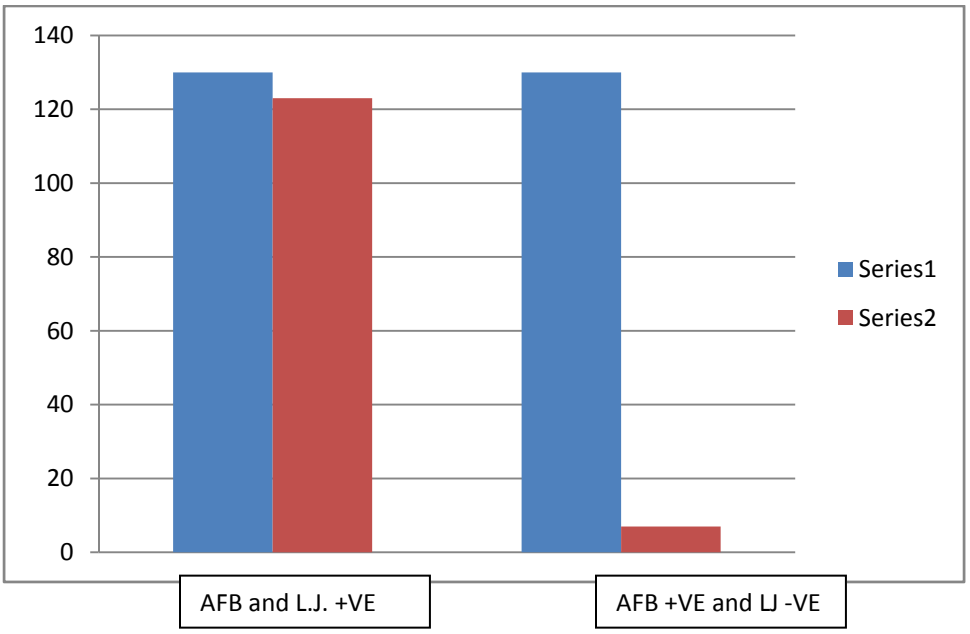


Figure 30: Histogram of Comparison of results of Microscopy versus Culture on L.J.

The average turnaround time for culture of *M. tuberculosis* on L.J. was 21-28 days.

4. Antibiotic Resistance pattern of *M. tuberculosis* isolates:

The H37RV strain and total 123 AFB and culture positive isolates were tested for drug susceptibility by **Proportion method on L.J.** medium to detect drug resistance pattern (whether MDR /XDR). For detection of MDR-TB strains, 1st line anti-tubercular drugs- Streptomycin (S), Isoniazid (I), Rifampicin (R) and Ethambutol (E) and for detection of XDR-TB strains 2nd line anti-tubercular drugs (Kanamycin, Amikacin, Ethionamide, D-Cycloserine, Clarithromycin, Ciprofloxacin, p-Amino salicylic acid, Rifabutin) were used.

As per the formula to be used to calculate the percentage of resistant cells i.e.

$$\frac{\text{No. of colonies on the drug medium}}{\text{No. of colonies on the control medium}} \times 100 = \% \text{ resistance.}$$

The resistance / susceptible pattern of the isolates against the, 1st line anti-tubercular drugs are depicted in Table 17 and 18.

Table 17

Resistance pattern and percentage of drug resistance of 123 isolates

Drug combination	No. of drug resistant isolates	Percentage of drug resistance
SIRE	10	8.13%
SIE	8	6.5%
SI	4	3.25%
SE	12	9.75%
RE	6	4.87%
S	2	1.62%
I	9	7.3%
R	3	2.43%
E	7	5.69%

S- Streptomycin, I- Isoniazid, R- Rifampicin, E- Ethambutol

Table 18

Number of Sensitive and Resistant isolates of *M. tuberculosis* for each drug

Drug		No. of isolates of *M. tuberculosis*	Percentage of drug susceptibility
Streptomycin	**Sensitive**	87	70.73%
	resistant	36	29.26%
Isoniazid	**Sensitive**	92	74.79%
	resistant	31	25.20%
Rifampicin	**Sensitive**	104	84.55%
	resistant	19	15.44%
Ethambutol	**Sensitive**	80	65.04%
	resistant	43	34.95%

Interpretation:

- 70.73% were sensitive to streptomycin.
- 29.26% were resistant to streptomycin.
- 74.79% were sensitive to Isoniazid.
- 25.20% were resistant to Isoniazid.
- 84.55% were sensitive to Rifampicin.
- 15.44% were resistant to Rifampicin.
- 65.04% were sensitive to Ethambutol.
- 34.95% were resistant to Ethambutol.

In the present study, out of 123, only 10 strains are resistant to both rifampicin and isoniazid, therefore the MDR-TB rate observed is 8.13%.

All these 10 MDR strains were then tested for drug susceptibility of 2nd –line drugs by proportion method on L.J. Table -19 depicts the resistance / susceptible pattern of these 10 MDR isolates against the 2nd line anti-tubercular drugs.

Table 19

Resistance pattern of MDR-TB isolates to second line anti-tuberculosis drugs

Drug	MDR-TB isolate numbers									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Kanamycin	-	+	+	-	-	-	-	-	-	-
Amikacin	-	-	-	-	-	-	-	-	-	-
Ethionamide	+	+	-	-	+	-	+	-	-	-
D-Cycloserine	-	+	+	+	-	-	+	-	-	-
Clarithromycin	-	-	-	-	-	-	-	-	-	-
Ciprofloxacin	+	-	-	-	-	-	-	-	-	-
P-amino salicylic acid	-	-	+	-	-	-	-	-	-	-
Rifabutin	-	+	+	-	-	-	-	-	-	-

'+' sign indicates the growth on drug containing L.J. media while '-' sign indicates no growth on L.J. media containing anti-TB drug.

Interpretation:

- All the MDR-TB isolates are sensitive to Amikacin - Clarithromycin.
- One is resistant to Kanamycin, D-Cycloserine, P-amino salicylic acid and Rifabutin while the other is resistant to Kanamycin, Ethionamide, D-Cycloserine and Rifabutin (i.e.) these are XDR strains.
- Monoresistance to Ethionamide and D-Cycloserine is observed in 2 isolates.
- Resistance to 2 drugs i.e. Ethionamide, Ciprofloxacin and Ethionamide, D-Cycloserine is observed in 2 isolates.

XDR rate:

Out of 10 MDR-TB isolates, only two were observed to be XDR-TB i.e. 20%.

Table 20

MDR-TB rate in HIV positive cases

MDR in HIV	1 (25%)
Sensitive to all drugs	3
Total HIV Positives	4

Table 21

MDR-TB rate in non- HIV cases

MDR in non HIV	9 (7.56%)
Sensitive to all drugs	110
Total HIV negatives	119

The present study found 4 cases among 123 cases tested for DST as HIV seropositive (Table 20 and 21). One of these had MDR-TB. Generally there is no significant difference in the DST pattern between HIV seropositive and seronegative patients. It is possible that the in-vivo action of the drugs may differ between the two groups. Due to decreased cell mediated immunity in HIV seropositive individuals, supportive lytic action is absent; hence the MDR-TB cases may have a poor prognosis [243,197,214].

Mutations associated with Drug Resistance:

Among *Mycobacterium tuberculosis* isolates, 8.13% were MDR-TB strains showing resistance to both INH and RIF (first-line anti-tuberculosis drugs). All these strains were subjected to line-probe assay (LPA) to detect mutation pattern in the respective genes. Table 22 and Figure 31 show different pattern of mutations in the genes of these 10 MDR-TB strains.

In 8 strains, RIF resistance was associated with the missing of *rpoB* WT8 gene while missing of *rpoB* WT7, 8 and *rpoB* WT4, 5 was observed in two different strains.

High level INH resistance was observed in 5 strains showing presence of *katG* MUT1 band while low level INH resistance was observed in 5 strains; showing presence of *inhA* MUT3A band in 2 strains and *inhA* MUT 3B band in 3 strains.

Table 22

Pattern of gene mutations detected by line probe assay (LPA) - MTBDR*plus* in *Mycobacterium tuberculosis* isolates (2-8)

Sample no.	Banding patterns of MDR-TB isolates
1	Negative control
2	*rpoB* WT8 missing and *rpoB* MUT3, *katG* MUT1 present
3	*rpoB* WT8 missing and *rpoB* MUT3, *katG* MUT1 present
4	*rpoB* WT8 missing and *rpoB* MUT2A, *inhA* MUT3A present
5	*rpoB* WT8 missing and *rpoB* MUT3, *inhA* MUT3B present
6	*rpoB* WT7and 8 missing and *rpoB* MUT3, *inhA* MUT3A present
7	*rpoB* WT4 and5 missing and *rpoB* MUT3, *katG* MUT1 present
8	*rpoB* WT8 missing and *rpoB* MUT3, *katG* MUT1 present
9	*rpoB* WT8 missing and *rpoB* MUT3, *katG* MUT3B present
10	*rpoB* WT8 missing and *rpoB* MUT3, *katG* MUT1 present
11	*rpoB* WT8 missing and *rpoB* MUT3, *katG* MUT1 present

Table 23

Location of mutations in the *rpoB, katG* and *inhA* genes of *M. tuberculosis* isolates

Name of the Gene	Band	Gene mutation region	No. of MDR *M. tuberculosis* isolates
rpoB	Missing of WT8 and presence of MUT3	S531L	7
rpoB	Missing of WT8 and presence of MUT2A	H526Y	1
rpoB	Missing of WT7, WT8 and presence of MUT 3	S531L	1
rpoB	Missing of WT4,WT5 and presence of MUT3	S531L	1
katG	MUT1	S315T1	5
inhA	MUT3A	T8C	2
inhA	MUT3B	T8A	3

LPA of the 10 isolates exhibited different band formations. Appearance of control bands validated the assay. The intensity of bands was compared with that of amplification control for positivity identification.

Resistance to Rifampicin was more commonly noticed by mutation in the 81 base pair region of *rpoB*. This region is called as the 'Hotspot' region in *Mycobacterium tuberculosis.* The mutations were commonly noticed between 516-533 regions of *rpoB* gene. Maximum mutations were observed at S531L locus in the present study. Resistance to INH was noticed by mutation in *KatG* S515T1 locus and mutations in the *inhA* T8C and T8A locus (Table 23). Further molecular and data analysis of MDR isolates from different countries worldwide may help in detection of a specific mutation responsible for drug resistance development in *Mycobacterium tuberculosis*. Further study may reveal newer sites of resistance and may also help in detecting newer sites for the action of new compounds including the herbal/ ayurvedic compounds[6].

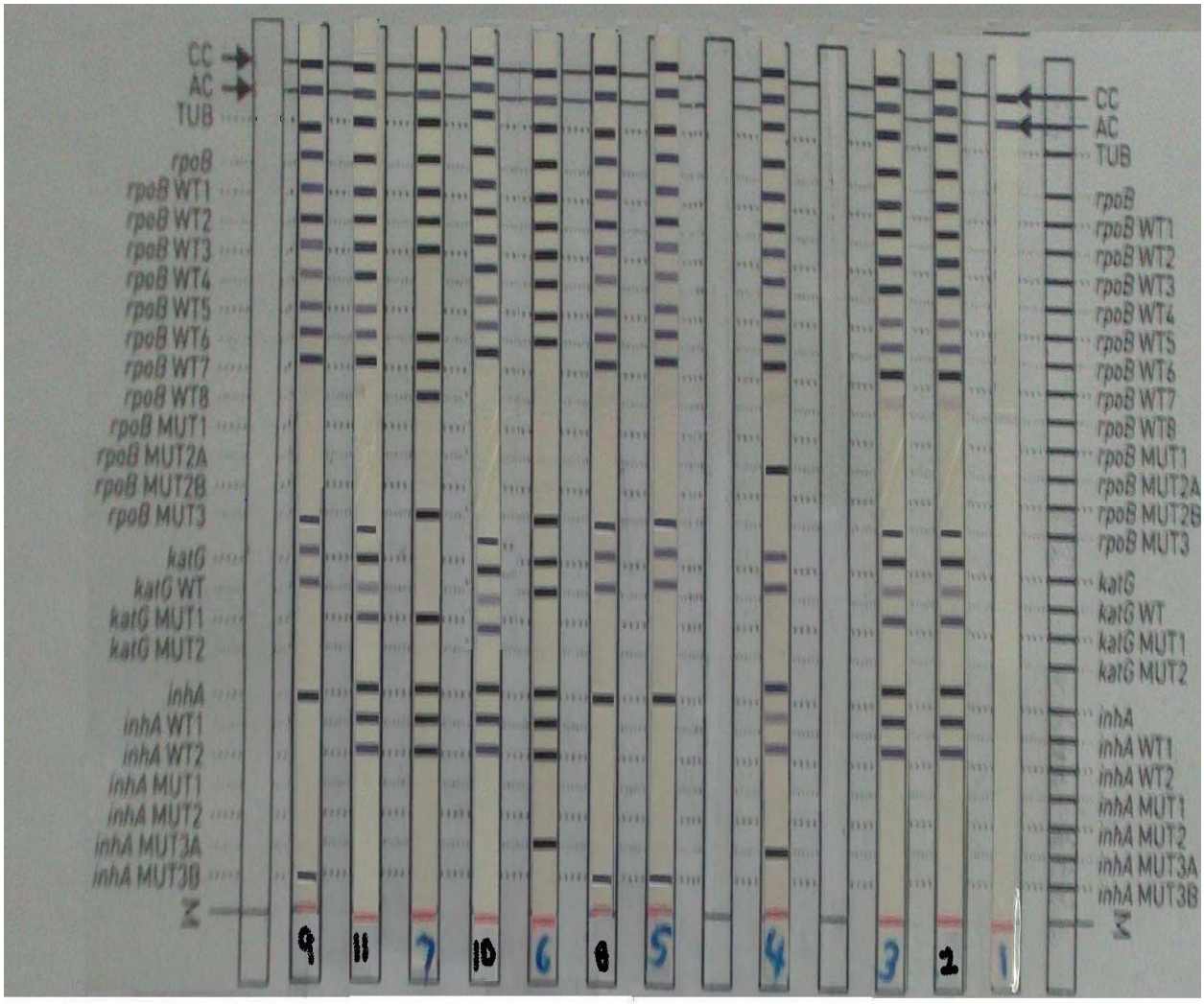
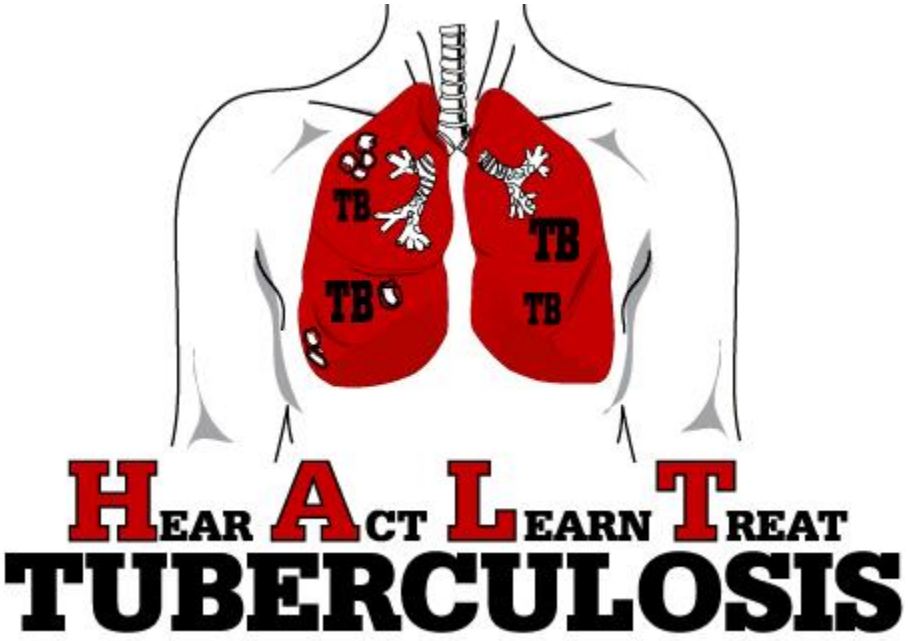


Figure 31: Images of Genotype MTBDR *plus* strips showing mutation bands

DRUG RESISTANT TUBERCULOSIS



DISCUSSION

DISCUSSION

Pulmonary tuberculosis is a disease of great antiquity. It has been referred to by different names like Pthisis and Rajyakshama. The number of Tuberculosis cases is alarming, increasing every day and annually 1.8 million cases worldwide. India accounts 5th of the world's new TB cases. Arrival of HIV has further increased the number of TB cases. Long duration of treatment, poor socioeconomic condition and poor quality of the drugs and indifferent policies of the government are responsible for the failure of Tuberculosis Control Program in developing countries. In 1993, WHO was forced to declare TB as a "Global Emergency" and instructed countries to take up drastic effective steps to control Tuberculosis. WHO has Stop TB project by 2015. Recently in May, 2012, TB is declared as a notifiable disease in India [258].

Incomplete treatment or usage of only one or two anti-tubercular drugs has led to the emergence of drug resistant strains of *M .tuberculosis*. This has further complicated the management and control of TB. In this regard an attempt is made to compare the drug resistance pattern of *M. tuberculosis* isolates in Pune area by using conventional Proportion method on L.J.

In the present study, 123 clinically suspected Pulmonary Tuberculosis cases admitted at Bharati Hospital, Pune, were studied.

1. Age :

Maximum number of patients suffering from TB was in the age group 21-30 years (24.4%) followed by 31-40 years (18%) and 16% were in the age group of41-50. Thus about half of the patients were in age group of 21-50 years. This is economically most productive age group in any society, resulting in reduction of manpower leading to economic loss. Findings in this research are similar to those of Grybowski *et al.* [105] who reported that, half of his patients were in this age group. Richard et al have also reported that majority of their patients (61%) were in this age group [232].

The reasons that make this age group vulnerable to TB are many. They are socially more active and are more exposed to an open case of TB than others.

2. Sex:

In this study, out of 123 patients there were 78 males and 45 females. Male to female ratio was 1.7:1. Many other investigators have also noted male preponderance in their

studies. Peter *et al.* [205] and Fandinho *et al.* [81] have reported male to female ratio of 1.8:1 and 1.6:1 respectively.

Likely reasons for male preponderance could be:

- In a male dominated society, usually he is the earning member. As he goes out for work, he is more likely to come in contact with an active TB case.
- Men are more likely to acquire habits like smoking and alcoholism which predispose to TB.

3. Microscopy :

Direct smear microscopy is the most commonly employed diagnostic tool in many countries. It is only the laboratory facility available in many laboratories. It is well known that smear for AFB has a lower sensitivity and it is further reduced in HIV patients. Smear examination is of no use in latent Pulmonary Tuberculosis cases. Even by employing fluorescent staining the sensitivity of smear study has not improved much. It is also affected by the quality of sputum sample submitted, efficiency of the technologist in smear making and efficiency of the microbiologist screening the stained sputum smears. In the present study sputum smear positivity rate was less (10.96%) while most of the other studies by Negi *et al*. [191] (34%), Bhargava *et al.* [23](59%), Githui *et al.*[99](65%), Rishi *et al*.[233] (28%) and Deshmukh *et al*.[69] (36%) have found higher sensitivity range. A number of technical and patient related factors can affect the sensitivity of direct smear microscopy. Also the sensitivity is affected by the nature of the specimen. A marginal increase of sputum AFB positivity can be achieved by use of Fluorescent staining [99].

4. Culture by Lowenstein-Jensen's medium:

a. Rate of isolation of *Mycobacterium tuberculosis* on L.J. medium:

In the present study, overall rate of isolation of *Mycobacteria* was 94.61%. Isolation of *Mycobacterium tuberculosis* by using conventional L.J. medium is a very difficult task. It needs strict quality work and trained personnel. Improper inspissation will result in contamination and liquefaction of L.J. medium. Another factor interfering with growth is anti-tubercular drugs. Sputum culture of a patient receiving ATT may yield no growth of *Mycobacterium tuberculosis.* Most of other studies by Jena *et al*. (85%) [121], Rishi *et al*.

(51%) [233], Negi *et al*. (49%) [191], Rodrigues *et al*. (29%) [235] have reported less rate of isolation of *M. tuberculosis* on L.J.

Rate of culture isolation of *Mycobacterium tuberculosis* is higher than that of Rodrigues *et al*., Negi *et al.* and others. But Jena *et al*. has reported 85% isolation rate.

Factors influencing the isolation rate of *Mycobacterium tuberculosis* are:

- The difference in the prevalence of *Mycobacteria* in different geographical areas.
- Criteria used for case selection
- Technical factors like number and the type of samples collected
- Type of media used for cultivation
- Sample collected before or after anti-tubercular treatment

Jena *et al.* [121] has included only clinically and radiologically proven cases of pulmonary tuberculosis and has used 3 consecutive sputum samples. Rodrigues *et al*., Rishi *et al*., Negi *et al*., all have used single early morning sputum sample and one single sample for other extrapulmonary cases. Also they have used clinically suspected cases as their subjects.

In this study, only clinically suspected cases are included. May be collection of more than one sample from each patient might have increased the isolation rate.

b. Duration of isolation:

In this study, the mean duration of isolation of *Mycobacteria* on L.J. media was 24.5 days. The shortest time taken by any strain was 17 days and longest time taken was 41 days.

Many authors have reported similar time period required for isolation of *Mycobacteria* on L.J. media. Negi *et al.* [191] and Rishi *et al.* [233] have reported 24.03 days and 28.81 days as mean duration of isolation respectively.

c. Identification of *Mycobacterium tuberculosis* isolates[184]:

Conventionally isolates are identified as *Mycobacterium tuberculosis* by putting up different conventional identification tests like nitrate reduction test, catalase test, niacin test. These tests were employed in the present study for identification of culture isolates of *Mycobacterium tuberculosis*.

1) **Niacin Production**: All positive cultures (rough, bough and tough colony producing) were tested for niacin production after they were well matured (50-100 colonies,

and3-4 weeks old). Strains of *M. tuberculosis* usually give positive Niacin test results; other *Mycobacteria* usually give negative results.

2) **Nitrate reduction**: *M. tuberculosis, M. kansasii, M. szulgai* and smooth colonies *M. fortuitum* are the clinically significant species which have the property of reducing nitrates to nitrites, though some saprophytic strains and *M. fiavescens* also reduce nitrates.

3) **Catalase activity, Semiquantitative test** [141, 89]: Nearly all *Mycobacteria*, with the exception of certain isoniazid resistant mutants of *M. tuberculosis* and *M. gastri,* possess catalase enzymes.

5. Antitubercular drug susceptibility :

The main objective of this research is to detect the drug resistance pattern and mechanisms in *Mycobacterium tuberculosis* isolates. In the present study for the detection of drug- resistant pattern 1st-line anti-tubercular drugs: Streptomycin, Rifampicin, Isoniazid, Ethambutol and 2nd-line anti-tubercular drugs: Kanamycin, Amikacin, Ethionamide, D-Cycloserine, Clarithrimycin, Ciproflixacin, P-amino salicylic acid and Rifabutin, gold standard conventional Proportion method on L.J. is used to detect the susceptibility/resistance to each of the 1st and 2nd-line drugs (Table-17,18, 19) .

Susceptibility pattern to 1st-line drugs was:

- Ethambutol and Streptomycin resistance was seen in majority of the isolates (34.95% and 29.26%).
- **Monodrug** resistance was observed for all 1st-line drugs like Streptomycin (1.62), Isoniazid (7.3%), Rifampicin (2.43%), and Ethambutol (5.64%).
- **Resistance to two** 1st-line drugs was observed for Streptomycin and Isoniazid (3.25%), Streptomycin and Ethambutol (9.75%), Rifampicin and Ethambutol (4.87%).
- **Resistance to combination of three** 1st-line drugs was observed for Streptomycin, Isoniazid and Ethambutol (6.5%).
- **Resistance to all 4** 1st-line drugs was observed by 8.13% cases.
- In this study, the resistance pattern to first line drugs was INH (25.20%), Rifampicin (15.44%), Streptomycin (29.26%) and Ethambutol (34.95%).

- Resistance to INH was seen in 25.20% in our study which is much lower than studies reported by Verma *et al.* (40.5%), Sureshkumar *et al.* (42%), Menon *et al.* (53.2%) and Barat *et a*l. (80%) [283, 285, 169, 18, 164].
- Resistance to Rifampicin was seen in 15.44% in our study which is much lower than studies reported by Verma *et al*.and Bhatt *et al*. (18%), Sureshkumar *et al.* (32%), Menon *et al.* (74.4%) and Barat *et al*. (76%)[283,24,255,169,18].
- Resistance to Streptomycin was seen in 29.26% in our studies which is much lower than the studies reported by Bhat *et al.* (62%) and Menon *et al.* (70%) [18,169].
- Resistance to Ethambutol was seen in 34.95% in our study, which is much higher than other studies reported by Bhat *et al*. (12%), Menon *et al.* (21.7%) and Verma *et al.* (27%) [18,169,283].

The present overall rate of MDR-TB in India is around 18-20%. In the present study, the MDR-TB rate is observed to be 8.13%. Rate of MDR-TB was higher in other studies of Filho *et al.* (78.8%), Barat *et al.* (72%), Verma *et al*. (66.6%) and Menon *et al*. (47.54%) [37,18,283,169]. Lesser MDR resistance was reported by Mulenga *et al.* (5%), 2.9% by Therese *et al*., 4% by Bhat *et al.* [182,263,18].

Susceptibility pattern of total 10 MDR-TB isolates to 2nd-line drugs was:

- All are susceptible to Amikacin and Clarithromycin.
- Mono-resistance to Ehionamide and D-Cycloserine was observed in 20% isolates.
- Resistance to two drugs i.e Ethionamide, Ciprofloxacinand Ethionamide, D-Cycloserine was observed in 20% isolates.
- Resistance to 4 drugs i.e Kanamycin, D-Cycloserine, P-Amino salicylic acid and Rifabutin was observed in10% isolates while resiatance to Kanamycin, Ethionamide, D-cycloserine and Rifabutin was observed in 10% isolates.
- In the present study pattern of drug resistance to second-line drugs was Kanamycin (20%), Ethionamide (40%), D-Cycloserine (30%), Ciprofloxacin (30%), P-amino salicylic acid (10%), Rifabutin (20%) and for Amikacin and Clarithromycin (0%).
- Zimenkov *et al.* (52%) saw resistance to Kanamycin in 20% in our study, which is much lower than the studies, Bonnet *et al.* (31.4%) [324, 27].

- Kim *et al.* (23.6%), Surehkumar *et al.* (32.26%), and Bonnet *et al.* (37.3%) saw resistance to Ethionamide in 40% in our study, which is much higher than studies studies[135, 255, 27].

The present overall rate of XDR-TB in India is 3.5 %[288] while 1.6% (20% of MDR) was observed in our study. Bonnet *et al.* (4.5%), Mondal *et al.* (7.4%), Jain *et al.* (8%) and Ajbani *et al*. (9.1%) have reported slightly higher XDR-TB rate [27, 179, 118, 5]. The increase in XDR rate can be attributed to the emergence of HIV co-infection, lack of awareness about treatment among the infected, non-availability of well-equipped diagnostic laboratory and family commitments.

Overall drug resistance pattern shows moderate rate of MDR-TB (8.13%) and XDR-TB (1.6%), which indicates the effective management of DOTS through RNTCP in the area.

6. Genotyping of Drug-Resistant strains of *Mycobacterium tuberculosis* by LPA:

This study aimed to provide preliminary data on the rate of resistance to first-line anti-tuberculosis drugs, especially INH and RMP and pattern of mutations in the genes responsible for drug resistance. For studying this aspect, the phenotypic Proportion method and the GenoType MTBDR*plus*- Hains Life science method of LPA was used.

7. Genotypic (LPA) Vs Phenotypic (proportion) methods:

It is reported that in recent years substantial progress has been made in our understanding of the molecular basis of *Mycobacterium tuberculosis* drug resistance. Molecular based assays are potentially the most rapid and sensitive methods for the detection of drug resistance. These assays detect all common drug resistance mutations. Some of these techniques include direct sequencing of PCR products, SSCP analysis, heteroduplex analysis, dideoxy fingerprinting, an RNA/DNA duplex, base-pair mismatch assay, Lucifer's mycobacteriophage strategy, a rRNA/DNA- bioluminescence-labled probe method, a reverse hybridization- based line probe assay and other strategies [248, 3, 278, 19 ,227, 224]. In the present study, GenoType MTBDR*plus* (LPA) was used which detects resistance to INH and RIF in clinical isolates based on the detection of the most common mutations *rpoB, katG* and *inhA* genes. It uses PCR and reverse-hybridization to probes immobilized on an assay strips [224, 323, 177, 151, 266, 108, 218].

All the 10 MDR- strains detected by phenotypic methods have shown the mutations in the *rpoB* gene (RMP- resistance) and *katG/inhA* (INH-resistance) by GenoType

MTBDR*plus* molecular method (Table 20). Thus, the sensitivity for MDR, RIF, and INH detection was found to be 100%. Kembhavi *et al.* (129) reported the same 100% sensitivity for MDR-TB detection by this method while lower sensitivity (92.5%) was reported by Farroqui *et al.* and 97.7% by Maurya *et al*. [178]. The commonest pattern seen was missing of WT8 and presence of MUT3 in *rpoB* gene in 9 (90%) strains while presence of *rpoB MUT2A* was seen in one strain. Low level INH resistance was observed in 50% strains while high level resistance was observed in 50% strains. Kembhavi *et al.,* Farroqui *et al.,* and Maurya *et al.* (Table 24) reported the similar pattern of mutations.

Table 24

Pattern of mutations in *rpoB* and *inhA* gene of *M. tuberculosis* by MTBDR*plus* assay in various studies:-

Study	**Technique used**	**MDR sensitivity**	**Mutations in RMP**	**Mutations in INH**
Farroqui *et al.* 2012	MTBDR*plus*	92.5%	531,533,S531L	*S315T:katG C15T:inhA*
Maurya *et al.*2013	MTBDR*plus*	97.7%	S531L	*S315T1:katG*
Kembhavi *et al.*	MTBDR*plus*	100%	531,526,516.S531L	*S315T1:katG C15T:inhA*
Present study	MTBDR*plus*	100%	S531L	*S315T:katG T8C:inhA, T8A:inhA*

Rifampicin resistance (RIF)

Rifampicin is a powerful bactericidal agent used as a first line anti-tubercular drug. Rifampicin resistance arises due to mutations in *rpoB* gene- producing DNA dependent RNA polymerase. The nature and frequency of mutations in the gene of RIF resistant isolates vary considerably according to geographical locations. Analysis of approximately 500 rifampicin resistant strains from global sources has found that 96% of rifampicin resistant clinical isolates of *Mycobacterium tuberculosis* have mutations in the 81- bp

core region *rpoB* gene, which encodes the β- subunit of RNA polymerase. Detection of mutations in the 81- bp core region correlated (100%) very highly in their study. Barnard *et al.* and Ravindran *et al.* (19,227) have reported similar findings. The sensitivity of RIF can be decreased if mutations are outside the 81-bp region of *rpoB* gene which cannot be detected by this assay. A higher (90%) proportion of RIF resistance due to S531L mutations was observed. Kembhavi *et al*. also found higher proportion (89.65%) of RIF resistance due to mutations in S531L in their study but lower proportion (62.3%) and (62%) of RIF resistance was reported by Maurya *et al.* and Farroqui *et al.* respectively[177, 300].

Isoniazide resistance (INH)

INH is also a bactericidal agent used as a first line drug for TB. Resistance to INH arises due to mutations in different genes including *katG*, *inhA,* and *ahpc* and other genes that remain to be established. In this study, *katG* mutations were found in 50% and50% in *inhA* genes. Kembhavi *et al.* had reported higher (75.86%) *katG* mutations and lower (20.66%) *inhA* mutations. Maurya *et al.* had also reported commonest INH mutations in *katG* (93.3%) and lower (28.9%) in *inhA* genes. Similarly Farooqi *et al.* had reported the higher proportion (66.1%) of mutations in *katG* genes and lower proportion (1.9%) of mutations in *inhA* genes. High prevalence of *katG* mutations has been reported to confer resistance in high prevalence countries [324] and for a much lower proportion in lower TB prevalence settings presumably due to ongoing transmission of these strains [263-265]. Lingala *et al.* studied geographical profile of *rpoB* mutations in *Mycobacterium tuberculosis.* [264] reported that 74% of RIF resistant isolates in their study includes common mutations at 531,526 and 516 region of *rpoB* gene. They have also reported the multiple silent mutations at 145-184 (outside the hot spot region) in their study [261-263].

LPAs are currently validated only for use directly from smear positive specimens, although reasonable performance in a small sample of smear-negative specimens was demonstrated by Barnard *et al*. Ongoing research into improved DNA extraction methods may enable LPAs to be performed directly from smear-negative sputum in future. However, cost-effectiveness of routine testing of smear-negative specimens would have to be carefully evaluated since the majority of specimens will be negative in most settings [6].

LPA is an appropriate tool for rapid screening for MDR-TB and has the potential to substantially reduce the turnaround time of DST results. However, WHO recommendations on infrastructure, training, quality assurance and other requirements should be followed to ensure high quality results.

Pooled sensitivity (98.1%) and specificity (98.7%) estimates for rifampicin resistance were very high. The accuracy for isoniazid was variable, with lower sensitivity (84.3%) and more inconstant than specificity (99.5%) [6].

8. HIV and TB:

TB is the commonest opportunistic disease in HIV positive persons in India and can develop at any stage of the disease. Hence one of the objectives of our study was to detect presence of go-existing HIV infection and Drug resistance pattern in HIV patients with TB.

All 123 patients were subjected to pretest counseling as per the NACO guidelines. All patients agreed to undergo HIV antibody detection by ELISA. All 123 cases proved pulmonary TB. 4 out of 123 blood samples tested were positive for HIV infection. 25% of HIV positive patients were MDR.7.56% were MDR in HIV negative patients. Percentage of MDR-TB among HIV sero-positives was higher than in HIV negative groups.

Even though the association of MDR-TB and HIV infection was not very significant in this study, it would not be too long before witnessing a rapid increase of MDR-TB among HIV patients if adequate measures are not taken [67].

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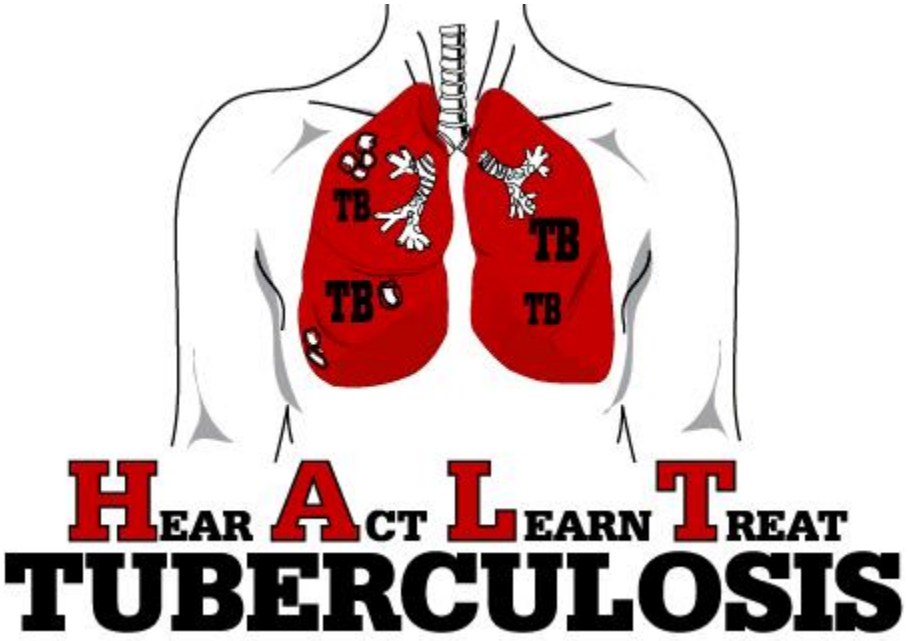
DRUG RESISTANT TUBERCULOSIS



SUMMARY

SUMMARY

According to WHO's latest report (2014), globally there were 8.6 million new TB cases and 1.3 million deaths due to TB. Out of these deaths, 170,000 were from MDR-TB; a relatively high as total compared with 4, 50,000 incident MDR-TB cases. Out all new TB cases 0.3 million are HIV positive cases. In 2013, S-E Asian region containing 11 countries accounts 58% of worlds TB cases. Out of that India accounts for 24% and China 11% and India ranks 2nd among 27 MDR-TB high burden countries worldwide after China (63000 cases emerge annually).It is estimated that about 40% Indian population is infected with TB bacilli. MDR-TB has reached all corners of the world while XDR-TB is reported in 92 countries including India.

The overall aim of this study was to detect the pattern and mechanism of drug resistant isolates of *Mycobacterium tuberculosis* from clinical samples of patients attending the Bharati Hospital, Pune. To detect the pattern of drug resistance DST was carried out by conventional proportion method on L.J. and to detect the mechanism of drug resistance, mutations in specific genes were detected by genotyping.

This is a cross-sectional study consisting of 130 clinically suspected pulmonary tuberculosis cases of which 123 were processed as per the standard methods.

Important observations are:

- Majority of patients (22%) were found in the age group of 21-30 years, followed by 17% in the 41-50 years and 14% were in the 31-40 years age group.
- Male preponderance was seen in this group of patients. Male to female ratio was 1.7:1.
- 10.96% of cases showed presence of AFB.
- Among 130 smear positive samples, 123 (94.61%) yielded growth of *Mycobacterium tuberculosis*.
- In our study, the mean duration of isolation of *Mycobacterium spp* on L.J. was 24.5 days; the shortest time taken by the strain was 17 days and longest time taken was 41 days.

Drug susceptibility pattern to 1st-line drugs was as follows:

- Ethambutol and Streptomycin resistance was seen in majority of the isolates (34.95% and 29.26%). Monodrug resistance was observed for all 1st-line drugs like Streptomycin (1.62), Isoniazid (7.3%), Rifampicin (2.43%), and Ethambutol (5.64%).
- Resistance to combination of two 1st-line drugs was observed for Streptomycin and Isoniazid (3.25%), Streptomycin and Ethambutol (9.75%), Rifampicin and Ethambutol (4.87%).
- Resistance to combination of three 1st-line drugs was observed for Streptomycin, Isoniazid and Ethambutol (6.5%).
- Resistance to all 4 1st-line drugs- streptomycin, isoniazid, rifampicin and pyrazinamide was observed by 8.13% cases.
- The present overall rate of MDR-TB in India is around 18-20%. In the present study the MDR-TB rate was observed to be 8.13%.

Drug susceptibility pattern of total 10 MDR-TB isolates to 2nd-line drugs was:

- All are susceptible to Amikacin and Clarithromycin.
- Mono-resistance to Ehionamide and D-Cycloserine was observed in 20% isolates.
- Resistance to combination of two drugs i.e. Ethionamide, Ciprofloxacin and Ethionamide, D-Cycloserine was observed in 20% isolates.
- Resistance to 4 drugs i.e. Kanamycin, D-Cycloserine, P-Amino salicylic acid and Rifabutin was observed in10% isolates while resistance to Kanamycin, Ethionamide, D-cycloserine and Rifabutin was observed in 10% isolates.
- The present rate of XDR-TB in India is 3.5%. In the study the XDR-TB rate was observed to be1.6% (20% of MDR).
- One (25%) isolate was MDR in HIV positive and 7.56% isolates were MDR in HIV negative patients. Percentage of MDR-TB among HIV seropositives was higher than HIV negative groups.
- Detection of drug resistance pattern by phenotypic methods is easy to perform for routine diagnosis as compared to genotypic tests which are very costly and requires more sophisticated infrastructure. These phenotypic tests will help in the

detection of resistance independent of the underlying mechanisms unlike molecular tests which can detect only those mutations which are screened for.

- Sensitivity (98.1%) and specificity (98.7%) estimates for rifampicin resistance by LPA were very high. The accuracy for isoniazid was variable, with lower sensitivity (84.3%) and more inconstant than specificity (99.5%).
- Use of automated, molecular assays will help in detection of drug-resistant cases and also helps to detect mutations. But these systems should be made affordable as the prices are higher than the conventional tests.
- This study will help us to initiate control measures by studying the antibiotic resistance pattern of *Mycobacterium tuberculosis* isolates and by documentation of it, one can understand the any change in resistance pattern when it occurs.

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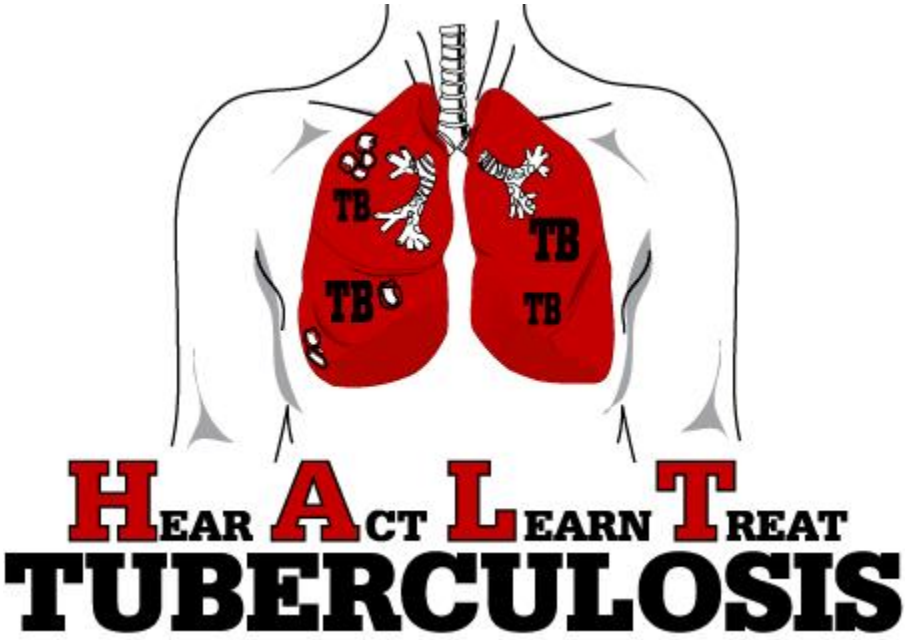
DRUG RESISTANT TUBERCULOSIS



CONCLUSION

CONCLUSION

- Pulmonary Tuberculosis is common among the population in and around Pune district. A mass population survey/screening would help to know the prevalence / incidence of pulmonary Tuberculosis and initiate an effective control measure.
- Culture of expectorated sputum in all clinically suspected pulmonary tuberculosis cases will help to detect the possible false negative cases reported by direct smear examination.
- Conventional, time consuming culture methods if replaced with automated and molecular assays will help in quick culture and will definitely help in revised national tuberculosis control program (RNTCP) control programs.
- Anti-tuberculosis drug susceptibility testing of all isolates of *Mycobacterium* spp. is very important to :

  1. initiate proper anti-tubercular therapy early so that the spread of resistant strains can be prevented
  2. detect the number of drug-resistant cases
  3. differentiate primary drug resistant cases from acquired drug resistant cases.
- Use of automated, molecular assays which will help in early detection of drug resistant cases and also help to detect the mutations. But these systems should be made affordable.
- MDR and XDR-TB cases are on the increase in many parts of the world and the rate of MDR and XDR-TB cases in the Pune district is also high as clinically suspected TB patients were selected. An effective monitoring system will definitely prevent the increase of MDR and XDR-TB cases.
- Regular drug susceptibility testing for newer/additional drugs against MTB cases during the forth- coming years will help in the early diagnosis of the disease.
- Emergence of HIV has increased the number of Tuberculosis cases. Periodical sputum examination for AFB among HIV positive patients is beneficial and helps in early diagnosis and prevention of transmission.
- MDR rate of MTB isolates is higher in HIV positives than HIV negative patients.

- In recent years, DR-TB has become a growing threat to global public health, a threat that has generated fear not only in the scientific and medical communities, but also among the general public. The primary message which must be delivered to everyone responsible for managing TB is that for all forms of DR-TB, cure is possible with optimal clinical and operational case management, including for those patients with a very long-standing pattern of resistance [(75)].
- In conclusion, we have come a long way in our fight against this deadly disease; we still have miles to go before we will make this planet TB free. WHO with its "STOP TB" strategy has given a vision to eliminate TB as a public health problem from the face of this earth by 2050 [(304)] .The need of the hour therefore is an easy access to appropriate diagnostic and therapeutic facilities to help us to meet this challenge. There is dire need to regulate the rational use of first- and second-line anti-TB drugs.

  Working association between physicians; private sector, religious bodies and other local nonprofit organizations, e.g. Lions Club, Rotary International, should be strengthened for better dissemination of awareness about diagnosis, management and control of the disease. Existing diagnostic laboratories need to be strengthened with routine training refresher courses for the involved personnel for better utilization of these already scarce resources. Better diagnostic tests for quick screening of this disease at field level should be developed and made available at the grass-root level. The links between primary health centers and DOTS centers should be strengthened and special attention should be given to prioritizing the groups which need to be followed first; utilizing human resources of related public health programs, e.g. programs for HIV/Malaria; promoting development of new drugs and vaccines against TB, and discouraging the use of homeopathy medicines for treating TB and HIV [(12)].

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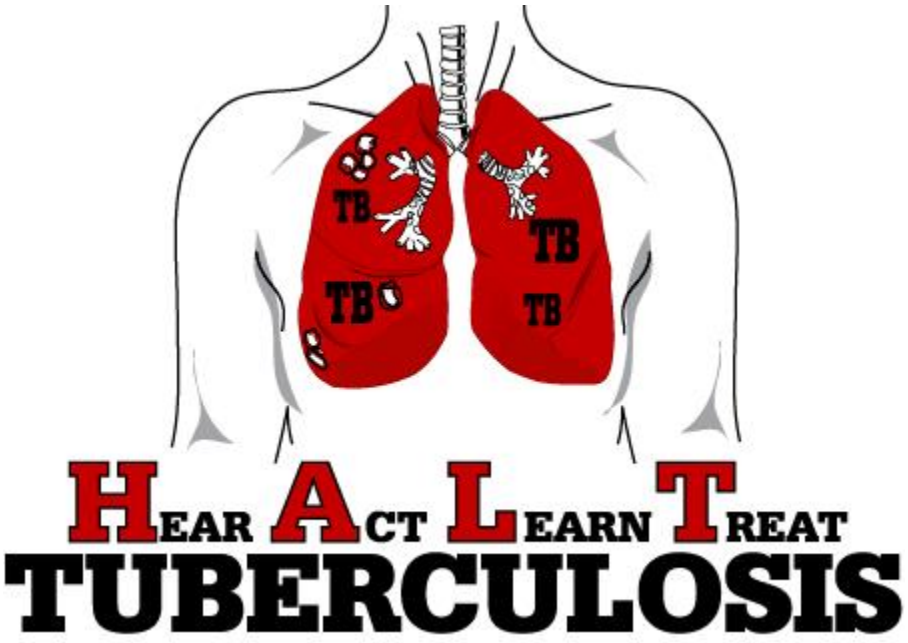
DRUG RESISTANT TUBERCULOSIS



BIBLIOGRAPHY

BIBLIOGRAPHY

1. Affolabi D, Sanoussi N, and Odoun M *et al*., Rapid detection of multidrug-resistant *Mycobacterium tuberculosis* in Cotonou (Benin) using two low-cost colorimetric methods: resazurin and nitrate reductase assays. J Med Microbiol 2008; 57:1024-1027.
2. Agarwal SP, Vijay S, Kumar P, Chauhan LS. The history of Tuberculosis Control in India: Glimpses through decades. In: Agarwal SP, Chauhan LS, editors. Tuberculosis Control in India; New Delhi, India: Directorate General of Health Services, Ministry of Health and Family Welfare. 2005, 15-22.
3. Ahmad S, Araj GF, Akbar PK, Fares E, Chung TD, Mustafa AS. Characterization of rpoB mutations in rifampicin resistant *Mycobacterium tuberculosis* isolates from the Middle East. DiagMicrobiol Infect Dis, 2000; 38:227-32
4. Ahmad Z, Shameem M. Manifestation of Tuberculosis in HIV infected patients. J Indian Acad Clin Med 2005; 6:302-5.
5. Ajbani K, Nikam C, Kazi M, Gray C, Boehme C, Balan K and Rodrigues C. Evaluation of Genotype MDRRsl assay to detect resistance associated with fluoroquinolones, aminoglycosides and ethambutol on clinical sediments. PLoS ONE, 7(11): e 49433.
6. Albert H, Bwanga F, Mukkada S *et al*., Rapid screening of MDR-TB using molecular Line Probe Assay is feasible in Uganda. BMC Infect Dis 2010; 10:41.
7. Alteri CJ, Cortes JX, Hess S, Olin GC, Giron JA and Friedman RL. *Mycobacterium tuberculosis* produces pili during human infection. Proc Natl Acad Sci USA. 2007 March 20; 104(12):5145-5150.
8. American lung association (2009) "Tuberculosis (TB)". Available at- www.lungusa.org
9. American Thoracic Society and Centers for Disease Control and Prevention. Diagnostic standards and classification of Tuberculosis in adults and children. Am J Crit Care Med. 2000; 16(4pt1):1376-1395.
10. Anand G and McKay B. Awakening to Crisis, India Plans New Push Against TB. The Wall Street Journal (2012). Retrieved April 3, 2013, from, from http://
11. Ancell, Henry (1852). A Treatise on Tuberculosis: The constitutional origin of consumption and scrofula. Longman, Brown, Green and Longmans.
12. ANI. Homeopathy dosen't helps in HIV, TB, and Malaria. The times of India. India: c2010. Available from: http://timesofindia.indiatimes.com/life-style.health/Homeopathy-dosen't-help-in-HIV-TB-malaria/articleshow/4918285.com.

103. Goyot-Revol V, Innes JA, Hackforth S, hinks T, Lalvani A. Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis. Am J Resp Crit Care Med. 2006; 173:803-810.
104. Grange JM. Tuberculosis. In: Husler WJ Jr., Sussman M, editors. Topley and Wilson's Microbiology and Microbial infections. 9th Edition. Vol 3. London: Amold; 1998; 391-417.
105. Grybowski S. Tuberculosis in the third world. Thorax; 46: 689-691.
106. Gupta M, Sing NP, Kaur IR. Evaluation of nitrate reductase assay for direct detection of drug resistance in *Mycobacterium tuberculosis*: rapid and inexpensive method for low-resource settings. Indian J Med Microbiol 2010; 28:363-365.
107. Gupta R, Kim JK, Espinal MA *et al*., Responding to market failures in TB control science 2001; 293:1049-51.
108. Heifets LB and Good RC. Current laboratory methods for the diagnosis of Tuberculosis, p.85-110, in B.R. Bloom (edi) 1994; Tuberculosis: pathogenesis, protection and control. American society for Microbiology, Washington, D.C.
109. Hillemann D, Rusch-Gerdes S, Richer E. Feasibility of the GenoType(R) MTBDRsl assay for fluoroquinolone, amikacin/capreomycin, and ethambutol resistance testing of *Mycobacterium tuberculosis* strains and in clinical specimens. J Clin Microbiol 2009; 47: 1767-1772.
110. Hippocrates, Aphorisms v.9 (http://classics.mit.edu/Hi[[ocrates/aphorisms.5.v.html)
111. Hoffner SE, Halie M, Kallenius G. A biphasic system for primary isolation of *Mycobacteria* compared to solid medium and broth culture. Ind J Med Microbiol, 1992; 37:332-334.
112. Hussy MA, Zayaitz A. Acid-fast stains protocols. American society for Microbiology. ASM MicrobieLibrary, 8thsep 2008. http://www.microbelibrary.org/component/resource/laboratory-test...
113. ICMR bulletin. What is new in the diagnosis of tuberculosis? Techniques for diagnosis of TB. August, 2002; 32(8).
114. Indian Council of Medical Research. Tuberculosis in India: A national sample survey 1955-58. Technical report series. New Delhi, India: Indian Council of Medical Research; 1959.
115. Interactive core curriculum on tuberculosis. Centers for Disease Control and Prevention. Web site, http://www.cdc.gov/tb/webcourses/CoreCurr/TB_Course/Menu/frameset_internet.htm.
116. Isalkar U. State records 155 XDR-TB cases in 3 years, 8 from Pune district. TNN. Jan 31, 2014. Available on http://timesofindia.indiatimes.com/city/pune/state-records-155-XD...

13. Anti-TB DR- in the world, report No.4. WHO/IUATLD. Global project on the anti- TB DR- Surveillance. WHO/HTM/TB/2008.394.
14. Aranaz A, Liebana E, Gomez-Mampaso E *et al*. "*Mycobacterium tuberculosis* subsp. Caprae subsp. Nov.: a taxonomic study of a new member of the Mycobacterium tuberculosis complex isolated from goats in Spain. Int J Syst Bacteriol 1999; 49:1263-73. "
15. Arora VK, Kumar SV. Pattern of opportunistic pulmonary infections in HIV ser-positive subjects: Observations from Pondicherry, India. Indian J Chest Dis Allied Sci 1999; 41:135-44.
16. Aufderheide, Arthur C; Conrado Rodriguez-Martin; Odin Langsjoen (1998). The Cambridge encyclopedia of human paleopathology. Cambridge University Press. ISBN 0521552036.
17. Banerjee R, Allen J, Lin SYG *et al*., Rapid drug susceptibility testing with a molecular beacon assay is associated with earlier diagnosis and treatment of multi-drug resistant tuberculosis in California. J Clin Microbiol 2010; 48: 3779-3781.
18. Barat D and Kumar G. Prevalence and pattern of acquired drug resistance in Tuberculosis including MDR-TB in Bihar. Journal- Association of Physician of India, 2003; 51:327.
19. Barnard M, Albert H, Coetzee G, O'Brien R, Bosman ME. Rapid molecular screening for multi drug resistant tuberculosis in a high volume public health laboratory in South Africa. Am J Respi Crit Care Med., 2008:787-792.
20. Bary MF, Coronel J, Gilman RH *et al*., The MODS method for diagnosis of tuberculosis and multidrug resistant tuberculosis. J Vis Exp 2008; 18.
21. BD. BACTEC^{TM} MGIT^{TM} 960 SIRE Kit for the anti-mycobacterial susceptibility testing of *Mycobacterium tuberculosis*. Franklin Lakes, NJ, USA: Becton Dickinson and Company; 2002.
22. Bemer P, Bodmer T, Munzinger J, Perrin M, Vincent V, Drugeon H. Multicenter evaluation of the MB/BacT System for susceptibility Testing of *Mycobacterium tuberculosis*. J Clin Microbiol. 2004 March; 42(3):1030-1034.
23. Bhargava A, Jain A. Comparison of liquid and solid culture media with radiometric system for detection of *Mycobacteria i*n clinical specimens. Ind J of Tuberculosis. 2001; 48:9-12.
24. Bhat S, Radhakrishna M, Kotian M and Rao S. Drug susceptibility profiles of *Mycobacterium tuberculosis* isolates at Manglore, Indian Journal of Medical Sciences,2010;64(3):99-103.
25. Blackmore R, Story E, Helb D *et al*., Evaluation of the analytical performance of the Xpert(R) MTB/RIF assay. J Clin Microbiol 2010; 48:2495-2501.

26. Blower SM, McLean AR, Porco TC, Small PM, Hopewell PC *et al.* The intrinsic transmission dynamics of tuberculosis epidemics. Nat. Med.1995; 1:815-821.
27. Bonnet M, Pardini M, Meacci F, Orru G, Yesilkaya H, Jarosz T and Varaine F. treatment of Tuberculosis in a region with high drug resistance outcomes, drug resistance amplification and reinfection. PloS ONE, 2011; 6(8) e23081.
28. Bourdelaris P, Bart KH (2006). Epidemics laid low: A history of what happened in rich countries. JHU Press. ISBN 080188294X.
29. Brennan PJ, Hunter SW *et al.*, 1990. Reapprasisal of the chemistry of *Mycobacterial* cell walls, with a view to understanding the roles of individual entitles in disease processes. In: Ayoub EM, Cassell GH *et al*., (edi), Microbial determinants of virulence and host response. Washington DC: American Society for Microbiology Press, 55-75.
30. Brock Thomas D. (1999). Milestones in Microbiology: 1546-1940. ASM Press.
31. Brock Thomas D. (1999). Robert Koch: A life in medicine and bacteriology. ASM Press. ISBN 0910239193.
32. Brossier F, Veziris N, Aubry A *et al*. Detection by GenoType MTBDRsl test of complex mechanisms of resistance to second-line drugs and ethambutol in multidrug-resistant *Mycobacterium tuberculosis* complex isolates. J Clin Microbiol 2010; 48:1683-1689.
33. Brunello F, Parvati F, Fontana R. Comparison of the MB/BacT and BACTEC 460 TB System for Recovery of *Mycobacteria* from various Clinical specimens. J Clin Microbiol April 1999; 37(4): 1206-1209.
34. Brzostek A, Dziadek B, Rumijowska-Galewicz A, Pawelczyk J. Cholesterol oxidase is required for virulence of *Mycobacterium tuberculosis*. FEMS Microbiol. Lett. (2007); 275:106.
35. Bwanga F, Hoffner S, Haaaile M *et al*., Direct susceptibility testing for multi drug resistant tuberculosis: a meta-analysis. BMC Infect Dis 2009; 9:67.
36. Caminero JA. Justification for the guidelines. Guidelines for clinical and operational management of drug-resistant Tuberculosis. International Union Against Tuberculosis and Lung Diseases, 2013; chapter-1:2
37. Campos Filho PC, Muniz-Sobrinho J.D.S., Da Silva TM, Nery JS, dos Santos GC, Mota C and Rios-Santos F. Accuracy of the polymorphisms in KatG, KasA and inhA genes for predicting isoniazide resistance in *Mycobacterium tuberculosis* isolates recovered from Brazillian population. International journal of Microbiology and Immunology Research, May, 2013; 2(5):044-050.
38. Casal M, Gutierraz J, Vaquero M. Clinical interest of a simple system for isolating *Mycobacterium tuberculosis*. Rev Clin Esp.1997; 197(3):148-151.

39. Cavendish R. Robert Koch's Nobel Prize for Medicine. History today, 2005;55(12)
40. Centers for Disease Control (1990). Outbreak of multidrug-resistant tuberculosis- Texas, California, and Pennsylvania. (http://www.cdc.gov/mmwr/preview/mmwrhtml/00001636.htm). Morb Mortal Wkly Rep39 (22):369-72. PMID2111434.
41. Centers for disease control (CDC) (August 1991). Nosocomial transmission of multidrug- resistant tuberculosis among HIV-infected persons- Florida and New York, 1988-1991. Morb Mortal Wkly Rep 40 (34):585-91. PMID 1870559 (//www.ncbi.nlm.nih.gov/pubmed/1870559).
42. Centers for disease control (CDC) (March1991). Transmission of multidrug-resistant tuberculosis from an HIV-positive client in a residential substance-abuse treatment facility- Michigan. Morb Mortal Wkly Rep 40(8):129-31.PMID 1900098
43. Centers for Disease Control and Prevention (1996). The role of BCG vaccine in the prevention and control of tuberculosis in the United States: a joint statement of Advisory Committee on Immunization Practices. MMWR Recomm Rep 45 (RR-4): 1-18. Pmid 8602127 (//www.ncbi.nlm.nih.gov/pubmed/8602127).
44. Centers for Disease Control and Prevention (12 September 2006). "2005 Sueveillance Slide Set" (http://www.cdc.gov/nchstp/tb/pubs/slidesets/surv2005/default.htm). Retrieved 14 October 2006.
45. Chandrasekaran S, Rao P. Diagnostic methods of Tuberculosis. Textbook of Tuberculosis. 2nd edition, Rao KN; Vikas Publishing House Pvt Ltd. 1981; 192-209.
46. Chandrasekaran S, Chauan MM, Rajalakshmi R, Chaudhri K, Mahadev B. Initial drug resistance to anti-tuberculosis drugs in patients attending an urban district tuberculosis centre. Ind J Tub 1990; 37:215-6.
47. Chandrashekaran SP, Jagota, Chandhur K. "Initial Drug resistance to Anti-tubercular drugs in urban and rural district Tuberculosis programme." Ind J Tub, 1992, 39, 171.
48. Chang JC *et al.* igr genes and *Mycobacterium tuberculosis* cholesterol metabolism. J.Bacteriol. (2009); 191:5232-5239.
49. Chaovavanich A,Chottanapand S, Manosuthi W, Sungkanuparph S, Thongyen S. Survival rate and risk factors of mortality among HIV/Tuberculosis-coinfected patients with and without antiretroviral therapy. J Acquir Immune Defic Syndr. 2006; 43:42-6.
50. Chitra C, Prasad CE, Evaluation of MGIT for primary isolation of *Mycobacteria* from clinical samples. Ind J Tuberculosis, 2001; 48:155-6.

51. Chiyoji ABE, Kazue Hirano, Tetsuo Tomiyama. Simple and rapid identification of *Mycobacterium tuberculosis* complex by Immunochromatographic assay using Anti-MPB64 Monoclonal antibodies. J Clin Microbiol. Vol 37 (11), 3693-3697.
52. Choong Park, Deborath Hixon, Carolyn Furguson *et al*., Rapid recovery of *Mycobacteria* from clinical specimens using Automated Radiometric technique. Am J Clin Path, March 1984; 81(3):341-345.
53. Choong Park. Deborah Hixon, Carolyn Furguson *et al*. Rapid recovery of *Mycobacteria* from clinical specimens using Automated Radiometric technique. Am J Clin Path. March 1988; 1(7):41-52.
54. Chretien J. Tuberculosis and HIV. The Cursed duet. Bull Int Union Tuberc Lung Dis, 1990; 65: 25-28.
55. Christopher SW Koehler. "Consumption, the great killer", MDD-Chemistry (February 2002); 5(2):47-49. Available on http://pubs.acs.org/subscribe/archive/mdd/v05/i02/html/02timeline.html.
56. Closs O, Harboe M *et al*., 1980. The antigens of Mycobacterium bovis, strain BCG, studied by crossed immunoelectrophoresis: a reference system. Scand J Immunol, 12,249-63.
57. COBAS-R TaqMan –R MTB Test. http://molecular.roche.com/assays/pages/COBASTaqManMTB Test.aspx
58. Cole ST. "Comparative and functional genomics of the *Mycobacterium tuberculosis* complex." Microbiology (2002); 148: 2919-2928.
59. Coronel J, Roper H, Caviedes L *et al*., MODS: a user guide (v 12.1). Microscopic observation drug susceptibility assay.
60. Coronel J, Roper M, Mitchell S *et al*., MODS accreditation process for regional reference laboratories in Peru: validation by GenoType(R) MTBDRplus. Int J Tuberc Lung Dis 2010; 14:1475-1480.
61. Crofton J, Chault P, Maher D. Guidelines for the management of drug-resistant Tuberculosis. Geneva, WHO; 1997:31-7.
62. Daniel TM and Janicki BW, 1978. *Mycobacterial* antigens: a review of their isolation, chemistry and immunological properties. Microbiol Rev, 42:84-113.
63. Daniels UJ, Washington DC. Blood picture in Tuberculosis. Journal of the National Medical Association.1918 (1):38-41.
64. Dash Muktikesh. Drug-resistant tuberculosis: A diagnostic challenge. J Postgrad Med 2013; 59:196-202.
65. David AJ, Moore, Daniel Mendoza, Robert H, Gillman *et al.*, Microscopic observational drug susceptibility assay, a rapid reliable diagnostic test for multidrug resistant tuberculosis suitable for use in resource poor settings. J Clin Microbiol. 2004; 42(10): 4432-37.

66. Deivanayagam CN, Rajasekaram S, Senthilnathan V, Krishnarajaseeekhar R, Raja K, Chandrasekhar C, *et al.* Clinicoradiological spectrum of tuberculosis among HIV sero-positives a Tambaram study. Indian J Tuber 2001; 48:123-7.
67. Deivanayagam CN, Rajasekaran S, Venkatesan R, Mahilmaran A, Ahmed PR, Annadurai S, Kumar S, Chandrasekar C, Ravichandran N,Penicillaiah R. Prevalence of acquired MDR-TB and HIV co-infection. Indian J Chest Dis Allied Sci. 2002; 44(4):237-42.
68. Demissie M,Lemma E, Gebeyhu M, Lindtjom B. Sensitivity to anti-tuberculosis drugs in HIV-positive and -negative patients in Addis Ababa. Scand J Infect Dis 2001; 33:914-9.
69. Deshmukh SR, Mantri SB, Kendre PB, Nagoba BS. A comparison of sputum examination for AFB by modified Schaffer and Fulton stain, Zeihl Neelsen stain and cold stain. Ind. J. Med. Reasearch, 1996; 103:294-95.
70. Dheda K, Booth H, Huggett JF *et al.* Lung remodeling in pulmonary tuberculosis. J Infect Dis. 2005; 192:1201-1210. Doi:10.1371/journal.pone.0057752 (http://dx.doi.org/10.1371%2Fjournal.pone.0057752).
71. Dolin, [edited by] Mandell GL, Bennett JE, Raphael (2010). *Mandell, Douglas, and Bennett's principles and practice of infectious diseases* (7th edi). Philadelphia, PA: Churchill Livingstone/Ejsevier.pp. Chapter 250.ISBN 978-0443068393.
72. Douglas AS, Strachan DP, Maxwell JD (1996). "Seasonality of tuberculosis: the reverse of the other respiratory diseases in the UK". *Thorax* 51:944-946
73. Drews S, Eshaghi A, Pyskir D, *et al*., The relative test performance characteristics of two commercial assays for the detection of *Mycobacterium tuberculosis* complex in paraffin-fixed human biopsy specimens. Diagn Pathol 2008; 3:37.
74. Dungra AY, Adjoh K, Tchaptchhhet Heunda S, Patassi AA, Sadzo D, Hetsu F *et al.* Prevalence of HIV-TB co-infection and impact of HIV infection on pulmonary tuberculosis outcome in Togo. Bull Soc Pathol Exot. 2011 Dec.; 104(5):342-6.
75. Earson DA Harries AD. Historical background and global epidemiology of *Mycobacterium tuberculosis* resistance. Guidelines for clinical and operational management of drug-resistant Tuberculosis. International Union Against Tuberculosis and Lung Diseases, 2013; chapter-2:7-8.
76. Edlin BR, Tokars JI, Grieco MH, Crawford JT, Williams JJ, Sordillo EM, Ong KR, Kilburn JO, Dooley SW, Castro KG, Jarvis WR, Holmberg SD. An outbreak of Multidrug-resistant Tuberculosis among hospitalized patients with the acquired immunodeficiency syndrome. New England Journal of Medicine, (1992), 326(23):1514-21.

77. Emarson DA, Rieder HL, Amadottir T, Trebcq A. Management of tuberculosis, a guide for union against tuberculosis and lung disease (IUATLD), Tuberculosis and HIV; 10-53.
78. Enrico Tortoli, Paola Cichero, Gabriella Chirillo *et al*., Multicentre comparison of ESP culture system II with BACTEC 460TB and with LJ medium for recovery of *Mycobacteria* from different clinical specimens, including blood. J Clin Microbiol.1998; 36(5):1378-81.
79. Eugen-Olsen J, Gustafson P, Idenius N *et al.,* 2002. The serum level of soluble urokinase receptor is elevated in tuberculosis patients and predicts mortality during treatment, a community study from Guinea-Bissau. Int J Tuberc Lung Dis, 6:686-92.
80. European concerted action- RIVM, Bithoven, the Netherlands. Beijing/W Genotype M. tuberculosis and Drug-resistance. Emerging Infectious Diseases-www.cdc.gov/edi. May 2006; 12(5): 737.
81. Fandinho FCO, Kritski AL, Hofer C *et al*., Drug resistance patterns among hospitalized patients in Rio De Janerio, Brazil. Mem. Inst. Oswaldo. Cruz. 1999; 94 (4): 543-547.
82. Farmer P. The major infectious diseases in the world- to treat or not to treat? New England Journal of Medicine. (2011), 345 (3):208-10.
83. Farnia P, Masjedi MR, Mohammadi F *et al*., Colorimetric detection of multidrug-resistant or extensively drug-resistant tuberculosis by use of malachite green indicator dye. J Clin Microbiol 2008; 46:796-799.
84. Ferguson JS, Weis JI, Martin JL, Schlesinger LS. Complement protein C3 binding to *Mycobacterium tuberculosis* is initiated by the classical pathway in human bronchoalveolar lavage and fluid. Infect Immun.2004; 72:2564-2573.
85. Fernandez L, Lawande D, Mesquita AM. "Prevalence of HIV infection in Tuberculosis patients in Goa." Ind J Tub, 2002; 49:235.
86. Ferra G *et al.* Use in routine clinical practice of two commercial blood tests for diagnosis of infection with *M.tuberculosis*: a prospective study. Lancet, 2006; 367(9519):1328-1334.
87. FinePEM, Cameiro IAM, Milstein JB, Clements CJ. Issues relating to the use of BCG in immunization programs. Geneva: WHO (1999)
88. Fischl MA, Uttamchandani RB, Daikos GL, Poblete RB, Moreno JN, Reyes RR, Boota AM, Thompson LM, Cleary TJ, Lai S. An outbreak of tuberculosis caused by multiple-drug-resistant tubercle bacilli among patients with HIV infection. Annals of Internal Medicine, (1992); 117(3):177-83.
89. Forbes BA, Sahm DF, Weissfeld AS. Mycobacteria. In: Bailey and Scott's Diagnostic Microbiology, 12th edition, Elsevier Science, United States of America. 2007; 480-481.

90. Frieden TR, Sterling T, Pablos Mendez A, Kilburn JO, Cauthen GN, Dooley SW. The emergence of Drug Resistant Tuberculosis in New York City. N Engl J Med 1993; 328:521-26.
91. Frieden TR, Sterling TR, Munsiff SS, Watt CJ, Dey C. Tuberculosis. Lancet 2003; 362:887-899.
92. Fukuda Mahito: The romantic images of Tuberculosis. (A Cultural history of tuberculosis in modern Japan), Nagoya University Press, 1995.
93. Gaby Pfyffer, Cornelia Cieslak, Hans-Matin, Welscher *et al*., Rapid detection of *Mycobacteria* in clinical specimens by using the automated BACTEC 9000 MB system and comparison with radiometric and solid culture systems. J Clin Microbiol. 1997; 35(9): 2229-34.
94. Gaby E, Pfyffer, Vincent V. MTB complex, *M. leprae* and other slow growing *Mycobacteria*, In: Borriello SP, Murray PR, Funke G editors, Topley and Wilson. Microbiology and Microbial infections. 10th edi, American Society for Microbiology. UK. 2005; 2:1181-1235.
95. Garza-Gonzalez E, Gonzalez GM, Renteria A *et al*., A pyrosequencing method for molecular monitoring of regions in the inhA, ahpC, and rpoB genes of *Mycobacterium tuberculosis*. Clin Microbiol Infect Dis 2010; 16: 607-612.
96. GenoType MTBDRplus, Version 1.0[product insert]. Germany: Hain Life Science, GmbH. Available from: http://www.hain-lifescience.de/en/products/microbiology/mycobacteria/genotype-mtbdrplus.
97. Gerri S Hall. Primary processing of specimens and isolation and cultivation of *Mycobacteria.* Clinical Mycobacteriology, Clinics in Lab Medicine. Sept 1996; 16(3):551-567.
98. Gerri SH. Isolation and identification of *Mycobacteria*. Clinical Mycobacteriology, Clinics in Laboratory Medicine; 1998; 18(3):851-867.
99. Githui W, Kitui F, Juma ES, Obwana DO, Mwai J, Kwamasga D. A comparative study on the reliability of the fluorescence microscopy and Ziehl Neelsen method in the diagnosis of Pulmonary Tuberculosis. East African Med J. 1993; 70:263-266.
100. Global Tuberculosis Control. Available at - (http://www.who.int/tb/publications/global_report/en/index.html), World Health Organization, 2011.
101. Goel D. Bedaquiline: A novel drug to combat multiple drug-resistant tuberculosis. J Pharmacol Pharmacotherapy 2014; 5:76-8.
102. Govindarajan KK, Chain FY. BCG adenitis- need for increased awareness. Mal J Med Sci (2011),18 (2):67-70.Malaysian Journal of Medical Sciences (http://www.mjms.usm.my/default.asp?tag=14andkod_volum=48)

117. Isenberg HD, Amito RF, Heifts L *et al*. Collaborative feasibility study of a biphasic system (Roche Septi Check AFB) for rapid detection and isolation of *Mycobacteria*. J Clin Microbiol.1991; 29:1719-22.
118. Jain A, Mondal R, Prasad R, Singh K, and Ahuja RC. Prevalence of multidrug resistant *Mycobacterium tuberculosis* in Lucknow, UP. Indian J Med Res. September 2008, 128:300-306.
119. James L Vossler. Culture media and isolation methods. *Mycobacterium tuberculosis* and Non-tuberculous *Mycobacteria*. Chapter 22, part-2, in Textbook of Diagnostic Microbiology, 2nd edition. Connie R, Mohan, George Manuselis. W.B.Saunders Company; 2000:667-708.
120. James P, Gupta R, Christopher DJ, Thankgunam B, Veeraraghavan B. MDR- and XDR-TB among suspected drug-resistant TB patients in a tertiary care hospital in India. Clin Respir J. 2011; 5(1): 19-25.
121. Jena J. Assessment of trisodium phosphate (TSP) penicillin transport medium and single step method of culture. Ind J of Tuberculosis. 1997; 3:176.
122. Jensen PA, Lambert LA, Lademarco MF, Ridzon R. Centers for Disease control and prevention. Guidelines for preventing the transmission of *Mycobacterium tuberculosis* in health-care settings. MWMR Recomm Rep. 2005; 54(RR-17):1-141.
123. Joe M, Bai Y, Nacario RC, Lowary TL. Synthesis of the docosanasaccharide arabinan domain of mycobacterial arabinogalactan and a proposed octadecasaccharide biosynthetic precursor. J Am Chem Soc. 2007, 129 (32) 9885-9901.
124. John Grange. Tuberculosis, Chapter 3.7 in Topley and Wilson's Principles of Bacteriology, Virology and Immunology, vol.III, 8thedition. Parker MT, Duerdev BI. Edward Arnold Ltd. London 1990; 93-122.
125. Johnson R, Streicher E M, Louw G E, Warren R M, Paul D, Victor T C. Drug Resistance in *Mycobacterium tuberculosis* Genomics and Mol Biol. January 2009 8: 97-112. Online journal at www.cimb.org.
126. Karakousis PC, Bishai WR *.et al. Mycobacterium tuberculosis* cell envelope lipids and the host immune response. Cell Microbiol 2004; 6(2): 105-116.
127. Karakousis PC. Mechanisms of Action and Resistance of antimycobacterial agents. In Antimicrobial drug Resistance. Mayers DL, Springer; 271-291.
128. Kebede D, Mitike G, Yeneneh H. HIV Infection and Antituberculosis Drug Resistance among Pulmonary Tuberculosis patients in Harar Tuberculosis center, Ethiopia. East African Med J. 1997; 74:154-7. Available from: http://cat.inist.fr/?aModele=afficheNandcpsidt=2791912.
129. Kembhavi P, Menon S, Hirani N, Wable V and Joshi A. Rapid and economical detection of multidrug resistant (MDR) tuberculosis: Can rifampicin resistance (RMP r) be a surrogate marker for MDR-TB? RRJMB, 2013; 2(3):78:84.

130. Kendall SL *et al.* A highly conserved transcriptional repressor controls a large regulon involved in lipid degradation in *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. Mol. Microbiol. (2007); 65:684-699.
131. Khanna A, Raj VS, Tarai B, Sood R, Pareek PK, Upadhyay DJ, Sharma P, Rattan A, Saini KS, Singh H. Emergence and molecular characterization of extensively drug-resistant *Mycobacterium tuberculosis* from clinical isolates from the Delhi region in India, Antimicrob Agents Chemother. 201; 54(11):4789-93.
132. Khatri GR, Frieden TR. The status and prospects of tuberculosis control in India. Int J Tuberc Lung Dis 2004; 4:193-6.
133. Kiet VS, Lan NTN, An DD *et al*., Evaluation of the MTBDRsl test for detection of second-line-drug resistance in *Mycobacterium tuberculosis*. J Clin Microbiol 2010; 48: 2934-2939.
134. Kim JH, Kim YJ, Kim CS *et al*., Evaluation of COBAS TaqMan(R)MTB PCR for the detection of *Mycobacterium tuberculosis*. J Clin Microbiol 2011; 49:173-176.
135. Kim H, Seo M, Park YK, Yoo JI, Lee YS, Chung TC and Ryoo S. Evaluation of MGIT960 system for the second-line drugs susceptibility testing of *Mycobacterium tuberculosis*. Tuberculosis Research and Treatment. 2013(2013), Article ID 108401.
136. Kochi A. The Global Tuberculosis situation and new control Strategy of the World Health Organization. Tubercle; 1991:72:1.
137. Kochi, Vareldzis AB, *et al.* Multidrug-resistant tuberculosis and its control. Res Microbiol 1993; 144(2):104-110.
138. Koef JE, Pynaert G, Tournoy K *et al.* Macrophage reprogramming by mycolic acid promotes a tolerogenic response in experimental asthma. Am J Respir Crit Care Med.2006; 174(2):152-160.
139. Koh GCKW, Hawthorne G, Turner AM, Kunst H, Dedicoat M. Tuberculosis incidence correlates with sunshine: an ecological 28-year time series study. *PLoS ONE*.
140. Kolyva AS, Karakousis PC. Old and New TB Drugs: mechanisms of Action and Resistance. In Understanding tuberculosis- New Approaches to Fighting Against drug resistance.2012; 209-232.
141. Koneman EW, Allen SD, Janda WM, Schrenkenberger PC, Winn WC. *Mycobacteria,* in- Colour Atlas and Textbook of Diagnostic Microbiology. 5th edition, Lippincott Williams and Wilkins, Philadelphia. 1997.
142. Korthals AH, Kremer K, Erkens C, Van Solingen D, Wallinga J. Tuberculosis seasonality in the Netherlands differs between natives and non-natives: a role for vitamin D deficiency? Int J Tuberc Lung Dis (2012); 16: 639-644.doi: 10.5588/ijtld.11.0680 (http://dx.doi.org/10.5588%2Fijtld. 11.0680).

143. Krishnaswamy Rahim *et al.* "Primary drug resistance in pulmonary Tuberculosis." Ind J Chest Dis 1976; 28:233-237.
144. Kumar V, Abbas AK, Fausto N, Mitchell RN (2007). Robbins Basic Pathology (8th edi). Saunders Elsevier. Pp. 516-522. ISBN 978-1-4160-2973-1.
145. Kumarasamy N, Vallabhaneni S, Flanigan TP, Mayer KH, Solomon S. Clinical profile of HIV in India. Indian J Med Res 2005; 121:377-94.
146. Lee RB, L I w, chatterjee D, Lee RE. Rapid structural characterization of the arabinogalactan and lipoarabinomannan in live mycobacterial cells using 2D and 3D HR-MAS NMR structural changes in the arabinan due to ethambutol treatment and gene mutation are observed. Glycobiology. 2005; 15(2:139-151).
147. Lemaire JF, Casenghi M. New diagnostics for tuberculosis: fulfilling patient needs first. J Int AIDS Soc 2010; 13:40.
148. Li Y, Petrofsky M, Bermudez LE. *Mycobacterium tuberculosis* uptake by recipient host macrophages is influenced by environmental conditions in the granuloma of the infectious individual and is associated with impaired production of interleukin-12 and tumor necrosis factor alpha. Infect Immun. 2002; 70:6223-6230.
149. Lin SYG, Desmond E, Bonato D *et al*., Multicentre Evaluation of BACTEC MGIT 960 system for second-line drug susceptibility testing of Mycobacterium tuberculosis complex. J Clin Microbiol 2009; 47:3630-3634.
150. Ling DI, Zwerling AA, Pai M. GenoType MTBDR assays for the diagnosis of multidrug-resistant tuberculosis: a meta-analysis. Eur Respir J 2008; 32:1165-1174.
151. Lingala MAL, Srikantam A, Jain S, Rao KVSM, Rao PVR. Clinical and geographical profiles of *rpoB* gene mutations in *Mycobacterium tuberculosis* isolates from Hyderabad and Koratput in India. J Microbio Antimicrobio. 2010; 2(2):13-18.
152. Lu D, Heeren B, Dunne WM. Comparison of the automated MGIT with Lowenstein Jensen media for recovery of *Mycobacteria* from clinical specimens. Am J Clin Patho. 2002; 118:542-3.
153. Mackie and McCartney. Practical Medical Microbiology, 13th Edition by Collee JG, Duguid JP, Fraser AG and Marmion BP; Churchill Livingstone, 1989.
154. Madkour, Monir M, Warrell DA (2004) "Tuberculosis" Birkhauser. ISBN 3540014411.
155. Mahler HT, Mohamed AP. Review of mass B.C.G. projects in India, (1955). (http://openmed.nic.in/04/). Ind.J. Tuberculosis 2 (3): 108-16.
156. Mak TK, Hesseling AC, Hussey GD, Cotton MF. Making BCG vaccination programs safer in the HIV era. Lancet (2008), 372(9641): 786-7. 218
157. Mandell GL, Benner JE, Dolin R. Principles and Practice of Infectious Diseases, 7th edition, 2010, 3129-63.

158. Marina AF, Laura IK, Andrea G, Julia SYG, Hector RM, Maria PS, Angel AC and Fabiana B. Virulence factors of *Mycobacterium tuberculosis* complex. Virulence, Jan 1, 2013; 4(1):3-66.
159. Mario C, Raviglione, Richard J, O'Brien, Mycobacterial Diseases. In : Fauci AS, Braunwald E,Kasper D, Hauser L, Jannison D editors, Harrison textbook of Medicine, 17th edition, Mc Graw Hill Companies, Boston, 2008; 1: 1006-1027.
160. Martin A, Portales F, Palomino JC. Colorimetric redox-indicator methods for the rapid detection of multidrug resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. J Antimicrob Chemother 2006; 59: 175-183.
161. Martin A, Von Groll A, Fissette K *et al.* , Rapid detection of *Mycobacterium tuberculosis* resistance to second-line drugs by use of the manual Mycobacterium Growth Indicator Tube system. J Clin Microbiol 2008; 46:3952-3956.
162. Martin A, Panaiotov S, Portales F *et al.,* The nitrate reductase assay for the rapid detection of isoniazid and rifampicin resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. J Antimicrob Chemother, 2008; 62:56-64.
163. Martineau AR, Nhamoyebonde S, Oni T, Rangaka MX, Marais, *et al.* (2011). "Reciprocal seasonal variation in vitamin D status and tuberculosis notifications in Cape Town, South Africa". Proc Natl Acad Sci USA ,108: 19999013-19017
164. Maurya AK, Kant S, Nag VL, Kushwaha RA, Dhole TN. Trends of anti-TB drug resistance pattern in new cases and previously treated cases of extrapulmonary tuberculosis cases in referral hospitals in northern India. J Postgrad Med.2012 Jul-Sep; 58(3):185-9.
165. Maurya AK, Singh AK, Kumar M, Umrao J, Kant S, Nag VL, Kushwaha R, Dhole TN. Changing patterns and trends of multidrug-resistant tuberculosis at referral centre in northern India: A 4 year experience. IJMM, 2013; 31(1):40-46.
166. Mazurek GH, Jereb J, Lobue P *et al*. Guidelines for using Quantiferon-TB Gold test for detecting *Mycobacterium tuberculosis* infection. MMWR54:15, 2005
167. Mc Carthy OR. The key to the sanatoria. J R Soc Med 2001:94; 413-7.
168. McKay B. Risk of deadly TB exposure grows along U.S. - Mexico Border. The Wall Street Journal (paper).p.A1.
169. Menon S, Dharmhale S, Chande C, Gohil A, Lilani S, Mohammad S, Joshi A, Chaudhari A, Bhardwaj R. Drug resistance profiles of *Mycobacterium tuberculosis* isolates to first line anti-tuberculosis drugs: A five years study. Lung India, 2012; 29(3):227-231.
170. Migliori G B, Loddenkemper R, Blasi F, Raviglione M C. 125 years after Robert Koch's discovery of the tubercle bacillus: the new XDR-TB threat. Is

"science" enough to tackle the epidemic? Eur Respir J, 2007; 29:423-427. Available on http://ersjournals.com/content/29/3/423.full.

171. Migliori GB, Matteelli A, Cirillo D *et al.,* Diagnosis of multidrug-resistant tuberculosis and extensively drug-resistant tuberculosis: current standards and challenges. Can J Infect Dis Med Microbiol 2008; 19: 169-172.
172. Migliori GB, Dheda K, Centis R *et al*. Review of multi-drug resistant and extensively drug-resistant TB: Global perspectives with a focus on sub-Saharan Africa. Trop Med Int Health, 2010; 15:1052-1066.
173. Minton J, Leung E, Menzies D *et al*., Microscopic observation, drug susceptibility and thin layer agar assays for the detection of drug resistant tuberculosis: a systematic review and meta-analysis. Lancet Infect Dis 2010; 10:688-698.
174. Miranda A, Morga M, Jamal L, Laserson K, Barreira D, Silva G *et al*. Impact of antiretroviral therapy on the incidence of tuberculosis; The Brazillian experience, 1995-2001. PloS One 2007; 2:e826.
175. Mishra G, Mulani J. Tuberculosis prescription practices in private and public sectors in India. NJIRM. 2013; 4(2):71-78. Available online at http://www.scopemed.org/?mno=36915. Accessed on 6/5/2013.
176. Mishra G, Ghorpade SV, Mulani J. XDR-TB:- An outcome of programmatic management of TB in India. Indian J of Medical Ethics, 2014; 11(1):47-52.
177. Mokrouusov I, Narvskaya O, Otten T, Limeschenko E, Steklova L, Vyshnevskiy B. High prevalence of KatG Ser315 Thr substitution among isoniazid-resistant *Mycobacterium tuberculosis* clinical isolates from northwestern Russia, 1996-2001. Antmicrob Agents Chemother, 2001; 46:1417-24.
178. Molecular line probe assay for rapid screening of patients at risk of multi-drug resistant Tuberculosis (MDR-TB): WHO policy statement, 27/6/2008. Available at http://www.who.int/tb/laboratory/line_probe_assays/en/index.html.
179. Mondal R and Jain A. Extensively drug-resistant *Mycobacterium tuberculosis*, India. Emerging Infect Dis. Sept2007, 13(9):1429-31.
180. Morcillo N, Imperiale B, Di Giulio B. Evaluation of MGIT 960 and the colorimetric-based method for tuberculosis drug susceptibility testing. Int J Tuberc Lung Dis 2010; 14:1169-1175. Reviews the value of the MGIT 960 system for DST.
181. Morgan M, Kalantri S, Flores L and Pai M. A commercial line probe assay for the rapid detection of rifampicin resistance in *M. tuberculosis*: a systematic review and meta-analysis. BMC Infect Disease, 2005 ;(5):62.
182. Mulenga C, Chonde A, Bwalya IC, Kapata N, Kakungu SM, Docx S and Rigouts L. Low occurrence of Tuberculosis drug resistance among pulmonary Tuberculosis patients from an urban setting, with a long-running DOTS

program in Zambia. Tuberculosis Research and Treatment, 2010 (2010); Article ID\938178.
183. Multi-drug and Extensively drug-resistant tuberculosis (M/XDR-TB), 2010. Global report on surveillance and response. WHO/HTM/TB/2010.3
184. Myer's and Koshi's Manual of Diagnostic Procedures in Medical Microbiology and Serology/Immunology. Faculty of Department of Clinical Microbiology, Christian Medical College and Hospital, Vellore, India. Kennedy nagar, Pondicherry, India: All India Press; 2001.
185. Myneedu VP, Visalakshi P, Verma AK, Behera D, Bhalla M. Prevalence of XDR-TB cases- a retrospective study from a tertiary care TB hospital. Indian J Tuberc. 2011; 58(2):54-9.
186. Narang P. Drug Resistance in Tuberculosis- Conventional Methods. CME: Drug Resistance in Tuberculosis, 2009; 27-30.
187. Narita M, Ashkin D, Hollander ES, Pitchenik AE. Paradoxical worsening of tuberculosis following antiretroviral therapy in patients with AIDS. Am Crit Care 1998; 158:157-61.
188. National AIDS Control Organization. Antiretroviral therapy guidelines for HIV-infected adults and adolescents including post-exposure prophylaxis (section 47). New Delhi, India: National AIDS Control Organization; 2007.
189. National HIV/AIDS and tuberculosis control programs. National Guidelines for TB/HIV co-management ministry of health and child welfare, Zimbabwe. Republic of Zimbabwe National Guidelines for TB/HIV co-management.
190. NCBI. 24 May 2007. Welcome Trust Sanger Institute. 2 June 2007. http://www.nicbi.nlm.nih.gov/sites/entrez?db=genomeprjandcmd=Retrieveanddopt=Overviewandlist_uids=224 .
191. Negi SS, Basir SF, Gupta S, Pasha ST, Khare S, Lal S. Comparison of the conventional diagnostic modalities, BACTEC culture and Polymerase chain reaction test for diagnosis of Tuberculosis. Ind J Med Microbiol 2005; 23:29-33.
192. Neonakis IK, Gitti Z, Baritaki S *et al*., Evaluation of Geno-Type *Mycobacteria* direct assay with Gen-Probe *Mycobacterium tuberculosis* amplified direct test and Geno-Type MTBDR plus for direct detection of *Mycobacterium tuberculosis* complex in clinical samples. J Clin Microbiol 2009; 47: 2601-2603.
193. News- Anon Increase in number of TB patients (TB Control Dept., Maharashtra state); Daily Sakal, 24th March, 2014.
194. NiCad LP. Immunology of Tuberculosis. Swiss Med Wkly.2007; 137(25-26):357-362.280
195. Nicol MP. New developments in the laboratory diagnosis of tuberculosis. Continu Med Educ 2010; 28:246-250

196. Ntiindia kar.nic.in [Internet]. India: National Tuberculosis Institute, Bangalore, India; available from: http;//www.theglobalfund.org/en/commitmentsdisbursements/lang=eng c2010.
197. Nwobu GO, Okodua MA, Tatfeng YM. Comparative study of HIV associated pulmonary tuberculosis in chest clinics from two regions of Edo state, Nigeria Online j Health Allied Scs. 2004; 3-4.
198. Pai M, Minion J, Sohn H *et al*., Novel and improved technologies for tuberculosis diagnosis: progress and challenges. Clin Chest Med 2009, 30:701-716.
199. Palomino JC, Martin A, Von Groll A *et al*., Rapid culture-based methods for drug-resistance detection in *Mycobacterium tuberculosis*. J Microbiol Methods 2008; 75:161-166.
200. Pandey AK and Sassetti CM. Mycobacterial Persistence Requires the Utilization of Host Cholesterol. Proc. Natl. Acad. Sci. U.S.A. (2008); 105:4376-4380.
201. Paramasivan CN, Bhaskaran K, Venkatraman, Chandrasekaran V, Narayanan PR. Surveillance of Drug resistance in Tuberculosis in the state of Taminadu. Ind J Tub 2000; 47:27.
202. Paramsivan CN. Surveillance of Drug resistance in India. In: Agarwal SP, Chauhan LS, editors. Tuberculosis Control in India. India, New Delhi, India: Directorate General of Health Services, Ministry of Health and Family Welfare; 2005:47-52.
203. Parrinello CM, Crossa A, Harris TG. Seasonality of tuberculosis in New York City, 1990-2007. Int J Tuberc Lung Dis (2012); 16: 32-37.
204. Pease, Stanley A. Some remarks on the diagnosis and treatment of Tuberculosis in Antiquity. Isis (April 1940), 31(2):380-393. JSTOR 225758 (//www.jstor.org/stable/225758).
205. Peter Eriki, Alphonse Okwera, Tom Aisu, Anne B. Morrissey, Errold J Ellner, Thomas M. Danniel. The influence of HIV infection in Kampala, Uganda. Am. Rev Respir. Dis. 1991; 143: 185-187.
206. Peter Rohmer, Beatrice Ninet, Catherine Metral *et al*. Evaluation for isolation of *Mycobacteria* from clinical specimens. J Clin Microbiol.1997; 35(12):3127-31.
207. Petran EL, Vera HD. Media for selective isolation of *Mycobacteria.* Health Lab. Sciences. 1971; 2238-2245.
208. Philipp WJ *et al.* An integrated map of the genome of the tubercle bacillus, *Mycobacterium tuberculosis* H37RV, and comparison with *Mycobacterium leprae*. Proc Natl Acad. Sci. USA.1996; 93: 3132-3137.
209. Piersimoni C, Olivier A, Benacchio L *et al*., Current perspectives on drug susceptibility testing of *Mycobacterium tuberculosis* complex: the automated non-radiometric systems. J Clin Microbiol 2006; 44:20-28.

210. Piersimoni C, Scarparo C, Callegaro A and Passerini C. Comparison of MB/BacT ALETR 3D System with Radiometric BACTEC system and Lowenstein- Jensen medium for recovery and identification of *Mycobacteria* from clinical specimens: a Multicentre study. J Clin Microbiol, 2000; 39(2); 651-657.
211. Pitchenik A, Burr J, Laufer M, Miller G, Cacciatore R, Bigler W, Witte J, Cleary T. Outbreaks of drug-resistant tuberculosis at AIDS centre. The Lancet, (1990); 336(8712):440-1.
212. Porth CM. Alterations in respiratory function: respiratory tract infections, neoplasms and childhood disorders. In: Porth CM, Kunert MP. Pathophysiology: Concepts of Altered Health States. Philadelphia, PA: Lippincott Williams and Wilkins; 2002:615-619.
213. Prasad H,Singhal A, Mishra A, Shah N, Katoch V, Thakral S ***et.al.*** Bovine tuberculosis in India; Potential basis for zoonosis. Tuberculosis 2005; 85:421-8.
214. Prasad R, Saini JK, Kannaujia RK, Sarin S, Suryakant, Kulshreshth R, Nag VL & Tripathi AK. Trends of HIV infection in patients with Pulmonary Tuberculosis in Luknow area. Ind J Tub, 2013; 50:39.
215. Prasad R. Multidrug and extensively drug resistance TB (M/XDR-TB): problems and solutions. Indian J Tuberc. 2010; 57(4):180-91.
216. Proceedings of 5th All India BCG Conference; 1962; Bangalore, India.
217. Proceedings of the Tuberculosis Association of India. New Delhi, India: Tuberculosis Association of India; 1939.
218. Purwar S, Chaudhary S. Katoch VM, Sampath A, Sharma P, Upadhyay P, Chauhan DS. Determination of drug susceptibility patterns and genotypes of *Mycobacterium tuberculosis* isolates from Kanpur district, North India. Infect Genet Evol. 2011 Mar; 11(2: 469-75).
219. Quality-assured laboratory methods for determining resistance to first and second-line anti-TB drugs. Guidelines for surveillance of drug resistance in Tuberculosis. WHO DST TB; 2007.
220. Raizada N, Sachdeva KS, Chauhan DS, Malhotra B, Reddy K ***et al.*** A multi-site validation in India of the line probe assay for multi-drug resistant tuberculosis directly from sputum. PLoS ONE, 9(2): e886. Available at-http://www.hain-lifescience.com/pdf/304xx_ppd.paf.
221. Raja A. Immunology of Tuberculosis. Indian J Med Res October 2004; 120:213-232 .
222. Rajshekaran S, Lima A, ***et.al.*** Trends of HIV infection in patients with Tuberculosis in Rural South India. Ind J Tub, 2000, 47,223.
223. Ramchandran R ***et.al.*** "Mycobacterium tuberculosis in patients with HIV infection". Ind J Tub 2002, 501-29.

224. Ramswamy S, Musser J. Molecular genetic basis of anti-microbial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tubercle and Lung disease. 1998; 79:3-29.
225. Rao KP. A cold staining method for tubercle bacilli using chloroform. Indian J Tuberc, 1966; 14:3-7.
226. Ratledge CR. The Biology of the Mycobacteria. Academic, San Diego, 1982:53-94.
227. Ravindran R, Wattal C, Oberoi JK, Goel N, Datta S, Prasad KJ. Utility of GenoType MTBDRplus assay in rapid diagnosis of multi drug resistant tuberculosis at a tertiary care centre in India. Indian J Med Microbiol, 2012; 30:58-63.
228. Rawat J, Sindhwani G, Juyal R, Dua R. Five year trend of acquired anti-tubercular drug resistance in patients attending a tertiary care hospital at Dehradun. Lung India.2009; 26(4): 106-8.
229. Revised National Tuberculosis Control Program. Training Manual for *Mycobacterium tuberculosis* Culture and Drug Susceptibility Testing, Central Tuberculosis Division, New Dehli;2009:1-76.
230. Revised National Tuberculosis Control Programme, Tuberculosis India, 2011. Available on www.tbfacts.org/**tb-india**.html.
231. Revised National Tuberculosis Control Programme. Guidelines on Programmatic Management of Drug Resistant TB (PMDT) in India. May 2012. Chapter 7: treatment of MDR and XDR-TB. Central TB Division, Directorate General of Health Services, Ministry of Health and Family Welfare, Nirman Bhavan, New Delhi-110011.
232. Richard SB, Louis ME, Nieburg P *et al.*, Impact of the HIV epidemic on trends in Tuberculosis in Abidjan, Cote d'lvoire. Tubercle and Lung Diseases, 1995; 76:11-16.
233. Rishi S, Sinha P, Malhotra B, Pai N. A comparative study for the Detection of *Mycobacteria* by BACTEC MGIT 960, Lowenstein Jensen's Media and Direct AFB smear examination. Ind. J of Med. Microbiol. 2007; 25(4):383-386.
234. Riska PF, Su Y, Bardarov S, Freundlich L, Sarkis G *et al*., Rapid film based determination of Antibiotic susceptibilities of Mycobacterium tuberculosis strains by using a Luciferase reporter phage and Bronx box. J Clin Microbiol 1999; 37:1144-1152.
235. Rodrigues CS, Shenai SV, Almedia D, Sadani MA, Goyal N, Vadher C, Mehta AP. Use of Bactec- 460TB system in the diagnosis of Tuberculosis. Ind J Med Microbiol 2007; 25:32-6.
236. Roggenkamp A, Hornef MW, Masch A, Aigner B, Autenrieth IB. Comparison of MB/BacT and BACTEC 460 TB Systems for Recovery of *Mycobacteria* in a Routine Diagnostic Laboratory. J Clin Microbiol 1999; 37(11):3711-3712.

237. Rohmer P, Ninet B, Metral C, Emler S, Auckenthler R. Evaluation of the MB/BacT System and comparison to the BACTEC 460 System and solid media for isolation of *Mycobacteria* from clinical specimens. J Clin Microbiol 1997; 35(12):3127-3131.
238. Rosencrantz I, Slayden RA, Crawford J *et al.* Hypoxic response of *Mycobacterium tuberculosis* studied by metabolic labeling and proteome analysis of cellular and extracellular proteins. J Bacteriol.2002; 184:3485-3491.
239. Rowland K. Totally drug-resistant TB emerges in India: Nature News and Comment. Nature Publishing Group: science journals, jobs and information. Retrieved April 3, 2013, from http://www/nature.com/news/totally-drug-resistant-tb-emerges-in-india-1.9797
240. Sandgren A, Strong M, Muthukrishnan P *et al*., Tuberculosis drug resistance mutation database. PLoS Med 2009; 6:1000002.
241. Sandhu GK. Tuberculosis: Current situation, challenges and overview of its control programs in India. J Global Infect Dis 2011:3:143-50.
242. Santos LC. Review: The Molecular Basis of Resistance in *Mycobacterium tuberculosis*. Open Journal of Medical Microbiology, 2012; 2: 24-36.
243. Sethi S, Mewara A, Dhatwalia SK, Singh H, Yadav R, Singh K, Gupta D, Wanchu A and Sharma M. Prevalence of multidrug resistance in *Mycobacterium tuberculosis* isolates from seropositive and seronegative patients with pulmonary tuberculosis in north India. BMC Infectious Disease'2013, 13:137.
244. Shah A R, Agarwal SK, Shah KV. "Study of Drug resistance in previously treated Tuberculosis patients in Gujarat, India." Int J Tuberc Lung Dis, 2002; 69(12):1098. 1
245. Sharma SK, Mohan A, Kadhiravan T. HIV-TB coinfection: Epidemiology, diagnosis and management. Indian J Med Res 2005; 121:550-67. 146
246. Sharma SK, Kaushik G, George N, Arora SK, Gupta D, Sing U, Hanif M, Vaishali RP. Prevalence of multidrug-resistant tuberculosis among newly diagnosed cases of sputum- positive pulmonary tuberculosis. Indian J Med Res. 2011; 133(3):308-11.
247. Sharma SK, Kaushik G, Jha B, George N, Arora SK, Gupta D, Sing U, Hanif M, Vaishali RP. Prevalence of multidrug-resistant tuberculosis among category II Tuberculosis patients. Indian J Med Res. 2011; 133(3):312-5.
248. Siddiqi N, Shamim MD, Jain NK, Rattan A, Amin A, Katoch VM *et al.* Molecular genetic analysis of multi drug resistance in Indian isolates of *Mycobacterium tuberculosis*. Mem Inst Oswaldo Cruz, ARio de Janeiro, 1998; 93(5):589-594.

249. Sikand BK, Pamra SP. Domiciliary treatment-results of antibiotic therapy. Proceedings of the 13th TB Workers Conference, 1956; Trivandrum, India: TB Association of Trivandrum; 1956: 179-213.
250. Skerman VBD, McGowan V and Sneath PHA (editors): Approved lists of Bacterial Names. Int.J.Syst.Bacteriol. 1980, 30:225-420.
251. Srivastava K, Chauhan DS, Gupta P, Singh HB, Sharma VD, Yadav VS *et al*. Isolation of Mycobacterium bovis and M. tuberculosis from cattle of some farms in north India- Possible relevance in human health. Indian J Med Res 2008; 128:26-31.
252. Stager CE, Libonati JP, Siddiquis SH *et al*. Role of solid media when used in conjunction with the BACTEC system for *Mycobacterial* isolation and identification. J Clin Microbiol, Jan 1991; 29 (1):154-157.
253. Stanford JL and Grange YM. 1974. The meaning and structure of species as applied to *Mycobacteria*. Tubercle, 55:143-52.
254. Sureshkumar D and Gopalkrishnan R. Drug susceptibility pattern of *Mycobacterium tuberculosis* isolated from patients attending a private hospital. Am J Infect Diseases, 2011:7:104-106.
255. Sureshkumar D, Gopalkrishnan R and Street R. Drug susceptibility pattern of *Mycobacterium tuberculosis* isolated from patients attending a private hospital. American Journal of Infectious Diseases, 2011; 7(4):104-106.
256. TB India, 2013; RNTCP- Annual status report. Available on www.tbindia.nic.in
257. TB India-2013, RNTCP Repot- Executive summary. Available on www.tb.**india**.nic.in/pdfs/tb%20**india**%20 **2013**. Pdf.
258. TB Statistics for India. http://www.tbfacts.org/tb-statistics-india.html
259. TB Statistics for India.(2012). TB Facts. Retrieved April 3, 2013, from http://www.tbfacts.org/tb-statistics-india.html
260. Telenti A, Honore N, Bernasconi C, March J, Ortega A, Heym B *et al.* J Clin Microbiol 1997; 35:719-23.
261. The sixteenth global report on tuberculosis". Available at - (http://www.who.int/tb/publications/global_report/2011/gtbr11_executive_summary.pdf).2011.
262. Theglobalfund, org [Internet]. The Global Fund to fight. Tuberculosis and Malaria: Available from: http://www.theglobalfund.or/en/commitmementsdisbursements/?lang=eng 2010
263. Therese KL, Gayathri R, Dhanurekha L,Sridhar R, Meenakshi N,Madhavan HN and Vinayagam AK. Detection of *Mycobacterium tuberculosis* directly from sputum specimens and phenotypic drug resistance pattern of *M. tuberculosis* isolates from suspected tuberculosis patients in Chennai. Indian J of Medical Research, May 2012; 135(5):778-82.

264. Thomas ST, Vander Ven BC, Sherman DR, Russell DG and Sampson NS. Pathway profiling in *Mycobacterium tuberculosis*: elucidation of cholesterol-derived catabolite and enzymes that catalyze its metabolism. The Journal of biological chemistry (2011); 286:43668-43678.
265. Todar K, Ph.D. *Mycobacterium tuberculosis* and Tuberculosis, page no.3.http://textbookofbacteriology.net/tuberculosis_3.html.
266. Toman K. World Health Organization, 1979. Tuberculosis, case finding and chemotherapy.
267. Tortoli E, Cichero P, Piersimoni C, Simonetti MT, Gesu G, Nista D. Use of BACTEC MGIT 960 for recovery *of Mycobacteria* from clinical specimens: Multicentre study. J Clin Microbiol, 1999; 37:3578-82.
268. Tortoli E, Mandler F, Bartolucci M *et al*. Multicentre evaluation of a biphasic system for recovery of *Mycobacteria* from clinical specimens. Eur J Clin Microbiol infect Dis. 1993; 12:425-429.
269. Trivedi SS, Desai SG. Primary Antituberculosis drug resistance and acquired rifampicin resistance in Gujarat, India. Tubercle. 1988 Mar; 69(1):37-42.
270. Trunz BB, Fine P, and Dye C. Effect of BCG vaccination on childhood tuberculous meningitis and military tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. Lancet ((2006), 367 (9517): 1173-80.
271. Tuberculosis- Causes, symptoms, Treatment, diagnosis. C-Health (2013). Retrieved April3,2013, from http://chealth.canoe.ca/channel_condotion_info_details.asp?disease_id=231 and channel_id=1020andrelation_id=71085
272. Udwadia Z F. India's Multidrug-resistant tuberculosis crisis. Ann N Y Acad Sci 2001 Dec; 953:98-105.
273. Udwadia Z and Vendoti D. Totally drug-resistant tuberculosis (TDR-TB) in India: every dark cloud has a silver lining. Journal of Epidemiology and community Health – BMJ Journals. Retrieved April 3, 2013, from http://jech.bmj.com/content/early/2012/11/14/jech-2012-201640.extract
274. Udwadia Z, Amale R, Ajbani K and Rodrigues C. Totally drug-resistant Tuberculosis in India. Oxford Journals,(2011);201(1). Retrieved April 3, 2013, from http://cid.oxfordjournals.org/content/early/2011/11/24/cid.cir889.full.pdf+html
275. Udwadia Z. MDR, XDR, TDR tuberculosis: ominous progression. Thorax, 2013; 67(4). Retrieved April 3,2013, from http://thorax.bmj.com/content/67/4/286.full.pdf+html
276. Uhia I, Galan B, Medrano FJ and Garcia JL. Characterization of the KstR-dependent promoter of the gene for the first step of the cholesterol degradative pathway in *Mycobacterium smegmatis*. Microbiology (2011); 157:267.

277. Vaishali P, Meharwal SK, Myneedu VP, *et al*., Evaluation of direct method of drug susceptibility testing of *Mycobacterium tuberculosis* to rifampicin and isoniazid by nitrate reductase assay in a national reference laboratory. Diagn Microbiol Infect Dis 2010; 66:148-152.
278. Valim A, Rossetti M, Ribeiro M *e .al*. Mutations in the rpoB gene of multidrug resistant *Mycobacterium tuberculosis* isolates from Brazil. J Clin Microbiol., 2003:3119-22.
279. Van Crevel R, Ottenhoff THM, Van der Meer JWM. Innate immunity to *Mycobacterium tuberculosis*. Clin Microbiol Rev.2002; 15:294-309.
280. Van der Geiz R *et al.* A gene cluster encoding cholesterol catabolism in soil actinomycetes provides insight into Mycobacterium tuberculosis survival in macrophages. Proc. Natl. Acad. Sci.U.S.A. (2007); 104:1947-1952.
281. Van Rie A, Page-Shipp L, Scott L *et al*. Xpert(R) MTB/RIF for point-of-care diagnosis of TB in high-HIV burden, resource-limited countries: hype or hope? Expert Rev Mol Diagn 2010, 10:937-946.
282. Velayati AA, Masjedi MR, and Farnia P, *et al.* Emergence of new forms of totally drug-resistant tuberculosis bacilli: Super extensively drug-resistant tuberculosis of totally drug-resistant strain in Iran. Chest 2009; 136: 420-425.
283. Verma JS, Rawat D, Hassan A, Capoor MR, Gupta K, Deb M and Nair D. The use of E-test for the drug susceptibility testing of *Mycobacterium tuberculosis*-A solution or an illusion? Indian journal of Medical Microbiology, 2010; 28(1):30-33.
284. Visalakshi P, Arora J, Behera D. Evaluation of nitrate reductase assay for rapid detection of resistance to second-line drugs in *Mycobacterium tuberculosis* in a tertiary care hospital. Diagn Microbiol Infect Dis 2010; 68:236-240.
285. Wallis RS, Pai M, Menzies D *et al*., Biomarkers and diagnostics for tuberculosis: progress, needs and translation into practice. Lancet 2010; 375:1920-1937.
286. Wheeler PR and Ratledge CR. Tuberculosis: pathogenesis, protection and control. Am. Soc. Microbiol., Washington DC, 1994 (ed.Bloom BR); 353-385.
287. WHO. Tuberculosis programme review-India. Geneva: WHO; 1992.
288. WHO/CDS/TB/2000.278. Anti-Tuberculosis drug-resistance in world, report no.2: Trends in Drug-resistance: 1996-99. 232
289. WHO. The WHO /IUATLD Global Project on Anti-tuberculosis Drug Resistance Surveillance. Anti-tuberculosis drug resistance in the world, Report No.2. Geneva: WHO: 2000.
290. WHO. Joint TB Programme review- India. Geneva: WHO, SEARO-TB-224. Geneva: WHO; 2000.
291. WHO. The WHO /IUATLD. Global Project on Anti-tuberculosis Drug Resistance Surveillance, Report No.3. Geneva: WHO; 2003.

292. WHO Guidelines for the Programmatic Management of Drug Resistant Tuberculosis. Geneva, Switzerland: WHO, 2006. Available on www.arfo.who.int/.../3306-guideline-for-the-programmatic-management-...World Health Organization 2006.
293. WHO. THE GLOBAL PLAN TO STOP TB 2006-2015: PART I Strategic directions. Geneva: WHO; 2006. Available from file://G:\tb-current situation.htm
294. WHO."Global Tuberculosis Control Report, 2006- Annex 1 Profiles of high-burden countries" (http://www.who.int/tb/publications/global_report/2006/pdf/full_report_correctedversion.pdf) (PDF). Retrieved 13October 2006.
295. WHO 2007. Revised BCG vaccination guidelines for infants at risk for HIV infection. Wkly Epidemiol Rec 82 (21): 193-6.
296. WHO. Use of Liquid TB culture and drug susceptibility testing (DST) in low- and medium-income settings Geneva: World Health Organization, 2007.
297. WHO. Molecular line probe assays for rapid screening of patients at risk of multidrug-resistant tuberculosis (MDR-TB). Geneva: World Health Organization: 2008.
298. WHO-Molecular Line Probe Assays for Rapid screening of patients at risk of Multiple Resistant Tuberculosis (MDR-TB). Policy statement, Geneva: World Health Organization, 2008.
299. WHO. Guidelines for the Programmatic Management of Drug-resistant Tuberculosis: Emergency Update 2008 (WHO/HTM/TB/2008; 402). Geneva: WHO; 2008.
300. WHO. Molecular line probe assay for rapid screening of patients at risk of multi-drug resistant tuberculosis. Policy statement. June 27, 2008. Available at-http://www.who.int/tb/dots/laboratory/lpa_policy.pdf.[lastaccessed on 2012 Aug18].
301. WHO. Monograph on integrated monitoring of TB/HIV –a case study in Malawi. Geneva: WHO; 2009.
302. WHO (2009). "Epidemiology". Available at - (http://who.int/entity/tb/publications/global report/2009/pdf/chapter1.pdf).
303. WHO (2009). "The Stop Strategy, case reports, treatment outcomes and estimates of TB burden" (http://who.int/tb/publications/global_3/en/index.html). *Global tuberculosis control: epidemiology, strategy, financing*. Pp. 187-300. ISBN 9789241563802. Retrieved 14 November 2009.
304. WHO. Global Tuberculosis Report. Geneva: WHO; 2009.
305. WHO- Guidance on Ethics of Tuberculosis, Prevention, Care and Control.2010. Available on www.who.int/tb/features-archive/ethics/en.

306. WHO, Framework for Implementing New Tuberculosis Diagnostics. Geneva: World Health Organization; 2010.
307. WHO, Multi-drug and Extensively drug-resistant TB (M/XDR-TB): 2010 Global Report on Surveillance and Response. Geneva: WHO; 2010.
308. WHO. Non-commercial culture and drug-susceptibility testing methods for screening of patients at risk of multi-drug resistant tuberculosis. Geneva: World Health Organization; 2010.
309. WHO. Fact sheet No.104: Tuberculosis. Geneva: WHO; 2010. Available from: http://www.who.int/mediacentre/factsheets/fs104/en/print.html
310. WHO. Global TB Control, 2011. Multi-drug resistant TB, Program management, program management- DOTS, Surveillance, Treatment. Available at http://www.who.int/tb/publications/global_report/2011/gtbr11_full.pdf
311. WHO Guidelines on the prevention and control of tuberculosis Geneva.
312. WHO. "Multidrug-resistant tuberculosis (MDR-TB) 2013 Update" (http://www.who.int/tb/challenges/mdr/MDR_TB_FactSheet.pdf). World Health Organization. Retrieved 14 June 2013.
313. WHO- Global Tuberculosis Report, 2014 – Executive summary (updated on 21st Nov 2014). Available on- www.who.int/tb/.../global_ report / gtbr14_executive summary.
314. WHO Report 2014 Tuberculosis control in the South-East Asia Region Annual Report 2014. Published on 21st March 2014.
315. WHO Tuberculosis-Fact Sheet No.104. Reviewed March 2014. Available on: http:/www.who.int/mediacentre/factsheets/fs 104/en/
316. William WM *et al.* The actinobacterial mce4 locus encodes a steroid transporter. J.Biol.Chem. (2008); 283:35368-35374.
317. Wilson GS, Miles AA. Tuberculosis in Topley and Wilson's principles of Bacteriology, Virology and Immunology, vol.II, 6th edition. Edward Arnold Publishers, London; 1975:1724-85.
318. Wilson ML, Stone BL, Hildred MV *et al*. Comparison of recovery rates of *Mycobacteria* from BACTEC 12B vials, Middlebrook 7H11 selective biplates and LJ slants in a public health laboratory. J Clin Microbiol, 1995; 33:2516-18.
319. Wood A J, Iseman MD. Treatment of multidrug-resistant Tuberculosis. New England Journal Medicine. (1993), 329 (11):784-91.
320. Wu X, Yang Y, Zhang J, Li B, Liang Y, Zhang C, Dong M. Comparison of antibody responses to seventeen antigens from *Mycobacterium tuberculosis*. Clin Chim Acta. 2010 Oct 9; 411(19-20):1520-8. Epub 2010 June 22.
321. Yang X, Dubnau E, Smith I and Sampson NS. RV 1106c from *Mycobacterium tuberculosis* is a 3beta-hydroxysteroid dehydrogenase. Biochemistry (N.Y.) (2007); 46:9058.

322. Zhang Y, Yew WW. Mechanisms of drug resistance in *Mycobacterium tuberculosis*. Int J Tuber Lung Dis, 2009; 13(11): 1320-1330.
323. Zhuang Y, He X, Zhang X. The studies on the molecular mechanism of rifampicin-resistant Mycobacterium tuberculosis. Zhonghua Jie He Xi Za Zhi, 2000; 23:711-4.
324. Zimenkov DV, Antonova OV, Kuz AV, Isaeva YD, Krylova LY, Popov SA and Gryadunov DA. Detection of second-line drug resistance in *Mycobacterium tuberculosis* using oliginucleotide microarrays.BMC Infectious Diseases, 2013; 13(1):240.
325. Zysk, Kenneth G. Medicine in the Veda: Religious Healing in the Veda. (1998), Motilal Banarsidas Publ. ISBN 8120814002.

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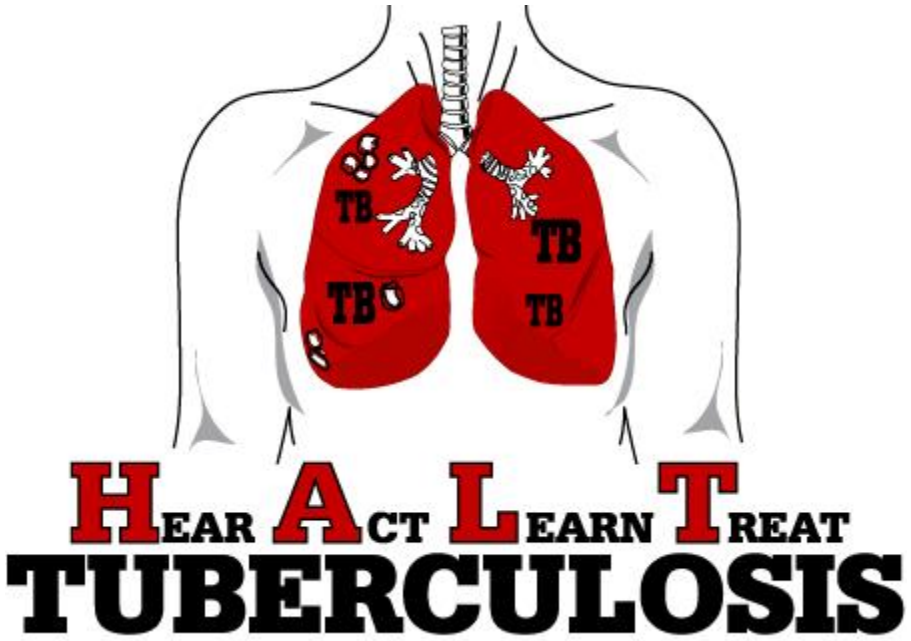
DRUG RESISTANT TUBERCULOSIS



ANNEXURES

ANNEXURE-I

Stains and reagents

I. Stains:

Stains used in Ziehl-Neelsen Staining (Acid-fast Staining):

1) Strong Carbol Fuchsin

Basic Fuchsine - 10gms

Phenol crystals - 50gms

95% Alcohol - 100ml

Distilled water - 1000ml

Basic fchsin is dissolved in alcohol and thnen combined with phenol solution.

2) 0.1% Methylene Blue

Methylene Blue - 1gm

Distilled water - 1000ml

Stock solution of 1% methylene blue was prepared by dissolving 1gm of powder in 100ml distilled water. It was further diluted by adding 900ml of distilled water.

II. Reagents:

1. 20% Sulphuric Acid

Concentrated Sulphuric acid - 200ml

Distilled water - 1000ml

Sulphuric acid was added to distilled water with constant mixing in a large boiling flask and stored in a stoppered glass bottle.

2. **4% NaOH:**

4% NaOH is used for decontamination. 10grams of NaOH pellets are weighed and dissolved in flask, sterilized in autoclave at 15 psi for 20 minutes.

3. **McFarland No.1 Standard Tube:**

(a) Barium chloride 1% solution:

Barium chloride - 1gm

Distilled water - 100ml

(b) Sulphuric Acid 1% solution:

Conc. sulphuric acid - 1ml

Distilled water - 100ml

Sulphuric acid is added to distilled water with constant mixing in a large boiling flask.

Store it in a stoppered glass bottle.

McFarland No.1 standard tube: add 0.1ml of 1% solution of anhydrous Barium chloride and 9.9ml of a cold 1% solution of pure sulphuric acid in a sterile glass test tube. Seal the tube and keep in a refrigerator.

4. **Reagents for Niacin test:**

1. 4% aniline in ethyl alcohol
2. 10% aqueous cynogen bromide

5. **Reagents for Nitrate reduction test:**

(a) Nitrate test substrate (0.01M) in M/45 phosphate buffer

$NaNO_3$ - 0.8gm

KH_2PO_4 - 1.17gm

Na_2HPO_4 - 1.93gm

H_2O - 999 ml

These chemicals are dissolved, pH adjusted to 7, autoclaved and dispensed in 2ml aliquots in sterile tubes at 2-8°C.

(b) 0.2% Sulphanimide: 0.1gm of sulphanimide was dissolved in 50ml of warmed water and stored in brown glass bottle at 2-8°C for 1 month.

(c)0.1% N-(1-naphthyl) ethylenediamine dihydrochloride: 0.05 gms of N-(1-naphthyl) ethylenediamine dihydrochloride was dissolved in 50ml of deionized water and in brown glass bottle at 2-8°C for 1 month.

6. **Reagents for Neutral red test:**

5ml of 0.002% neutral red (Merk AG, Darmstadt, Germany) in barbital buffer (1% sodium barbital in 5% NaCl [pH 9.8])

7. **Reagents for Catalase test:**

1. Phosphate buffer, 0.067 mol/lit, ph 7.0
2. Hydrogen peroxide- 30%
3. Tween 80 solution-10%

Mix the equal quantity of 30% hydrogen peroxide and10% Tween 80 (freshly prepared).

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ANNXRE-II

Culture Media

1. <u>Lowenstein Jensen media</u>:

L.J. medium containing glycerol favours the growth of *M .tuberculosis* while L.J. medium without glycerol but containing pyruvate encourages the growth of *M. bovis*.

Ingredients:

Potassium dihydrogen phosphate anhydrous (KH_2PO_4)	: 2.4 gms
Magnesium sulphate anhydrous	: 0.2 gms
Magnesium citrate	: 0.6 gms
Aspargine	: 3.6 gms
Glycerol (reagent grade)	: 12 ml
Malachite green, 2% solution*	:20 ml
*Malachite green solution-2%	
Malachite green dye	: 2.0 gms
Distilled water	: 100 ml

Dye is dissolved in distilled water completely. Filtered and stored in refrigerator.

Ingredients are dissolved in order in about 300ml distilled water by heating. Glycerol, Malachite green solution is added and made up to 600ml with distilled water. This solution was sterilized by autoclaving at 121°C (15 psi) for 30 minutes. Cooled to room temperature. If required, this solution was stored in the refrigerator.

Homogenized whole eggs:

Fresh hen's eggs which were not more than 7 days old were cleaned by scrubbing thoroughly with a hand brush in water and soap. Rinsed thoroughly in running water and soak them in 70% ethanol for 15 mins. Contents of the eggs were poured into a sterile beaker and were properly blended.

Preparation of complete medium:

The following ingredients were aseptically pooled in a large, sterile flask and mixed well:

Mineral salt solution with malachite green	: 600 ml
Homogenized eggs	: 1000 ml

The blend was distributed in McCartney universal bottles (20 ml capacity) in 6-8 ml quantity. Caps were tightened and placed slanting in an inspissator and inspissated at 85°C for 30 mins on 3 successive days.

Sterility check:

After inspissations, the entire batch of prepared L.J. medium was checked for sterility by incubating at 37°C overnight. Any of the medium showing visible bacterial colony formation was considered as contaminated and discarded. Among the remaining slopes, 5% of the slopes was picked up randomly and continued incubation for further 14 days to check for fungal contamination.

QC: In both sterility check methods, rate of contamination was <10%.

Storage: The L.J. medium was dated and stored with the batch number in the refrigerator. One batch of prepared L.J. medium slants was used within 4 weeks from the date of preparation.

2. <u>Middlebrook 7H9 broth</u>:

Potassium dihydrogen phosphate, KH_2PO_4	:	1.0g
Disodium hydrogen phosphate, anhydrous, Na_2HPO_4	:	2.5g
L-sodium glutamate	:	0.5g
Ammonium sulphate	:	0.5g
Tri-sodium citrate ($2H_2O$)	:	0.1g
Ferric ammonium citrate (green)	:	1.0ml of 8% aq. Soln
Magnesium sulphate ($7H_2O$)	:	1.0ml of 5% aq. Soln.
Calcium chloride ($2H_2O$)	:	1.0ml of 0.05% aq. Soln.
Zinc sulphate ($7H_2O$)	:	1.0ml of 0.1% aq.soln.
Cupric sulphate ($5H_2O$)	:	1.0ml of 0.1% of aq. Soln.
Pyridoxine hydrochloride	:	1.0ml of 0.1% of aq.Soln.
Biotine	:	1.0ml of 0.05% of aq.Soln.
Tween80(10% soln)	:	5.0ml
Distilled water, to	:	1 liter

Dissolve the ingredients (base) and autoclave.

With sterile precautions, add the following:

Bovine albumin-dextrose complex	:	100ml
Bovine albumin, fraction V	:	5g
Glucose	:	2g
Sodium chloride, 0.85% solution	:	75ml

Make up to 95ml with water. Mix well till dissolved by leaving it in 4°C. Filter by 0.22µ membrane filter.

Before use for every 900ml base 100ml bovine albumin dextrose solution should be added. Distribute in 5 ml aliquots in sterile McCartney bottles.

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ANNEXRE-III

PROFORMA OF THE PATIENT

Name: OP/IP No:

Age/Sex: Lab. No.:

Address: Sr. No.

Presenting complaints:

History of present illness:

Past History:

Treatment History:

Personal History: h/o smoking

Contact with known case of TB

General physical examination:

Systemic examination:

Respiratory system:

Cardiovascular system:

Central nervous system:

Per abdomen examination:

Provisional diagnosis:

Investigations:

(a) Blood: Hb%, TC, DC, ESR

(b) Urine: Sugar, Protein, Microscopy

(c) Chest X-ray

(d) HIV status detection by ELISA

(e) Any other

Microbiological investigations:

(1) ZN stain: positive/negative, Grading of smear

(2) Culture: L.J. MBBacT

(a) Date of inoculation

(b) Date of appearance of growth

(c) Duration of growth

(d) Contamination testing(Grams staining)

(e) Identification (biochemical testing)

(f) Speciation(MTB/NTM)

(3) DST: (1^{ST}-line and 2^{nd}-line drugs) L.J. MBBacT

(a) Sensitive

(b) Resistant

(4) DST(INH and RIF) and detection of mutations Line probe assay

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ANNEXRE-IV

MASTER CHART OF CLINICAL SPECIMENS

Sr.NO.	Hospital IP/OP	Gender	Age	Direct Smear	L.J. Culture
1	7/9616	M	54	positive	positive
2	7/9894	M	55	positive	positive
3	Ayurved	M	40	positive	positive
4	4/400027	F	17	positive	Positive
5	Pulm.med.	M	25	positive	positive
6	Pulm.med.	M	28	positive	positive
7	4/10572	F	55	positive	positive
8	7/10624	M	20	positive	positive
9	7/10715	M	50	positive	positive
10	Pulm.med.	M	23	positive	positive
11	Pulm.med.	M	49	positive	positive
12	7/11036	M	48	positive	positive
13	4/11333	F	52	positive	positive
14	4/11273	F	20	positive	positive
15	7/11366	M	45	positive	positive
16	Pulm.med.	M	65	positive	positive
17	Pulm.med.	M	56	positive	positive
18	Opd.	M	25	positive	positive
19	Pulm.med.	M	60	positive	positive
20	Ayurved	M	35	positive	positive
21	4/11899	F	20	positive	positive
22	Pul.med.	M	27	positive	positive
23	4/11942	F	50	positive	positive
24	Npw/12o67	M	10	positive	positive
25	4/12191	F	38	positive	positive

26	Ayurved	M	35	positive	positive
27	Puml.med.	F	21	positive	positive
28	Pulm.med.	F	18	positive	positive
29	7/12574	M	50	positive	positive
30	Pulm.med.	M	50	positive	positive
31	7/12438	M	48	positive	positive
32	7/12723	M	17	positive	positive
33	OPD	F	26	positive	positive
34	Pulm.med.	F	55	positive	positive
35	Pulm.med.	M	40	positive	positive
36	4/13311	F	65	positive	positive
37	Pulm.med.	M	45	positive	positive
38	Pulm.med.	F	26	positive	positive
39	OPD	M	38	positive	positive
40	Pulm.med.	M	56	positive	positive
41	Pulm.med.	F	48	positive	positive
42	Pulm.med.	M	21	positive	positive
43	OPD	F	54	positive	positive
44	Pulm.med.	M	27	positive	positive
45	Ayurved	M	26	positive	positive
46	4/15246	F	18	positive	positive
47	Pulm.med.	M	36	positive	positive
48	7/15924	M	60	positive	positive
49	Ayurved	M	27	positive	positive
50	Pulm.med.	F	17	positive	positive
51	7/16440	M	55	positive	positive
52	Pulm.med.	M	15	positive	positive
53	Pulm.med.	F	17	positive	positive
54	7/17242	M	40	positive	positive
55	Pulm.med.	M	50	positive	positive

56	4/10827	F	25	positive	positive
57	7/17998	M	70	positive	positive
58	4/18159	F	60	positive	positive
59	Pulm.med.	M	44	positive	positive
60	Pulm.med.	F	21	positive	positive
61	Pulm.med.	F	40	positive	positive
62	Medicine	M	42	positive	positive
63	Pulm.med.	M	50	positive	positive
64	Pulm.med.	M	65	positive	positive
65	PICU/59566	F	7 months	positive	positive
66	OPD	F	48	positive	positive
67	OPD	M	49	positive	positive
68	OPD	M	53	positive	positive
69	OPD	M	55	positive	positive
70	OPD	M	65	positive	positive
71	Ayurved	M	41	positive	positive
72	Ayurved	M	65	positive	positive
73	11/1079	F	70	positive	positive
74	4/1342	F	29	positive	positive
75	7/1422	M	27	positive	positive
76	Ayurved	F	45	positive	positive
77	Ayurved	F	50	positive	positive
78	Pulm.med.	M	21	positive	positive
79	7/3607	M	60	positive	positive
80	Pulm.med.	F	28	positive	positive
81	Pulm.med.	F	18	positive	positive
82	Pulm.med.	M	26	positive	positive
83	2/4117	F	28	positive	positive
84	7/4270	M	43	positive	positive
85	Pulm.med.	F	33	positive	positive

86	Pulm.med.	M	20	positive	positive
87	7/5463	M	20	positive	positive
88	4/5762	F	62	positive	positive
89	Ayurved	M	59	positive	positive
90	Ayurved	M	20	positive	positive
91	Ayurved	F	26	positive	positive
92	Pulm.med.	M	60	positive	positive
93	Pulm.med.	M	30	positive	positive
94	4/5762	F	62	positive	positive
95	Ayurved	M	59	positive	positive
96	Ayurved	M	20	positive	positive
97	Ayurved	F	26	positive	positive
98	Pulm.med.	M	60	positive	positive
99	Pulm.med.	M	30	positive	positive
100	4/5762	F	62	positive	positive
101	OPD	M	30	positive	positive
102	OPD	F	34	positive	positive
103	OPD	M	35	positive	positive
104	OPD	M	24	positive	positive
105	OPD	F	28	positive	positive
106	OPD	F	40	positive	positive
107	Pulm.med.	M	65	positive	positive
108	OPD	M	38	positive	positive
109	Pulm.med.	M	39	positive	positive
110	OPD	M	60	positive	positive
111	OPD	M	19	positive	positive
112	OPD	F	55	positive	positive
113	OPD	M	33	positive	positive
114	OPD	M	24	positive	positive
115	OPD	F	13	positive	positive

116	OPD	M	17	positive	positive
117	OPD	F	15	positive	positive
118	OPD	M	30	positive	positive
119	OPD	M	32	positive	posotive
120	OPD	F	36	positive	positive
121	OPD	F	33	positive	positive
122	OPD	F	34	positive	positive
123	Pul. Med.	M	37	positive	positive

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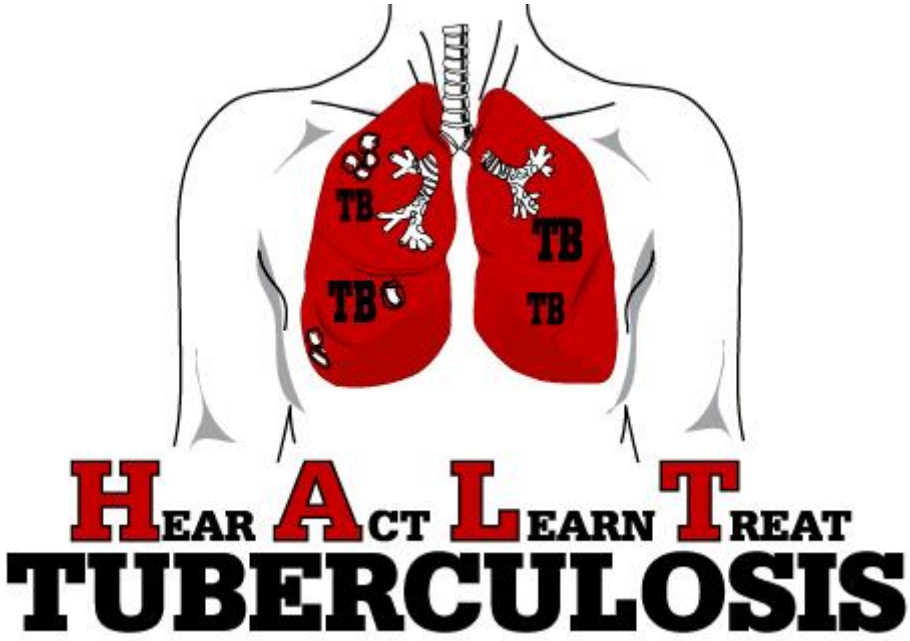
DRUG RESISTANT TUBERCULOSIS



PUBLICATIONS and PRESENTATIONS

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PUBLICATIONS:

1. Patil S D, Angadi K M, Modak M. and Bodhankar M G. Studies on drug-resistance pattern by phenotypic methods in *Mycobacterium tuberculosis* isolates in a tertiary care hospital. International Journal of Microbiology Research, 30th Oct.2013;5(6)497-501. **[IF: 7.2]**

2. Patil S D, Angadi K M, Modak M. and Bodhankar M G. Molecular line probe assay: Genotype MTBDRplus for rapid detection of primary drug resistance in *Mycobacterium tuberculosis* isolates from a tertiary care hospital in Western India. Research J of Pharmaceutical, Biological & Chemical Sciences. Oct-Dec-2013; 4(4)1612-1620. **[IF: 4.0]**

3. Patil S D, Modak M and Bodhankar M G. Molecular mechanisms of drug-resistance in MDR- strains of *Mycobacterium tuberculosis*. Integrated Journal British, Jan-Feb 2015;2 (1) 71-80. **[IF: 3.32]**

PRESENTATIONS:

1. Oral and Poster presentation on "Drug-resistance pattern in *Mycobacterium tuberculosis* isolates" in National conference on Challenges and Opportunities in Life Sciences (COLS-2013) at (Shivaji University) Kolhapur held on February 8th and 9th, 2013.

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