PREVALENCE, CHARACTERIZATION AND CLINICAL EVALUATION OF INDIGENOUS MYCOBACTERIUM TUBERCULOSIS.

BY

Taha Nazir

DEPARTMENT OF MICROBIOLOGY
QUAID-I-AZAM UNIVERSITY
ISLAMABAD

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TAHA NAZIR

DEPARTMENT OF MICROBIOLOGY
QUAID-I-AZAM UNIVERSITY
ISLAMABAD

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DECALARATION

The material contain within this thesis are my original work and have not been previously submitted to this or any other university

Taha Nazir

CERTIFICATE

This thesis by **Mr. Taha Nazir,** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, as satisfying the thesis requirement for the degree of Doctor of Philosophy in Microbiology

External Examiner : ____________________

External Examiner : ____________________

Supervisor: ____________________________
Prof. Dr. Abdul Hameed

Chairman: _____________________

Dated: ____________

DEDICATED TO

FATHER, MOTHER (LATE),

SONS (ABDULLAH, ARSLAN, ALI),

WIFE (Dr. NIDA TAHA) AND TEACHERS

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunity Deficiency Syndrome
AK	Amikacin
ANOV	Analysis of Variance
ahpC	Alkyl hydroperoxide reductase
Apr	April
AFB staining	Acid Fast Staining
Aug	August
BACTEC	Becton Dickinson System
BCG	Bacillus Calmette Guirine Vaccine
CDCP	centre of Disease Control and Prevention
CIP	ciprofloxacine
CPC-NaCl	Cetylpyridinium Chloride Sodium Chloride
CSF	Cerebero Spinal Fluid
CTX	Cefotaxium
Dec	December
DRS	Drug Resistance Surveillance
dNTPs	Deoxynucleoside Triphosphates
DNA	Deoxyribose Nucleic Acid
DOTS	Direct Observed Treatment Strategy
Feb	February
EESBM	Egg Enriched Sheep Blood Media
ELIZA	Enzyme Linked Imuno Assay
EmbCAB	Arabinosyl Transferase
ESR	Erythrocyte Sedimentation Rate
ETB	Ethambutole
EPTB	Extra-Pulmonary *TB*
Gyra	DNA Gyrase Sub-unit A
HCWs	Health Care Workers
HIV	Human Immuno-deficiency Virus

HPLC	High Performance Liquid Chromatography
inh A	Synthesis, Enoyl-ACP Reductase
ISN	Isoniazide
ISSB	International Committee on Systematic Bacteriology
IUATLD	International Union Against Tuberculosis & Lung Diseases
IID	International Immune Diagnostic
IUATLD	International Union Against Tuberculosis and Lung Diseases
Jan	January
KatG	Catalase Peroxidase
LRM Test	Luciferase Reporter Mycobacteriophage Test
LJ medium	Lowenstein Jensen medium
Mar	March
MBC	Minimum Inhibitory Concentration
MIC	Minimum Inhibitory Concentration
MDR-TB	Multi Drug Resistant Tuberculosis
MGIT	Method Microbial Growth Indicator Tube Method
MOTT	Mycobacterium Ohter Than Tuberculosis
MTB	*Mycobacterium tuberculosis*
NCCLS	National Committee for Clinical Laboratory Standards
NIBGE	National Institute of Biotechnology & Genetic Engineering
Nov	November
NTBGSN	National TB Genotyping and Surveillance Network
OADC	Oleic Acid-Albumindextrose- Catalase
Oct	October
OFX	Ofloxacin
OTC	Over The Counter
PAS	Para-Amino-Salicylic Acid
PcnA	Amidase
PCR-SSCP	Polymerase Chain Reaction – Single Strand Conformational Polymorphism
PHRI	Public Health Research Institute
PMRC	Pakistan Medical Research Centre
PTB	Pulmonary *TB*

PCR	Polymerase Chain Reaction
PZA	Pyrazinamide
PZase	Pyrazinamidase
RFP	Rifampicin
RFPCR	Restricted Fragment Polymorphism PCR
rpoB	B subunit of RNA Polymerase
rpsL	Ribosomal Protein S12
rrs	16s rRNA
RRDR	Rifampicin Resistance–Determining Region
Sep	September
Spp	Species
SEA	Southeast Asia
SSCP PCR	Single Stranded Confrontation Polymorphism in Conjunction with PCR
ssDNA	Single Strand DNA
TB	Tuberculosis
TNF	Tumer Necrosis Factor
TST	Mantoux Tuberculin Skin Test
UTI	Urinary Tract Infection
UK	United Kingdome
USA	United State of America
USP	United State Pharmacopoeia
WAPDA	Water & Power Development Authority
WHO	World Health Organization
%	Percentage
Δ	Heat
≠	Not equal to
≤	Lesser than and equal to
≥	Greater than and equal to
∞	Infinity
×	Multiplication
÷	Divided by
±	Plus – minus
˚C	Degree Celsius

Symbol	Meaning
@	At the rate of
=	Equal to
<	Lesser than
>	Greater than
# or n	Number
-	Subtraction
/	Divided by
µg	Micro gram
mg	Milligram
gm	Gram
µL	Micro litre
£	Pound sterling
$	Dollar
¢	Cent
α	Alpha
β	Beta
γ	Gamma
λ	Lambda
π	Pi
Ω	Omega

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Taha Nazir

ABSTRACT

A total six thousands five hundred and seventy three (6573) indigenous pulmonary and extra-pulmonary specimens were collected from tuberculosis suspicious patients of 17-67 years age group during November, 2004 to December, 200. The sputum, pus and bronchial washings were collected from five different sources, labeled and processed for initial screening. One hundred and seventy two (172) 2.616% of total (6573) tuberculosis diagnosed (AFB positive) patients were selected from six different sources. The patients were selected, regardless of their age, gender and previous therapeutic profile. The specimen comprised of 85% sputum, 10.5% puss and 4.5% bronchial washing. We considered 29.% female and 71% males with 84.% pulmonary (sputum, bronchial washing & puss) and 16% extra-pulmonary (puss & bronchial washing) specimens. Sixty six (66) resistant *Mycobacterium tuberculosis* strains were further studied to determine the highest level of resistance (in % age) . The clinical isolates were collected from cultured growth on Lowenstein Jensen media supplemented with antitubercular drugs at minimum inhibitory concentration (MIC) level. The parameters of study were the pattern of sensitivity/ resistance of *mycobacterial TB* against rifampicin, isoniazid, ethambutol and pyrazinamide, overall pattern of resistance, resistance percentages with respect of number of colonies, overall trend of resistance during Jan. - Dec. 2005, resistance pattern in percentage against five different levels (µg/ml) above their respective critical concentrations, therapeutical interpretation of drugs to evaluate the pharmacological credibility and molecular study of Pnc A gene of *Mycobacterium tuberculosis* responsible of resistance against pyrazinamide. The data obtained from this study showed 37 (21.5%) strains resistant and 135 (78.5%) strains sensitive to rifampicin, 25 (14.5%) strains resistant and 147 (85.5%) strains were sensitive to isoniazid, 10 (5.8%) resistant and 162 (94.2%) strains founded sensitive to Ethambutol, 47 (27.3%) resistant and 125 (72.7%) strains were founded sensitive to Pyrazinamide of total 172 clinical isolates of *Mycobacterium tuberculosis.* The resistance of *Mycobacterium tuberculosis* noted on basis of growth pattern (number of colonies) over the mycobacterial specific Lowenstein Jensen medium.

Overall mono-resistance pattern was observed as 25.71% resistant to rifampicin, 8.57% resistant to isoniazid, 2.85% resistant to ethambutol and 62.85% resistant to pyrazinamide out of 20.34% mono-resistant isolates of total 172 *Mycobacterium tuberculosis* strains. Poly resistance profile

obtained was as 19.35% *Mycobacterium TB* strains resistant to rifampicin & isoniazid, 22.58% resistant to isoniazid & pyrazinamide, 3.22% resistant to ethambutol & pyrazinamide, 6.45% resistant to isoniazid & pyrazinamide, 22.58% resistant to rifampicin, isoniazid and pyrazinamide, 3.22% resistant to rifampicin, ethambutol and pyrazinamide and 22.58% resistant to all of the four 1st line drugs.

The resistant *Mycobacterium TB* having an ultimate highest level of resistance against the first line antitubercular drugs. Which were interpreted therapeutically to study the pharmacological suitability of dosage and regimen. It was observed that no any rifampicin strain inhibited at 1st and 2nd drug levels. 40.54% resistant *Mycobacterium -TB* strains inhibited at 3rd rifampicin level of 120ug/ml. Practically it is not feasible to maintain a plasma concentration higher than therapeutic range of 6.5±3.5ug/ml (Joel *et al*., 2001). It was observed that no any isoniazid strain inhibited at 1st, 2nd and 3rd drug levels. There 28% resistant *Mycobacterium-TB* strains inhibited at 4th isoniazid level 9ug/ml. Maximally plasma concentration that can be maintained in body is -4ug/ml (Richard *et al*., 2006), therefore it can not be used in actual practice. It was observed that no any ethambutol strain inhibited at 1stand 2nd drug levels 2ug/ml and 4ug/ml. 50% resistant *Mycobacterium TB* strains inhibited at 3rd level of 6ug/ml. The maximum plasma concentration (Cmax) that can be maintained in tuberculosis patient during treatment protocol are described by other researchers as 3-5ug/ml (Bertram G. Katzung, 2004), 2-5ug/ml (Leon et al., 2004) and 4-6ug/ml (Richard et al., 2006). It was observed that no any pyrazinamide resistant strain inhibited at 1st and 2nd drug levels100ug/ml and 200ug/ml. 27.66% pyrazinamide resistant *Mycobacterium TB* strains were inhibited at 3rd pyrazinamide level of 300ug/ml. The maximum plasma concentration than can be maintained in human body reported by different researchers are 9-12ug/ml (Joel et al, 2001), 19ug/ml (Leon et al., 2004), 30-50ug/ml (Bertram, 2004), 37-40ug/ml (Richard et al., 2006).

The genomic DNA of pyrazinamide resistant *Mycobacterium TB* extracted by mechanical method and examined on gel. PCR for *Mycobacterium TB* is specific for *Mycobacterium TB* complex DNA. By using the SSCP (Single Strand Conformational Polymorphism), we were able to show most divers pattern. The resistant 17.44% showed different pattern than sensitive

samples. Which indicate the mutation in this domain, while 9.88% did not show any difference in mobility in comparison to sensitive samples.

Key word: Tuberculosis, resistance, Minimum Inhibitory Concentrations, PncA gene

CHAPTER No. 1

INTRODUCTION

INTRODUCTION

1.1 Statement of the Problem

Antituberculosis drugs can be divided into two main classes; 1st line drugs having greater level of efficacy, acceptable degree of toxicity & cheep in price than 2nd line therapy having lesser efficacy, higher toxicity and more expensive. A majority of the patients with tuberculosis can be treated with these drugs. But microbial resistance make it essential to resort to second line drugs or reconstitute the first line treatment protocol. The prevailing resistance of *Mycobacterium* to each class of compound employed in its chemotherapy required clinical evaluation. The increase in tuberculosis rate associated with HIV infection has been halted but tuberculosis remains the number one cause of death worldwide. The slow growth, disease chronicity, patient compliance, drug toxicity and development of microbial resistance present especial therapeutical complication. It is therefore required to understand the drugs used for tuberculosis treatment and therapeutical strategies evolving as resistance to available agents. A numbers of *Mycobacterium tuberculosis* can lead to serious infection of lungs, ganito-urinary tract, skeleton and menings. *Mycobacterium* infection presents therapeutical problems during treatment. Because of slow growth, the *Mycobacterium* is treated for six months to two years. Resistant organisms readily emerge, particularly in patients who have had prior therapy or who failed to adhere to treatment protocol. *Mycobacterium tuberculosis* can be dormant and therefore completely resistant to many drugs. It can be killed only very slowly and particularly by selective drugs. The lipid rich mycobacterial cell wall is impermeable to many agents. Substantial proportions of *Mycobacterium* reside intracellularly within microphages and become inaccessible to poorly penetrating drugs. It also has capability of getting genetic mutation to develop resistance to any single drug. Combination therapy therefore carried to overcome these obstacles and to prevent emergency of resistance during course of therapy. The treatment must be carried for months to years depending on drugs used because of slow chemotherapy response. A feasibility required to be developed to use directly observed therapy to improve the outcome of tuberculosis treatment regimens. Some mycobacterial strains have been identified to resistant to as many as seven anti-tubercular agents (Bertram et al., 2004). Therefore treatment regimens vary in duration and in the agents employed. They always include a minimum of two drugs preferly with both being bactericidal. The multidrug regimen is continued well beyond the disappearance of clinically symptoms to eradicate any persistent organisms. Two months or short term chemotherapy of

tuberculosis includes rifampicin, pyrazinamide and isoniazid and the rifampicin and isoniazid for next continuation phase of four months . The compliance often lowered when multidrug schedule last for six months or longer. It is constantly needed to design more effective, less toxic and comparatively short treatment regimens. The recent increase in the incidence of tuberculosis (TB) in certain parts of the world and the emergence of multi-drug resistant (MDR) strains, has urged the need for its rapid diagnosis (Nagwa et al., 2004). The delayed susceptibility and identification of *Mycobacterium* drug resistant or failure to isolate contagious patients appropriately had helped much in transmission of MDR *Mycobacterium TB*.

1.2 Objectives of the Study

It is the need of time to interpret therapeutical advantages of first line treatment regimen instead of practicing the less effective, more toxic and comparatively limitedly studied 2nd line drugs. In this study project we have envisaged the pre-treatment protocol with maximally increases regimen of first line drugs to get a maximum therapeutical benefits. Robert Koch made an observation; more than a century before that the victim of tuberculosis are significance higher than any other disease, even more than the fearful infectious ailments of bubonic Asiatic cholera, malaria, plague. That must rank far behind than tuberculosis. Thus; after even more than hundred years, this disease is still remains a major causes for mortality and morbidity. (Herendra et al., 1998) This research project comprised of three main phases; clinical evaluation *Mycobacterium tuberculosis*, determination of level of resistance of resistant *Mycobacterium* and genetic elucidation/ characterization of pyrazinamide resistant *M. tuberculosis*. The major objective of this study were as give below, 1 Study of the source, number and type of specimens collected form the tuberculosis diagnosed patients. 2 Comparatively study of the pulmonary & extra-pulmonary tuberculosis and gender wise distribution of patients. 3 Determination of the pattern of resistance of already resistant *Mycobacterium tuberculosis* against 1stline anti-TB drugs- isoniazid, ethambutol, pyrazinamide and rifampicin. 4 investigaiton of resistance pattern of indigenous *Mycobacterium TB* strains on basis of quantity of growth/ number of colonies observed over Lowenstein Jensen media incorporated with 1st line antitubercular drugs. 5 Study the overall resistance profile of indigenous *Mycobacterium tuberculosis* against 1st line antitubercular drugs. 6 Study of overall resistance profile of *Mycobacterium tuberculosis* with

respect of quantity of growth/ number of colonies observed over 1st line antitubercular drugs containing Lowenstein Jensen media. . 7 Study of the mono-resistance pattern of indigenous pulmonary/ extrapulmonary *Mycobacterium TB* against 1st line anti-TB drugs. 8 Study of the poly-resistance profile of indigenous pulmonary/ extrapulmonary *Mycobacterium TB* strains categorized on basis of resistance against two, three or all of the four 1stline anti-TB drugs. 9 Study of the overall trend of resistant prevalence of indigenous pulmonary/ extrapulmonary *Mycobacterium tuberculosis* during January- December, 2005 against 1stline anti-TB drugs. 10 Therapeutical interpretation of 1stline anti-tuberculosis drugs against *Mycobacterium tuberculosis*. 11 Molecular level elucidation of pyrazinamide resistant *Mycobacterium TB*.

1.2 Significances of the Study

Tuberculosis is a great concern of human society. The cases of this disease are increasing with the most serious aspect of developing the multidrug resistant, that poses major public health hazard and requires rapid intervention. Emergence of new antimycobacterial agents has gained an impetus after the spurt of resistance. Better knowledge about diagnosis, toxicities and monitoring the chemotherapy and further reduction in duration of therapy will certainly make life more comfortable for the patient (Herendra et al., 1998). While; world's one third population is infected, eight million got active infection and three million are died annually with this mortal disease. It means twenty thousand new cased introduced each day and someone did after every fifteen seconds. There is about 98% tuberculosis patients (mostly the young adults) died in developing countries affecting during their most productive age. Moreover; it is the primary killer amongst young women, especially in Africa. Tuberculosis particularly affected the malnourished and poor people more vulnerably. It is worldwide virulent disease, however the African countries have highest rate per capita outbreak (a quarter of all tuberculosis patients). While; half of all new tuberculosis patients are enrolled in six Asian countries; China, India, Pakistan, Bangladesh, Philippines and Indonesia. WHO has recently surveyed that MDR-TB (Multi Drug Resistant Tuberculosis) virtually exists in all countries of the world with miscellaneous intensity and form. Moreover; approximately 425,000 new MDR tuberculosis patients enrolled every year with the highest rates in China and former USSR. Where up to forty percent of all new cases are do not respond to the standard Antituberculosis therapy. It is

therefore the need of time to explore most accurate, effective and minimal toxic regimen. In this study project the 1st line anti-tuberculosis drugs; isoniazid, rifampicin, pyrazinamide and ethambutol that are more intensively studied and having long surveillance period are extrapolated to explore an accurate treatment protocol with increased regimen. Prisons are another pump of the tuberculosis epidemic in different countries with highest imprisonment rate i.e. USA, Russia. Prisons are referred as "Ebola with wings" because of providing breeding ground for MDR-TB. The constant in and out movement of people endangers the health of general populating. MDR-TB and drugs abusing in prisons quickly death sentencing to prison sentenced individuals. The huge suffering and death, especially in developing countries required an urgent response on humanitarian grounds. Tuberculosis not only hurdle the financial development and growth of living standards but also destabilized the local political and commercial structure. It destroys the social set up and diminished the human cultures of vulnerable nations. In this study project the tuberculosis epidemiology calculated to propose the future targets. The susceptibility rate, epidemiological trends and treatment credibility determined to assure a successful eradication of this mortal disease.

CHAPTER No. 2

REVIEW OF LITRATUE

REVIEW OF THE LITERATURE

2.1 Historic Profile of Tuberculosis

Phthisis – Greek word indicating the character of “wasting away”, scrofula – swelling of the lymph nodes of the neck, white plague – Tb epidemic term introduced in Europe during the 18 th century and tuberculosis – the presence or product of tubercle bacilli are all words used for tuberculosis, making specific points in history. This invisible enemy continues to challenge man’s knowledge and mock his efforts. The “captain of the men of death” continues to march forth leaving behind a trial of human misery, economic chaos and death. Since antiquity *tuberculosis* presents in human population. The spinal column fragments from Egyptian mummies 2400 BCE show explicit evidence of this disease. In 2000BC it was subject of hymn in an Indian sacred text. There were also some reference of lung scaring in preserved bodies (mummies) resemble to modern tuberculosis drive toward that tuberculosis was exist among the Americans since 2000 BCE. The term “phthisis” meaning consumption appears first in Greek literature. Around 460 BCE Hippocrates identified phthisis as the most widespread disease of the time and noted that it was almost always fatal. During 2500 -1500 BC TB was recognized in main Europe countries and made the earliest confirmation of the tuberculosis. While; in Britain it came from grave dating of 55BC- 800 AD through the roman occupation. Thus; in mid 17th century in London, one in five deaths; as recorded in mortality bills, were b because of the consumption (the term used for tuberculosis at that time). Tuberculosis was also known as “white plague” in Europe because of massive death and whitish skin coloration of patients. Recently; the DNA of *Mycobacterium tuberculosis* identified in pulmonary lesion of 1000 year old human remains in Peru. Thus; the exact anatomical and pathological depiction of tuberculosis started from identifying the characteristic modifications and appearance of actual tubercle in lungs or other body parts of patients. The development to abscesses and cavities also were described. Tuberculosis was also more commonly referred as vampire during the era of industrial revolution; when one infected member of family lost his health while other passed away because of this disease. Furthermore tuberculosis patient’s exhibited red swollen eyes; coughing blood and pale skin were supposed be the need of replenishment. This may be the medication of the common mythology of the tuberculosis vampire origination. In 1865 Jean Anthonio demonstrated the transmission of TB by inoculating the sputum of patient to rabbit.

The bacillus causing TB was identified and described on 24 th March,1882 in monthly meeting of Berlin physiological society by Robert Koch . He also isolated the tubercle bacillus. Same year Paul Ehrlich discovered the staining method of *Mycobacterium tuberculosis*. Robert Koch was awarded the Nobel Prize in 1905 (physiology and medicines) for his discovery. 25th March celebrated every year to commemorate this glorious achievement. By the turn of 19th century, there as seven million estimation of worldwide TB death rate per year along with 50 million per year pulmonary TB rate. This may lead to the dreadful situation of destruction of European civilization by the end of the 19th century. While; in 1939, Schonelein gave the name "tuberculosis" to this disease. In 1942, two American scientists Dr. Hinshaw and Feldman reported the 1st remedial medicine trailed (Promin) in tuberculosis treatment. But unluckily; this trial was failed because of sever adverse effects. Dr. schatz, Bugie and Waksman; in January 1944 to declared to the world streptomycin discovery. A 21 year old tuberculosis patient girl 'Patricia' was treated successfully with this medicine. Dr. Lehmann in 1946 discovered para-amino-salicylic acid (PAS) for treatment of tuberculosis. In 1952, a new wondered medicine "isoniazid" was published in paper by Dr. Robizek and Selikoff. It was presented in New York to use against tuberculosis by Dr. Robizek and Selikoff. In France; the vaccine BCG (bacillus calmette guirine) was introduced following a 50,000 children survey that indicated 80% decline in infant rate. Dr john Crofton an expert of tuberculosis at University of Edinburgh has proposed a combination of drug, PAS, streptomycin and isoniazid to completely cure the tuberculosis in 1960. Then; he announced "all out war" to defeat the tuberculosis. In the USA; a survey was conducted (1961- 68) to investigate susceptibility of drugs among hospitalized tuberculosis patients. That showed 3.5% resistance against anti-tuberculosis drugs in 1970. WHO affirmed TB as global emergency in 1993. That causes more morbidity and motility than any curable infectious disease. In 1993-94 WHO was recommend the DOTS – Directly Observed Treatment Strategy. In 1997 International Union Against Tuberculosis & Lung Diseases (IUATLD) and WHO launched DRS (anti-tubercular drug resistance surveillance) a worldwide plan. Thus; WHO and several partners conceived the DOTS Plus and working group for multidrug resistant tuberculosis.

2.2 Epidemiology/ Prevalence of Tuberculosis

WHO estimated that one third of world's population; about 1.7 billion people are carrier of tubercle bacillus and 8 million new patients of tuberculosis causing 3 million deaths to every year (World Health Organization, 1992). WHO projects that, by the year 2000 AD, the annual figures instead of going down will further grow up to ≥ 3 million new tuberculosis patients along with 3 .7 million causalities (Raviglione M.C, 1995). Thus; the problem is more aggravated by pandemic of increased incidence of multidrug resistant tuberculosis and AIDS (Herendra et al., 1998)

2.2.1 World Wide Prevalence Tuberculosis (TB)

Tuberculosis is the main cause of death that cause about 3 million deaths every year) (Bloom BR & Murray, 1992). There are about 8.8 million new TB patients are enrolled in 2003 and 80% of these patient belong to only twenty two countries of world. More than 80% of tuberculosis patients belong to the most important age group of 15 - 49 years. Thus; approximately 252,000 tuberculosis patients passed away every weekly that make ≥7,000 each day. However; the death rates is big threat for mankind in this only glob. It is curable but leading killer among HIV infected people. ¼ of a million tuberculosis causalities belong to the HIV and majorly from the African countries. Tuberculosis is the leading case of mortalities in young women especially in Africa. If tuberculosis is left unrestricted, it may kill further thity five million people within next twenty 20 years. Thus; tuberculosis is still growing at the rate of 1% yearly due to fast increase in Africa. one in ten tuberculosis bacilli carrier people are infected with this mortal disease. According to a recent WHO and partner survey MDR tubreculosii exist in almost all (109) countries of the world. Approximatly 10425,000 New MDR-tubeculosis patients produced every year with the highest rate in china and Ex USSR; where about 14% of all new patient are MDR (WHO, 1997) The deprived socioeconomic strucgture and emergence of AIDS has major contribution in diseases resurgence especialty in most industrialized area of the world (Barnes P, 1991). While; in developing countries, the disease has always been widespread. Moreover; the global HIV has increased its ruthlessness. There are rapid pandemic and extensive social restructuring noticed due to

rapid industrialization. Thus; tuberculosis becomes a major worldwide public health problem. It seems like an emergency in glob (Ashok Rattan, 1998). Moreover; tuberculosis is the leading cause of mortality in adults and make a 26 % of all avoidable adult demises worldwide. World Health Organization reported 8 million tuberculosis (TB) patients each year, resulting in 3 million caulatities (Raviglione & Sinder, et el. 1995). The resistance of *Mycobacterium tuberculosis* against Antituberculosis drugs have been recovered from both immunocompromised and immunocompetent patients (Cohn & Bustreo , 1997)

The new advancements in health care facilities, progressive policies and rational treatment protocols particularly in developed part of the world has offered the goals to eliminate tuberculosis by the end of the 20th century. But it is reemerged because of the epidemic of global human immunodeficiency virus (HIV). Additionally; the tuberculosis control programs partially disrupted because of the entrapement of huge populaliton in poverty. However, 95% of tuberculosis patients are seen inn in developing countries (Pablos- Méndez et al. 1998). Thus tuberculosis has remained endemic for many decades (Maria Ines Moura Friexo, 2004). It kills a big populalitn in thieri most productice young age. That contribute an economically defcit inn society. Approximately 9 million of new tuberculosis patietns produced every year. While; 2 million active patients died; that makes it the biggest killer after HIV.

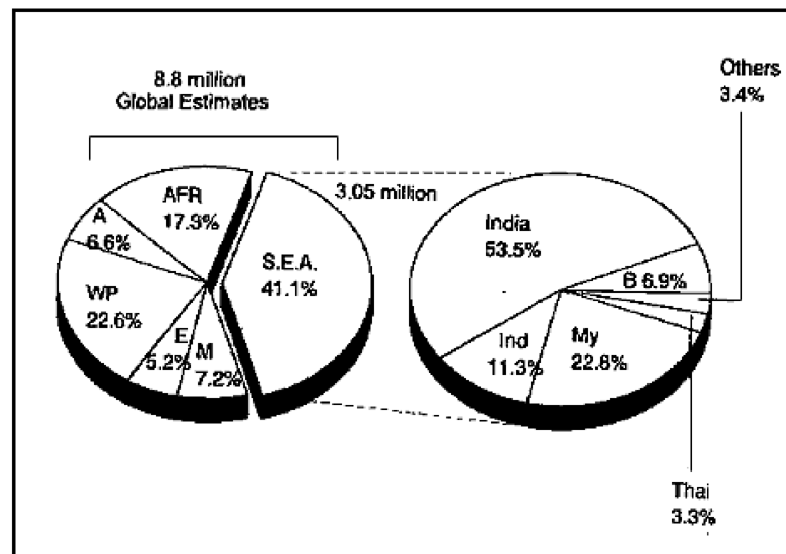


Figure 1. Tuberculosis global incidence estimated 8.8 million patients; India has approximately 53.3% of those cases; ≥ 40% of the patients are in Southeast Asia;

A – Americas; Afr - Africa; WP - Western Pacific; E - Europe; SEA - Southeast Asia; Ind - Indonesia; M - Eastern Mediterranean; B – Bangladesh; Thai – Thailand; My - Myanmar; *Others include Bhutan, 0.05%; Nepal, 1.2%; Maldives, 0.001%; Sri Lanka, 1%; DPR Korea, 1.2%.

(Ashok, et al., 1998)

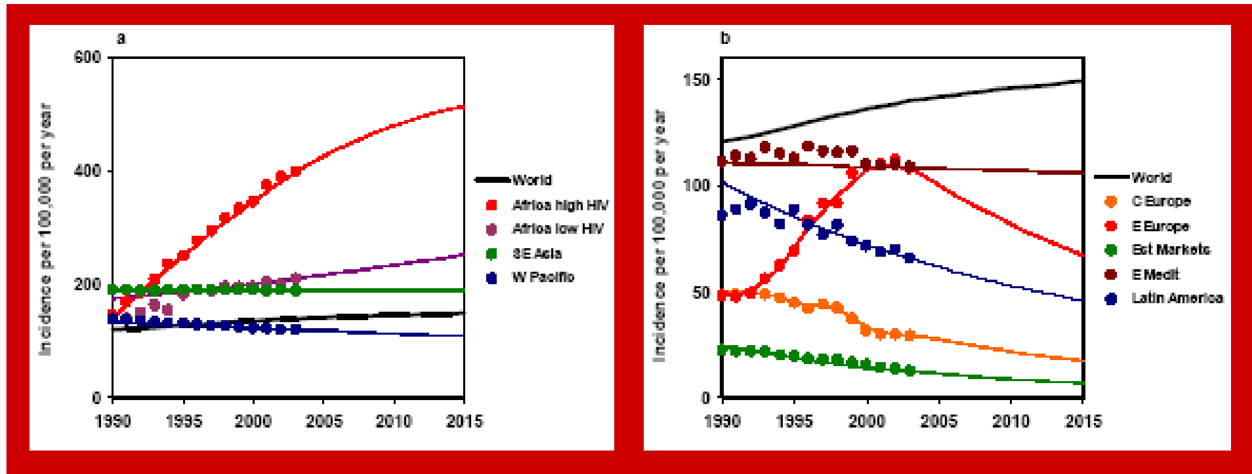


Figure 2. World wide epidemiology of tuberculosis.

Thus; the poorly implemented DOTS provides an increase in resistance of tubercle bacilli. The pervasiveness of resistant *Mycobacteria* demands treatment through DOTS plus program. There are two major problems faced in Tuberculosis control programmes; HIV and drug resistance. 2.5 million HIV patients out of the 6 million people are infected with tuberculosis. Approximately 60% of AIDS patients develop tuberculosis indicating the most frequent life threatening opportunistic infection associated with HIV (WHO, 2004)

2.2.2 Local Prevalence

Approximately 38% of the world tuberculosis burden is in the South East Asian countries. Where 750 000 patinet died annually because of tuberculosis.that makes 1500 every day, or 1 after every minute. Five out of the 22 highest burden countries are in South East Asian region (WHO, 2004).

2.2.3 Tuberculosis Transmission

The risk for an individual of becoming infected with tubercle bacilli depends on the concentration of organisms in the source case, the duration of exposure to air contaminated with tubercle bacilli and the aerodynamics of the droplet nuclei. (Karin, et al., 1997). Thus; the contacts person should be investigated for resistant because of having exposure of patient with Mulit drug resistant tuberculosis. That may get infected and should be examined to assess the likelihood of the actual infection. That might be a mutli resitatant strain of *Mycobacterium tuberculosis*. The considerable factors that may contribute are as under;

1 The probability of exposure of persons with drug susceptible/ resistant tuberculosis patient.

2 Closeness type and intensity with source infectious patient.

3 Duration of sharing the air space with tuberculosis patient and type of sharing e.g. household member, hospital roommate etc that possess more risk for infection than those with a short exposure.

4 The closeness and intensity of the exposure;

5 The pattern and level of resistantce of sourc infectious MDR patient.

6 Exposure in an enclosed, small and poorly ventilated space is more likely to transmit this bacillus as compared with an open, large and well ventilated space. Thus; in conclusion, the exposure during cough inducing procedures e.g. bronchoscopy, sputum induction etc may seriously increase transmission (C. Karin Weyer et al., 1997)

2.2.3.1 Nosocomial transmission

Implicit and confirmed community and nosocomial exposure can be investigated by reviewing the medical record of the treatment and residential facilities. Apparently; the nosocomial transmission was considered if the newly identified patient having the same section of the institution. Usuallay symptoms of the tuberculosis are grown up at least after 30 days of infection. While in most of the cases; disease arrest immunity multiplication and prevent clinical outcomes. The 20% - 50% of immunocompetent

prsons may get infected if exposed to tuberculosis for a long period (hours or days rather than minutes). While; among 90% of infected persons the *M. tuberculosis* may become dormant and will bot produce the clinical symptmes of disease. 5% of person may develop an early stage of active tuberculosis (usually within two years after infection). Moreover; the further 5% individuals may develop tuberculosis at some point during their resting life time. Mostly the suppression of immune system because of any physical or emotional stress may contribute to get infection with this disease (C. Karin Weyer et al., 1997)

2.2.3.2 Community transmission

The community transmission of tuberculosis is considered if any of the following has taken place:

1 The patient exposed to another patient having same strain of *Mycobaterium tuberculosis* and was infectious at least thirty days before illness beginning in the following patient. The exposure may occur in single room occupancy hotal., at home, homeless shelter or other non- institutional surroundings.

2 The patient contacts another patient, whose *Mycobacterium tuberculosis* has the identical DNA pattern or mulit drug resistance profile, but DNA genotyping test results are not obtainable.

The patients should have an epidemiologic link, if investigation of community ir nosocomial transmission was confirmed. While; the source patient is not be considered to cofirm the epidemiologic link. The computerized outpatient clinic records and indormation of hospital ward, bed and floor can be used to analyze the spatial and temporal overlap of cases. The Medical records can be reviewed for patients who breach the isolation protocol during their hospitalization. Moreover; the additional demographic and social information may be collected through questionnaires. Particularly, the patients were ivestigatd for where and with whom they spent significant time along with the additional personal and background inforamtions. The infected patients may also be asked about how and where they think be get exposed for tubreculosis (Sonal et al.,

1997). The adults pulmonary tuberculosis are the main infectious source of *M. tuberculosis* that may also contribute the mtulilt drug resistant tuberculosis. The droplets or particles of infective patient are generally resulting from moist forced expiration through the mouth discharged into the air. Mostly the sneezing, spitting, coughing or by procedures of liberating aerosols from nose or throat evnentually produce othr tubercular patients. Approximately 1-5 microns in size, the infective particles are produced if an aerosolised material dried out to form droplet nuclei in lungs. The trapped and inhaled resident alveolar macrophages droplet nuclei remained airborne to initiate the lung infection (Karin et el. 1997).

2.2.3.3 Molecular Epidemiology

The extensive transmission of multi drug resistant *M. tuberculosis* strains take place during the outbreak of 1980s and in beginning of 1990s. The epidemic was identified in many hospitals. The subsequently these outbreaks were associated with one strain known as the "W" strain of tuberculosis. While; in many of the strains wre resistant to rifampicin, isoniazid, streptomycin and ethambutol. Though, other multi drug resistant strains were linked with epidemic and transmission during that time. A survey of molecular epidemiology conducted in New York City indicagted that multi drug resistant tuberculosis was linked with clustered strains of Mycobacterim tuberculosis. That suggests a current transmission of the bacilli. Since 1992; when an enhanced Tuberculosis Control Program was implemented, the incidence of susceptible and resistant tuberculosis has been reduced rapidly. By 1994; 21.5% of the tuberculosis patients are decreased. While; the 60% of MDR tuberculsis cases are reduced. In 1995; Tuberculosis Control Program was started by DNA genotyping of new MDR tuberculosis strains to understand betterly the epidemiology of MDR tubereculosis in New York City. The main objective of this study was to provide the descriptive molecular epidemiology of MDR tuberculosis in during 1995 – 1997. That may also help to recognize major MDR strains exist during that time (Sonal et al., 1997).

2.2.4 Risk Factors Contributing the Transmissions

Short term therapy is the backbone of pahrmacotherapy of tuberculosis (Kochi, 1993). The patient compliance and rational prescriptions always assure the treatment. In fact, tuberculosis incidences were progressively decreasing in most of the industrialized part of the world; until the trend was reversed (Bell RT.1992). The significantly contributing or associated factors are as under; lower education, female sex, more than six instances of health seeking encounters, visiting a private doctor/ traditional healer and outpatient diagnosis. Ladies are most likely; more vulnerable and susceptible because of fear of stigma, combined mobility, additional workload and limited financial resources. Thus; in netshell; the financiel burden produce barriers in prompt diagnosis particlulary among women. Significant financial obstacles including cost of 'special food', transportation expenditure and lost income are main hurdles in successful eradication of tuberculosis. These obstacles can be reduced by certain interventions including the deceasing the travelilng distance, duration of illness before diagnosis and number of health visits. Additionally, 58% of tuberculosis mothers disclosed the inability to feed their kids, get proper education and fulfil the daily needs of their life. The parental disease affects the continuation of 11% school childres; 8% rural, 13% urban. Moreover; 8% of the school children join employment to support their families. Whiel; 34% of patients, parents are not able to buy enough clothing or food or books for their kids because of the heavy expenditure spent on tuberculosis treatment. Thus; the mothers with tuberculosis affect the total family circle especially the children. The family unit disrupted because of loss of earnings and reduced routine family leisure activities. The socioeconomic effect of tuberculosis over individual families is an important factor to decrease the the earning competence. Thus; the risks of families' disturbance increased in society. That's why; World Health Orgenisation has introducd the DOTS program to improve positivel the overall well being of the family by reinstating the working ability through treatment protocols and reducing the wastage of revenue. Food consumption, resources and assets can be preserved considerably by implementing the DOTS program. That renders the family financial and physical resources. The likelihood of death of the sick factory laborer and rickshaw puller may be reduced significantly by improving the family welfare over the long run (Emanuele & Narain, 2002). The major contributing "epidemic" reasons to mulitd drug resistance in certain parts of west are the outbreak of

Human Immuno-deficiency Virus, overcrowding leading, homelessncss and drug addiction. Multi drug resistance tuberculosis occures either through drug resistant mutant's collection of innovative susceptible strain or by already patient with resistant *Mycobacterium tuberculosis*. Those are developed in result of inadequate therapy or poor patient adherence (acquired resistance) (Karin, 1997).

2.2.5 WHO Contribution (DOTS Programme)

World Health Organization has major contribution to control tuberculosis worldwide. Its monogram is mentioned hare and services can be divided into following headings,

2.2.5.1 WHO Targets for TB

The two major targets to TB controls are,

1 World Health Assembly has targets in 2005 to identify at least 70% of tuberculosis infections cases (latest data 45%) and simnilarly provide treatment successfully to 85% of detected cases (82%).

2 While; the goals of millennium development has been targeted for 2015 by associated stop TB partnership; that aimed the reversal of tuberculosis incidence at the target of halv of the prevalence and death by 2015 in compassion to 1990.

2.2.5.2 DOTS Strategy

Higher than 20 million tuberculosis cases are treated under DOTS program. Wile; 82 countries have taken up the DOTS strategy. Eventhogh; a quarter of the world's population is sill seeking access to DOTS program. The DOTS strategy was offered in 1995 consisting of 1) Government commitment to tuberculosis control. 2) Diagnosis through bacteriology and an effective laboratory network. 3) Standardized short term therepay with complete patient

support. 4) Continuous supply of quality assured drugs. 5) Reporting and recording to measure the patient and programme outcome.

2.2.5.2 New global stop TB strategy

World Health Orgenisation has developed a new world wide stop tuberculosis strategy with aims of to reach all patients and strengthen the tuberculosis control activivities. That have six key importat components 1) Addressing tuberculosis, MDR-TB and HIV; 2) Pursuing the quality DOTS extension; 3) Contributing to strengthen the health care programs; 4) working groups; 5) Empowering the communities and patients; 6) Enabling and supportting the research activities. World Health Orgenisation stop tuberculosis department develops policies, standards to support the efforts and strategies in collobaration of the country offices and regional health care programs. The participant provinces and states measure the progress towards tuberculosis assesses and targets toward the national programmes performance, financing impact and partnerships facilities, advocacy and communication. While; the stop TB partnership secretariat is housed by WHO; that is an association of 400 stakeholders. That coordinates, bold and assign the responsibilities to all health care providers. They do advocate for communication social mobilization, DOTS plus, DOTS expansion, new drugs, MDR-TB, new vaccines new diagnostics, and tuberculosis HIV. The drug facility run by stop tuberculosis partner world wide is growing its access to drugs for DOTS; that has scaled up in just four years to provide 4 million successful tuberculosis treatments.

2.2.5.3 Green light committee

Green light committee work throth DOTS plus project for access to quality Multi Drug Resistance tuberculosis at reduced cost in some cases by as much as 99%.

2.2.5.4 World wide TB strategy:

The blueprint recommendations for TB control, particularly in Africa during 2006-2007 are incorporated into development agendas to strengthen the DOTS

programmes. Tuberculosis activities are expanded in partnership of HIV treatment. WHO declared tuberculosis as an emergency in Africa in 2005. While; the WHO's director for European region has warned the tuberculosis emergency in Europe. Eventhough; G8 world leaders offered to meet the needs to identify the stop TB partnership in 2005 in Africa. The development of new drugs and vaccines were encouraged in meeting of the financing needs of the world to fund in fighting against TB, AID and malaria.

2.2.6 Tuberculosis and prisoners

According to a susceptibility test a high occurrence of resistance to the 1st line tuberculosis drugs sre were experientialed. There were only 21% patients had *M. tuberculosis* strains sensitive to all antibiotics, and 23% of patients with strains of multidrug resistance. Thus; overall, there was only 42% sputum conversion rate. Those are ranging 10 to 29% (MDR strains) to 61% (sensitive strains). The overall 54% treatment rate despites an excellent compliance. Certain operational limitation were notices as under; 1). premature release or transfer of prisoners, 2). late identification of cases with consequent high death rate, 3). Convincing staff to directly observe patients while swallowing antituberculois medcicaion or lack of proper inspiration, and 4) disease advancement. The existence of highly resistant tuberculosis strains in prisons is sginficant to affected prisoners; But also the hazardous for for prison staff, their inmates, and for their families. Which should be considered especially in possible diffusion to the general population. While; the active tuberculosis patints in prisons can be controlled by early detection to improving outcome and decerase circulation. Antituberculosis therapy should be given to the patients when they are transferred or released; referred to DOTS centers or DOTS in all prisons (Emanuele and Narain, 2002).

2.2.7 Antitubercular organization

World Health Organization declared TB as a worldwide public health (Julio, 1997). The Center for Disease Control and Prevention National Surveillance Systems

registered tuberculosis patients, including MDR-tuberculosis (Moore M.. et al., 1997). There certain other local/ international, governmental/ non governmental anti-TB organization/ programmes launched are Pakistan Medial Research Council for Pulmonary & Chest Diseases (PMRC), National TB control programme, the NTP manager; National players, the National Drugs and Therapeutics Committee; the National TB Advisory Committee; the Procurement Office; the National Drug Regulatory Authority; the office responsible for inspection of stocks and distribution; the Legislative Office; the Financing Office and; the Pharmacovigilance Network.

2.2.8 Tuberculosis and AIDS

The out breakd and registration of smear positive tuberculosis patients are increased and most of these patients are sanctioned to the HIV pandemic. The relapse of tuberculosis or incidence of reappearance in a population with high level of coinfection with HIV observed intensively. Thus; being the most favorable period of tuberculosis management for individuals infected with HIV (human immunodeficiency virus) still undere discussion. The frequency of reversion in patients with HIV coinfected was compared with HIV negative individuals. Tuberculosid reappearance was defined as having positive < 30 days culture after the very last theratpy date. While; the reversion having a positive culture at least 30 days after the last therapy. Thus; individuals HIV infected were more probable than non-infected to have recurrence or relapse. Individualsl HIV infected received more close to 36 weeks of therapy was more possible than those received higher than 36 weeks to have reappearance. In netshal., physicians, pharmacists and clinicians should be attentive of the likelihood of reappearance of tuberculosis in 6-9 months after the begining of therapy. Moreover; in compariable settings, sputum examination to make sure alleviation or appraisal of three months following completion of treatment should be done among individuals with HIV infection or have received the shorter regimen. The patients of HIV infection can be effectively cured with regimens of DOT anti-TB. Though, excess mortality of patients infected with HIV despite the successful treatment is different challenged faced in clinical practic. Therefoe; if it emerges significant to HIV infected patients treatment, then necessarily be further

investigate to study the causes of high mortality (most probably becusue of opportunistic infections of other than HIV) to identify potential and successful interventions. (Emanuele and Narain, 2002). Presently; the human immunodeficiency virus is the most significant threat for disease development and following infection. Human Immunodeficiency Virus kills T4 lymphocytes or T-helper cells. That decreases the defense against infection of *Mycobacterium tuberculosis*. Therefore; Human Immunodeficiency Virus infection raised the danger of reactivation of inactive TB infection and the risk of progressive disease following new infection as well (Karin, et al., 1997).

2.2.9 World Wide Ideas

To Highlight Tuberculosis The top ten ideas recognised to highlight the tuberculosis are as follow, i) Education/ training. ii) Lobbing for funding. iii) Convincing/ discussion with thing tanks/ deciders Parades/ rallies. iv) Food & cloths for TB patient's. v) Recognizing/ obliging the workers & partners. vii) Commemorative by postage stamps. viii) Poster competition. ix) Charity sports/ games. x) New DOTS clinics 2.3 General features of *Mycobacterium tuberculosis Mycobacterium* has distinct general and molecular features. It can particularly be identified on basis of these specifications. 2.3.1 General features of M-TB. It is non-motile, non-spore/ capsule forming, rode shaped & slow growing *Mycobacterium*. It have Waxy mixture of lipid rich resistant cell wall, polysaccharides and remain dormant for decades. More than 50 species currently accepted by ICSB and 20 Species capable to cause human disease 2.3.2 Species of M-TB Forty one species of mycobacte4rium currently accepted by the international committee on systematic bacteriology (ISSB). The species that are capable of causing human diseases are referred as obligate and opportunistic pathogens. In developing countries, the major pathogenic species are M. leprae, *M. tuberculosis*, and M. ulcerans. There is very little information about the incidence of opportunistic mycobacterial pathogens in most developing countries. *Mycobacterium tuberculosis* (Koch bacillus) is the most important *Mycobacterium* species that cause tuberculosis. Tuberculosis also caused by M.bovis, M.africanum and occasionally by opportunistic mycobacteria.

Some scientist proposed a subdivision of *M. tuberculosis* for epidemiological purpose into five variants,

1 M. TB var classical human (major type causing human tuberculosis).
2 M. TB var classical bovine (bovine type M. bovis associated with cattle)
3 M. TB var asian human (human type of Asian origin)
4 M. TB var afrincan I (west African type of M. africanum)
5 M. TB var afrincan II (east African type of M. africanum)

Table 1. Mycobacterial opportunistic and obligate strains with respective pathogenicity.

OPPORTUNISTIC PATHOGENS		OBLIGATE PATHOGENS	
M. Kansasi	Pulmonary infection	*M. tuberculosis*	Tuberculosis
M. Szulgai	Pulmonary infection	M. Africanum	Tuberculosis
M. Xenopi	Pulmonary infection	M. Bovis	Tuberculosis
M. Avium	Cervical adenitis	M. Leprae	M.TB Complex MAC Leprosi
M. Scrofulaceum	Cervical adenitis	M. Ulcerans	M.TB Complex MAC Skin ulcer
M. Fortuitum	Skin infection		
M. Marinum	Skin infection Superficial		

M.bovis and M.africanum have been given their own individual species names, if the criteria used to classify other organisms were applied, including DNA homology.

2.3.3 Atypical/ anonymous/ environmental

These terms were formerly used to describe those organisms that were not tubercle bacilli. Most of these anonymous organisms have low been given species names and each organism is typical of its own species. It is now recommended, therefore that the terms atypical and anonymous should be discontinued, and the term environmental mycobacteria should be used instead.

2.3.4 Acid fast staining of M-TB

This is the most important characteristic of mycobacteria. It means that when mycobacteria are stained with carbol fuchsin in Ziel-Neelsen technique. They are able to retain (hold fast to) their red color when washed with an acid solution. Other organisms, with the exception of some nocardia species and some corynebacteria, are decolorized by the acid.

2.3.5 Cultural characteristics of mycobacteria: With the exception of M. Leprae, mycobacteria can be grown aerobically using a protein-enriched culture media. Many saprophytic mycobacteria and the opportunistic pathogens M.fortuitum and M. chelonei are able to grow on non-enriched medium i.e. nutrient agar. An atmosphere which contains an increased amount of carbon dioxide has been shown to improve the growth of some mycobacteria. The culture of specimens for the isolation of M.tuberculosis is described in subsequent subunits. Some of the cultural features which are used to differentiate *Mycobacterium* species include, a) Rate at which growth occur b) Temperature at which growth occur c) Production of pigment in light and darkness.

2.3.6 Partial resistance to acid or alkali of *Mycobacterium tuberculosis*

Mycobacteria have a much grater resistance to acid and alkali than other bacteria. Specimens such as sputum and urine that contain contaminations can be treated with as acid or alkaline reagent before being cultured for *M. tuberculosis*. Such treatment is intended to kill the commensales with the loss of as few tubercles bacilli as possible. The strength of acid or alkali used must not exceed more than is required to decontaminate the specimen otherwise large numbers of mycobacterial will be killed.

2.3.7 Identification of *Mycobacterium*

The following investigations together with the cultural features provide a basis for identifying the medically important mycobacteria:

1. Growth on medium containing 500 ug/ ml 4(p)-nitro-benzoic acid.
2. Nitrate reducing test
3. Growth on medium containing 10 ug/ ml thiacetazone,
4. Tween 81 hydrolysis test
5. Arylsulphatase test

2.3.8 Other identification tests for M.TB

Some other typical identification tests preformed to particularize the specific *Mycobacterium tuberculosis* species are as given below,

Table 2. Typical identification tests used to specify the mycobacterial species.

ACID FAST ORGANISM FORM LJ MDIUM	FIRST TEST			IDENTIFICATION
	Pigment	25° C	PNB	
Slow grower 36°C Colonies:2-6weeks	N	-	-	*M. tuberculosis* Complex
	N	-/+	+	Growth at 44°C Tween 80 hydrolysis test Possibly: M.Xenopi, M.avium intracellular complex, or saprophytes.
	P	+	+	Tween 80 hydrolyses test Growth on thiacetazone Nitrate rduction test Possibly: M. Kensi, M. marinum or M. simiae
	S	+	+	Tween 80 hydrolysis test Nitrate reduction test Arylsulphatase test Passibly: M. scrofulaceum M. szulgai or saprophytes
Slow grower 32°C No growth at 36°C Colonies:2-6weeks	N	-	-	M. ulcerans
	P	+	+	M. marimun Note: on subculture M. marinum will grow at 36°C
Rapid grower at 36°C	N or S rarely P	+	+	Nitrate reducitontest Arylsulphatase test Passibly: M.chelonee, M.fortuitum or saprophytes.

In zeil-neelsen stained smears, *M. xenopi* appears as a long pale-staining acid bacillus and *M. avium*-intracellular appears as a small acid fast *coccobacillus*.

Key: N- Nonchromogen, S-Scotochromogen, P-Photochromogen, PNB- 4(p)-Nitro-benzoic acid

2.3.9 Normal habitat of *Mycobacterium TB*

The main reservoirs for M.tuberculosis are infected humans, the organism also occur as a pathogen in animals. It mainly transmitted by infected persons coughing and spitting out bacilli which are then inhaled by others. M. bovis is found merely as a pathogen in cattle and occasionally in other animals. Human can become infected by close contact with infected cattle or by ingesting the bacilli in mild form infected animals. Person to person transmission of bovine strains may also occur. The incidence of M. bovis in developing countries is thought to be low. M. africanum has been found in several countries in East and West Africa.

2.4 Molecular Features of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis isolates invetigated genotypically for the purposes of as under;

1 Confirm the source or the origin of outbreaks (Mokrousov, et al., 2002),

2 explore transmission in public (Van Rie et al., 1999),

3 confirm cross contamination in the laboratory,

4 identify patient's of exogenous reinfection (S Jasmer, 2002) and

5 study clonal expansion of strains.

Clonal expansion is studied comprehensively in some laboratories. Moreover; an appraisal of the phylogenetic reconstruction and epidemiology has been compiled and published (Bifani, P. J., 2002).

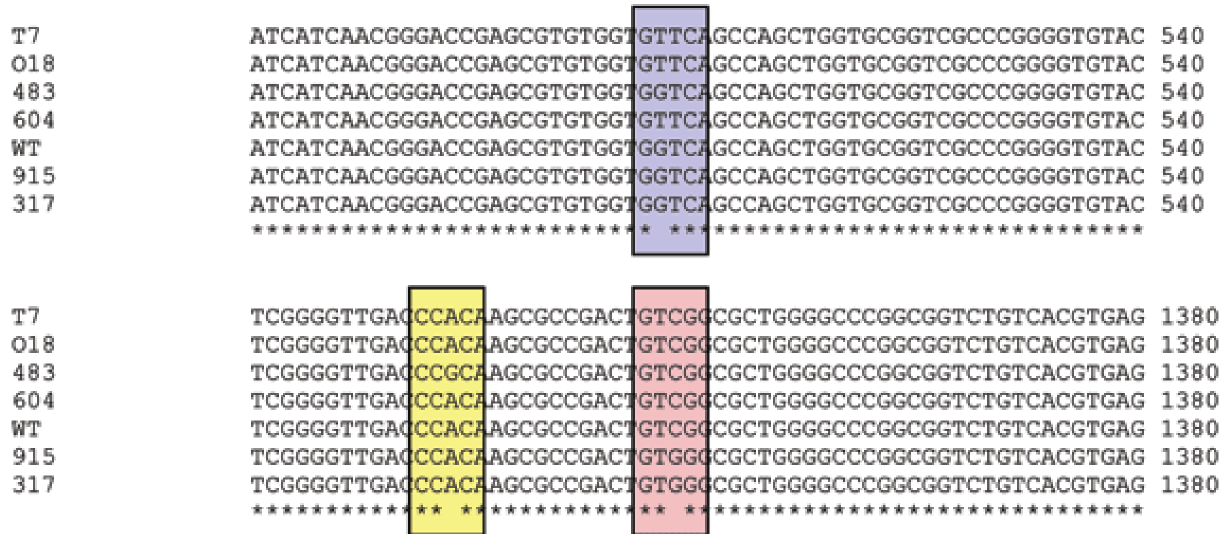


Figure 4. Genotyping of Rifampicin resistant gene of *Mycobacterium TB*.

2.5 Pathogenesis of Tuberculosis

Infection is usually a localized multiplication of the organism in the lung and nearby lymph glands. In children there is often a marked enlargement of the lymph glands. This first multiplication of the organism is referred a to as the primary tuberculosis infection. Occasionally the primary infection is in the tonsil or intestinal tract. In most people the primary infection is self-healing. The healed area often becomes calcified. Pulmonary tuberculosis can occur when the primary infection does not heal completely and there is either continued multiplication or reactivation of the organism in the lung several months or years late. Reactivation may occur due to poor health, malnutrition or defective immune response. It may also be useful to be aware of the tuberculosis pathogenicity to conceive all the relatd hazards. Majority of the people usually not get tuberculosis illness, because specific cell mediated immunity usually produced within 2-10 weeks after the initial infection (Karin et al., 1997).

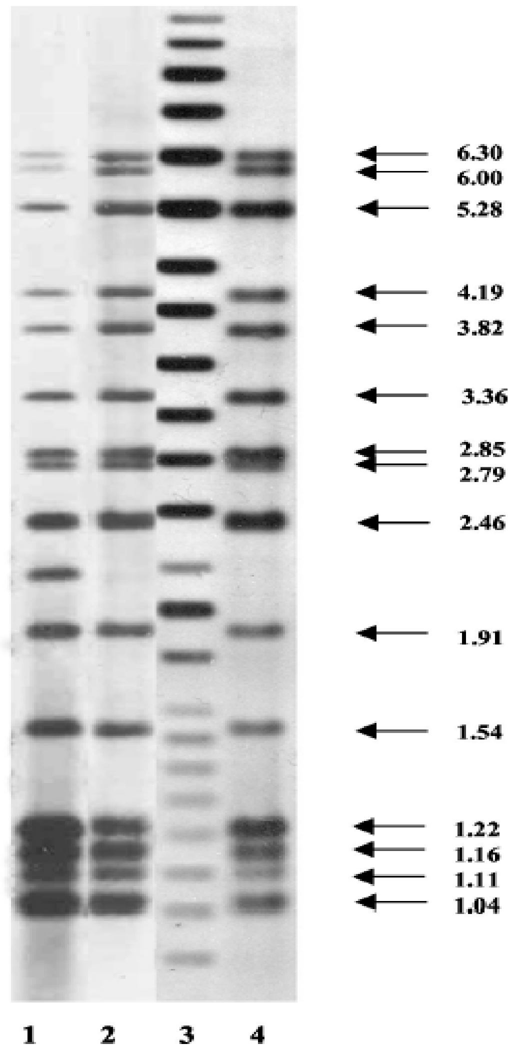


Figure 3. RFLP genotyping patterns based on IS*6110* of N family strains Seattle and New York City. Lanes 1 and 2, New York City strains N2 and N4. Lane 4, Seattle outbreak strain SBR19. Lane 3, molecular size standards (in kilobases) (S.Joy Milan et al., 2004)

2.5.1 Granulomatous Lesions and Tissues Destructions

The pulmonary tubercular lesion can be divided into five major types,

- Epitheloid granuloma with necrosis.
- Epitheloid granuloma without necrosis.
- Chronic non-specific inflammation.
- Chronic non-specific granuloma.
- Abscess.

2.5.2 Categorization of Patients (on basis of smear results)

- 1stCategory: Pulmonary tuberculosis positive patients, Pulmonary/ Extra- Pulmonary – positive & seriously ill patients
- 2 nd Category: Pulmonary positive patients having previous treatment failure/ relapse/ defaulting history.
- 3 rd Category: Pulmonary / Extra- Pulmonary – positive & not seriously ill patients

2.5.3 Disease Classification (on basis of area of origin)

Tuberculosis can be divided into two major classes – pulmonary and Extrapulmonary tuberculosis as given below,

2.5.3.1 Pulmonary Tuberculosis In the lung, M.tuberculosis causes an inflammatory reaction leasing to a liquefied destruction of lung tissue with caseation i.e. breakdown of the diseased tissue into a cheese like mass. The yellow pieces of caseous material contain tubercle bacilli and are often coughed up by the patient in sputum. Patients with advanced infection have difficulty in breathing due to cavities in there lungs. The main symptoms include chronic cough with mucoid/ mucoplurulent sputum which may contain blood (haemoptysis). In later stages there is loss of weight, fever, sweating especially during sleeping, tiredness, chest pain and anemia, enlargement of lymph, emphysema. Erythrocyte sedimentation rate (ESR) is raised due to an increase in immunoglobulin (mainly IgG and IgA). Rheumatoid factor is occasionally detected in the serum. Complications of pulmonary tuberculosis include dry pleurisy or pleural effusion, lung collapse, acute miliary tuberculosis and occasionally the tuberculous meningitis.

2.5.3.2 Extra-Pulmonary Tuberculosis

The tuberculosis that observed other than lungs referred as extra- pulmonary tuberculosis.

2.5.3.2.1 Tuberculosis of Menings

The tubercle bacilli reach the menings in the blood. Tuberculosis meningitis occurs more frequently in infants and young children, as a complication of primary tuberculosis infection. The condition is usually rapidly fatal unless treated at an early stage. It is therefore important for the laboratory to exam carefully the cerebrospinal fluid fro acid fast bacilli, especially when the fluid contains lymphocytes.

2.5.3.2.2 GIT & Lymph Tuberculosis

Continued active infection in the lymph glands can lead to the bacilli spreading by way to the lymphatic system and blood circulation to the lungs, pleural cavity, kidneys, bones and joints. An enlarged lymph gland may rupture into one of the bronchi (tubes leading to the lung) and cause acute tuberculosis of the affected lung.

2.5.3.2.3 Tuberculosis of renal and urinogenital tract and kidney

Tubercle organisms reach the kidney and genital tract by way o the blood circulation. Infection is often suspected when repeater urine specimens are isolated by routine urine culture. In the late stages of infection there is frequency in passing rune and often hematuria. There is usually a recurring fever. Tuberculosis of the genital tract may cause infertility and pelvic inflammatory disease.

2.5.3.2.4 Tuberculosis of Bones and joints

Tuberculosis that is observed by accumulation of *Mycobacterium* into bones and joints. It is not very sufficiently observed but exists with marked symptoms of pain, inflammation activation (TNF).

2.5.3.2.5 Miliary tuberculosis

Patients are often acutely ill with fever but a chronic form of the disease can also occur. The disease is usually recognized by seeing widespread fine nodules on the chest X-ray. The infection may be accompanied by a low white cell count or occasionally by a leukemoid reaction especially in elderly patients. The liver, spleen and lymph glands may be enlarged and the meninges may also become infected.

2.6 Resistance of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis Resistant is a serious threat in its successful eradication and control programmes (Victor, et al, 1997). There are so many epidemiological, genetic, social and unavoidable reasons of this resistance. Therefore, the spontaneously occuracne of multidrug resistance is rare. But; the likelihood of naturally mutant's resistant is very low. A large bacterial load in lung cavities required to be treated for MDR -TB strains to emerge (Karin et al, 1997).

2.6.1 Epidemiology of Resistant *Mycobacterium*

The turbeculosis has alarming dimensions in view of emergence of multidrug resistant tuberculosis (Herendra and Shah, 1998). Drug resistant tuberculosis is a high prevalent phenomenon. In the former Soviet Union, the isoniazid primary resistance rates are as high as 32%; while, the primary multidrug resistance is nearly 15%. The increased infection and death rates has posed an urgent challenge to detect patients rapidly and make sure the successful eradiction (Iseman, et al., 1992). The Multi Drug Resistance tuberculois patients need treatment with 2nd line drugs; that incurring higher costs due to prolonged hospitalization, more toxicity and remained in illness longer period of time (Cohn, et al., 1997).

2.6.2 Factors Contributing to the Developing the Resistance

There are clinical, biological, social and epidemiological risk factors that contribute to develop drug resistance. Suboptimal doses, omission of one or more prescribed agents and poor absorption are the main reasons for emergence of drug resistance.

Antituberculosis medicines are freely obtainable in the market. That leads to self treatment and improper regimens. While; the appearance of acquired drug resistance is primarily because of the inaccurate regimes application. Monotherapy, poor drug absorption, sub-optimal doses of the drugs and insufficient active agents in the regimen leads to reversions of one or multiple drugs resisitn from susceptible within a span of a few months. Acquired drug resistance may be caused due to selection of pre-existing mutants and their eventual proliferation because of inaccurate chemotherapy (Herendra Thakker & Sah JR, 1998).

Clinical factors

Unreliable treatment regimens - Inadequate dosage/duration of drugs. - Free availability of anti-TB drugs over the counter, poor drug supply at treatment centers, and delay in diagnosis.

Biological factors

Biological factors are large bacillary population, Genetic predisposition, local factors in host, cavitary lesion, and insufficient drug concentrations in tissues.

Social factors

Failure of public health system, neglect of disease, irregular or inadequate intake of drugs, ignorance, poverty are the major social factors that contribute in development of resistance.

Epidemiological risk factors for drug resistance

History of previous anti-tuberculois therapy, length of previous chemotherapy, contact with a known case of Multi Drug Resistance tuberculois, living in an area with high occurrence of drug resistant tuberculosis - HIV infection, and social instability (Herendra Thakker and Shah JR, 1998)

2.7 Molecular Basis of Resistance

The likelihood of mutation or resistance is directly proportional to the bacterial load. The 10^9 bacillary load will contain numerous mutants resistant to anyone of tubercular drug (Bodmer T, et al., 1995). Since the mutations impart resistance against drugs are chromosomal, therefore; the probability of mutation is simultaneously occurred in more than one drugs. Thus; the likelihood of MDR is multiplicative (Miriam Bobadilla, et al., 2001). The molecular and genetic mechanisms may involve in the acquisition of *Mycobacterium tuberculosis* resistance against drugs. That is concomitant with the development of differtn molecular strategies of rapid detection of multi drug resistance tuberculois (Ashok et al., 1998).

This antitubercular drug was introduced in 1972. In resistance; it is extremely effective against *Mycobacterium tuberculosis*. Rifampicin has MICs of 0.1µg - 0.2µg (Mitchison DA., 19985). Rifampicin with isoniazide forms the backbone of short term chemotherapy because of its high bactericidal action (Kochi, et al., 1993). It was a highly effective bactericidal first line tuberculosis medicin and key component of the initial anti-tuberculous regimen. The clinical isolates of *Mycobacterium tuberculosis* usually having the rpoB rifampin resistant gene in the 81-bp core region that encodes the B subunit of RNA polymerase (Snider DE & Roper WL, 1992). In susceptible organisms these mutations are not notices. Even though; insignificant discrepancies are reported in general, that has a strong correlation with MIC and specific amino acid substitutions. Codons 513, 526, or 531 missense mutations that occurred in elevated Rifampin resistance level. While; the change in amino acid position at 514 or 533 usually produce in low Rifampin resistance levels. In 4% of Rifampin resistant tuberculosis isolates, the molecular mechanism of resistance observed is the lack RRDR unknown changes (Pearson ML, et al., 1992). This is investigated that approximately 90% rifampin resistant clincla isolates in various areas are resistant to isoniazid, making resistance against rifampin a helpful substitute marker for multidrug resistance. That also representative of an urgent requirement of 2nd and 3rd line drugs for susceptible *Mycobacterium tuberculosis* (Edlin, et al., 1992). RNA polymerase, a complex oligomer composed of ß, ß' and, encoded by rpoA, rpoB, rpoC, and rpoD; four different subunits, is highly conserved among bacterial species (Ovchinnikov, et al., 1981).

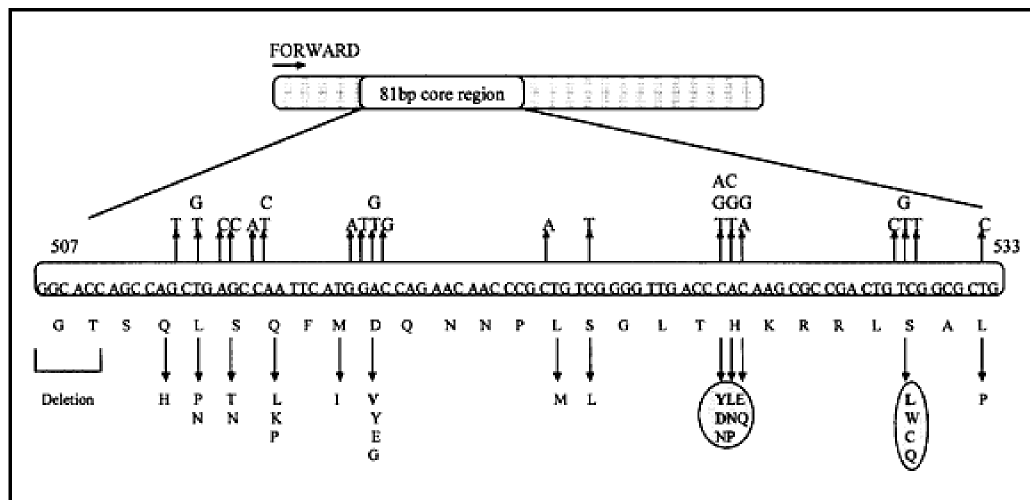
Table 3. Mechanisms of Drug Resistance in *Mycobacterium tuberculosis*

Antimycobacterial agent	Mechanism of action	Resistant Gene	product	Mechanism of resistance
Isoniazid	Inhibition of mycolic acid biosynthesis	(1) KatG (ii) inh A synthesis, (iii) ahpC	Catalase Peroxidase Enoyl-ACP reductase Alkyl hydroperoxide reductase	(i)Mutations in KatG result in failure to generate an active intermediate of EMH. (ii) Overexpression of inhA allows continuation of mycolic acid (iii) ahpC mutations m as a marker for lesions in KatG
Rifampicin	Inhibition of transcription	rpoB	B subunit of RNA Polymerase	Mutations in rpoB prevent interaction with Rifampicin
Streptomycin	Inhibition of protein synthesis	(I) rpsL (ii) rrs	Ribosomal protein S12 16s rRNA	Mutations prevent interaction with Streptomycin. Overexpression or mutation of
Etharabutol	Inhibition of arabinogalactan biosynthesis	EmbCAB	Arabinosyl transferase	embAB allows continuation of arabinan biosynthesis.
Pyrazinamide	Unknown	PcnA	Amidase	Loss of pyrazinamidase activity results in decreased conversion of Pyrazinamide to pyrazinoic acid, the putative active moiety (?)
Fluoroquinolones	Inhibition of the DNA gyrase	Gyra	DNA gvrase sub-unit A	Mutations in gyreA prevent interaction with fluoroquinolones.

Pyrazinamide is analogue (structurally) of nicotinamide; used as a 1st tuberculosis drug. In 1952, it was considered as antituberculosis, however; in the mid- 1980s, it became significant part of short term therapy. Pyrazinamide is not affected by any other drug and active against semidormant bacilli. It possesses strong synergy with rifampicine and isoniazide and reduces the chemotherapeutic program for antitubercular therapy six months from nine to twelve months. Depending on the conditions applied, MICs of pyrazinamide is being different from 8µg/ml - 60µg/ml. It has no important bactericidal results and is mainly supposed a "sterilizing drug"

(Heifets and Lindohlm, 1992). The acidic environment is supposed to be phagolysosomes the tubercle bacilli make pyrazinamidase. That enzyme produces pyrazinoic acid from pyrazinamide, the active derivative of this compound. The *Mycobacterium tuberculosis* pncA gene encoding pyrazinamidase is sequenced to describe the molecular based mechanism of pyrazinamide resistance. The outcomes provided proofs of pncA mutations gave pyrazinamide resistance. The DNA of pyrazinamide resistance clinical isolates sequenced to identify mutations at codons, 162, 141, 138 and63. While on othe hand; susceptible *Mycobacterium tuberculosis* has sequences of wild type. In 28% of pyrazinamide resistant isolates lack of pncA mutations recommended the presence of minimally one extra gene contributing the resistance. An amazingly broad range of pncA mutations resultant in structural modifications in the PncA has recognized in higher than 70% of drug resistant specimens. It is supposed that these structural modifications detrimentally change role of enzyme. In this manner; pyrazinamide altered to its bioactive form (Mitchison DA. 19985).

Figure 5. Substitutions of single amino acid in the *rpo*B gene's 81 bp core region give resistance against rifampicin. Single letter abbreviations represented Amino acids. Alterations in codon His526 and Ser531 considered for ≥70% mutations with rifampicin resistance depicted in shaded ellipses.



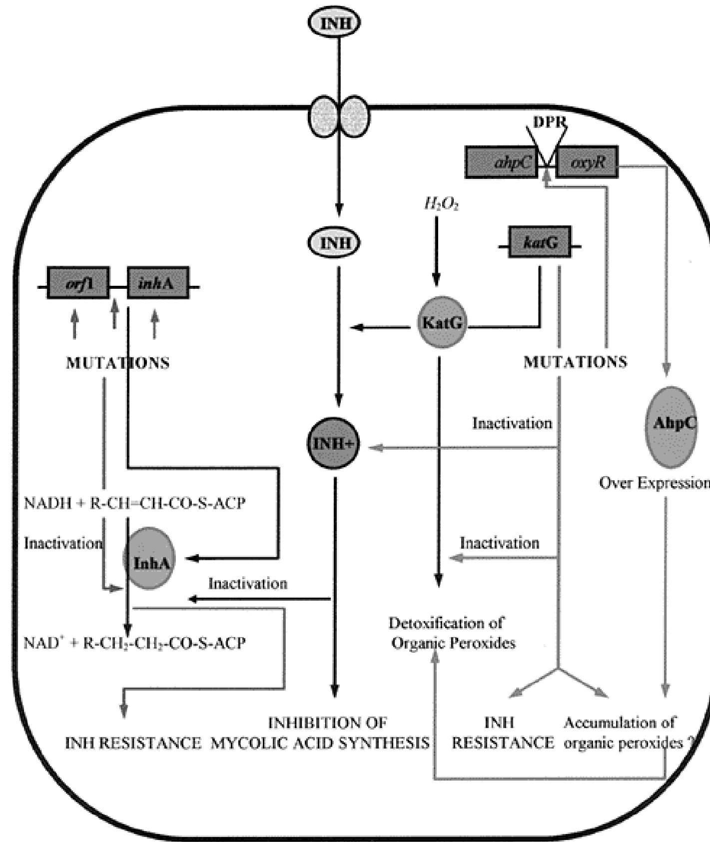


Figure 6. Resistance acquisition mechanism and combating oxidative stress by isoniazid. Divergent Promoter Region (DPR).

2.7.1 Prevention of the Development of Resistance.

Some valuable steps are taken to cure and reduce the resistance. That can assume alarming proportions. Planned and supervised therapy with judicious surgical interference is the only way to ensure cure for turbeculosis patient or prevent the spread of drug resistance (Herendra and Shah, 1998).

2.7.2 Test Procedures Used for Drug Sensitivity/ Resistance for MTB There certain methodologies used to determine the susceptibility of *Mycobacterium tuberculosis*. Which are given below,

2.7.2.1 Radiometric method

Radiometric BACTEC (Becton Dickinson) method of detection of mycobacterial growth by sophisticated gamma camera

2.7.2.2 Agar proportion method

Miscellaneous proportions of drug incorporated in media to calculate the MIC of particular antitubercular drug. 2.7.2.3 MGIT method: Microbial growth indicator tube method used to identify the susceptible/ resistant *Mycobacterium tuberculosis*. 2.7.2.4 LJ Proportion method: Lowenstein Jensen medium that is specific for mycobacterial growth is used to evaluate the susceptibility/ resistance of *Mycobacterium*. It may also be used to determine the level of resistancy of resistant *Mycobacterium tuberculosis*.

2.7.2.5 Disk elusion method

The disks with different drug concentrations used to determine the accurate MIC of specific antitubercular drug. It is usually not recommended to mycobacterial species because of some technical discrepancies.

2.7.2.6 Microtitre method

A minute quantity of mycobacterial/ antigenic substance used to produce a reaction with a given volume of substance to characterize/ determine the specific parameter.

2.7.2.7 BACTEC 450

In Becton Dickinson system a liquid medium prepared to grow the *Mycobacterium tuberculosis* rapidly that contains the radio-labelled palmitate as the sole carbon source. The growing/ multiplying

Mycobacterium use and break down the palmitate and librates radiolabeled CO2. Using the BACTEC system MTB can be detected in 9-16 days rather than more the four weeks while using conventional media.

Figure 8. Growth pattern (colonies) of *Mycobacterium tuberculosis* on LJ medium

2.7.2.8 Luciferase Assay

Luciferase is rapid assay for drug resistant bacteria. It enzyme is obtains from fireflies that produce flashes of light in ATP presence. Less amount of ATP will be produced and less light be flashd if organism is sensitive to medicine.

2.8 Diagnosis and Prognosis

Application of accurate chemotherapy and early diagnosis are important because as under;

1 Development of resistance;

2 Cause unnecessary drug toxicity;

3 Decrease chances of cure; and

4 Increase the cost of therapy (Herenra and Shah, 1998).

More than a century back; the discovery of the causative agent and the bacteriological diagnosis of tuberculosis were main obstacles in the control and treatment of tuberculosis. Becsase of its worldwide occurrence, the methods for diagnosis were under rapid development (Jain, 1996).

2.8.1 Patients personal and medical history

A prompt diagnosis and implementation of effective chemotherapy is the mainstay in the drug resistant management of tuberculosis. Any history of contact with known drug resistant patient should arouse suspicion. The drugs history (previously taken medicaction) should be recorded vigilantly, because most of the time an accurate history play a key rol in diagnosis. Drug history recorded under the heading of (a) drugs used irregularly and inadequately or as monotherapy (b) drugs used regularly in adequate doses for short periods © drugs never used (d) cross resistant dings. If drugs have been used irregularly in inadequate doses or as monotherapy, the chances of patients becoming drug resistant are high. If drugs were used regularly in adequate combination and doses, the chances are that the patient is still sensitive to drugs.

2.8.2 Patient's Physical (Symptomatic)

Examination Physical symptoms include anorexia, weight loss, fever, sweating, weakness, sputum with/ with out blood, respiratory complications, chest pain etc.

2.8.3 Clinical evaluation

Presence of drug resistance should be suspected when (i) No clinical improvement is evident in the patient inspite of adequate chemotherapy and regularity during three months; (ii) Presence of giant/ multiple cavities and lung destruction; (iii) Sequential x-rays showing deterioration or no improvement after three months of therapy (iv) Fall and rise phenomenon may occur in which direct sputum smear examination initially shows a fall in number of bacteria due to killing of susceptible strains. This is followed by a gradual rise in count due to growth of resistant organisms that remain unaffected by the

drug. The presence of this phenomenon indicates drug resistance, (v) Suspect presence of drug resistance when patient is on regular and adequate chemotherapy but sputum for Acid Fast Staining Bacilli (AFB) by direct smear is affirmative even after five months of tuberculosis therapy, (vi) Immunocompromised person or person with HIV positive infection may develop drug resistance. The clinical parameters arc more important and informative for diagnosis instead of exclusive dependence on sophisticated and costly laboratory investigations. Traditional culture and sensitivity test: The drug resistance is confirmed by obtaining the culture and sensitivity test. The sensitivity culture test of the *Mycobacterium tuberculosis* can be determined with the assistance of either LJ Media or 7H10, 7H11 Middle brook. Therefore; if person is smear positive. Then; it is gon through the determinatioin of pattern of resistance by incorporating the drug into the LJ medium. Eventually; the results are obtained within 6-8 weeks. While; in non tuberculosis or sputum negative patients, culture of the *Mycobacterium* is necessary and susceptibility tests of cultured bacilli ar carried out. This generally requires 12-16 weeks. While; the traditional techniques delay the treatment and time taking; thus at least need 2-4 months. The rapid and newer methods of sensitivity and culture have been established.

Slide culture

Mycobacteria are fixed on slide and then transferred to the liquid medium containing the drug. This is incubated at 37° degree C for one week and the growth is examined microscopically. It gives sensitivity results within 8 to 10 days.

Egg enriched sheep blood media (EESBM)

In this, egg enriched sheep blood medium is used instead of LJ medium for rapid isolation of organisms by using slide culture, when sputum positive samples are used, the outcomes of drug susceptibility are obtainable within 8-10 days. The results of culture and sensitivity tests of mycobacteria by using EESBM are almost at par with those of traditional culture as LJ medium.

BACTEC System

It is radiometric detection of mycobacterial growth with the help of sophisticated gamma camera by using radioactive14C. It gives results of culture and sensitivity within 10-14 days

Luciferase Reporter Mycobacteriophage Test (LRM Test)

This is one of the the newer and rapid methods used for accurate culture and susceptibility testing. This test uses mycobacteriophage, a virus that infects *M. tuberculosis*, and has a cloned gene for production of luciferase reporter enzyme. The LRM (Luciferase mycobacteriophage) when mixed with culture of bacterial cells results in production of light. The procedure of the test includes growth of mycobacteria in the drug treated medium to which LRM particles are added and then emitted light is measured. If drug kills the mycobacteria no light is produced, revealing sensitivity of the organism to the drug. If *Mycobacterium* is resistant to the drug, the cell remains unaffected and light will be produced. This test is extraordinarily sensitive, specific and rapid. It provides results of sensitivity of M Tuberculosis to various drugs within 48 to 72 hours.

Restriction fragment length polymorphism (RFLP)

RFLP is used to classify and compare the clinicall isolates of *Mycobacterium tuberculosis*. Patients isolates can be compared and can be categorized into drug sensitive/multiple drug resistant and treatment can be started on that basis.

DNA Finger printing

It has been confirmed that during development of resistance against drus, DNA is usually be changed.

Single stranded Confrontation Polymorphism in conjunction with PCR (SSCP PCR)

This test is very useful for primary drug sensitivity. isoniazide, RMP, SM resistance can be per- formed directly and gives results in 48 hours. Currently this test is being tried.

Ligase chain reaction

The enzyme DNA Ligase is utilizes in this test to join two strands of DNA as double strand. Thus; ligase chain reaction method it is possible to detect mismatch of nucleotide. (Jain, 1996)

2.8.4 Radiological Examination (X- Rays Abnormalities)

X ray evaluation used to determine the outcome the treatment protocol/ regimen. If certain abnormalities retained, the therapeutic profile reviewed and may be changed.

2.8.5 Acid Fast Staining/ Microscopic Examination

The pulmonary tuberculosis can be diagnosed be made by the identification of acid fast bacilli by microscopy method, using fluorochrome stain and/or carbol fuchsin stain. Microscopic method is a rapid but be deficient in adequate sensitivity. Therefore; it does not differentiate among various mycobacteria species. The microscopy sensitivity is not often ≥ 25-40% as compared with culture. However; under ideal environment, it may be possible to attain 60-70% rate. Microscopy method is still the diagnostic and preventive base for tuberculosis control programme in developing countries. The carbol fuchsin stain method is not supposed be better thatn fluorochrome stain technique; however it is less specific, expensive and not suitable to use as a everyday procedure (Jain, 1996). The sputum from the tuberculosis suspected patient processed to acid fast staining. These acid fast bacilli examined under microscope. The growth pattern and number of colonies are counted.

Fluorescent staining

In this type fluorescent staining of staining, Auramine Stain is used which can be visualized by florescence microscopy.

False acid staining

There is no actual tuberculosis but the specimen indicates a positive test results. False negative AFB staining: The negative AFB test result of a tuberculosis positive patient called false acid fast staining.

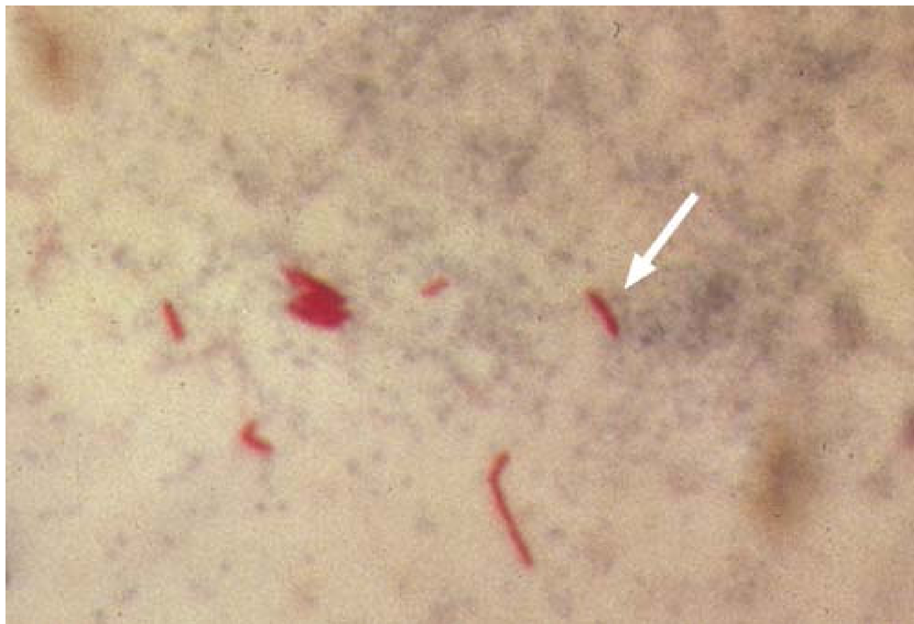


Figure 9. The microscopic view of acid fast bacilli stained *Mycobacterium tuberculosis*

2.8.6 Cultivation and Identification

For the identification of tubercle bacilli, culture is considered as the reference technique. But; culture of *Mycobacterium tuberculosis* is expensive, slow and laborious. However, not more than 50% of clinically diagnosed patients are confirmed by culture. Conventional culture on LJ egg medium acquires two to six weeks. Therefore; looking for some other media for tubercle bacilli culture, i.e. 7H10 and 7H11 agar resulted in a higher and faster rate of identification. These media are are more costly and usually used

in advanced countries of the world. The radiometric respirometry technique (BACTEC) can drastically reduce the time needed for detection and more sensitive. Moreover; the median time being around 4 weeks both for sensitivity testing and detection. But; because of its specificity, imitations in terms of cost and biohazards; it is not appropriate for third world countries. Therefore; this method is usually used in the United States (Jain, 1996).

2.8.7 Automated Detection System

Radiometric BACTEC Method, Microbial Growth Indicator Tube (MGIT) and Detection of Microbial Antigen are used to diagnose the disease as automated detection system.

2.8.8 Genodiagnosis

For Multidrug-resistant *Mycobacterium tuberculosis*, the demand of an urgent detection is rapidly growing from clinician's side. Thery need exact molecular and rapidly diagnose.The involvement of various deletion, missense mutations and insertion within hypervariable region of rpoB, the gene encoding theß -subunit of the DNA dependent RNA polymerase of Mycbacterium tuberculosis to investigate the development of resistance to rifampicin (Vitali, et al., 1999). Description of the molecular level mechanisms of antituberclosos drug resistance has contributed in the application and development of different PCR-based strategies designed for rapidl detection of mutations linked with respective drug's resistance. The molecular assays are potentially more sensitive and rapid methods for drug resistance detection. They are hypothetically able to offer a similar day diagnostic report of clinical isolate. The usefulness of these tests is dependent on their capability to identify all ordinary mutations relagted to drug resistance.

2.8.8.1 DNA probes

Developments of molecular genetics of *Mycobacterium* have made it possible to recognize specific DNA sequences that are particulalr for individual *Mycobacterium* species. These distinctive DNA sequences can be identified by

means of labeled oligonucleotides, whick are accurately complementary to the nucleotide sequence in the *Mycobacterium* genomic DNA. These DNA probes can recognize genus with species specific bacterial DNA sequences or high specificity. While; such probes have revealed the great specificityand sensitive, when utilized in research laboratories, these may some lose sensitivity and specificity if clinical samples used directly (Jain, 1996). *Mycobacterium tuberculosis* isolates can be tested with Inno-LiPA (Innogenetics Belgium), a line probe resistance determining hybridization assay, as explained in the manufacturer's directions. That surrounding the RRDR or 81-bp region of the rpoB gene (Vaire, et al., 2005). The assay's sensitivity may be enhanced if used on fresh samples. The *Mycobacterium* can be damaged by deep freezing, causing the release of DNA that can be washed away.

2.8.8.2 PCR

Presently; a sufficient attention is given to the usage of polymerase chain reaction (PCR). The principle of the PCR technique is based on the amplification of a given DNA sequence to a large number of copies that can be identified by separation on gel electrophoresis and, subsequently, either with or without probing with a labeled oligonucleotide specific for the amplified DNA fragment. Approximately; all PCR laboratories are facing the contamination problem, because there are millions of suitable templates contain in product of the reaction that can be carried back to the next assay on pipettes, finger tips, in aerosols or clothing. The extraction of DNA from the cells of *Mycobacterium* is another difficulty. This is one of the significant restraining factors in evaluating the sensitivity of PCR test for *Mycobacterium tuberculosis* (Jain, 1996).

2.8.8.3 PCR-SSCP

To screen the gene mutations associated with resistance, PCR-SSCP and sequencing techniques were used. To confirm the identity of DNA fingerprint, an

analysis made on sequential isolates. While; the treatment had a deep effect on modifications in drug resistance blueprints, the MIC for a specific agent remained constant in follow up isolates. Fingerprinting and mutational analysis of DNA demonstrated that the genome of MDR strains of *Mycobacterium tuberculosis* is comparatively stable throughout the way of treatment. The rpoB gene was the mainly mutated structural gene concerned a novel C to T mutation upstream of open reading frame (ORF)1 of the inhA operon was 51 detected and in drug resistance. There were no clues established for the existence of strain W in New York in this group of MDR strains (Victor, et al., 1997).

2.8.9 Serodiagnosis

The ELIZA and IID one step tuberculosis tests can be performed for diagnostic purpose.

2.8.9.1 ELIZA test

Like all other bacteria, the tubercle bacillus, is rich in antigens that stimulate antibody production. A number of tests are established in the last one century to identify particular antibody response in supposed patiets. These assays are used either for fragments of AFB or whole bacteria, purified antigens both by chromatography, culture filtrates, partially purified antigens and by DNA recombinant technology. Different methods are used to carry out the assays, i.e radio-immuno-assay, immunoblot and ELISA. Furthermore, instead of looking for particular antibodies, efforts have been made to identify antigens in clinical specimens using particuallr monoclonal and polyclonal antibodies. There are a number of inherent complexities in hunting for antibodies to diagnose tuberculosis. Response of antibodies may persist for years specifically if an individual immune system identified a dormant bacilli population. While; in elevated occurrence regiond, it may be hard to differentiate existing from old illness. But, currently, no test is suitable for the tuberculosis diagnosis. Many physicians use the tuberculin test for the diagnosis purpose. It is not a diagnostic test as it disclosed only whether a person is infected or not with *Mycobacterium tuberculosis*. However; can't distinguish between disease and infection. In

countries with high endemic of tuberculosis, this assay is of no use at all, because a big part of the population is already infected with the bacilli (Jain, 1996).

2.8.9.2 International immune diagnostic (IID) one step tuberculosis test

The serodianostic test is limitedly used in research and investigation. It is not yet trust worthy and may be developed in future for rapid tuberculosis diagnosis. 2.8.10 Gas Liquid Chromatography and HPLC It is used to detect the specific biomaterial of MTB in given patients specimen. It also detect the AFB fragments culture filtrates, partially purified antigen/ purified antigen of MTB to produce a conclusive clinical report. It is the chromatographic elucidation of *Mycobacterium* or its metabolites for diagnostic or research purpose.

2.8.11 Susceptibility Evaluation

In the treatment of difficult tuberculosis cases, the role of drug sensitivity testing should not be underestimated. Hence; this is an additional significant area where the laboratory has significant function. Antituberculosis drug therapy depends on the susceptibility of the tubercle bacilli. Drug resistance is the potential of tuberculosis strains to grow and survive even if exposed to optimum drug concentrations that kill or inhibit the parental bacilli, and shift this trait to its progeny. Drug susceptibility testing should be preffered for the the individuals as undr;

1. Patients that remain smear (sputum) positive even after 2-3months' of intensive therapy
2. Interruption or treatment failure cases
3. Patients having tuberculosis symptoms and are in close contacts of MDR tuberculosis cases

4 Patient having the symptoms of tuberculosis and are directly with tuberculosis patients e.i. prisoners, laboratory workers health and care workers (Karin , et al., 1997).

The quality of laboratory susceptibility testing has impacts directly on tuberculosis treatment and is of vital significance. Reporting and laboratory methodology must be caliberated, standardized and controled appropriately.

Table 6. Antiturberculosis critical concentrations (μ/ml) used in susceptibility assay

Drug	Middlebrook 7H10	Löwenstein-Jensen	Radiometric BACTEC 12B
Rifampicin	1.0	40.0	2.0
Streptomycin	2.0	4.0	2.0
Isoniazid	0.2	0.2	0.1
Ethambutol	5.0	2.0	2.5

(Karin, et al., 1997).

Critical drug concentration can be defined as a sufficient degree of drug to make it rationally sure that the concerned strain is unlike from a tubercle bacilli sample that have not at all come in contact with the drug. Currently; the known mechanism drug resistance acquisition of *Mycobacterium tuberculosis*'s is spontaneous mutation (Jain, 1996). There were various techniques; E test, bioluminescence, polymerase chain reaction single strand conformational polymorphism (PCR-SSCP) etc used for drug susceptibility testing of tubercle bacilli in past. The proportion method that use Lowenstein Jensen (L-J) medium is most widely accepted.

Drug susceptibility

The clinical isolates of *Mycobacterium tuberculosis* used for susceptibility testin of antituberculous drugs rifampicin, isoniazide, ethambutol, ciprofloxacin and streptomycin. The proportion methods use the Middlebrook 7H11 agar and

LJ medium to executed experiments with accordance of the established standards or procedures (Sachdeva , et al., 2002).

Table 7. Miscellaneous MIC,s of anti-TB drugs against *M. tuberculosis*.

#	Origin	RFP µg/ml	PZA µg/ml	ISN µg/ml	ETD µg/ml	CIP µg/ml	STP µg/ml
1	Dept. of Respiratory Medicines, Semmelwies University, Budapest, Hungry.	>32					
2	A.K. Praharaj et.el, Armed Forces Medical College, Pune, India.	>64	>100	>1	8		
3	Memorias Do Insti. Oswaldo Cruz, NCCLS, 2002	40		0.2-1	2		4
4	Dept. of Clinical Microbiology, Christian Medical College, Velore, India.	64		0.4			
5	American Society of Microbiology J of Clinical Microbiology. (Middle Brook 7H10, BACTEC 460)					≤0.5	
6	Pakistan Medical Research Centre for Tuberculosis & Chest Diseases, KEMC/ Mayo Hospital, Lahore, Pakistan.	40	100	0.2	2		4
7	Arneimittelforschung, Germony	4-32					
8	WHO/CDS/TB/2001.288			0.1-1			2-8
9	CDC/, USA, 2004		100-		1-5		
	Resistant to higher INH concentration	1-50	400	0.1-	1-7.5		1-10
	Resistant to Ethambutol	5-50	1-400	1-10	1-5		1-10
	Resistant to low INH concentration	1-50	1-400	10-	1-5		1-10
	Fully susceptible	1-50	1-400	100			1-10
				1-100	0.5-		
10	Richard J., JR Wallace et.al; 1986,	0.125-16	0.25-32		64		0.5-64
11	Ahmed Yelmiaz et.al; 2004 LJ	40	0.2-1		2		4
12	Public Health Agency, Canada, (BMM)	0.25-16	3.2-0.05		0.5-32		0.5-32

E test susceptibility

E test strips placed on the surface of the agar, the plates sealed with cling films and again incubated at 37°C in CO2 jar for 7-10 days. E test strips containing gradient concentrations of the INH, RIF, EMB, STM and CIP used. Minimum

inhibitory concentrations (MICs) recorded and reported as sensitive or resistant as per AB Biodisk manufacturer's guidelines (Hoffner SE, et al., 1994)

2.9 Treatment of Tuberculosis

The tuberculosis is treated by certain drugs (chemotherapy), radiation and surgical methods. The detail of all treatment strategies as given below,

2.9.1 Chemotherapy of Tuberculosis

There are four major 1stline drugs – rifampicin, isoniazid, pyrazinamide and ethambutol available to cure the tuberculosis. There also 2 nd line drugs available to treat the resistant *Mycobacterium tuberculosis*.

2.9.1.1 1stline therapy of susceptible *Mycobacterium tuberculosis* (MTB)

There are toxicity induction, variance in dose and regimen, poor supervision, clinical complication and resistance developments, are the major hurdles in the chemotherapy of susceptible and resistant *Mycobacterium*. The first line therapy comprised of four or some time five drugs – rifampicin, isoniazid, pyrazinamide, ethambutol and streptomycin. The dose, protocol and regimen are decided on basis of severity & type of disease and pathological & personnel history. The clinical record of body temperature, weight, cough and general well being is more important than many sophisticated investigations to assess the success of retreatment regimens. During the initial therapy, bacterial count in sputum specimen should be done every week to assess mycobacterial burden. It should be followed by sputum smear examination for AFB at monthly intervals till sputum conversion. Development in the results of bacteriological assays of sputum is the specific marker of the success of a chemotherapy regimen. Sputum culture for *Mycobacterium* should be done every three months till

the end of the therapy. Improvement assessed by X-ray is a non-specific and less reliable parameter and should be done every third month. (Herendra & Shah, 1998). Directly observed treatment all over the chemotherapy course is important with objective of 18-24 months of therapy.

There are so many patients successfully treated with DOT's ambulatory therapy provided to ensured eradication of tuberculosis illness. Therefore; preventing treatment interruption, enabling patients to remain in employment, release up scarce beds, decreasing costs and therefore. Thus; tuberculosis sick individuals are trained for fundamental infection control procedures: sputum disposal, seperate sleeping place, safer coughing, sunlight and ventilation. It must be kept in mind that the individual has previously had contact over an elongated period of time with persons he/she lives with has extra risk for this disease. The patient should be admitted, if any of the following decisive factors are involved; 1) Previous treatment interrupter, 2) Poor clinical condition, 3) Major adverse drug reactions, 4) Complications ie haemoptysis, and 5) Poor social circumstances.

Characterization of to manage the Multidrug Resistant tuberculosis patients can be done as by under;

1. Provision of a social worker for support and counsel,
2. Rationalizin the drug susceptibility examination of specimens from MDR tuberculosis patients
3. Keeping registers updated,
4. Provision of key nursing staff to provide direct and continuity observation of therapy,
5. Therapeutical drug compliance monitoring,
6. Improving the motivation and education of patients,
7. Developing measures for rapid recall if patients interrupt their therapies,
8. Evaluating and tracing contacts rapidly (Karin, et al., 1997).

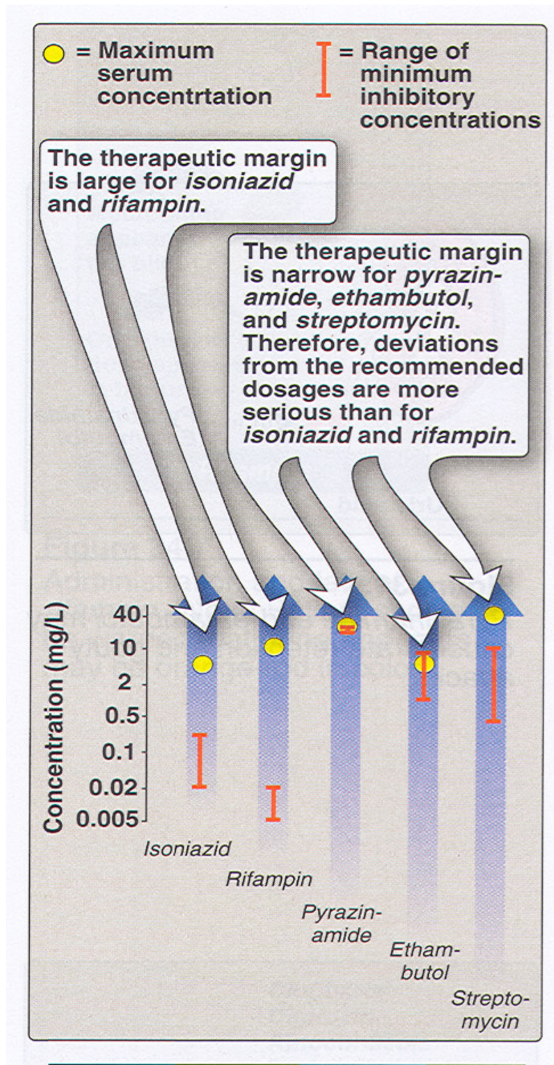


Figure 10. One of several recommended Multidrug schedule for the treatment of tuberculosis.

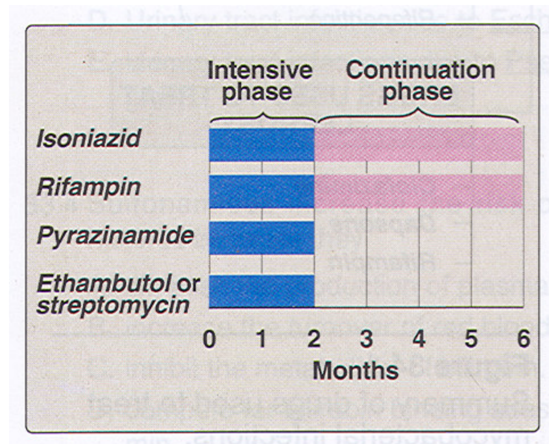


Figure 11. The therapeutical margin for different antitubercular drugs.

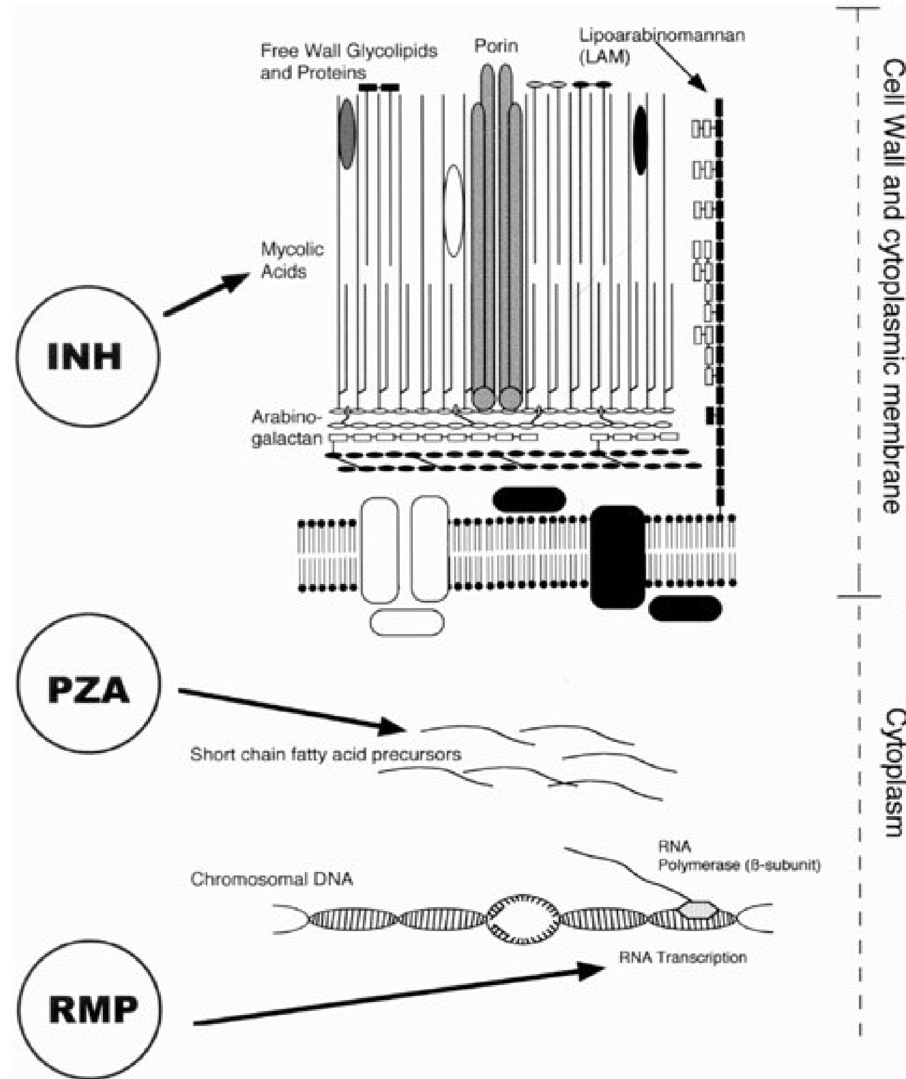


Figure 12. Molecular level mechanism of action of isoniazid, pyrazinamide and rifampicin.

Table 8: Recommended duration of therapy of tuberculosis

No.	**Regimen**	**Months**
1	Isoniazid, Rifampicin, pyrazinamide	6
2	Isoniazid, Rifampicin	9
3	Rifampicin, Ethambutol, Pyrazinamide	6
4	Rifampicin, Ethambutol	12
5	Isoniazid, Ethambutol	18
6	Others	24

#

8: **2.9.1.1.1 Pharmacological of Rifampicin.**

It having broader antimicrobial activity than isoniazid and isolated from the mold streptomyces soil. Rifampicin has been found application in the treatment of number of different bacterial infections. Since resistant strains quickly emerge during treatment it is never recommend as a single agent in the threpy of active patients of tuberculosis.

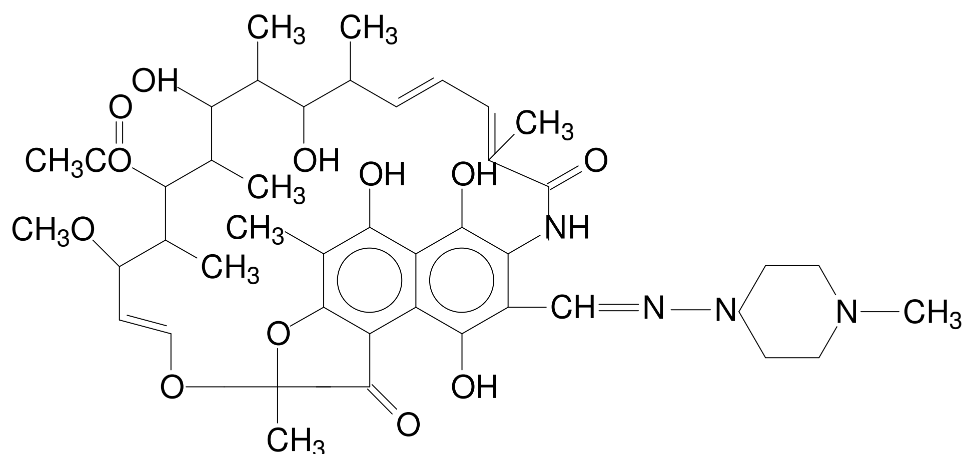


Figure 13. Molecular structure of Rifampicin.

Mechanism

Rifampicin inhibits the transcription by interacting with the β subunit of bacterial (rather thatn human) DNA depending RNA polymerase. Rifampicin is specific for prokaryotes and inhibits RNA synthesis by suppressing the initiation step. Resistance: Résistance to rifampicin can be caused by a mutation in the affinity of the bacterial DNA polymerase for the drug or by decreased permeability. Pharmacokinetics:

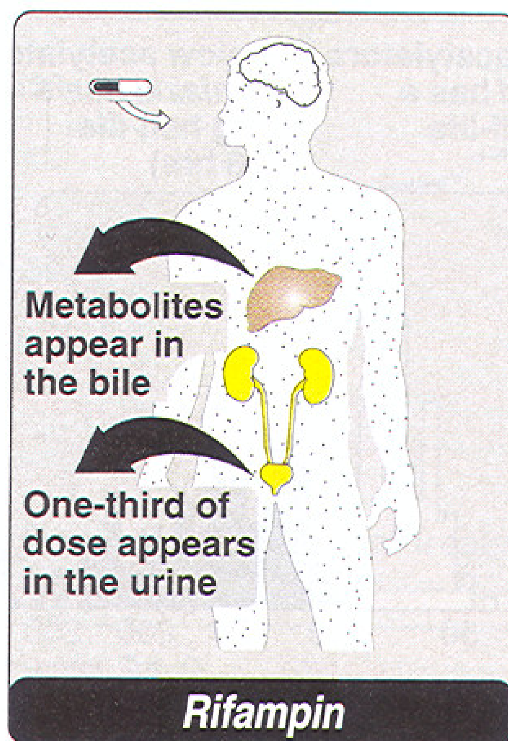


Figure 14. Administration and fate of rifampicin

Rifampicin is moderately absorbed after oral administration. Distribution of rifampicin occurs to all by fluids and organs. Adequate levels are attained in the CSF even in absence of inflammation. The drug is taken up by the liver and undergoes enterohepatic cycling. Rifampicin itself can induce the hepatic mixed-function oxidases, leading to a shortened half life. Elimination of metabolites and the parent drug is via the bile into the feces or via the urine. There is orange-red coloration of urine and feces and tear may permanently stain the soft contact lens. Adverse effects: Rifampicm can produce the nausea, vomiting, rashes and fever. There is jaundice introduce the chronic liver disease, alcoholic and elderly patients. Interaction: Rifampicin induces a number of cytochrome P450 enzymes as mentioned in figure. It can decrease the half lives of drugs that are co-administered and metabolized this system.

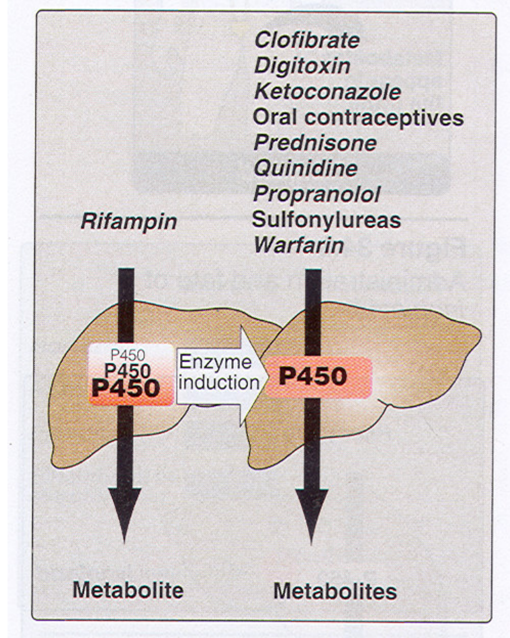


Figure 15. Rifampicin potentiates the adverse effects of Phenytoin.

2.9.1.1.2 Pharmacological of isoniazid

The hydrazide of isonicotinic acid or isoniazide is a synthetic analog of pyridoxine. This drug is not at all given as single therapeutical agent in the treatment of active tuberculosis. It is also the most effective the antituberculosis drugs. Isoniazide has revolutionized the successful eradication of of tuberculosis.

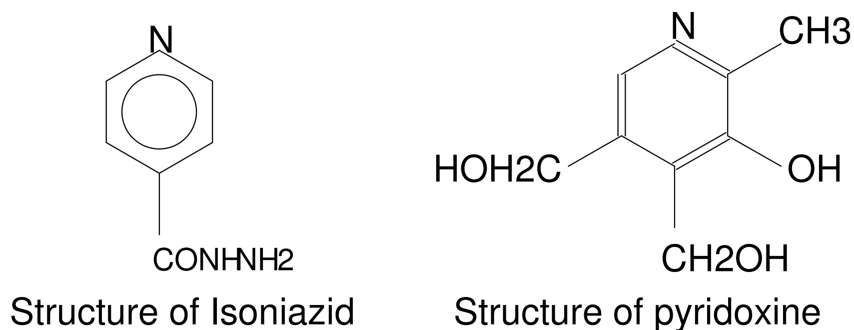


Figure 16. Molecular structure of Isoniazid and Pyridoxine.

Mechanism: This pro-drug is activated by mycobacterial catalase-peroxidase (KatG). The biochemical and Genetic evidence has

implicated at least two dissimilar target enzyme concerned in the synthesis of mycolic acid. Mycolic acid is a unique class of very long chain, β-hydroxylated fatty acid found in mycobacterial cell wall. Decreased mycolic acid synthesis corresponds with the loss of acid fastness after exposure to isoniazid. The targeted enzymes are enyol a β-ketoacyl-ACP synthase (KasA) and acyl carrier protein reductase (InhA).The activated drug covalently binds to and inhibits these enzymes, which are essential for the synthesis of mycolic acid Resistance: This is associated with several different chromosomal mutations, each of which results in one of the following; mutation or deletion of KatG (producing mutation incapable of prodrug activation), varying mutation of the acyl carrier proteins, or overexpression of InhA. Cross-resistance does not occur between isoniazid and other antitubercular drugs. Pharmacokinetics: Isoniazid undergoes hepatic N-acetylation that is regulated genetically, with the fast acetylator trait being autosomally dominant. Chronic liver disease decreases metabolism, and dose must be reduced. Excretion is through glomerular flirtation, predominantly as metabolites. Slow acetylators excrete more of the parent compound. Severely depressed renal function result in accumulation of the drug, primarily in slow acetylators.

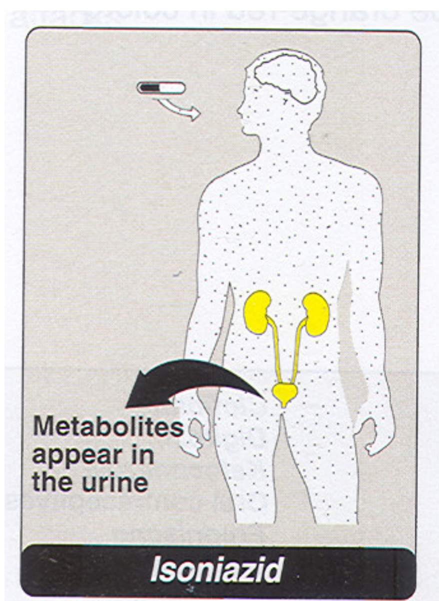


Figure 17. Administration and fate of isoniazid

Adverse effects: The adverse effects are fairly low except hypersensitivity. The adverse effects are related to dosage and duration of administration.

Peripheral neuritis: Manifested as paresthenia which is the most common adverse effect, appears to be due to a relative pyridoxine deficiency. It is corrected by pyridoxine (vitamin B6) supplementation and therefore particularly recommended to lactating women to intake vitamin supplementation. Hepatitis and idiosyncratic hepatotoxicity: potentially fatal hepatitis is the most sever adverse effect. That is caused by a toxic metabolite of monoacetylhydrazine, formed during the metabolism of isoniazid. Incidences are further aggravated in aged, rifampicin taking and alcohol drinking patients. Other adverse effects: Metal abnormality, convulsion in patient prone to seizures, optic neuritis and hypersensitivity reactions including rashes and fever. Interactions: The drugs that interfere the P-450 hepatic microsomal isozyme i.e. Phenytoin may potentiates the unwanted effects profile.

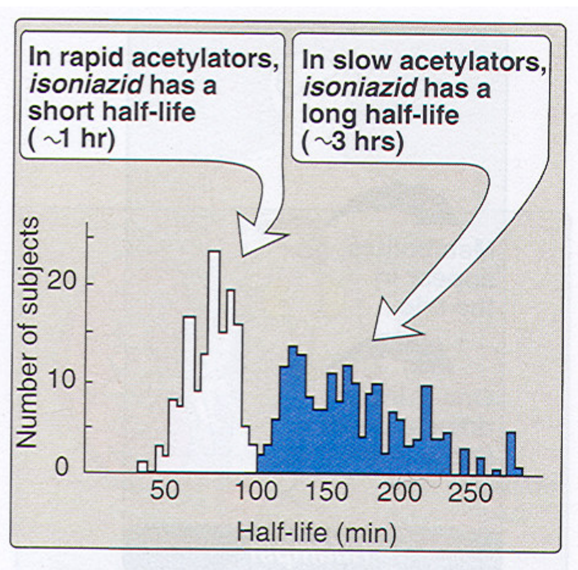


Figure 18. Bimodal distribution of isoniazid half lives caused by rapid & slow acetylation of drugs

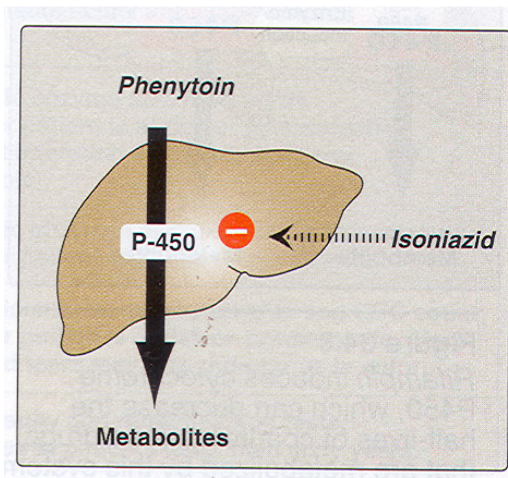


Figure 19. Isoniazid potentiates the adverse effects of Phenytoin

2.9.1.1.3 Pharmacological of Ethambutol

This drug is bacteriostatic and specific for most of *M. tuberculosis* and M. kansasi. Ethambutol inhibits arabinosyl transferase - an enzyme that is important for the synthesis of the mycobacterial arabinogalactan cell wall. While; the resistance is not a serious problem if the drug is employed with other antitubercular agents. Ethambutol can be used in combination with pyrazinamide, isoniazid and rifampicin to treat tuberculosis.

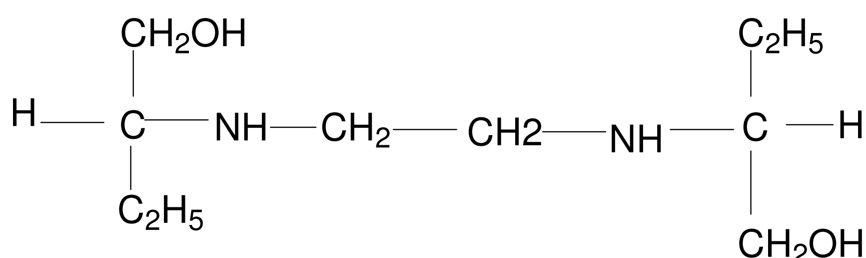


Figure 20. Molecular structure of Ethambutol

Absorbed on oral administration ethambutol is well distributed through out the body. Diffusion into the central nervous system is therapeutically sufficient in meningitis tuberculous. Both; the metabolites and parent drug

are excreted by tubular secretion and glomerular filtration. Optic neuritis is the most significant adverse effects, which results in diminished visual acuity and ability to differentiate between green and red lost. Visual acuity should be periodically examined. Discontinuation of the drug results in reversal of the toxic symptoms. In addition, urate excretion is decreased by drug; thus gout may be exacerbated as mentioned in figure. Antitubercular have a therapeutic margin, as with any drug, is difference between the maximum concentration that can be given without provoking drug toxicity and minimum drug concentration required to inhibit the growth of *Mycobacterium tuberculosis*. This therapeutic margin varies for the various 1st line medicines (Richard, et al., 2006).

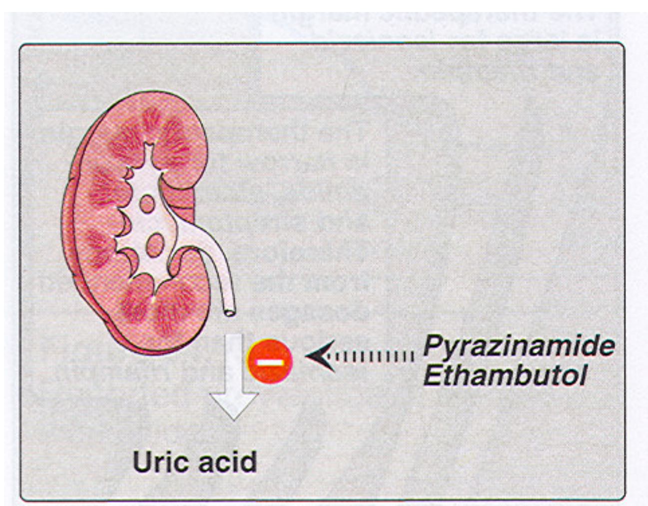
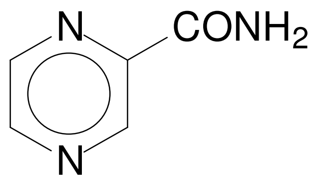


Figure 21. Pyrazinamide & ethambutol cause urate retention & gout attack.

2.9.1.1.4 Pharmacological of Pyrazinamide

Pyrazinamide is a synthetic, orally effective, bactericidal antitubercular agent used in combination with isoniazid and rifampicin. It is bactericidal to actively dividing organisms, but the mechanism of its action is unclear. Pyrazinamide have to be enzymatically hydrolyzed to pyrazinoic acid, that is the active drug form of the Pyrazinamide. Some resistant strains lack the pyrazinamidase. Pyrazinamide is active against tubercle bacilli in the acidic environment of lysosomes as well as in macrophages.



Structure of Pyrazinamide

Figure 22. Molecular structure of Pyrazinamide

Pyrazinamide distributes all through the body, penetrating the CSF. It undergoes widespread metabolism. About one to five percent of patients taking 65 isoniazid, rifampicin and pyrazinamide may experience liver dysfunction. Urate retention can also occur and may precipitate a gouty attack.

Table 9: Some characteristics of first line drugs used in treating tuberculosis

Drug	Adverse effects	Comments
Ethambutol	Optic neuritis with blurred vision, red-green color blindness.	Establish baseline visual acuity and color vision, test monthly.
Isoniazid	Hepatic enzyme elevation, hepatitis, peripheral neuropathy.	Take baseline hepatic enzyme easements, repeat if abnormal or patient i9s at risk or symptomatic clinically significant interaction with Phenytoin and antifungal agents. (azoles).
Pyrazinamide	Nausea, hepatitis, hyperuricemia, rashes, joint ache, gout (rare) .	Take baseline hepatic enzyme and uric acid measurements. Repeat it abnormal or patient is at risk or symptomatic.
Rifampicin	Hepatitis, GI upset, rash, flu like syndrome, significant interaction with several drugs.	Take baseline hepatic enzyme measurements an CBC count repeat if abnormal or patient is at risk or symptomatic. Warn patient that urine and tears may turn red-orange in colour.

2.9.1.2 Second line therapy of Resistant MTB

The fundamental principles of therapy of multi drug resistant tuberculosis are as under;

1. Use of 1st line drugs should be preferred since they are less toxic and more effective
2. Drugs chosen for the therapy should not be used in past or used regularly for shorter time,
3. Medicines are to be given in enough doses and for sufficient period of time (Herendra & Shah, 1998).

Directly observed therapy throughout the intensive phase of therapy is should be considered as national policy. Good adherence throughout the intensive treatment phase, that reduces the time of the total bacterial load in the patient. This also is critical to the prevention of Multidrug resistnace tuberculosis. This is particularly factual for sputum smear positive patients having higher bacterial load. During the the follow up phase; DOT is significant to facilitate stop reversion. The continuous supply of tuberculosis medicines to cure points is critical in the prevention of resistance against drugs(Karin , et al., 1997). The resistant strains of *Mycobacterium tuberculosis* against a particular agent emerge during treatment with single drugs. Therefore multidrug therapy is employed when treating tuberculosis in an attempt to prevent or delay the appearance of resistant strains. Management of patients, whose treatment with commonly used antitubercular drugs has failed, is problematic and so one has to depend on less potent, potentially more toxic and costly drugs in various combinations. The prohibitive cost and nonavailability of drugs further deteriorate the situation. Moreover, it is advantageous to use four or more medicine for such patients for a minimum of tow years.

2.9.1.3 Second Line treatment protocol

Treatment of drug resistance in pulmonary tuberculosis is a great challenge for treating physicians. INH should be used in all the anti-tubercular regimens as it is one of the highly effective drugs with least toxicity and also costs less. Cross resistant drugs should be avoided as they are not effective and increase toxicity. Drugs with one-way cross

resistance Viomycin and Capreomycin Viomycin and Kanamycin Kanamycin and Streptomycin Thiocetazone and Para-aminosalicylic acid. Preferably three new drugs should be given along with first line drugs. If second line or newer drugs are prescribed, then at least 4 or 5 drugs to be given. Addition of a single drug in failing regimen is contra- indicated. Considering the toxicity of reserve drugs, doses should be increased in time with optimal tolerance of the patient to achieve best results. Retreatment should always be given preferably in hospital under strict supervision for close observation of adverse drug reactions commonly associated with the reserve drugs. Intermittent chemotherapy is normally not effective and should not be given in multi drug resistant tuberculosis. An early surgical interference can be planned if disease is limited and patient's general condition permits (Herendra & Shah, 1998).

2.9.1.4 Newer drugs

Effective new regimens have been developed by using newer drugs in combination with other ones. The new drug delivery system and rapid strides in molecular biology and biotechnology will soon make their impact in the thereapyt of multidrug resistant tuberculosis. These medicines are Rifampicin derivatives - Rifabutin/ Rifapentin (CGP 29861/CGP 7040), Quinolones - Ciprofloxacin, Ofloxacin, Pefloxin, Lomfloxacin, Sparfloxacin, Clofazinamine - dye- Sulphone derivatives, Macrolides - Roxithromycin/Clarithromy- ucin/Azithromycin, 5. Betalactum Antibiotics with Clavulanic acid, Cephalosporins – Cefornide, Folate Antagonists - Trimethoprim derivative: Brodinoprim, Metioprim, Miscellaneous - CQQ (Gangamycin), Fusidic acid, Immunomodulators - Interferon Y, Interleukin -2, TNF.

2.9.2 Laser Therapy

Laser has been tried in some countries of the world to manag the tuberculosis. It is believed to be valuable in disease of multicavitary with profound bacterial load, particularly when there are improved alterations of medical treatment failure. Laser is supposed to be used in fast killing of bacteria. Thus; it reduces the bacterial load. Secondly, laser helps in early cavitary closure and increase the incursion of antitubercular medicines in walled off lesions. Because of the tubercular granuloma, it has proven benefit in the bronchial and tracheal stenosis. A variety of types of laser have been applied in tuberculosis resistant case that undergoes surgical treatment. Therapeutic lasers (Helium-Neon, Surgical (CO2 and YAG), Ultra Violet and semiconductive are used for this purpose. Thus; laser decreases the trauma of the surgery and volume to decreases the post operative complications and raise the effectiveness of surgical treatment.

2.9.3 Radiotherapy

Chest X-ray deterioration can be a sign of failure but deterioration may be because of one of the three reasons: 1) supervening carcinoma, 2) Pulmonary embolism, 3) Intercurrent pneumonia. After two to three weeks, a repeat chest X-ray may possibly demonstrate development in the case of pulmonary embolism or intercurrent pneumonia. Apparently; if radiological deterioration is not accompanied by bacteriological corrosion, is less likely to be contributed by tuberculosis.

2.9.4 Surgical Resection

In view of the frequent treatment failure inspite of the planned and supervised chemotherapy in multidrug resistant tuberculosis, the surgical interference is now increasingly used as an adjuvant to medical treatment. The main aim of surgery in patients with multidrug resistant tuberculosis is to decrease the bacillary load. Surgical treatment is mainly shown in cases with continual destroyed lobe or cavitation or lung in which medical therapy is likely to fail or increased the probability of reversion. Surgical treatment can be used when; (a) disease is limited to one anatomical area so that removal of the lung will not involve extensive lung resection. (b) Pulmonary function is adequate.

(c) drug therapy should be continued after operation also. Before the advent of chemotherapy, surgical intervention was widely practiced. Eventually; it was became evident that drug therapy alone was sufficient to trdat most cases in the chemotherapy era. Moreover; it was emphasized that the MDR tuberculosis therapy is 1st and leading chemotherapeutic.

2.9.5 Chemoprophylaxis

Those in contact with multidrug resistant tuberculosis patients are ideal candidates for Chemoprophylaxis. It is the only proved treatment normally recommended and efficacious for the avoidance of tuberculosis. But in contacts of INK resistant cases, Rifampicin can be used which has efficacy equal or even greater than INH as a chemo-prophylactic agent. In contacts of cases resistant to both Rifampicin and INH, Pyrazinamide (Z) + Ethambutol (E), Pyrazinamide with or without Ciprofloxacin/Ofloxacin can be given.

2.10 Prevention of Tuberculosis

Tuberculosis control programmes are mainly concern with its prevention of transmission. The community and nosocomial transmission minimized by certain preventive measures. The precautionary principle include a). Receiving ongoing training and education of the consequences of MDR tuberculosis, disease transmission and tuberculosis pathogenesis; b). Persistent awareness of risk situations and their avoidance should be stressing. c). Informing about the raisd risk of acquiring the tuberculosis and MDR illness) may become HIV positive. Secret HIV assay and substitute employment are obtainable for HIV positive patients. d). Worldwide infection prevention measures execution in all health care facilities, including secure waste clearance. e). Severely controlling the coughing behavior and collecting sputum in especially containers.

2.10.1 Prevention of Susceptible/ MDR Tuberculosis

Two major approaches are used to stop multidrug resistance: (a) Identification of cases with infection of tubercular and their prophylactic treatment, (b) Identification and

treatment of multidrug resistant tuberculosis patients. The aim is to identify their disease and to prevent further transmission. The objective is to avoid the 5-10% danger of following expansion of illness.

2.10.2 Vaccination

Bacillus Calmett Guirin (BCG) vaccination status and causal health conditions may enhaunce susceptibility of community to disease. While; previous contact with tuberculosis confirmed cases also help in sickness. A Mantoux tuberculin skin test (TST) and a baseline chest x-ray can be conduced to understand the exacnt situation.

2.10.3 Heath Care Awareness/ Education

The danger of tuberculosi infection depends on exposure time, intensive exposure to this case and the severity of disease in the source case. Therefore, all Health Care Workers (HCWs) are not at equal danger of acquiring disease. For numerous HCWs cadres the hazard is approximately identical to that of the general population.

High risk: Health Care Workers are at high risk that are close contact with smear positive MDR tuberculosis patients or infectious cases i.e. other medical staff and nursing staff in tuberculosis centres and wards. HCWs involved in aerosol producing procedures i.e. respiratory technicians, pulmonary physicians and other medical staff involved in sputum induction, bronchoscopy, aerosolised pentamidine therapy, autopsy procedures and tracheal intubation.

Medium risk: Staff concerned in collection of sputum from tuberculosis suspected patients are in close contact for a prolonged period of time at medium risk.

Low risk: People concerned in management of tuberculosis patients, providing the facilities and supporting staff i.e. cleaners, administrative staff and porters.

CHAPTER No. 3

MATERIAL AND METHOD

MATERIALS AND METHODS

This study project conducted at Pakistan Medical Research Centre (PMRC), Mayo Hospital/ King Edward Medical College, Lahore, Pakistan and National Institute of Biotechnology & Genetic Engineering (NIBGE), Faisalabad, Pakistan and Microbiological Research Laboratory, Dept. of Microbiology, Quaid-e-Azam University, Islamabad, Pakistan during Feb. 2003 to January, 2007. The isolated *Mycobacterium tuberculosis* evaluated clinically to study the resistance profile, epidemiological trend of resistant and level of resistance of resistant *M. tuberculosis* against 1st line anti-TB drugs; rifampicin, isoniazid, pyrazinamide and ethambutol. These drugs also studied therapeutically to interpret their certain pharmacological parameters i.e. Cmax (maximum plasma concentration), minimum inhibitory concentrations, adverse reactions etc. This scientific approach evolved to treat the resistant *Mycobacterium tuberculosis*. The resistant isolate also genetically characterized to elucidate the molecular basis of resistant against pyrazinamide.

3.1 Experimental Requirements

The Chemicals, reagents, apparatuses, instruments and equipments involved in this study project as given below,

1.1.1 Chemicals and Reagents

Alcohol Hydrochloric Acid P-Nitro Benzoic Acid Sodium Hydroxide Antiseptic Agent Cleansing Agent/ Detergent

LJ media: Magnesium Sulfate (MgSO4.7H2O) L- Asparagines Magnesium Citrate (Mg-Citrate) Potassium Dihydrogen Sulfate (KH2PO4) Malachite Green Eggs Distilled Water

1.1.2 Apparatuses and Glass Wares

Burner Bijoux Bottle of 25ml capacity Glass Flask of 250, 500 & 1000cc Glass Beads Metallic Knife 3.1.3 Equipments and Instruments Incubator Autoclave Centrifuge Machine Electric Stirrer Oven and Laminar Flow Hood of Biosafety Level III with UV light

1.2 Location

The present study was conducted at the Pakistan Medical Research Centre for Tuberculosis & Chest Diseases, King Edward Medical College/ Mayo Hospital, Lahore, Pakistan, Health Biotechnology Division, National Institute of Biotechnology & Genetic Engineering (NIBGE), Faisalabad, Pakistan and Microbiological Research Laboratory, Department of Microbiology, Faculty of Biological Sciences, Quaid-I-Azam University, Islamabad, Pakistan.

1.3 Experimental Period

The exploration was last upto five years. It was started in February, 2003 and completed in January, 2008.

1.4 Experimental drugs

The first line antitubercular drugs isoniazid, rifampicin, ethambutol and pyrazinamide were studied to explore the treatment protocol with highest possible regimen. There are definite proposed doses of these drugs, exceeding from these specific regimens (therapeutic window or index) may produce sever adverse effects. The pure chemicals of these four drugs were collected from the Schazoo Laboratories (Pvt.) Ltd., 75 Km

GT Road, Lahore, Pakistan. These pure drugs were used to study their antimycobacterial activities.

1.4.1 Rifampicin

The rifampicin (rifampin, rifabutin, rifapentine) is structurally a group of similar complex macrocyclic antibiotics (Bertram, et al., 2004). It is produced by Streptomyces mediterranei. Rifampicin has large molecular Wight 823 and is a complex of semi-synthetic derivatives. It is a fine powder of dark orange color and freely soluble in organic solvent and in water at acidic pH.

1.4.2 Isoniazid

Isoniazid (isonicotinic acid hydrazide) is the main mdicine used for tuberculosis therapy. Its discovery is somewhat fortuitous. In 1945, nicotinamide was reported for possessing the tuberculostatic action. Further studies revealed that many pyridine derivatives possess tuberculostatic activity. Isoniazid (1-isonicotinyl-2-isopropylhydrazide) is the hydrazide of isonicotinic acid and isopropyl derivative. It inhibits the multiplication of tubercle bacillus. It is potent monoamines axidase inhibitor and too toxic for use in human beings.

1.4.3 Pyrazinamide

Pyrazinamide is stable slightly water soluble, quiet inexpensive and the synthetic pyrazine analog of nicotinamide. It exhibits the bactericidal activity at a slight acidic pH (5.5). Activity at acidic pH is ideal for *Mycobacterium tuberculosis* resides in as acidic pathosome within the macrophage. The target of pyrazinamide appears to be the mycobacterial fatty acid synthase gene, concerned in biosynthesis of mycolic acid.

1.4.4 Ethambutol

Ethambutol is a water soluble and heat stable compound. It suppresses the growth of most isoniazid and streptomycin resistant tubercle bacilli. Resistance to ethambutol develops very slowly. It blocks arabinosyl transferases involve in cell wall biosynthesis. Resistance developed by single amino acid changes in the EmbA gene when ethambutol is given in absence of other effective agents.

1.5 Patient Selection

A total six thousands five hundred and seventy three (6573) indigenous pulmonary and extra-pulmonary specimens were collected from tuberculosis suspicious patients of 17-67 years age group during November, 2004 to December, 2006. The sputum, pus and bronchial washings were collected from five different sources, labeled and processed for initial screening. One hundred and seventy two (172) 2.616% of total (6573) tuberculosis diagnosed (AFB positive) patients were selected from six different sources; Indoor and outdoor of Mayo Hospital, Jinnah Hospital, DOTS and WAPDA Hospital Lahore, Pakistan. The patients were selected, regardless of their age, gender and previous therapeutic profile.

Table 12: Source and number of samples of M-TB strains

Source	Frequency	Percent
Mayo Hospital Outdoor	41	23.8
Mayo Hospital Indoor	110	64
Jinnah Hospital	14	8.1
DOTS	6	3.5
WAPDA Hospital	1	0.6
Total	172	100

The patients of all age group, regardless of their age, gender and previous therapeutic profile i.e. defaulters, relapse, failure etc were selected The patient sample comprised of 370.9% (122) males and 29.1% (50) females out of the total 172 patients.

Table 13: Gender wise patient's distribution

Gender	**Frequency**	**Percent**
Female	50	29.
Male	122	71
Total	172	100

Gender Frequency Percent Female 50 29. Male 122 71 Total 172 100

1.6 Specimen Collection

There three different types of samples- sputum, bronchial washing and puss collected from 172 pulmonary and extrapulmonary tuberculosis diagnosed patients.

Table 14: The number and types of samples

Specimen	**Frequency**	**Percent**
Bronchial Washing	8	4.5
Puss	18	10.5
Sputum	146	85
Total	172	100

The specimens collected in screw caped bottle, 5-20cc disposable syringe or plastic container. The early morning (without mouth wash) well coughed up sputum collected preferably at laboratory or in ward by standard method as mention in Appendix 1. The containers were properly labeled with patient particulars, registration number and date of receiving the sample.

1.7 Experimental Design

A total six thousands five hundred and seventy three (6573) specimens were collected from tuberculosis suspicious patients of 17-67 years age, during November, 2004 to December, 2006. The sputum, pus and bronchial washings were collected from five different sources, labeled and processed for initial screening. One hundred and seventy two (172) 2.616% of total (6573) tuberculosis diagnosed (AFB positive) patients were selected from six different sources; Indoor and outdoor of Mayo Hospital, Jinnah Hospital, DOTS and WAPDA Hospital Lahore, Pakistan. The patients were selected, regardless of their age, gender and previous therapeutic profile. The 172 tuberculosis diagnosed patients samples were treated to determine the sensitivity against all of the four 1stline antitubercular drugs - rifampicin, isoniazid, ethambutol and pyrazinamide. The sensitivity of strains was evaluated by the standard drug proportions method. The concentration of rifampicin, isoniazid pyrazinamide and ethambutol incorporated in LJ media as mention in Table No. 17. The 66 resistant *Mycobacterium tuberculosis* strains were studied to determine their highest level of resistance. Five drug levels above of their respective critical concentrations were prepared by incorporating into LJ media. The highest drug levels at which the mycobacterial growth inhibited noted for all of the four drugs. The determined levels of resistance against resistant *M. tuberculosis* were studied to interpreted the therapeutical credibility of anti-TB drugs. For this purpose, the pharmacokinetic and Pharmacodynamic profile studied to maintain accuracy for each antitubercular drug. The 47 pyrazinamide resistant *Mycobacterium tuberculosis* strains were studied at molecular level to elucidate the genetic basis of resistance against pyrazinamide. The genetic DNA isolated by mechanical method and used to detect the pyrazinamid resistant gene Pnc A. That gene was purified and identified by PCR for detection of mutation in Pnc A gene. The SSCP analysis conductedto confirm the molecular basis of resistance.

1.8 Experimental Parameters

The isolated *Mycobacterium tuberculosis* strains were investigated qualitatively and quantitatively to study the following parameters, I. Pattern of sensitivity/ resistance of *Mycobacterium TB* against rifampicin, isoniazid, ethambutol and pyrazinamide. II. Overall resistance pattern of *Mycobacterium TB* to 1stline drugs III. Resistance percentages of

Mycobacterium TB with respect of number of colonies. IV. Overall epidemiological trend of resistant tuberculosis during Nov. 2004 - Dec. 2005. V. *Mycobacterium TB* resistance pattern (%) against five different levels (µg/ml) of rifampicin, isoniazid, pyrazinamide and ethambutol above of their respective critical drug concentrations. VI. Therapeutical interpretation of all of four 1stline anti-TB drugs to evaluate their pharmacological credibility. VII. Isolation, purification and amplification of Pnc A gene, responsible of resistance against pyrazinamide. Single Strand Conformational Polymorphism (SSCP) - PCR analysis used to elucidate molecular basis of resistance against pyrazinamide.

1.9 Preparation of LJ (Lowenstein Jensen) Media

The Lowenstein Jensen media is specific for the mycobacterial organisms. There were standard nutrients and there respective proportions maintained to prepare the effective media. 3.9.1 Ingredients of LJ media The Lowenstein Jensen medium contained the miscellaneous ingredients, which required by *Mycobacterium tuberculosis*. These ingredients are as given below,

Table 15: Ingredients of Lowenstein Jensen media.

#	Ingredients	1000ml	1800ml
1	Magnesium sulfate (MgSO4.7H2O)	0.15 g	0.27 g
2	Magnesium citrate (Mg - Citrate)	0.375 g	0.674 g
3	L- Asparagines	2.20 g	4.05 g
4	Potassium Dihydrogen Sulfate (KH2PO4)	1.5 g	2.7 g
5	Glycerin	7.5 g	13.5 ml
6	Malachite green	12.5 g	22.5 g
7	Distilled Water	375 g	675 g
8	Eggs	625 ml	1125 ml

3.9.2 Stock Solutions

The stock solution prepared as mentioned follow,

Table 16: Preparation of stock solution

#	Drugs	RFP	ISN	ETB	PZA
1	Weight of drugs (mg)	82	20	20	100
2	Ethylene glycol ml (EG)	10	-	-	-
3	Sterile distilled water ml (DW)	-	40ml	100ml	10ml
4	Dilution in distilled water	-	2ml in 50ml	-	-
5	Concentration mg/ ml	8000	20	200	10,000
6	Quantity used for 100 ml	0.5	1	1	1
7	Critical concentration µg	40	0.2	2	100

According to the given quantity of drugs the rifampicin 82 mg, isoniazid 20mg, ethambutol 20mg and pyrazinamide 100mg weighed and dissolved in 10ml ethylene glycol, 40 ml, 100ml and 10ml distilled water respectively. A further dilution of isoniazid prepared by adding 2ml of above solution in 50ml of distilled water.

Table 17: The final concentration of antitubercular agents incorporated in LJ media.

#	Drug	Concentration
1	Rifampicin	40 µg/ ml
2	Isoniazid	0.2 µg/ ml
	Pyrazinamide	100 µg/ml
4	Ethambutol	2 µg/ ml

The resultant stock solution contained 800, 20, 200 and 10,000 µg of rifampicin, isoniazid, ethambutol and pyrazinamide respectively.

3.9.3 Weighing and Mixing

Magnesium Sulfate (MgSO4.7H2O), Magnesium Citrate (Mg-Citrate) and L- Asparagines weighed accurately by Micro Digital Balance. These were then dissolved in 360ml of distilled water taken in 1000ml capacity flask. The ingredients shaken slightly to dissolve. Then Potassium Dihydrogen

Phosphate (1.5ml) and glycerol (7.5ml) were added. The solution mixed to dissolve all ingredients and finally tightly closed with a cotton plug.

3.9.4 Sterilizing/ Autoclaving of the Apparatus and Glass Wares

The above prepared solution autoclaved at 121o C for 15-20minutes and cooled to room temperature. All the apparatus and glassware i.e. bijoux bottle of 25ml capacity, glass flask of 250, 500 & 1000cc, glass beads, metallic knife etc autoclaved and sterilized.

1.9.5 Egg Homogenizing

18-20 fresh hen eggs collected washed and cleaned by some cleansing agent (detergent, soap etc) in warm water. The eggs were not more than 7 days old. These were then soaked in 70% ethanol for 15 minutes and cracked with sterilized metallic knife into the sterilized glass flask. That was homogenized by adding the glass beads and vigorous shaking. Finally it was filtered by draining through sterile gauze.

1.9.6 Preparation of Malachite Green Solution 2%

A fresh solution was prepared by adding 2g of malachite green in 100ml of distilled water into the sterilized mixing flask. It was mixed well and kept in incubator for 1-2 hours. There was freshly prepared solution used in LJ medium every time.

1.9.7 Final Mixing to Prepare the Media

The homogenized eggs of 625ml and malachite green (2%) 12.5ml added into the flask containing 360ml salt solutions and mixed well.

3.9.8 Filling and Capping the Media into the Bijoux Bottles Approximately the 15ml of the completed Lowenstein Jensen media poured into the autoclaved and sterilized 25cc Bijoux Bottle/ Mc Cartney vial. The bottles were then closed with the sterilized silver capes and kept at the specific wooden stand (that can hold 25 bottles). 3.9.9 Coagulation/ Hardening the Media (slanting) by Water Bath These wooden stands along with the bijoux bottles placed in as inspissator at 85 ℃ in slanting position for 45 minutes. The medium was solidified/ hardened. A high temperature and long exposure of water bath may destroy the media. Therefore bottles were let out from the water bath as the given temperature and time overed.

1.9.10 LJ medium Containing 4(p)-Nitrobenzoic Acid (PNB) 500 µg/ ml

Lowenstein Jensen medium containing the 4(p)- Nitrobenzoic acid (PNB) 500µg/ml having following ingredients, Table 18: Ingredients of LJ medium containing Nitrobenzoic acid. Para-nitrobenzoic acid 200mg Sodium hydroxide solution 4% (1N) 2 ml Sterile distilled water 20 ml Phenolphthalein Hydrochloric acid A glass flask of 100ml capacity took for Para-Nitrobenzoic Acid. Sodium Hydroxide solution 4% was added and shacked to dissolve. Then 15 ml of distilled water was added and shacked. All the ingredients mixed after adding a drop of phenolphthalein until it turned pink. By adding Hydrochloric acid drop wise, the solution was neutralized till it appeared colorless. Any precipitation if formed was dissolved by Sodium Hydroxide. The solution was kept in screw caped bottle. It was kept at 115 ℃ for 10 minutes. Then cooled, labeled and stored at 2- 8 ℃ temperature. 5ml of this solution was used for 100ml of LJ medium.

1.10 Specimen Treatment/ Processing

The sputum, puss and bronchial washing collected form tuberculosis diagnosed patient as per standard method described in Appendix #1 and processed for sensitivity evaluation. 3.10.1 Decontamination & Homogenization of Specimens by Modified Petroff Method The NaOH 40g/ L (4%w/v) was use as decontaminating agent. It inhibits the growth of commensale organisms. This NaOH also function as buffer. 3.10.2 Concentrating Tubercle Bacilli by Centrifugation The liquefied sputum puss or bronchial washing containing tuberdule bacilli centrifuged at force of 2000 g for 15 mints. The organisms were sedimented. A small quantity of distilled water added to lower specific gravity and increases the number of bacilli sedimentation. The supernatant fluid was discarded to obtain the residual concentrated specimen of MTB. 3.10.3 Transport Media for Preserving *Mycobacterium TB* The Cetylpyridinium Chloride Sodium Chloride (CPC-NaCl) used as transport medium when there was a more than 24 hours delay in sputum reaching to laboratory. It inhibits the growth of commensal organisms and slowly liquefies the thick & viscid specimen. The tubercle organisms remain in the solution for upto 8 days. The specimen protected from direct sunlight because the viability of tubercle bacilli is affected by sunlight.

1.11 Inoculation and Incubation of Strain for Primary Culture

The concentrated residue of sputum, puss or bronchial washing obtained after discarding the supernatant fluid of centrifuged sample was used for primary culture. The Micro-Pippetman used to take the sample and poured it at the centers of the LJ media slides. The slide was then being moved up and down to spread the sample homogenously over the media. This primary culture kept in incubator at 35-37 o for 4 weeks. Ultimate growth of *Mycobacterium TB* further used for sensitivity testing 3.12 Sensitivity/ Resistance Testing of *Mycobacterium tuberculosis* The drugs sensitivity testing of patient's specimen primary culture was performed within 1-2 weeks after the growth of *Mycobacterium tuberculosis*. The sensitivity of *Mycobacterium TB* evaluated against for four 1stline antitubercular drugs rifampicin, isoniazid, pyrazinamide and ethambutol by drugs proportion method as recommended by WHO and IUATLD (International Union Against Tuberculosis and Lung Diseases). The patient's samples were studied in batches of 10-15 specimens. There were three controlled and two drug containing LJ medium slides used to gain maximally authenticated outcomes for each patient.

3.12.1 Standard Drug Concentrations

The Lowenstein Jensen media containing the critical drug concentration as mentioned in Table No. 17 to determine the sensitivity of *Mycobacterium tuberculosis*. The medium was divided into four portions to inoculate the four different 1stline drugs - rifampicin, isoniazid, pyrazinamide and ethambutol. These drugs containing media was filled into the bijoux bottles to evaluate the sensitivity of each strain against all of the mentioned incorporated antitubercular drugs. For this purpose there were the stock solutions of each drug used as mentioned in Table 16.

1.12.2 Incorporation of Drug in LJ Media

A large quantity of (1000ml) of LJ medium was prepared and just before inspissation, 100ml of the medium was placed in each of the three separate sterile flasks labeled as rifampicin, isoniazid and ethambutol and for each testing drugs. There was a 200ml of the media saved for the pyrazinamide drug and control media that was acidified by adding 5-8 ml 1N HCl to maintain the pH 4.85 and then divided equally into flasks labeled as PZA and PZA control. The stock solution of each drug was added to each corresponding labeled flask and mixed well. With the help of occupenser 15-20ml of medium was poured into 28ml Bijoux Bottle previously labeled with name and critical concentration of the drug. The rest of the drugs free media poured into Bijoux Bottle as control. The media inspissated at 85o C for 50 minutes in a slanting position. The caps were kept loose. After inspissation, the bottles cooled down, tightly closed and preserved in 2-8 o C. 3.12.3 Preparation of Required 10 3 & 10 5 Dilutions of *Mycobacterium TB* The over seeding of a drug containing medium was prevented by standardization of inoculums that mitigate the possibility of miss judgment of considering the resistant to a susceptible strain. Approximately 1mg wet weight bacilli/ ml are estimated to vary between ≥ 10 6 and 10 8 CFU. By using the spatula a representative sample of growth (containing

minimally 50 colonies) taken from primary culture and placed into a screw caped sterile Mc Cartney vial containing 1ml of distilled water and 5 glass beads. This was then be homogenized by vigorous stirring by Vertex Mixer/ Electric Stirrer for 1-3 minutes. It was then left in safety cabinet. The opacity of the suspension was adjusted by the addition of sterile distilled water to that of a Mac Farland standard No.5 (alternately a standard suspension of 1.0 mg/ ml of BCG can be used). Fourserial 10 fold dilutions of inoculums were made i.e. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} in tubes labeled as 1, 2, 3 and 4 respectively. Each tube contained 9 ml of distilled water. 1ml of standard inoculums was added to tube No.1. 1ml from tube No. 1 to tube No.2 and so on. The tube No.3 of dilution inoculums of 10^{-3} and tube No. 5 of dilution inoculum 10^{-5} were used to culture for sensitivity evaluation.

1.13 Inoculation and Incubation

Two bottles of LJ medium and one bottle of LJ medium containing Para-Nitrobenzoic Acid used for each sample. The bijoux bottles labeled with patient culture number, date and type of medium. By micropipette 2-3 drops of the deposit obtain by modified petroff method placed at the centre of slope of culture media. The culture bottle slight rocked and rolled to spread the inoculums. The media incubated in horizontal position for 24 hours at 37oC in an incubator and thereafter in upright position for 4-8 weeks. Then caps of the bottles kept loose for one week and then tightened to prevent drying of medium. The caps were loosened for a few seconds once or twice a week for aeration. Whole of the process required a strong surveillance for accurate outcomes.

1.14 Identification of *Mycobacterium TB* Complex

The Atypical *Mycobacterium* other than tuberculosis (MOTT) were identified by Lowenstein Jensen medium containing 4-(p) Nitro-benzoic Acid (PNB) (500ug/ ml). The confirmation and identification of *Mycobacterium tuberculosis* was carried by the criteria as given below, I No pigmentation on exposure to sunlight or in dark incubator. II Slow growth rate, no growth within

three days at 37 o C. III No growth on PNB containing LJ medium IV Colonial morphology, rough, buff and tough colonies V AFB staining of smear made from growth, red colored, straight or slightly curved or beaded AFB seen against blue background.

1.15 Reading and Recording the Results

The Bijoux Bottles inspected weekly for appearance of growth. When the growth was evident on LJ medium, colonial morphology was noted. One culture bottle took and exposed to day light for one hour and re-incubated. On the following day it was examined for pigmentation. Observations were being recorded accordingly. The cultures with no growth were discarded after 8 weeks of incubation. The presence and amount of growth in term of number of colonies on control and drug mediarecorded. The results interpreted for resistance on the basis of percentage of colonies on drug media in comparison to the growth on drug free medium. The criterion for resistance was 10% of growth for Pyrazinamide and 1% of growth for the rest four drugs. The strains showing susceptibility were again incubated and examinedat 6 weeks before declaring as sensitive. The results were noted and interpreted as given at annexure 2.

Table 19. The number colonies and respective resistance of *Mycobacterium TB*.

Codes	Stand for	Remarks
10	10 colonies	Might be resistant.
25	25 colonies	Mild resistant
50	50 colonies	Resistant
1+	More than 100 colonies	Severe Resistant
2+	More than 200 colonies	Highly resistant
3+	More than 300 colonies	Very highly resistant

1.16 Determination of Elevated MIC,s of Resistant *Mycobacterium TB*

The Sixty Six (66) resistant *Mycobacterium TB* strains separated to determine the level of resistance. This part of the research project was conduced at National Institute of Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan.

3.16.1 Preparation of LJ media and inoculums dilutions

The media was prepared by standard methodology as mentioned before. The cultures that were used to determine the sensitivity considered as the primary culture for this part of the research project. The 10^{-3} and 10^{-5} inoculums dilutions were prepared by the method previously described.

3.16.2 Profile of Elevated Drug Levels

There were five drug levels above MIC prepared by incorporating the drugs into the LJ medium.

Table 20. Preparation of five different dilutions / proportions of rifampicin, isoniazid, pyrazinamide and ethambutol inoculated in LJ-media for determination of level of resistance of resistant M-TB.

	RFP µg/ ml	**ISN** µg/ ml	**PZA** µg/ ml	**ETH** µg/ ml
MIC (µg/ ml)	**40**	**0.2**	**100**	**2**
1st Dilution	80	3	200	4
2nd Dilution	120	6	300	6
3rd Dilution	160	9	400	8
4th Dilution	200	12	500	10
5th Dilution	200+	12+	500+	10+

RFP- Rifampicin ETB- Ethambutol PZA- Pyrazinamide
ISN-Isoniazid MIC- minimum inhibitory concentration

The pharmaceutical graded antituberculosis drugs rifampicin, isoniazid, ethambutol and pyrazinamide provided by Schazoo Laboratory Pvt. Limited, GT Road, Lahore. The quality and purity of these drugs was assured by analyzing according to the specifications of United State Pharmacopoeia (USP) in the Quality Assurance Department of Schazoo Laboratory, Lahore.

3.16.3 Inoculation and Incubation

The 10 -3 and 10 -5 diluted inoculums spread over the control and drug containing Lowenstein Jensen media by Micro-Pippetman. These bijoux bottles were then be place in incubator at 35-37 0 C for four weeks.

3.16.4 Reading and Recording the Results

The results were noted weekly basis. The growth pattern, number of colonies and contamination were analyzed carefully.

3.17 Molecular basis of resistance of *Mycobacterium-TB* against Pyrazinamide:

The *Mycobacterium tuberculosis* Genomic DNA was isolated and amplified. The Single Strand Conformation Polymorphism (SSCP) analysis conducted to elucidate the molecular basis of resistance against the pyrazinamide. It was purified and sequenced by PCR for detection of mutation in PncA gene.

3.17.1 Isolation of DNA of Mycobacterial Tuberculosis:

The mechanical method used to extract the DNA of *Mycobacterial tuberculosis*, collected from the primary culture of TB diagnosed Acid Fast Staining positive patients. The wooden loop used to pick the *Mycobacterium TB* colonies from Lowenstein Jensen media. It was suspended in 1ml of TE buffer pH 8.0 and heat inactivated for 30 minutes at 80 C. Eppendorff micro centrifuge used for centrifugation of mycobacterial suspension at 13000 rpm for 15minutes. 100 ul glass beads added along with pellet suspended in 200 ul of TE buffer. The mycobacterial cells disintegrated mechanically in a Mickle Apparatus by centrifugation for 10 minutes at 13000 rpm. The genomic DNA of *Mycobacterium TB* lies in supernatants, transferred to new Eppendorff tubes.

3.17.2 Quantitative analysis of *Mycobacterial* Genomic DNA:

There two methods gel estimation method and photometric method used to analyse

quantity of *Mycobacterium* genomic DNA.

Gel Estimation Method:

3ul of bromophenol blue used as loading dye to load 10ul of isolated DNA on 2% agar gel. 90V maintained for 1 hour in 0.5 X TBE buffer to run the sample and observed on UV transilluminator.

Photometric Method:

1ul of *Mycobacterium TB* genomic DNA analyzed quantitatively by spectrophotometer at wavelength of 260nm. The concentration of nucleic acid in sample calculated at the reading of 260nm. The formula applied to determine DNA quantity was as follow,

$$\text{Amount of DNA} = OD260 \times \frac{\text{Volume of total solution in tube}}{\text{Volume of sample DNA}} \times 0.05$$

3.17.3 PCR Amplification of *Mycobacterium TB* Genomic DNA:

Different fragments of pyrazinamide resistant gene Pnc A of the DNA of *Mycobacterium TB* was amplified for PCR and SSCP analysis.

Amplification and Identification of *Mycobacterium TB* Genomic DNA:

Mycobacterial species identified by using the primers homologous to the specific mycobacterial insertion sequence IS987 TB-1 and TB-3, 20 bases primers corresponding to bp 105 to 124 (5'-GTG CGG ATG GTC GCA GAG AT-3') and 626 to 645 (5'-CTC GAT GCC CTC ACG GTT CA-3') respectively of IS987 sequence.

Preparation of Reaction Mixture:

Reaction Components	Volume/ Reaction (µl)
10 X PCR buffer	2.0
MgCl2	1.2

DNTPS (1mM)	1
TB-1 Primer (10P mol)	1
TB-3 Primer (10P mol)	1
DH2O	9.3
Taq-polymerase (2 u/ul)	0.5
Total	16 (µl)
Isolated DNA	04 (µl)
Total reaction vol.	20 (µl)

Procedure:

A 16 ul of above mixture added into 0.2 ml PCR reaction tube. 4ul of isolated DNA of each sample added and 4ul dH2O (Negative control) added into corresponding tubes and mixed thoroughly.

PCR Conditions:

Run the PCR cycling profile as follow,

One cycle	
95°C	4min
58°C	2min
35cycle	
95°C	40 Sec
58°C	40 Sec
72°C	1 min
Final Extension	
72°C	10 min
End	
4°C	α

Detection of PCR Products

The amplified 10µl of PCR product and 2ul of 2 X loading dye loaded into each well of 2% agarose gel in 01 X TBE buffer containing 1ug/ ml final concentration of ethidium

bromide. The gel was run with 50 bp marker in 0.5XTBE. The fragment 541 ~bp was visualized and compared to marker by UV transilluminator.

3.17.4 PCR of fragments of Pyrazinamide resistant PncA gene and SSCP analysis:

Certain fragments of Pnc A gene are responsible of mycobacterial TB resistance against pyrazinamide i.e. 215 bp, 217bp, 179 bp. The PCR of these fragments was conducted and analyzed by SSCP.

3.17.4.1 PCR of the 215bp fragment from Pnc A Gene and SSCP analysis

The specific primers designed and synthesized to amplify the 215 bp region from the Pnc A gene of pyrazinamide resistant *Mycobacterium tuberculosis* by using the PCR. The conditions for PCR were optimized. The specific primers, P1 (5'-GTCGGTCATGTTCGCGATCG 3') and P2 (5'-TCGGCCAGGTAGTCGCTGAT 3') were used for PCR.

PCR reaction mixture

Reaction Component	Volume/ Reaction (ul)
10 X PCR buffer	2.0
MgCl2 (50mM)	1.2
DNTPs (500uM)	1
P1 (10P mol)	1
P2 (10P mol)	1
DH2O	9.3
Taq polymerase (2u/ ul) 0.5	
Total	16 (ul)
Isolated DNA	04 (ul)
Total reaction Vol.	20 (ul)

PCR Cycles:

One Cycle:

95°C	2 minutes
56°C	1 minute
30 cycles: 94°C	1minute
56°C	1 minute
72°C	1.30 minute
Final extension:	
72°C	140 minutes
End	
4°C	α

PCR conducted for reaction mixture consist of 4ul (20-50ng) of isolated DNA from the samples and amplified 215 bp fragment from Pnc A gene in 16 ul of reaction buffer containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.2 mM deoxynucleotide Triphosphatase (dNTPs), 10 pmol of each primer and one unit of taq polymerase enzyme.

3.17.4.2 PCR of the 179 bp Fragment from Pnc A Gene and SSCP Analysis:

179bp region from the Pnc A gene of pyrazinamide resistant *Mycobacterium tuberculosis* was amplified by PCR with specifically designed primers. Conditions were optimized for PCR of this gene. The specific primers, P3 (5’ - ATCAGCGACTACCTGGCCAGA 3’) and P4 (5’ - GATTGCCGACGTGTCCAGAC 3’) were used for PCR.

PCR reaction mixture:

Reaction components Volume/ Reaction (ul)

10 X PCR buffer 2.0

MgCl2 (50mM)	1.2
dNTPs (500uM)	1

P3 (10p mol)	1
P4 (10p mol)	1
DH2O	9.3
Taq polymerase (2u/ ul)	0.5
Total	16 (ul)
Isolated DNA	04 (ul)
Total reaction volume	20 (ul)

PCR cycles:

One cycle:

95°C	2 minutes
56°C	1minute

30 Cycles:

94°C	1 minute
56°C	1 minute
72°C	1.30 minute

Final extension:

72°C	10 minute

End:

4°C	α

PCR conducted for reaction mixture consist of 4ul (20-50ng) of isolated DNA from the samples and 179 bp fragment from Pnc A gene amplified in 16 ul of reaction buffer containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.2 mM deoxynucleotide Triphosphatase (dNTPs), 10 pmol of each primer and on unit of taq polymerase enzyme. As the sample mixed thoroughly, spun and shifted to thermocycler.

3.17.4.3 PCR of the 217 bp Fragment from Pnc A Gene and SSCP Analysis

217 bp region from the Pnc A gene of pyrazinamide resistant *Mycobacterium*

tuberculosis was amplified by PCR with specifically designed primers. Conditions were optimized for PCR. The PCR was don with the specific primers, P5 (5'- CCACCGATCATTGTGTGCGC3') and P6 (5'-GCTTTGCGGCGAGCGCTCCA3').

PCR Reaction Mixture:

Reaction Components	Volume/ Reaction (ul)
10 X PCR buffer	2.0
MgCl2 (50mM)	1.2
dNTPs (500uM)	1
P5 (10p mol)	1
P6 (10p mol)	2
DH2O	9.3
Taq polymerase (2 unit/ ul)	0.5
Total	16 (ul)
Isolated DNA	04 (ul)
Total reaction volume	20 (ul)

PCR Cycles:

One cycle:

95°C	2 minutes
56°C	1minute
30 Cycle:	
94°C	1 minute
56°C	1 minute
72°C	1.30 minute

Final extension:

72°C	10 minute

End:

4°C	α

PCR conducted for reaction mixture consist of 4ul (20-50ng) of isolated DNA from the samples and amplified 217 bp fragment from Pnc A gene in 16 ul of reaction buffer containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.2 mM deoxynucleotide Triphosphatase (dNTPs), 10 pmol of each primer and one unit of Taq polymerase enzyme.

3.17.5 Detection of PCR Amplified Products

1 ul of PCR product was mixed with 3ul of dH2O and 1 ul of loading dye (bromophenol blue/ xyelene cyanol) and electrophoresed on a 12% non-denaturing page in a folding page apparatus at 160 V for one hour and silver stained to see the amplification of a (217, 215, 179bp) fragment from Pnc A gene.

3.17.5.1 Silver Staining Method

The detection of PCR amplified products was carried by silver staining method. 7.5% Glacial Acetic Acid solution (fix/ stop solution) used to fix the gel. It was agitated for at least 20 minutes or until the tracking dyes were no longer visible. Some time the gel was stored in a fix/ stop solution overnight without shaking. Fixed/ stop solution was removed and kept at 4°C to stop the reaction after developing the gel. The distilled water was used to rinse the gel for 3 times with continuous agitation. The stained solution (1 % AgNO3 and 0.15% of the 37% formaldehyde) added for 25 minutes in complete darkness. The staining solution discarded and the gel rinsed in dH2O for 5-10 seconds (the time is critical as long rinse resulted in weak or no signal). The gel placed in pre-chilled developing solution (3% Na2CO3, 0.15% of the 37% formaldehyde and 2ug/ ml of sodium thiosulphat) and agitated unit the bands started to develop. The developing solution replaced with the fresh pre-chilled and developing continued till all the bands were visible. The gel rinsed twice time with dH2O.

3.17.5.2 SSCP Analysis of Pnc A Gene

There different sequences exhibited by single stranded DNA or RNA strands because of different mobility during electrophoresis in non-denaturing polyacrylamide gels. It was carried by simplifying the target gene by PCR and then compares the mobility of the denatured DNA with that of reference fragment of known sequence. A single strand point mutations within the sequence lead to a change in running behavior in the gel. It is very suited for the detection of mutation in the DNA segment. Single stranded DNA exhibits a folded structure under non-denaturing conditions, which is determined by intra molecular interactions and thus by the sequence. The changes in the DNA sequence by mutation result in different configuration and mobility in the gel. Thus, in the SSCP analysis, the mutation is determined by the mobility shift of single strand DNA (ssDNA).

To optimize SSCP conditions, different percentages of polyacrylamide gel, percentage of TBE buffer and DNA, starting and running voltage, buffer temperature and running time recorded. 4 ul of the PCR product mixed with 6ul SSCP dye and kept at 30°C for 2 hours or 37°C for 1hour. The sample were then heat denatured at 98°C for 5minutes in thermal cycler immediately chilled on ice. The sample was electrophoreses in a 20% (39:1 acrylamide: bis-acrylamide) non-denaturing page (80 X 80 X 1 mm) and 1.5 X TBE buffer in a cold room (4°C). Starting voltage was 25 V till the sample entered in the gel, then the voltage was reduced to 15V and gel was run for 4 hours at this voltage in folding gel apparatus. The SSCP bands were visualized by silver staining techniques. The mobility of SSCP bands in normal control DNA was compared with the mutant bands in the samples.

3.17.6 PCR of the 611 bp Fragment from Pnc A Gene for Sequencing Analysis:

The specific primers designed and synthesized to amplify the 611 bp region from the Pnc A gene of pyrazinamide resistant *Mycobacterium tuberculosis* by conducting the PCR. The conditions for PCR were optimized. The specific primers, P1 (5’-GTCGGTCATGTTCGCGATCG 3’) and P6 (5’- GCTTTGCGGCGAGCGCTCCA3’)

used for PCR.

PCR Reaction Mixture

Reaction	Volume/ Reaction (ul)
10 X PCR buffer	2.0
$MgCl2$ (50mM)	1.2
DNTPs (500uM)	1
P1 (10P mol)	1
P2 (10P mol)	1
DH2O	9.3
Taq polymerase (2u/ ul)	**0.5**
Total	16 (ul)
Isolated DNA	04 (ul)
Total reaction Vol.	20 (ul)

PCR Cycles:

One Cycle:

95°C	2 minutes
56°C	1 minute
30 cycles:	
94°C	1minute
56°C	1 minute
72°C	1.30 minute

Final extension:

72°C	140 minutes

End

4°C	α

PCR conducted for reaction mixture consisted of 4ul (20-50ng) of isolated DNA from the samples and amplified 611 bp fragment from Pnc A gene in 16 ul of reaction buffer containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.2 mM deoxynucleotide Triphosphatase (dNTPs), 10 pmol of each primer and one unit of Taq polymerase enzyme.

3.17.7 Purification of Amplification Product:

The PCR products were purified putting into 1.5 ml eppendorf tube. The concentration of 95% ethanol adjusted to 60% in the mixture. The tube and vortex were closed briefly. The tube was left for 8 minutes at room temperature, more time may precipitate the small product. The orientation of the tube adjusted in micro-centrifuge and spin for 20 minutes at 14000 rpm. Supernatant pipet out carefully so that pellet shout not be disturbed. 70% ethanol added (250ml) and mixed thoroughly and vortex briefly. The orientations of tubes adjusted in micro centrifuge and spin for 10 minutes at 14000 rpm. Supernatant pipette out carefully so that pellet should not be disturbed and pellet was dried. It was then be resuspended in distilled water and concentration was measured.

3.17.8 Sequencing PCR for the Detection of Mutation in Pnc A Gene:

The mutation in 611 bp region from the PncA gene of pyrazinamide resistant *Mycobacterium tuberculosis* detected by conducting the sequencing PCR with specifically designed and synthesized primers. The conditions for PCR were optimized for this gene. Then their sensitivity and specificity were increased. The specific primers, P1 (5'- GTCGGTCATGTTCGCGATCG3') and reverse P6 (5' - GCTTTGCGGCGAGCGCTCCA3'), P4 (5'- GATTGCCGACGTGTCCAGAC3') were used for sequencing PCR.

PCR Reaction Mixture

Reaction Component	Volume/ Reaction (ul)
5 X PCR buffer	1
Primer (3.2 P mol)	1
DH2O	5.0
Big Dye	**1**
Total	08 (ul)
Purified DNA	02 (ul)
Total reaction Vol.	10 (ul)

PCR Cycles:

One Cycle:

96°C	20 minutes
35 cycles:	
96°C	20 seconds
50°C	15 seconds
60°C	04 minute
Final extension:	
60°C	05 minutes
End	
4°C	α

PCR was conducted for reaction mixture consisted of 2ul (50ng) of purified DNA from the 611 bp fragment from Pnc A gene in 10 ul of reaction and 3.2 pmol of each primer and one ul of big dye. After mixing, the samples were spun and shifted to thermocycler.

3.17.9 Purification of Sequencing PCR:

The 10ul of sequencing PCR product pipet into 1.5 ml eppendorff tube and wrap the tube into aluminum foil. 40 ul of 75% ethanol add to adjust 60 % in mixture. The tube closed and vortex briefly. The tube left at room temperature for 20 minutes more time may precipitate the smaller products. The orientation of tubes adjusted in microcentrifuge and spin for 20 minutes at 14000 rpm at 4°C. The supernatant carefully pipet out so that pellet should not be disturbed. 70% ethanol (250ul) add, mix well and vortex briefly. The orientation of tubes adjusts in microcentrifuge and spin for 10 minutes at 14000 rpm at 4°C. The supernatant carefully pipet out so that pellet should not be disturbed and the pellet dried.

3.17.10 List of Primers

Table 21: List of Primers:

Primer	Sequence	Tm	Product	Annealing	Position
TB-1 TB-3	5’-GTG CGG ATG GTC GCA GAG AT-3’ 5’-CTC GAT GCC CTC ACG GTT CA-3’	62°C 64°C	541 bp	56	105-124 & 626-645 of IS987
P7 P8	5’- TGCGGGCGTTGATCATC-3’ 5’-CAGGAGCTGCAAACCAACTC-3’	56°C 56°C	541 bp	52	1-17 & 521-541
P1 P2	5’-GTCGGTCATGTTCGCGATCG3’ 5’- TCGGCCAGGTAGTCGCTGAT 3’	64°C 64°C	215 bp	56	From 105bp upstream & nucleotide position 110-91
P3 P4	5’ – ATCAGCGACTACCTGGCCAGA 3’ 5’-GATTGCCGACGTGTCCAGAC3’	64°C 64°C	179 bp	56	Nucleotide position 91-110 & nucleotide position 270-25
P5 P6	5’-CCACCGATCATTGTGTGCGC3’ 5’ – GCTTTGCGGCGAGCGCTCCA3’	64°C 74°C	217 bp	56	401-420 & 60bp downstream of the stop codon

CHAPTER No. 4

RESULTS

RESULTS

This experimental study comprised of 172 clinical isolates of *Mycobacteiumnl tuberculosis* collected during November, 2004 to December, 2005 from TB diagnosed (AFB positive) patients of 17-67 years age group. Sixty six (66) of resistant *Mycobacterium tuberculosis* strains were further studied to determine their highest level of resistance (in % age) of resistant *mycobacterial TB*. The clinical isolates were collected from culture growth over Lowenstein Jensen media containing the respective 1st line antitubercular drugs at an optimum drug concentration. The parameters of study were the pattern of sensitivity/ resistance of *mycobacterial TB* against 1st line drugs - rifampicin, isoniazid, ethambutol and pyrazinamide, overall resistance pattern, resistance percentages with respect of number of colonies, overall trend of resistance during Nov. 2004 - Dec. 2005, resistance pattern in percentage against five different levels (µg/ml) of rifampicin, isoniazid, pyrazinamide and ethambutol above of their respective critical concentrations and therapeutical interpretation of all of four drugs to evaluate their pharmacological credibility and molecular study of Pnc A gene of *Mycobacterium TB* responsible of resistance against pyrazinamide. The results obtained were recorded after four weeks as mentioned at Appendix II.

4.1 Collection of Clinical Isolates of *Mycobacterium tuberculosis*:

Mycobacterium tuberculosis strains collected from primary culture of TB diagnosed patients during November 2004 to December 2005. A total 172 samples of sputum, puss and bronchial washing collected from six different local sources; 41 (23.8%) from Outdoor, Mayo Hospital, Lahore, 110 (64%) form Indoor, Mayo Hospital, Lahore, 14 (8.1%) from Jinnah Hospital, Lahore, six (3.5%) from WAPDA Hospital, Lahore, Pakistan. (Figure 27 and Table 31).

The specimen comprised of 146 sputum (84.9%), 18 (10.5%) puss and 8 (4.7%) bronchial washing. (Figure 27 and Table 32).

4.2 Gender wise distribution of study sample:

Data showed 122 (70.9%) males and 50 (29.1%) female of total 172 clinical isolates from TB diagnosed patients of 17- 67 years age group. (Figure 29 and Table 33).

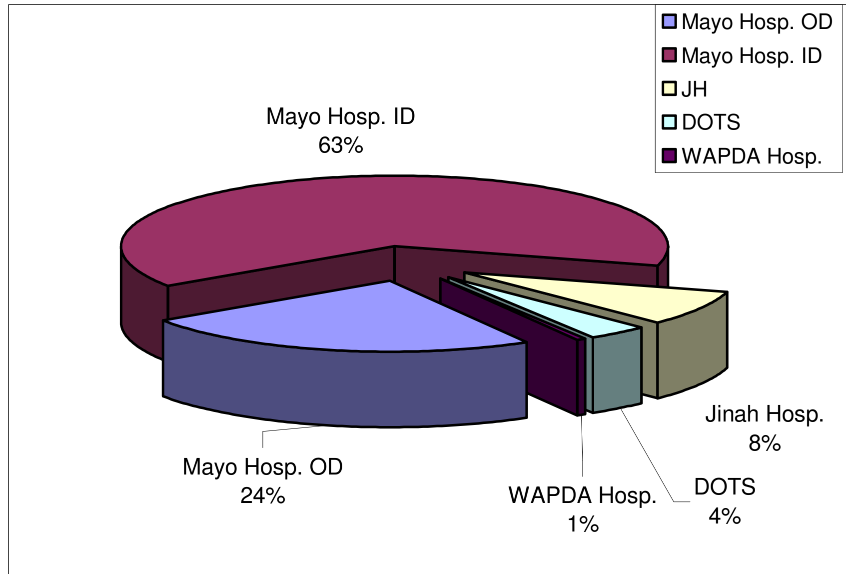


Figure27. The number of samples of MTB strains from TB positive patients and their respective sourced institutions/ hospitals

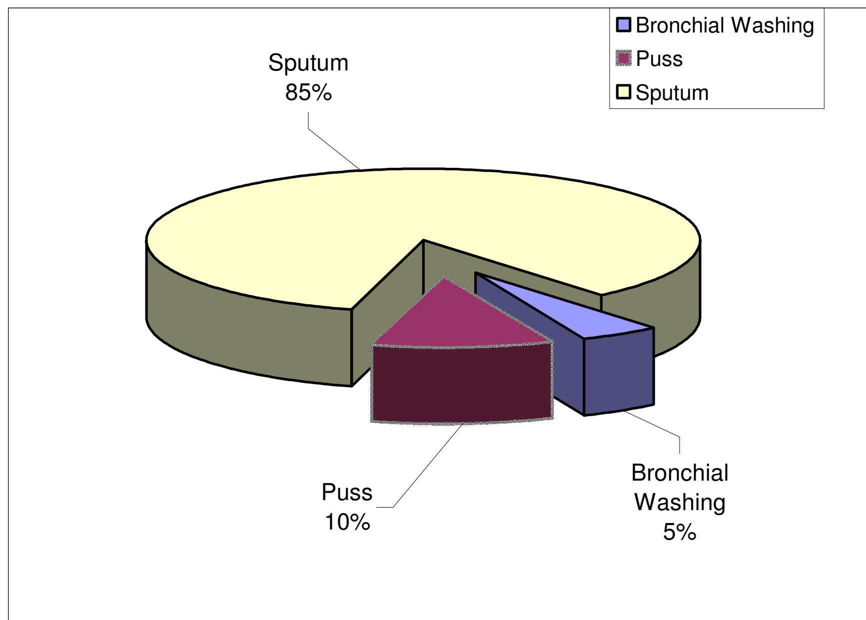


Figure28. The number and types of samples collected from Tuberculosis (AFB) positive patients.

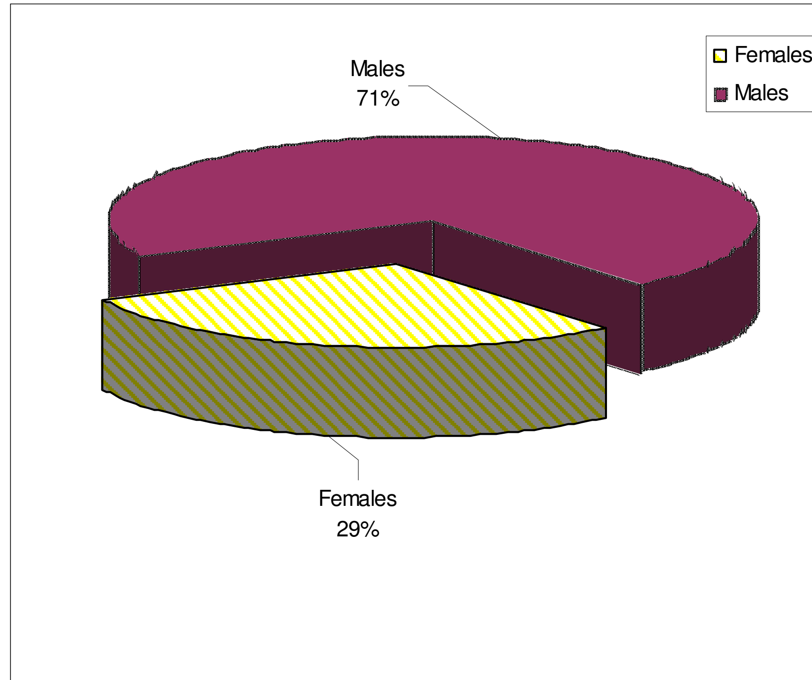


Figure 29. Comparison of male and female TB positive (AFB positive) patients.

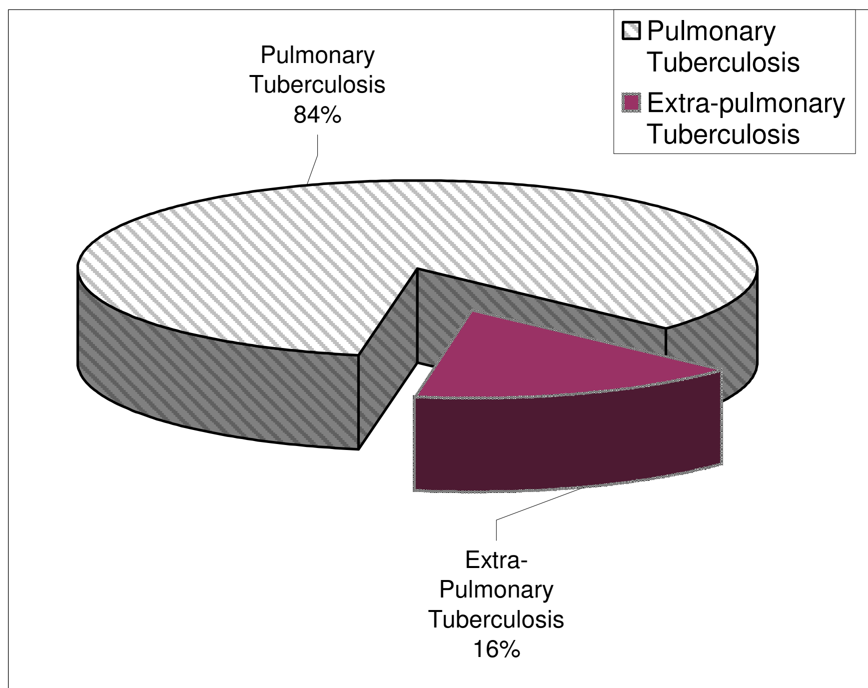


Figure 30. Distribution of the pulmonary and extra-pulmonary tuberculosis specimens:

4.3 The pulmonary and extra-pulmonary specimen's distribution:

We have collected 145 (84.30%) pulmonary (sputum, bronchial washing & puss) and 27 (15.69%) extra-pulmonary specimens (puss & bronchial washing) from tuberculosis diagnosed (AFB positive) patients of 17-68 years age group. (Figure 30 and Table 34).

4.4 Pattern of resistance of *Mycobacterium TB* strains against 1st line antitubercular drugs:

The *Mycobacterium tuberculosis* strains collected from primary culture of TB diagnosed patients. Lowenstein Jensen medium was used in standard drug proportion method to determine the sensitivity of *Mycobacterium tuberculosis*. The final drug's concentrations of antitubercular drugs rifampicin, isoniazid, ethambutol and pyrazinamide incorporated in media to consider as border line for declaration of sensitive or resistant mycobacteria were 40 µg/ml, 0.2µg/ml, 2µg/ml and 100µg/ml respectively.

4.4.1 Rifampicin resistance pattern of *Mycobacterium TB* strains:

Standard drug proportion method used to determine the sensitivity of *Mycobacterium tuberculosis* against rifampicin. The final drug concentration of rifampicin incorporated in Lowenstein Jensen medium media to consider as border line for declaration of sensitive or resistant *Mycobacterium* was 40 µg/ml. Data of this experimental study showed 37 (21.5%) strains resistant and 135 (78.5%) strains sensitive to rifampicin of total 172 clinical isolates of *Mycobacterium tuberculosis*. (Figure 31 and Table 35).

4.4.2 Isoniazid resistance pattern of *Mycobacterium TB* strains:

For determination of isoniazid susceptibility against *Mycobacterium tuberculosis,* Lowenstein Jensen medium used in standard drug proportion method. The final drug concentration of isoniazid, maintained in media to consider as border line for declaration of sensitive or resistant *Mycobacterium* was 0.2 µg/ml. According to the results obtained from this experiment study 25 (14.5%) strains resistant and 147 (85.5%) strains were sensitive to isoniazid of total 172 clinical isolates of *Mycobacterium tuberculosis*. (Figure 32 and Table 36).

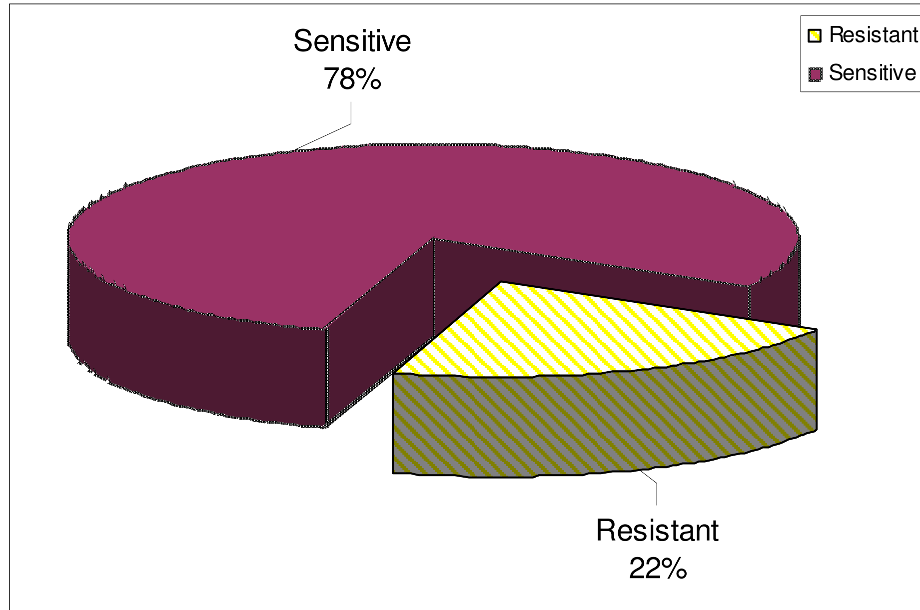


Figure31. Rifampicin resistance pattern of indigenous *Mycobacterium TB* strains collected from primary culture of TB diagnosed (AFB positive) patients.

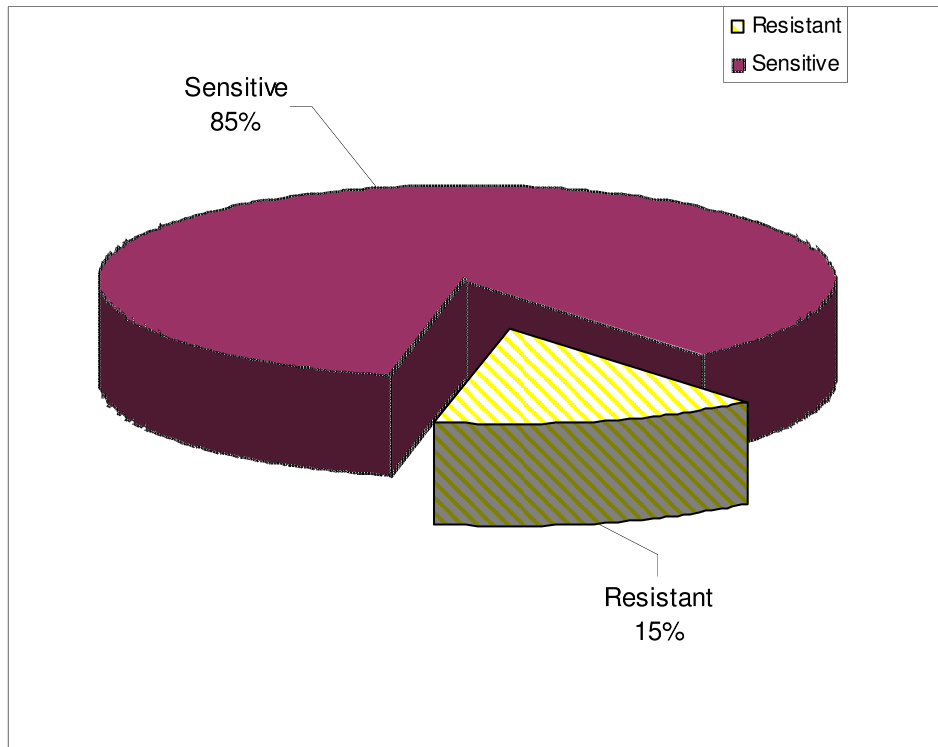


Figure32. Isoniazid resistance pattern of indigenous *Mycobacterium TB* strains collected from primary culture of TB diagnosed (AFB positive) patients.

4.4.3 Ethambutol resistance pattern of *Mycobacterium TB* strains:

Susceptibility of *Mycobacterium tuberculosis* against ethambutol was determined by drug proportion method. The final drug concentration of ethambutol incorporated in media was 2µg/ml. In this experimental, it was observed that 10 (5.8%) strains resistant and 162 (94.2%) strains sensitive to ethambutol of total 172 clinical isolates of *Mycobacterium tuberculosis*. (Figure 33 and Table 37).

4.4.4 Pyrazinamide resistance pattern of *Mycobacterium TB* strains:

Lowenstein Jensen medium was used in standard drug proportion method to determine the sensitivity of *Mycobacterium tuberculosis* against pyrazinamide. The final drug concentration of pyrazinamide maintained in media was 100µg/ml. The finding of this experimental study showed 47 (27.3%) resistant strains and 125 (72.7%) sensitive strains against pyrazinamide of total 172 clinical isolates of *Mycobacterium tuberculosis*. (Figure 34 and Table 38).

4.5 Quantitative (number of colonies) based pattern of resistance of indigenous *Mycobacterium TB* strains:

The drug proportion method was used to determine the resistance of *Mycobacterium tuberculosis* on basis of growth pattern (number of colonies) over the mycobacterial specific Lowenstein Jensen medium. The mycobacterial growths were divided into six categories on basis of number of colonies; 10 colonies, 20 colonies, 30 colonies, 50 colonies, 50 - 100 colonies and 100 - 200 colonies. The final concentrations of antitubercular drugs; rifampicin, isoniazid, ethambutol and pyrazinamide incorporated in LJ media to consider as border line for declaration of sensitive or resistant were 40µg/ml, 0.2 µg/ml, 2 µg/ml and 100 µg/ml respectively.

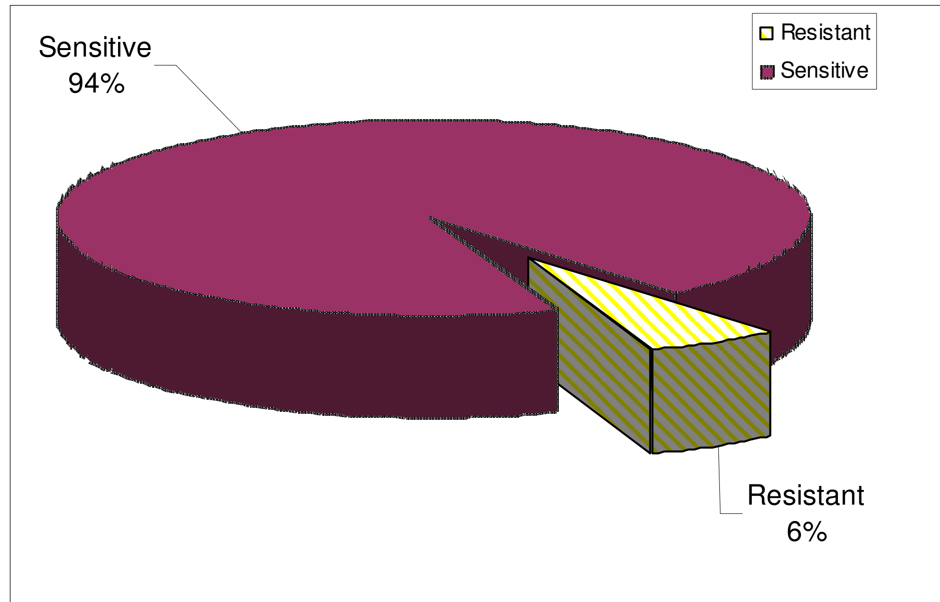


Figure33. Ethambutol resistance pattern of indigenous *Mycobacterium TB* strains collected from primary culture of TB diagnosed (AFB positive) patients.

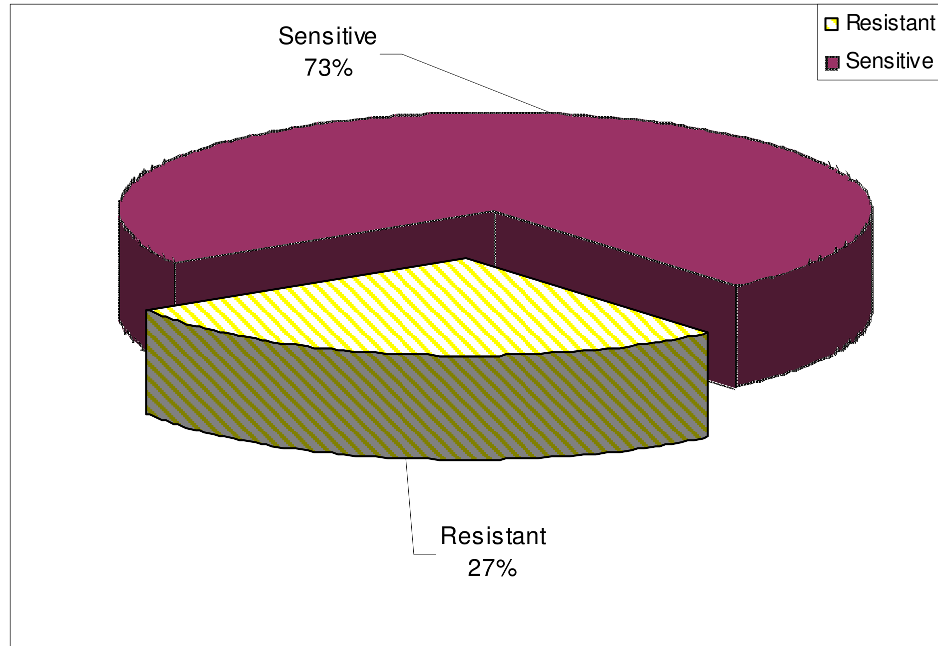


Figure34. Pyrazinamide resistance pattern of indigenous *Mycobacterium TB* strains collected from primary culture of TB diagnosed (AFB positive) patients.

4.5.1 Comparison of Rifampicin resistance percentage with quantity of growth/ number of colonies of *Mycobacterium TB:*

The resistance of *Mycobacterium tuberculosis* against rifampicin was determined on the basis of number of colonies over the Lowenstein Jensen medium. Five categories of mycobacterial growth were observed on the basis of the number of colonies; 20 colonies, 30 colonies, 50 colonies, 50 - 100 colonies and 100 - 200 colonies. The final drug concentration of rifampicin incorporated in media was 40 µg/ml. Data of this experimental study showed 1 (2.70%) resistant strain has 20 colonies, 3 (8.10%) resistant strains have 30 colonies, 6 (16.20%) resistant strains have 50 colonies, 26 (70.30%) resistant strains have 100 colonies and 1 (2.70%) resistant strains has 200 colonies out of 37 (21.51%) rifampicin resistant clinical isolates of total 172 *Mycobacterium tuberculosis* strains. (Figure 35 and Table 39).

4.5.2 Comparison of isoniazid resistance percentage with quantity of growth/ number of colonies of *Mycobacterium TB:*

Four categories of mycobacterial growth were observed on basis of the number of colonies; 30 colonies, 50 colonies, 50 - 100 colonies and 100 - 200 colonies. The final drug concentration of isoniazid incorporated in media was 0.2µg/ml. According to the results obtained from this experimental study, 1 (4%) resistant strain has 30 colonies, 1 (4%) resistant strains has 50 colonies, 21 (84%) resistant strains have 100 colonies and 2 (8%) resistant strains have 200 colonies out of 25 (14.53%) isoniazid resistant clinical isolates of total 172 *Mycobacterium tuberculosis* strains. (Figure 36 and Table 40).

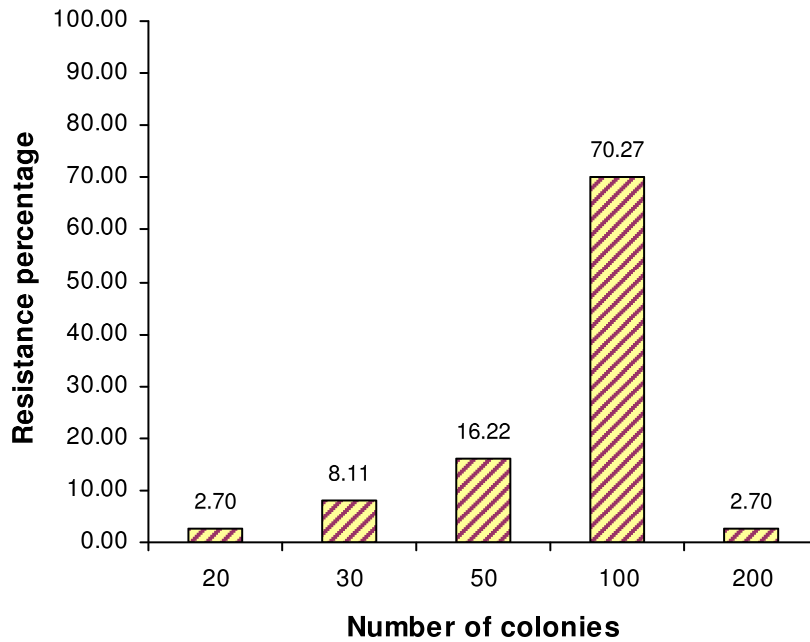


Figure35. Comparison of resistance percentage and quantity of growth of indigenous *Mycobacterium TB* in rifampicin incorporated Lowenstein Jensen media.

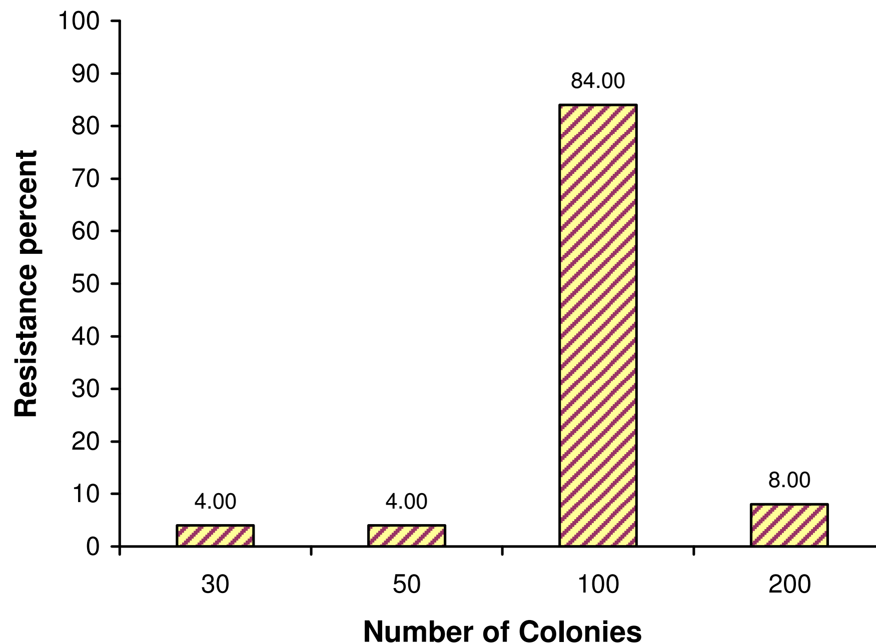


Figure36. Comparison of resistance percentage over quantity of growth of indigenous *Mycobacterium TB* in Isoniazid incorporated Lowenstein Jensen media.

4.5.3 Comparison of ethambutol resistance percentage with quantity of growth/ number of colonies of *Mycobacterium TB:*

Two categories of mycobacterial growth were observed on basis of the number of colonies; 10 colonies and 100 colonies. The final drug concentration of antitubercular drug ethambutol maintained in media was 2µg/ml. The findings of this experimental study showed 1 (10%) resistant strain has 10 colonies, 9 (90%) resistant strains has 100 colonies out of 10 ethambutol resistant clinical isolates of total 172 *Mycobacterium tuberculosis* strains. (Figure 37 and Table 41).

4.5.4 Comparison of pyrazinamide resistance percentage with quantity of growth/ No. of colonies of *Mycobacterium TB:*

Four categories of mycobacterial growth were observed on basis of the number of colonies; 30 colonies, 50 colonies, 100 colonies and 200 colonies. The final drug concentration of pyrazinamide maintained was 100µg/ml. Data of this experimental study showed 1 (2.13%) resistant strains has 30 colonies, 4 (8.51%) resistant strains have 50 colonies, 39 (82.98%) resistant strains have 100 colonies and 3 (6.38%) resistant strains have 200 colonies out of 47 (27.32%) pyrazinamide resistant clinical isolates of total 172 *Mycobacterium tuberculosis* strains. (Figure 38 and Table 42).

4.6 Over all growth based resistance pattern of *Mycobacterium TB* against 1st line antitubercular drugs,

Over all growth (number of colonies) based resistance pattern of *Mycobacterium TB*, was determined against 1st line antitubercular drugs. The overall resistance of *Mycobacterium tuberculosis* was evaluated by comparing the percentage of resistance of resistant *Mycobacterium TB* to the number of colonies (growth). There were the six categories of mycobacterial growth observed on basis of the number of colonies; 10 colonies, 20 colonies, 30 colonies, 50 colonies, more than 100 colonies and more than 200 colonies. According to the results obtained from this experimental study, 10% ethambutol resistant strains were under the category of 10 colonies, 2.7% rifampicin resistant strain under the category of 20 colonies, 8.11% rifampicin resistant, 4% isoniazid resistant strain and 2.13% pyrazinamide resistant strains under the category of 30 colonies, 16.22% rifampicin resistant strains, 4% isoniazid resistant strains and

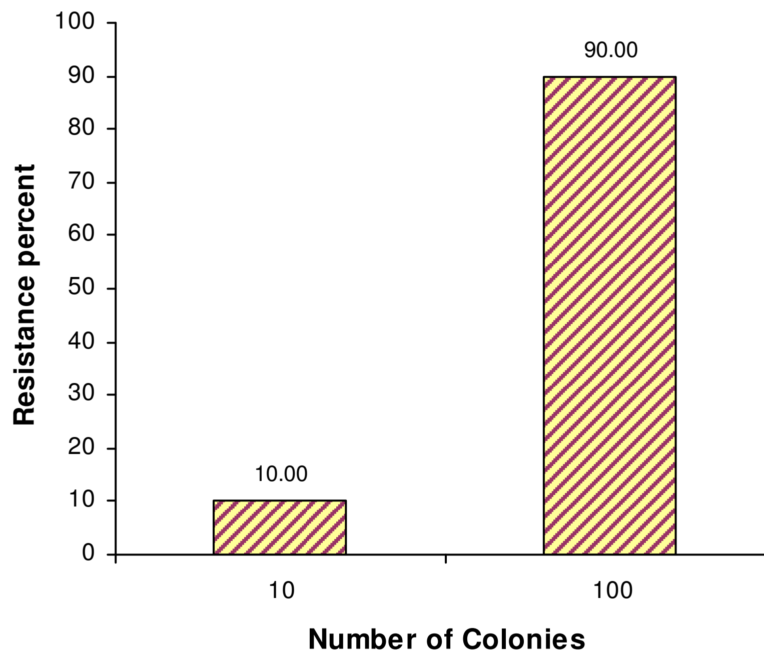


Figure37. Comparison of resistance percentage and quantity of growth of indigenous *Mycobacterium TB* in ethambutol incorporated Lowenstein Jensen media.

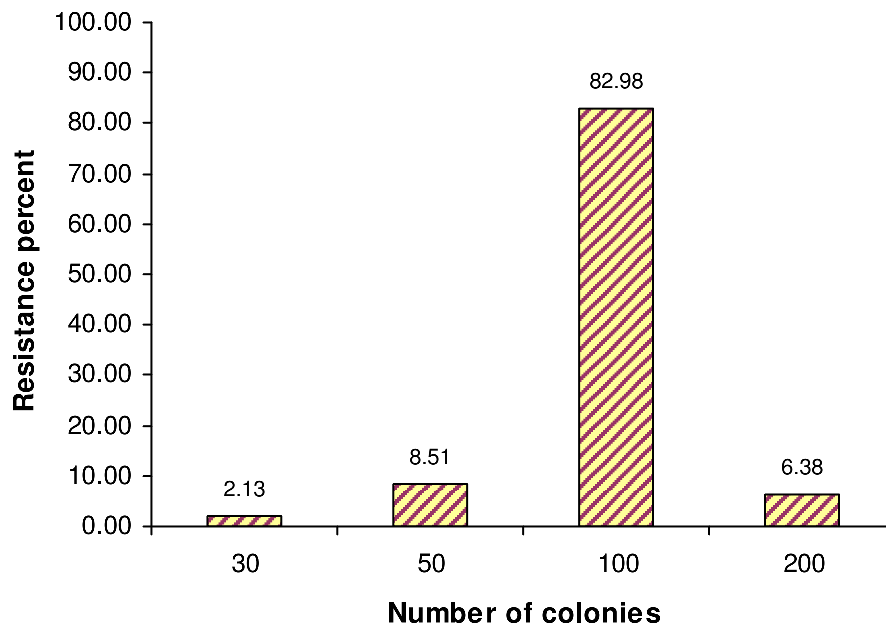


Figure38. Comparison of resistance pattern (percentage) and quantity of growth of indigenous *Mycobacterium TB* in pyrazinamide incorporated Lowenstein Jensen media

8.51% pyrazinamide resistant strains under the category of 50 colonies, 70.27% rifampicin resistant strain, 84% isoniazid resistant strains, 90% ethambutol resistant strains and 82.983% pyrazinamide resistant strains under the category of 100 colonies and 2.70% rifampicin resistant strain, 8% isoniazid resistant strains and 6.38% pyrazinamide resistant strains under the category of 200 colonies. (Figure 39 and Table 43).

4.7 Overall mono-resistance pattern of *Mycobacterium TB* against 1st line antitubercular drugs:

Overall mono-resistance pattern of *Mycobacterium TB* was determined against 1st line antitubercular drugs. By the overall comparison of anti-TB drugs and there respective percentage of growth, it was observed that 9 (25.71%) clinical isolates resistant to rifampicin, 3 (8.57%) resistant to isoniazid, 1 (2.85%) resistant to ethambutol and 22 (62.85%) resistant to pyrazinamide out of 35 (20.34%) mono-resistant isolates of total 172 *Mycobacterium tuberculosis* strains. (Figure 40 and Table 44).

4.8 Poly-resistance profile of *Mycobacterium TB* strains categorized on basis of resistance against two, three or all of the four 1st line antitubercular drugs.

Poly resistance profile of this indigenous *Mycobacterium tuberculosis* strains was studied against antitubercular drugs. According to the results obtained 6 (19.35%) strains were resistant to rifampicin & isoniazid, 7 (22.58%) strains resistant to ethambutol & pyrazinamide, 1 (3.22%) strains resistant to ethambutol & pyrazinamide, 2 (6.45%) strains resistant to isoniazid & pyrazinamide, 7 (22.58%) strains resistant to rifampicin, isoniazid and pyrazinamide, 1 (3.22%) strains resistant to rifampicin, ethambutol and pyrazinamide and 7 (22.58%) strains resistant to all of the four 1st line drugs. Poly resistance profile of *Mycobacterium tuberculosis* strains was also studied on basis of resistance against one, two, three or all of the four 1st line antituberculosis drugs. Data showed 61.63% (106) susceptible strains, 20.35% (35) strains resistant against one drug, 9.30% (16) strains resistant against two drugs, 4.65% (8) strains resistant against three drugs and 4.07% (7) stains resistant against all of the four anti-TB drugs. (Figure 41 & 42 and Table 45 &b 46).

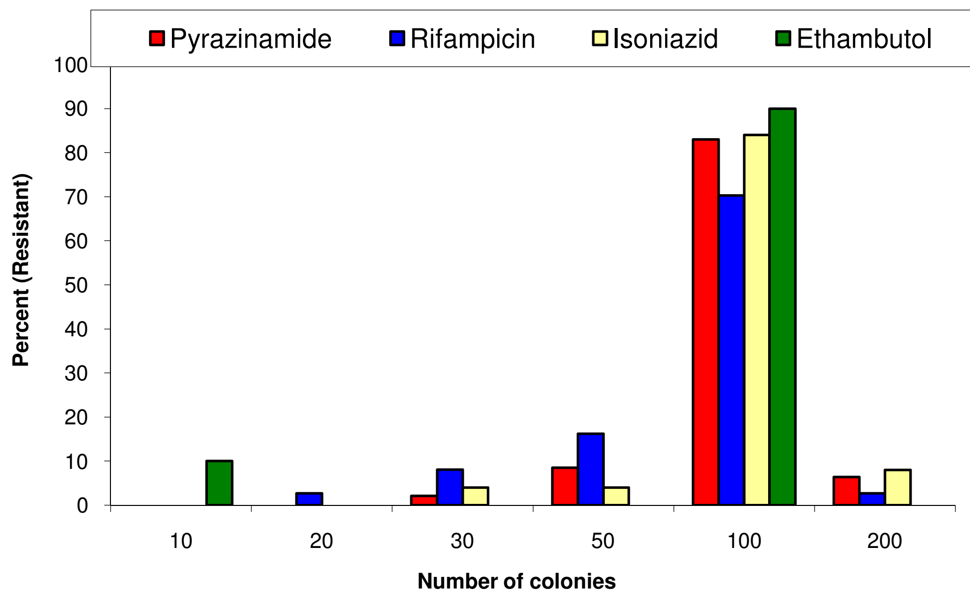


Figure39. Over all growth (No. of colonies) based resistance pattern of indigenous pulmonary/ extrapulmonary *Mycobacterium TB* against 1st line antitubercular drugs

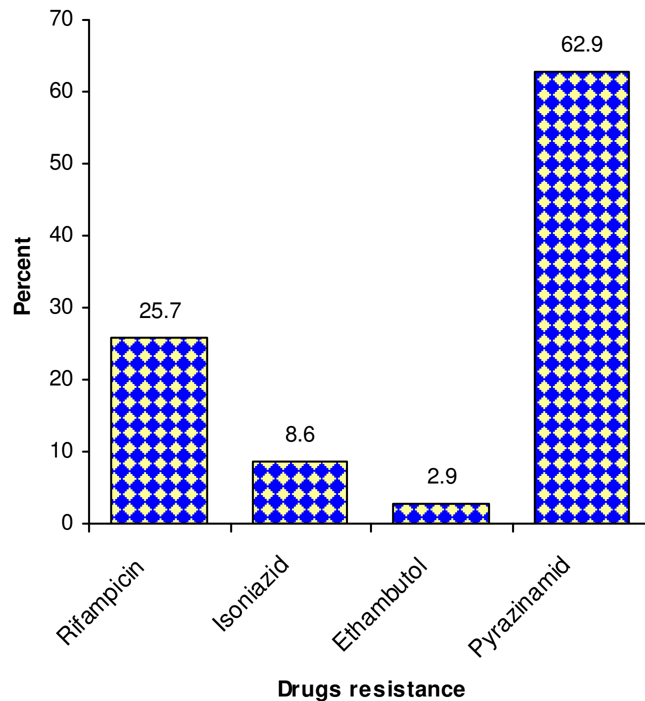


Figure 40. Overall mono-resistance pattern of indigenous pulmonary/ extrapulmonary *Mycobacterium TB* against 1st line antitubercular drugs.

N= 35

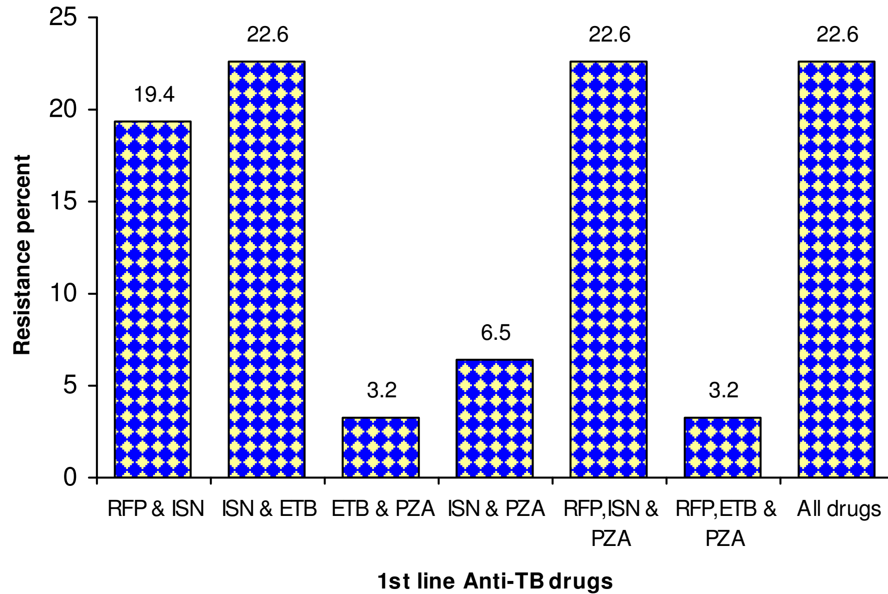


Figure 41. Poly-resistance profile of indigenous pulmonary/ extrapulmonary *Mycobacterium TB* strains categorized on basis of resistance against one, two, three or all of the four 1st line antitubercular drugs. N = 31

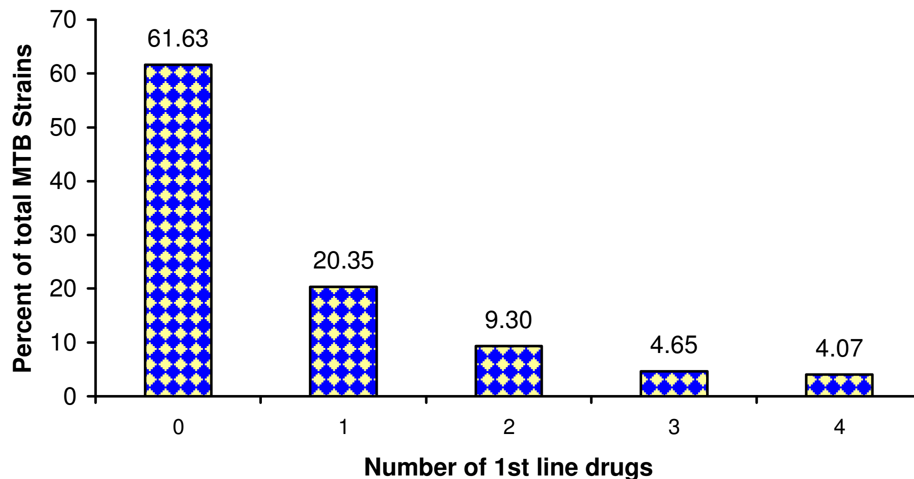


Figure42: Poly-resistance profile (in percentage) of indigenous pulmonary/ extrapulmonary *Mycobacterium TB* strains categorized on basis of resistance against one, two, three or all of the four 1st line antitubercular drugs. N=172

4.9 Overall trend of resistance of *Mycobacterium TB* during January - December, 2005, against 1st line antitubercular drugs.

The *Mycobacterium TB* strains were collected from primary culture of TB diagnosed patients. Which were studied for evaluation of overall trend of resistance prevalence against all of the four 1st line antitubercular drugs, during November, 2004 to December, 2005. The total 172 *Mycobacterium TB* strains were divided into four segments (each segments comprised of 43 *Mycobacterium TB* strains) and chronologically compared over number of resistance drugs. In 1st segment there were 11 *Mycobacterium TB* strains resistant to one drug, 6 strains resistant to two drugs, 3 strains resistant to three drugs, 4 strains resistant to the entire four of the drug and 20 strains were sensitive to all of the four drugs. In 2nd segment there were 11 *Mycobacterium TB* strains resistant to one drug, 4 strains resistant to two drugs, 5 strains resistant to three drugs, 4 strains resistant to the entire four of the drug and 19 strains sensitive to all of the four drugs. In 3rd segment there were 9 *Mycobacterium TB* strains resistant to one drug, 3 strains resistant to two drugs, no any strain resistant to three drugs and entire of the four drug and 31 strains sensitive to all of the four drugs. In 4th segment there were 4 *Mycobacterium TB* strains resistant to one drug, 3 strains resistant to two drugs, no any strains resistant to three or entire of the four drugs and 36 strains sensitive to all of the four drugs. (Figure 43).

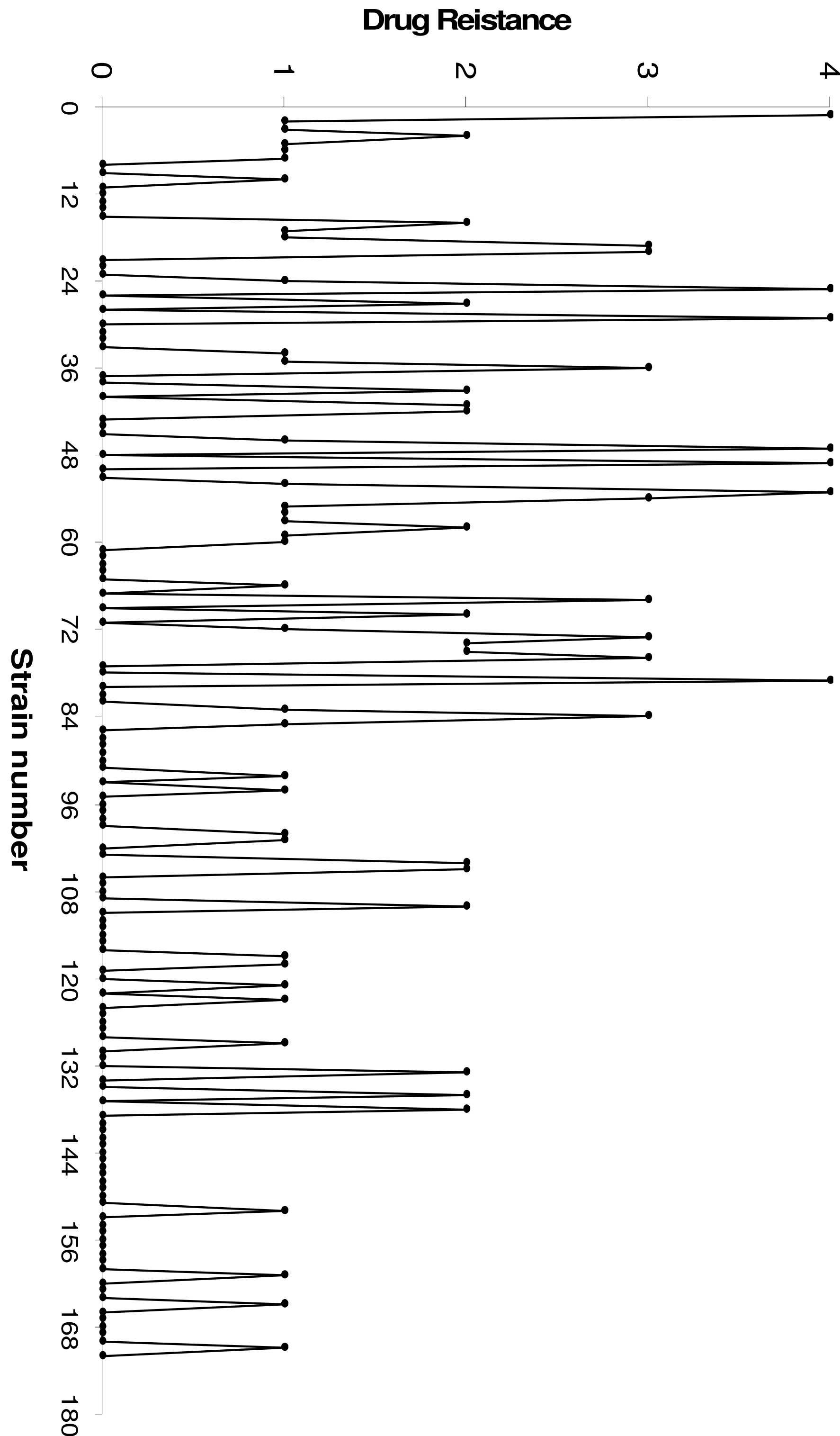


Figure43. Overall trend of resistance prevalence of indigenous *Mycobacterium -TB* during November, 2004- December, 2005. N=172

4.10 Determination level of resistance of resistant *Mycobacterium TB:*

The resistant *Mycobacterium TB* having an ultimate highest level of resistance against the first line antitubercular drugs - rifampicin, isoniazid, pyrazinamide and ethambutol which were studied by preparing the Lowenstein Jensen media with five drug levels above of their respective optimum concentrations. The highest level of resistance obtained from experimental study against all of the four 1st line antitubercular drugs were as follow,

4.10.1 Level of resistance of rifampicin resistant *Mycobacterium TB:*

The rifampicin resistant *Mycobacterial TB* strains were collected from culture growth over Lowenstein Jensen media containing the rifampicin at minimum inhibitory concentration level of 40µg/ml that was used for sensitivity evaluation. Minimum fifty colonies collected in eppendroff vial to prepare the required 10^{-3} and 10^{-5} dilutions. It was then inoculated over the Lowenstein Jensen media containing the five rifampicin levels - 40 µg/ml, 80 µg/ml, 120 µg/ml, 160 µg/ml and 200 µg/ml respectively. These drug levels were in ascending order; increased by adding 40 from 1st level (40ug/ml) to highest 5th level (200ug/ml). The final results collected on data collection sheet (Appendix II) after four weeks (28 days). Data of this experimental study showed 37 (21.51%) *Mycobacterium TB* strains resistant to rifampicin of total 172 clinical isolates. All of the 37 (100%) *Mycobacterium*-TB strains were resistant to 1st rifampicin level of 40 µg/ml and 2nd rifampicin level of 80 µg/ml. 15 (40.54%) rifampicin resistant *Mycobacterium -TB* strains were resistant upto 3rd rifampicin level of 120 µg/ml, 13 (35.13%) rifampicin resistant *Mycobacterium -TB* strains resistant upto 4th rifampicin level of 160 µg/ml, 7 (18.91%) rifampicin resistant *Mycobacterium -TB* strains resistant upto 5th rifampicin level of 200 µg/ml and 2 (5.40%) rifampicin resistant *Mycobacterium -TB* strains resistant to higher than 5th rifampicin level of 200+ µg/ml. (Figure 45 and Table 46).

4.10.2 Level of resistance of isoniazid resistant *Mycobacterium TB:*

The isoniazid resistant *mycobacterial TB* collected from culture growth over

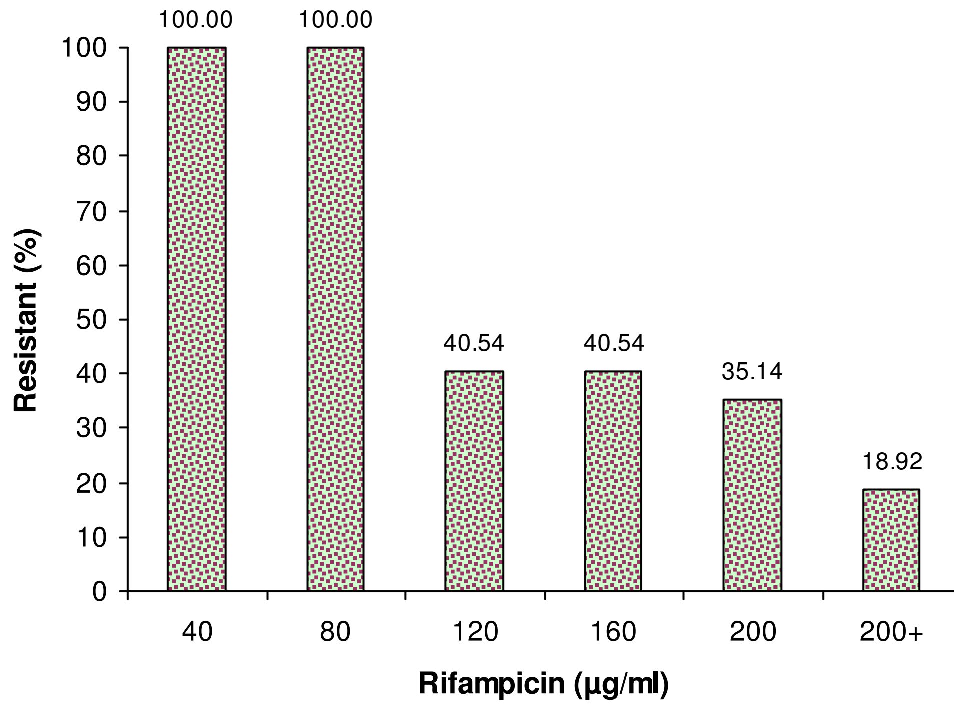


Figure 45. Level of resistance (in % age) of rifampicin resistant *Mycobacterium TB*

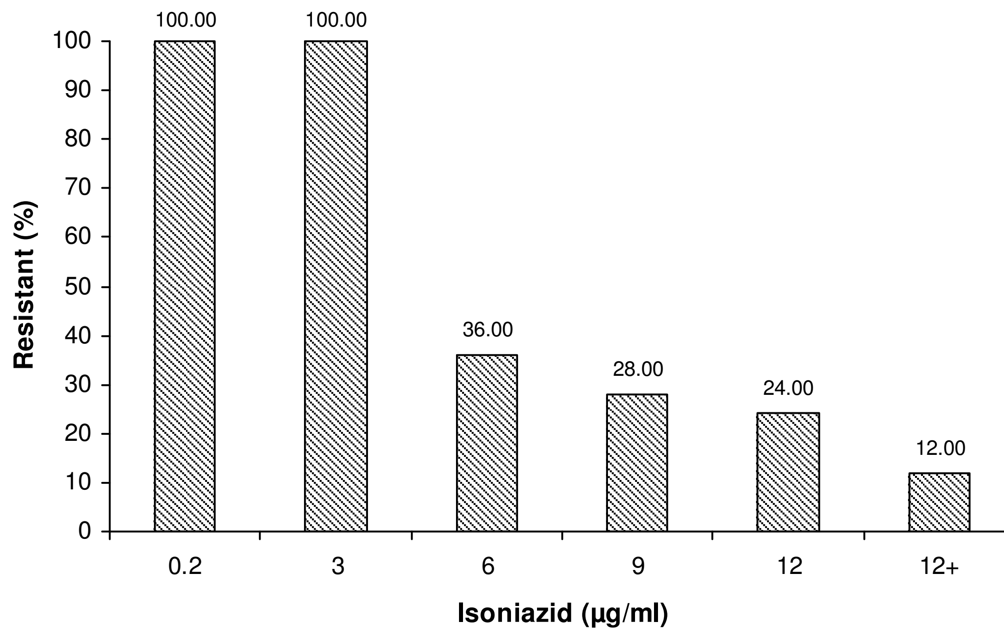


Figure 46. Level of resistance (in % age) of isoniazid resistant *Mycobacterium TB*

Lowenstein Jensen media containing the isoniazid at minimum inhibitory concentration level of 0.2µg/ml. Minimum fifty colonies collected in eppendroff vial to prepare the required 10^{-3} and 10^{-5} dilutions. Which were then inoculated over the Lowenstein Jensen media containing the five isoniazid levels - 0.2µg/ml, 3 µg/ml, 6µg/ml, 9µg/ml and 12µg/ml and 12+ ug/ml respectively. 25 (14.53%) *Mycobacterium TB* strains were resistant to isoniazid of total 172 clinical isolates. According to the final results obtained from this experimental study, 25 (100%) *Mycobacterium-TB* strains were resistant to 1st level 0.2µg/ml and 2nd level 3µg/ml. 9 (36%) isoniazid resistant *Mycobacterium -TB* strains were resistant upto 3rd level of 6µg/ml, 7 (28%) isoniazid resistant strains resistant upto 4th level of 9µg/ml, 6 (24%) isoniazid resistant strains resistant upto 5th isoniazid level of 12µg/ml, 3 (12%) isoniazid resistant strains resistant to higher than 5th isoniazid level of 12+ µg/ml. (Figure 46 and Table 47)

4.10.3 Level of resistance (in % age) of ethambutol resistant *Mycobacterium TB:*

The ethambutol resistant *Mycobacterial TB* collected from culture growth Lowenstein Jensen media containing the ethambutol at minimum inhibitory concentration level of 2.0µg/ml. Minimum fifty colonies collected in eppendroff vial to prepare the required 10^{-3} and 10^{-5} dilutions. Which were then inoculated into Lowenstein Jensen media containing the five ethambutol levels - 2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml and 10 µg/ml respectively. These ethambutol levels were in ascending order; increased by adding 2 from 1st level (2ug/ml) to highest 5th ethambutol level (10ug/ml). 10 (5.81%) *Mycobacterium TB* strains were resistant to ethambutol of total 172 clinical isolates. The final results obtained for determination of level of ethambutol resistance were as 10(100%) *Mycobacterium-TB* strains resistant to 1st ethambutol level of 2µg/ml and 2nd ethambutol level of 4µg/ml. 5 (50%) ethambutol resistant *Mycobacterium TB* strains were resistant upto 3rd isoniazid level of 6µg/ml, 3 (30%) ethambutol resistant *Mycobacterium* -TB strains resistant upto 4th ethambutol level of 8µg/ml, 2(20%) ethambutol resistant *Mycobacterium* -TB strains resistant upto 5th ethambutol level of

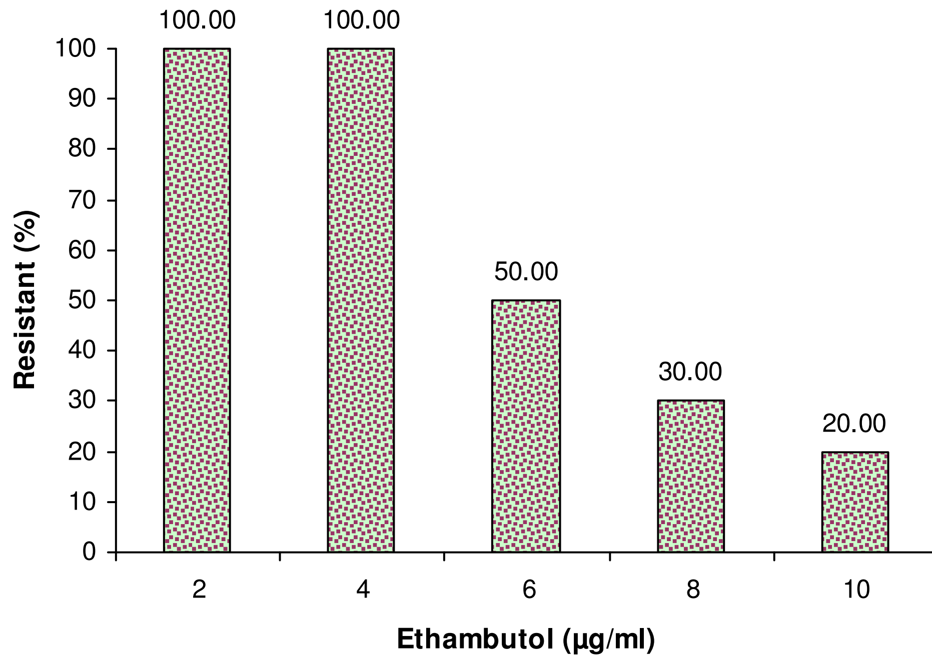


Figure 47. Level of resistance (in % age) of Ethambutol resistant *Mycobacterium TB*

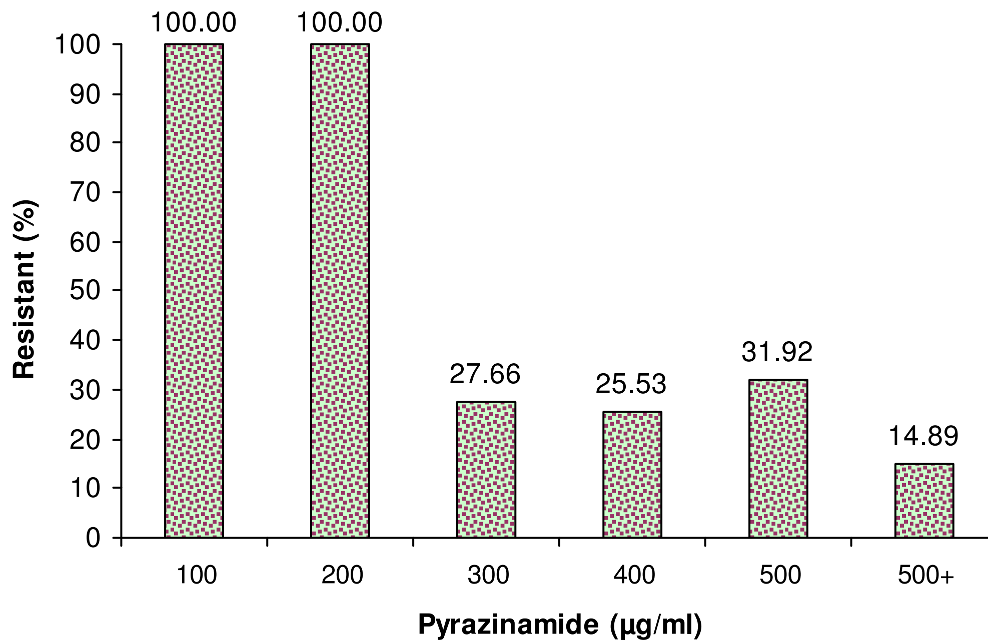


Figure 48. Level of resistance (in % age) of Ethambutol resistant *Mycobacterium TB*

10µg/ml and there no any ethambutol resistant M-TB strains observed at higher than 5th ethambutol level of 10+ µg/ml. (Figure 47 and Table 48).

4.10.4 Level of resistance (in % age) of pyrazinamide resistant *Mycobacterium TB:*

The pyrazinamide resistant *Mycobacterial TB* collected from culture growth over Lowenstein Jensen media containing the pyrazinamide at minimum inhibitory concentration level of 100µg/ml. Minimum fifty colonies collected in eppendroff vial to prepare the required 10^{-3} and 10^{-5} dilutions. Which were then inoculated into Lowenstein Jensen media containing the five pyrazinamide levels - 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml and 500µg/ml respectively. These ascending ordered drug levels were increased by adding 100 from 1st (100ug/ml) level to highest 5th pyrazinamide level (500ug/ml). 47 (27.32%) *Mycobacterium TB strains were* resistant to pyrazinamide of total 172 clinical isolates. The findings of this experimental study showed 47 (100%) *Mycobacterium-TB* strains resistant to 1st pyrazinamide level of 100µg/ml and 2nd pyrazinamide level of 200µg/ml. 13 (27.66%) pyrazinamide resistant strains were resistant upto 3rd pyrazinamide level of 300µg/ml and 12 (25.53%) pyrazinamide resistant strains resistant upto 4th pyrazinamide level of 400µg/ml, 15 (31.91%) pyrazinamide resistant strains resistant upto 5th pyrazinamide level of 500µg/ml and 7 (14.89%) pyrazinamide resistant strains resistant to higher than 5th pyrazinamide level of 500+ µg/ml. (Figure 48 and Table 49).

4.11 Overall Level of resistance (in % age) of resistant *Mycobacterium TB:*

A total 66 resistant *Mycobacterial TB* strains selected out the total 172 clinical isolates, from the growth over *mycobacterial* specific Lowenstein Jensen media containing the respective antitubercular drugs - rifampicin, isoniazid, ethambutol or pyrazinamide at their respective invitro MIC,s levels. Data of this experiment study showed 100% resistance against all of the four 1st line antitubercular drugs at 1st and 2nd drug levels. 40.54% resistance against rifampicin, 36% resistance against isoniazid, 50% resistance against ethambutol and 27.22% resistance against pyrazinamide was observe at 3rd drug level. 35.13% resistance against rifampicin, 28% resistance against isoniazid, 30% resistance against ethambutol and 25.53% resistance against pyrazinamide seen at 4th drug level. 18.91% resistance against rifampicin, 24% resistance against

isoniazid, 20% resistance against ethambutol and 31.91% resistance against pyrazinamide seen at 5th drug level. 5.40% resistance against rifampicin, 12% resistance against isoniazid and 14.89% resistance against pyrazinamide seen at the higher than 5th drug level. (Figure 49 and Table 50).

4.12 Therapeutical interpretation of 1st line antitubercular drugs

1st line antitubercular drugs - rifampicin, isoniazid, pyrazinamide and ethambutol were interpreted therapeutically to understand the suitability of dosage and regimen. The pharmacodynamic parameters - Cmax (maximum plasma concentrations), therapeutic index and maximum regimens were also considered to explain the comprehension of anti-TB drugs.

4.13 Molecular basis of pyrazinamide resistance of *Mycobacterium tuberculosis*:

The genomic DNA of *Mycobacterium TB* extracted by mechanical method and examined on gel. PCR for *Mycobacterium TB* is specific for *Mycobacterium TB* complex DNA and does not detect non-tuberculosis *Mycobacterium* (NTB) or *Mycobacterium* other than tuberculosis. NTB can cause a variety of infection resembling tuberculosis. The clinical and radiological features of these diseases are similar to tuberculosis. The negative test indicates the absence of *Mycobacterium TB* complex but not absence of NTB. The medical experts should make judgment on basis of patient's clinical investigations and other findings. This test has sensitivity of 70-90 % and specificity 95-97%. By using the SSCP (Single Strand Conformational Polymorphism), we were able to show most divers pattern. 30 (17.44%) resistant *Mycobacterium* showed different pattern than sensitive samples. Which indicate the mutation in this domain, while 17 (9.88%) did not show any difference in mobility in comparison to sensitive samples. This indicates that there is no mutation in this region but it should be confirmed by sequencing. Pyrazinamide is an important therapeutic agent of 1st line therapy.

CHAPTER No. 5

DISCUSSION

DISCUSSION

Present study was aimed to characterize the resistant *Mycobacterium tuberculosis*. The clinical isolates of Mycobacterial tuberculosis of TB diagnosed patients were studied for susceptibility against the 1stline anti-TB drugs. The resistant *Mycobacterium tuberculosis* further studied to determine their highest level of resistance. The parameters of the study were the pattern of resistance of Mycobacterial tuberculosis against rifampicin, isoniazid, ethambutol and pyrazinamide, overall trend of resistance prevalence during Jan. - Dec. 2005, resistance pattern in percentage against five different levels (µg/ml) of aforesaid anti-TB drugs above their respective MIC,s and mathematical determination of highest possible regimen of all of four drugs to evaluate the pharmacological credibility of newly designed invivo doses.

5.1 Gender comparison

Data showed 122 (70.9%) males and 50 (29.1%) females of total 172 clinical isolates of TB diagnosed patients of 17- 67 years age group. Gender comparison depicts greater percentage of male (70.9%) than females (29.1%) of total clinical isolates. These findings are substantiated by Uplekar et al., (2001), who reported a seventy percent (70%) the worldwide excess of male over female tuberculosis cases each year. The exact reasons of this difference are still unclear. Normally, the ladies of poor world confront higher hurdles as compared with men in health care services accessibilities. Thus far, the impact of gender inequalities is not explained to accessing the health cares. Limited reviews obtained in literature and tuberculosis programmes offering approaches and recommend a structure to invstigate the gender variation in tuberculosis. The role of gender at different is level in effective turbculosis care is quite important. The findings of this study are consistent with Haq et al., (2002), who reported 68% males and 32% females tuberculosis patients. Our findings are in conformity with WHO/ IUALTD Report No. 2, (2000), who reported approximately 67% of males tuberculosis patients. These findings are in agreement with the results of Bitar et el., (2001), who reported70% males with a M:F sex ratio of 2.8. Age ranged 3from 1 to 91 years (median = 35 years). For several other demographic and clinical characteristics information was missing for an important proportion of cases. The possible reasons may lie in the facts reported by Jiménez, (2006), the most of the poor countries have double of the tuberculosis cases in men as compared with women. This

variationis is attributed by epidemiological and biological characteristics alongwith cultural barriers and availability of socioeconomic access to control this disease.

5.2 The pulmonary and extra-pulmonary specimen's distribution

We had collected 145 (84.30%) pulmonary (sputum, bronchial washing & puss) and 27 (15.69%) extra-pulmonary (puss & bronchial washing) specimens from tuberculosis patients. These findings are in conformity with Bitar et el., (2001), who reported the majority of cases (n=607; 89%) pulmonary TB including 16 cases (2%) presenting with associated extra- pulmonary TB (EPTB). There 39 cases presenting EPTB only (6%) and 37 cases for whom site of disease was unknown (5%).

5.3 The drug concentration incorporated in LJ media

The final drug concentration of antitubercular drugs rifampicin 40 µg/ml, isoniazid 0.2µg/ml, ethambutol 2µg/ml and pyrazinamide 100µg/ml incorporated in media to consider as border line for declaration of sensitive or resistant *M. tuberculosis*. Similar concentrations were used by Ahmet et al., (2004) for drug susceptibility testing of *Mycobacterium tuberculosis*. The guid lines of National Committee for Clinical Laboratory Standards were followed in drug proportion method performed (NCCLS 2002). Antitubreculosis drugs incorporatd in the Lowenstien Jensen medium were as isoniazid 0.2-1ug/ml, rifampicin 40 ug/ml and ethambutol 2ug /ml. These inhibitory concentrations are in conformity with those used by C Karin, (1997) in drug concentration susceptibility testing. He described it as paramount importance and impacts on tuberculosis treatment. Each drug should be tested at its critical concentration. The critical drug concentration for routine susceptibility testing incorporated in LJ medium were rifampicin 40 µg/ml, isoniazid 0.2µg/ml, ethambutol 2µg/ml and pyrazinamide 100µg/ml. These concentrations used to date are consistent with international data. The minimum inhibitory concentrations are in agreement with the CCDR, (2004), who reported ethambutol 2.5ug/ml, rifampicin 2.0ug/ml and isoniazid 0.1ug/ml, Pyrazinamide 100 ug/ml. The MIC for pyrazinamide also in accordance with Hirano et el., (1998), who used pyrazinamide ≥400 ug /ml,

pH 6.0 for *Mycobacterium tuberculosis* PZase negative. While; the clinical islolates of *Mycobacterium tuberculosis* with PZase positive were equal to or ≤ 200 g/ml. These MIC's are substantiated by the MIC's reported by Sumathi & Srivastava, (2004), who stated the minimum inhibitory concentration for rifampicin as 40 ug/ ml.

5.4 Sensitivity/resistance of *M. tuberculosis* against 1st line Antituberculosis drugs

The standard minimum inhibitory concentration of antitubercular drugs; rifampicin, isoniazid, ethambutol and pyrazinamide incorporated in media to consider as border line for declaration of sensitive or resistant mycobacteria were 40 µg/ml, 0.2µg/ml, 2µg/ml and 100µg/ml respectively. The complications i.e extensive cavitation and emphysema allow big inhabitants to make in a partition into which the medicine may not able to go through. This big mycobacterial group raises the number of gentetic mutations. That contributes in deprived diffusion to improved probability of resistance occurance. A similar condition may occure in case with poor immunity or extensive disease (Stephen, 2002). There are still challenges in Pakistan to control tuberculosis resistance. Inadequate therapy is major factor that contribute the developmet of tuberculosis resistance; that is worse than not giving treatment. It is confirmed that the incomplete eradication of tuberuculosis may increase the rates of creation of resistance development and failure of treatmenet (Rizwan et al., 2003). A dialy increase in resistant agaisn antituberculosis drugs is an important risk to tuberculosis control programs (Bengisun et al., 2000). The connection in the drug resistance and treatment duration may result of failure of treatment in drug resistant cases. Moreover; it leads to resistance against drugs and irregulate the treatment (Espinal et al., 2001).

5.4.1 Sensitivity/ resistance of *Mycobacterium tuberculosis* against Rifampicin

Mycobacterium tuberculosis isolated from miscellaneous clinical samples of tuberculosis diagnosed patients. In this study project the susceptibility of patients toward 1stline anti-TB drug rifampicin was found as 37 (21.5%) strains resistant and 135 (78.5%) strains sensitive to rifampicin of total 172 clinical isolates of *Mycobacterium tuberculosis*.

The findings of this study are in conformity with the work of WHO/ IUALTD, (2000), who reported the rifampicin resistance in India 25%.and in Estonia 39%. The resistance against antituberulosis agents complicates the work to prevent tuberculosis (Haq et al., 2002). Our study supported the work of Herendra & Shah, (1998), who conceded 20-30% rifampicin resistance. Findings of study under discussion substantiated by the findings of Rizwan et al., (2003), who reported the study of five drugs isoniazid, rifampicin, ethambutol, pyrazinamide, and streptomycin given against *Mycobacterium tuberculosis* to study their pattern of sensitivity or resistance. There were total 53% of Mycobactrium tuberculosis strains showed resistant against one or more drugs; 28% to rifampicin, 16% MDR, 16% primary and 44% acquired resistance. There was a significant statistical difference noticed in acquired and primary resistance. Thus; the DOTS's worldwide expansion is most appropriate strategy for rapid and effective tuberculosis control. Miah et al., (2000), reported an 30% over resistnace in Bangladesh. That indicate 5% MDR and 11% resistance against rifampicin. Similar findings were reported by Sumathi Muralidhar & Srivastava L., (2004), who carried out a study to establish a rapid and accurate method of susceptibility testing for *Mycobacterium tuberculosis* using three methods; proportion method by agar dilution on Middlebrook 7H11 agar, proportion method using the conventional Lowenstein-Jensen (L-J) medium and E test strip method. 67 (89.3%) isolates were sensitive to rifampicin by the three methods respectively and 6 (8.0%) isolates seen resistant to rifampicin by all the three methods. Of the 75 samples, 52 (69.3%) were sensitive to all the 5 drugs by the L-J medium proportion method. 49 (65.3%) were sensitive to all the five drugs by all the three methods. One (1.3%) isolate alone was resistant to all the five drugs by the three methods. The possible reason of difference in results may lie in the facts of the priority of research objectives, methodology adopted and proposed protocol to conduct the project. There also demographic and socio-economic factors influence the research outcomes.

5.4.2 Sensitivity/ resistance of *Mycobacterium tuberculosis* against Isoniazid

0.2µg/ml was an optimum isoniazid concentration incorporated in media to consider as border line for declaration of sensitive or resistant *Mycobacterium*. According to the

results of this study project, 25 (14.5%) strains resistant and 147 (85.5%) strains were sensitive to isoniazid of total 172 clinical isolates of *Mycobacterium tuberculosis*. Our findings are supported the work of WHO/ IUALTD, (2000), who reported the 19.4% isoniazid resistance in Russian Federation, 11.3 % resistance in china and 15.4 % resistance in India. Study by CCDR (Canada Communicable Disease Report), (2004), reveals similar finding of 9.3% most common type of drug resistance against isoniazid. The possible reason of difference of results may lie in the facts of the differences in living standards, health facilities, over all hygienic condition and socio-economic structure. These findings are in agreement with the results of Herendra & Shah, (1998), who reported 20-30% isoniazid resistance. These findings are in conformity with Stephen, (2002), who reported cetain physiological conditions that might contribute to produce multiple resistances and expand the hypermutable circumstances. Thus; the hypothesis of lower risk of resistant strain thatn than wild type is rejected. Because, the initial strength is attenuated by adaptation of various means of access. These findings are in consistent with Miah et al., (2000), who reported 15.8% resistance against isoniazid and 5% to MDR in Bangladesh. Our finding are in line with Sumathi Muralidhar & Srivastava L, (2004), who reported a study to establish a rapid and accurate method of susceptibility testing for *Mycobacterium tuberculosis* using three methods proportion method by agar dilution on Middlebrook 7H11 agar, proportion method using the conventional Lowenstein- Jensen (L-J) medium and E test strip method. A total of 56 (74.7%) isolates were sensitive and 11 (14.7%) were resistant toisoniazid by the three methods. 11 24(14.7%) isolates were seen resistant to isoniazid by all the three methods. 49 (65.3%) were sensitive to all the five drugs by all the three methods. One (1.3%) isolate alone was resistant to all the five drugs by the three methods. Findings of the study under discussion substantiated by the findings of Rizwan et al., (2003), who reported drug resistance in confirmed tuberculosis clinical isolates of *Mycobacterium tuberculosis*.

5.4.3 Sensitivity/ resistance of *Mycobacterium tuberculosis* against Ethambutol

The critical concentration of Ethambutol maintained in media to consider as border line for declaration of resistant was 2µg/ml. The final results of this study project showed 10 (5.8%) resistant and 162 (94.2%) sensitive strains to ethambutol of total 172 clinical isolates of *Mycobacterium tuberculosis*. The findings are consistent with the work of WHO/ IUALTD, 2000, who reported ethambutol resistance as 5.8% in Iran and 4.1% resistance in china. These finding are also in conformity with those reported by other researcher Bitar et el 2001, who reported 4.4% resistant against ethambutol. Our findings are similar to earlier study by Khan et el (2001), who reported increased drug resistance (except 8% to pyrazinamide and 7% to ethambutol) in Saudi Arabia during the last five years. While; Miah et al., (2000), reported 2.7% resistance against ethambutol in Bangladesh. These findings are in agreement with Sumathi Muralidhar & Srivastava L., (2004), who reported a study to establish a rapid and accurate method of susceptibility testing for *Mycobacterium tuberculosis*. 70 (93.3%) isolates were sensitive and 2 (2.7%) isolates were resistant to ethambutolby all the three methods. Our study also supported the work of Bertram, (2004), who reported an established resistance of resistant *Mycobacterium tuberculosis*. 5.4.4 Sensitivity/ resistance of *Mycobacterium tuberculosis* against Pyrazinamide: In this study project the susceptibility of patients toward pyrazinamide was found as 47 (27.3%) resistant and 125 (72.7%) sensitive of total 172 clinical isolates of *Mycobacterium tuberculosis*. These finding are in conformity with Bitar et al., (2001), 128 who reported 25% of resistant to pyrazinamide (one-third of cases with missing information). The findings of this study are in line with the work of Joal., (2001), who reported the resistance prevalence and molecular level comprehension of resistance against pyrazinamide.

5.5 Mono-resistance profile against 1stline antituberculosis drugs

In this research project the mono-drug resistance of *Mycobacterium tuberculosis* was studied against 1stline anti-TB drugs. By the overall comparison it was observed that 9 (25.71%) clinical isolates resistant to rifampicin, 3 (8.57%) isolates resistant to isoniazid, 1 (2.85%) isolate resistant to ethambutol and 22 (62.85%) isolates resistant to pyrazinamide out of 35 (20.34%) mono-resistant isolates of total 172 *Mycobacterium tuberculosis* strains. These findings are

consistent with Helen, (2002), who reported similar findings in 2002. They studied 385 cases of tuberculosis. Mycobacteriology Reference Laboratories reportd 268 (69.6%) culture positive cases. Susceptibility testing of the results of antimicrobial was obtaind for all 268 specimens, consisting of four M. bovis and 264 *Mycobacterium tuberculosis* isolates. The percentage of resistant was 1.5% to rifampicin, 9.7% to isoniazid, 4.1% to pyrazinamide and 1.9% to ethambutol. While; an increase in the trend of resistance was noticed against streptomycin (p - 0.0078). there was a fluctuated in pyrazinamide and isoniazid resistance observed. Whereas; the ethambutol and rifampicin resistance were comparatively stable. There significant differences observed in resistance profile of rifampicin and pyrazinamide. The possible reasons may lie in the fact of difference in living standards, health facilities, over all hygienic condition and socio-economic structure of Auckland, New Zealand and Pakistan. Findings of the study under discussion are substantiated by Rizwan et al., (2003), who reported tuberculosis (TB) control still faces major challenges. The resistant *Mycobacterium tuberculosis* strains wre subjected to antituberculosis drugs. The five 1st line Antituberculosis drugs were investigated fro their resistance/ sensitivity pattern.

5.6 Poly resistance profile against two three or all of the four 1stline antituberculosis drugs

Poly resistance profile of this indigenous *Mycobacterium tuberculosis* strains was studied on basis of resistance against two three or all of the four 1stline antituberculosis drugs. According to the results obtained, 20.35% *Mycobacterium TB* strains resistant to any single drug, 9.30% strains resistant to any two antituberculois drugs, 4.65% strains resistant to any three anti-TB drugs and 4.07% strains resistant against all of the four 1st line Antituberculosis drugs. 6(19.35%) *Mycobacterium TB* strains were resistant to rifampicin & isoniazid, 7 (22.58%) strains resistant to isoniazid & pyrazinamide, 1 (3.22%) strain resistant to ethambutol & pyrazinamide, 2 (6.45%) strains resistant to isoniazid & pyrazinamide, 7 (22.58%) strains resistant to isoniazid, pyrazinamide, and rifampicin, 1 (3.22%) strain resistant against rifampicin, pyrazinamide and ethambutol 7 (22.58%) strains resistant to all of the four 1stline drugs. 11.62% *Mycobacterium tuberculosis* strains were multidrug resistant (minimally resistant to rifampicin and isoniazid). These finding are consistent with the findings of CCDR, (2004), who reported 12.5% resistance against one or more 1st line antitubercular drugs. 1.5% multi

drugs resistance tuberculosis (MDR-TB) strains. The reasons of difference of indigenous and Canadian tubercular resistance prevalence may lie in facts of excellent Canadian drug monitoring system, chemotherapeutic surveillance and an appreciable standard of health. Our study supported the work of Isik Johnsson et al., (1995), because of approximately similar pattern of resistance. These findings are in agreement with Rizwan et at., (2003), who reported the % age of resistant clinical isolates as two or more antitubercular drugs alongwith comparison of acquired and primary drug resistance. The poly resistance profile of *Mycobacterium tuberculosis* is as 14.6% (99) resistance to any two drugs, 8.11% (55) resistance to any three drugs, 5% (34) resistance to any four drugs and 4.71% (32) resistance to any five drugs. A 5% of tuberculosis cases were resistance against four drugs that is frightening. Findings under discussion are substantiated by finding of Hemvani et al., (2001), who reported the alarming patternof resistance as; 25% to rifampicin, 8.1% MDR, that is low the 19 .4% of current study in India. The overall 37% drug resistance in Estonia is equivalent with 38.37% of present study. Espinal et al., (2001), reported the overall Multidrug rsistance 10.8% in Henan province of China, 9% in Latvia and 9% in Russia; while 5% in Iran, 6.5% in Tomsk and 4.5% in Zhejiang province in China. Nevertheless, in United States and France MDR has significantly decreased. The possible difference may lie in difference of health facilities and socio- economic structure. Our findings are in consistent with WHO/ IUALTD, (2000), who reported single antitubercular drug resistance as 13.7% in china, 18.1% in Russian federation and 12.9% resistance in Estonia, resistance against any two antitubercular drugs as 11.6% in china, 10.5% resistance in Russian Federation, 9.3% resistance in Estonia, resistance against any three anti-tubercular drugs as 5.6% resistance in India, 4.7% resistance in Russian Federation, 3.4% resistance in china and resistance against all of the four antitubercular drugs observed in Iran as 3.7%, in china as 5.7%, in Russian federation as 6.2% and in china as 3%. The multidrug resistant cases were as 12.3% in Russian federation, 9.4% in china and 10.8% resistance in S. Mexico. Findings of study under discussion substantiated by Helen Heffernan (2002), who reported antituberculosis drug resistance surveillance in the Mycobacteriology Reference Laboratories, Auckland, Waikato Hospitals and Wellington. The susceptibility assay results available for all isolates of M. bovis and *Mycobacterium tuberculosis*. There was 32 (11.9) resistant to any 1 agent, 11 (4.1) resistant to any 2 agents, 1(0.4) resistant to any three drugs, 1(0.4) resistant to all of the 4 agents. There is is more treatment failure in case of the short treatment course; especially if patient infected with

rifampicin resistant organism (Mitchison & Nuna, 1986). There was an increase in resistant of four times higher than earlier reported against rifampin and isoniazid during 1995 in Mexico (Sifuentes-Osornio J et al., 1995). The Multi Drug Resistance strain of tuberculosis stain is rare in New Zealand. 0.7% (14 cases) average annual incidence recorded during the eight years started in 1995. The possible reason of difference of obtained results may lie in the fact of big differences in living standards, health facilities, over all hygienic condition and socio-economic structure.

5.7 Overall trend of resistance prevalence of *Mycobacterium TB* against 1st line anti-TB drugs during Nov. 2004 to Dec. 2005.

The *Mycobacterium TB* strains studied for evaluation of overall trend of resistance prevalence against all of the four 1stline antitubercular drugs, during Nov. 2004 to Dec. 2005. The total *Mycobacterium TB* strains were chronologically compared over number of resistant drugs. There was a gradual decline observed in resistance profile of *Mycobacterium tuberculosis* from January to December, 2005. These findings are in consistent with the work of WHO/ IUALTD, (2000), who reported a decrease in overall resistant prevalence against *Mycobacterium tuberculosis*. Finding of study under discussion substantiated by the findings of CCDR, (2004), who reported the %age of clinical isolates representative of any type of resistance against drug was unchanged during two reporting years and proportion of MDR tuberculois was the same. Study by Rizwan et al., (2003), revealed the drug resistance of multidrug resistnace and other drugs for the year of 1998 - 2002. The possible reason may lie in the fact of gape of approximately five years that made the increasing trend of resistance static. Our finding are consistent with Xianyi, (2002), who reported the rapid DOTS implementation in wide range in various countries that target 85% worldwide cure rate. Our findings are in agreement with Helen Heffernan (2002), who reported similar findings in 2002. Antimicrobial susceptibility testing results were available for all 268 isolates, which comprised 264 *Mycobacterium tuberculosis* and four M. bovis isolates.17Over the last eight years, there has been a trend of increasing streptomycin resistance (p=0.0078). Isoniazid and pyrazinamide resistance has fluctuated, while rifampicin and ethambutol resistance has been relatively stable and remained ≤2%. There some differences observed in resistance profile against 1stline antitubercular drugs. The possible reason may lie in the fact of big

differences in living standards, health facilities, over all hygienic condition and socio-economic structure.

5.8 Therapeutical interpretation of resistant *Mycobacterium TB* against 1stline antituberculosis drugs

The resistant *Mycobacterium TB* having an ultimate highest level of resistance against the first line antitubercular drugs - rifampicin, isoniazid, pyrazinamide and ethambutol, which are interpreted therapeutically to study the pharmacological suitability of dosage and regimen. The pharmacodynamic parameters - Cmax (maximum plasma concentrations), therapeutic index and standard dose, maximum regimens, unwanted effects etc considered for accurate scientific outcomes. Different combination of 1stline anti-TB drugs used for miscellaneous time period; isoniazid, rifampicin and pyrazinamide for six months, isoniazid and rifampicin for nine months,68rifampicin, ethambutol and pyrazinamide for sixmonths, rifampicin and ethambutol for twelve months, isoniazid and ethambutol for eighteen months and others for two years (Richard et al., 2006). It is an exact evidence of effectiveness of lower plasma concentration of drugs used in combination therapy than standard MIC used in mono drug therapy. The standard dose given in single drug therapy is higher than the dose of same drug given in combination therapy. The drugs used in combination therapy act by different mechanisms to cure the disease. A similar study of reasoning the quantitative difference of doses required in individual and combination therapy was conducted by Stephen H. Gillespie 2002, who reported resistant against anti tuberculosis drugs that cause a important danger to human health.

5.8.1 Therapeutical interpretation of rifampicin against its resistant *Mycobacterium TB*

There were five rifampicin levels prepared to determine the highest possible level of resistance. It was observed that not` any rifampicin strain inhibited at 1stand 2 nd drug levels. 40.54% resistant *Mycobacterium* -TB strains inhibited at 3rd rifampicin level of 120ug/ml. Practically it is not feasible to maintain a plasma

concentration higher than therapeutic range of 6.5±3.5ug/ml (Joel et al., 2001). 35.12% resistant *Mycobacterium TB* were inhibited at 4 th rifampicin level 160 ug/ml, 18.91% inhibited at 5th rifampicin level 200 ug/ml and 5.40% inhibited at higher than 5th rifampicin level 200+ ug /ml. These final concentrations 160ug/ml and 200ug/ml incorporated in LJ media also exceed the therapeutic index, that's why can not be used in actual practice. If we exceed this therapeutic window or therapeutic index, it will produce sever unwanted effects i.e. nausea, vomiting, rashes, hepatitis, GI upset, flu like syndrome and fever. Jaundice may be introduced in chronic hepatic patient and alcoholic symptoms seen in elderly patients (Joel et. al., 2001). Patient is warned for red-orange in colored urine and tears. (Richared et al 2006). If these determined optimum concentrations decreased proportionately because of combination therapy with other anti-TB drugs. Even then it exceeds the therapeutic range and can not be used in actual practices. The findings of this study are in line with the work of Richard et al., (2006), who reported the MIC as 0.005-0.2ug/ml, maximum plasma concentration (Cmax) 6 ±3.5 ug/ml, 98% plasma protein binding, 0.9L /Kg volume of distribution and standard therapeutical dose 600mg with maximum regimen of 1100mg per day. Our findings correlate with the work of Bertram, (2004), who reported 1.0ug/ml minimum inhibitory concentration, 5-7ug/ml maximum plasma concentration and 600mg daily dose of rifampicin. Our findings are agreement Venkatesan D. (1989), who reported the peak serum concentration of rifampicin for adults 7 to 9 µg/mL after single 600mg oral regimen. These findings substantiate by the work of Dikinson & Mitchison, (1981), who reported the routine dosage of rifampicin 6mg/kg at a maximum regimen of 600mg per day. It is a generally well-tolerated drug, although it produces reddish coloring in the urine and other fluids, and it can cause hepatoxicity. Another side effect is the appearance of a pseudo-flu syndrome. These results are consistent with the findings of Juan, (2006), who reported rifampicin oral or IV administration with bactericidal effect. Its daily dosage for adults is 10–20mg/kg and for children 8–12mg/kg. The maximum administrable regimen is 600mg. This drug acts on the rapid-growth populations of bacilli as well as on slow-multiplying populations. The findings of the study under discussion are substantiated by AK Praharaj et al., (2004), who reported rifampicin resistance >64ug/ml. These findings are in conformity with the work of

CDCP, 2004, who reported MIC of rifampicin sensitive *Mycobacterium tuberculosis* in the range of 1-50ug/ml. Our finding are also in line with Karin et al., 1997, Grim, 1978, Rizwan et al., 2003 and CCDR, 2003, who reported the optimum rifampicin concentration for declaration of sensitive or resistant *Mycobacterium tuberculosis* as 40 ug/ml.

5.8.2 Therapeutical interpretation of isoniazid against its resistant *Mycobacterium TB*

There were five isoniazid levels - 0.2ug/ ml, 3ug/ml, 6ug/ml, 9ug/ml, 12ug/ml and 12+ ug/ml prepared to determine the highest possible level of resistance. It was observed that no any isoniazid strain inhibited at 1st, 2 nd and 3 rd drug levels. There 28% resistant *Mycobacterium*-TB strains inhibited at 4 th isoniazid level 9ug/ml. Maximally plasma concentration that can be maintained in body is - 4ug/ml (Richard et al., 2006), therefore it can be used in actual practice. The maximum isoniazid regimen is 400mg (Leon Shargel et al, 2004). 24% resistant *Mycobacterium TB* strains were inhibited at 5 th level of 12ug/ml, 48% resistant strains inhibited at higher than 5 th level of 12+ug/ml. These concentrations of 12ug/ml and 12+ug/ml incorporated in LJ media also exceed the therapeutic index. Therefore should not be maintained in actual tubercular treatment. If we exceed this therapeutic window or therapeutic index, the sever unwanted effects i.e. hypersensitivity, paresthenia, hepatic enzyme elevation, hepatitis and peripheral neuropathy, nausea, vomiting, epigastric distress, agranulocytosis, thrombocytopenia, fever, various rashes, dermatological and rheumatoid and lupoid syndromes may produce serious health hazards. The adverse effects are related to dosage and duration of administration. Paresthenia is the most common adverse effect, appears to be due to a relative pyridoxine deficiency. It is corrected by pyridoxine (vitamin B6) supplementation and therefore particularly recommended to lactating women to intake vitamin supplementation. The findings of the study under discussion substantiated by the work of Richard et al., 2006, who reported the standard MIC as 0.2-0.4ug/ml, plasma concentration 4ug/ml, maximally allowed plasma concentration 6ug/ml, the standard therapeutical dose 300mg with maximum regimen of 300mg. Our findings are

in conformity with the work of Joel et al., (2001), who reported 0.2ug/ml MIC, 300mg standard dose, maximum plasma concentration in slow acetylation 5.4 ±2.0ug/ml and in fast acetylation 7.1±1.9ug/ml. These findings are in consistent with Holdiness, (1984), who reported the peak serum concentration of isoniazid 3 to 7 mcg/mL (21.9 to 51 micromoles per L) after a single 300-mg oral dose. 0.57 to 0.76 L/kg is the volum of distribution (Kergueris, 1983). Isoniazide is widely distributed to all fluids and tissues, including CSF, pleural and ascitic fluids, skin, sputum, saliva, lungs, and muscle. It crosses the placenta and is distributed into breast milk. The protein binding of isoniazid is very low (0 to 10%). These finding are in agreemetn with the findigns of Venkatesan D. (1989), who reported the time to peak serum concentration of isoniazid - 1 to 2 hours and peak plasma concentratioin (Cmax) as 4ug/ml. Findings of study under discussion substantiated by the findings of Juan M Broquetas Doñate, 2006, who reported oral or IM administered of isoniazid with bactericidal effect. Its daily dosage for children is 5–10mg/kg and for adult is 5mg/kg. The maximum daily administrable regimen is 300mg. These results are in consistent with the findings of WHO, (2001), who reported 100% minimum 11.11% median and 88.88% maximum resistance at 1stdrug level of 0.2 ug/ml and 100% minimum, 12.52% median and 87.5% maximum resistance at 5 th drug level of 12 ug/ml. Finding of the study under discussion are substantiated by the findings of WHO/ IUALTD, (2000), who reported 58.33% resistance at 1stdrug level 0.2ug/ml, 8.33% resistance at 2 nd drug level 3ug/ml and 33.33% resistance at 5 th drug level 12ug/ml. Our study is also supported the work of Jesudason et al., (2003), CCDR, (2004) and Victor et al., (1997), who reported the resistance of *Mycobacterium tuberculosis* above of an optimum drug concentration of 0.2ug/ml.

5.8.3 Therapeutical interpretation of ethambutol against its resistant *Mycobacterium TB*

There were five ethambutol levels - 2ug/ ml, 4ug/ml, 6ug/ml, 8ug/ml and 10ug/ml prepared to determine the highest possible level of resistance. It was observed that no any ethambutol strain inhibited at 1stand 2 nd drug levels 2ug/ml and 4ug/ml. 50% resistant *Mycobacterium TB* strains inhibited at 3 rd level of 6ug/ml. The maximum plasma

concentration (Cmax) that can be maintained in tuberculosis patient during treatment protocol are described by other researchers as 3-5ug/ml (Bertram G. Katzung, 2004), 2-5ug/ml (Leon et al., 2004) and 4-6ug/ml (Richard et al., 2006). It is very obvious from given data, exceeding the mentioned plasma concentration may introduce toxicity. The most important adverse effect of ethambutol is optic neuritis, which results in diminished visual acuity and loss of ability to discriminate between red and green (optic neuritis with blurred vision, red-green color blindness). Visual acuity should be periodically examined. Discontinuation of the drug results in reversal of the toxic symptoms. In addition, urate excretion is decreased by drug; thus gout may be exacerbated. (Richard, et al., 2006). Drug fever, abdominal pain, headache, dizziness and confusion also observed (Leon et al., 2004). 3(30%) ethambutol resistant *Mycobacterium TB* were inhibited at 4 th ethambutol level 8ug/ml, 2 (20%) ethambutol resistant strains inhibited at higher than 5 th level of 10ug /ml. The respective plasma concentration at 4th level of 8ug/ml and 5 th level of 10ug/ml can not be maintained without the risk of sever patient's health hazards. Therefore regimens are rejected to use in actual chemotherapeutical practice. The findings of this study are in line with the work of Richard et al., 2006, who reported 1-8ug/ml minimum inhibitory concentration (MIC), 4-6ug/ml maximum plasma concentration (Cmax) and 6ug/ml maximally permitted plasma concentration. Our findings also correlate with the work Joel et al., (2001), who reported minimum inhibitory concentration 1- 5ug/ml, maximum plasma concentration (Cmax) 2-5ug/ml, standard dose 400mg or 25mg/kg/day and maximally regimen 1100mg. Similar findings reported by Myambutol (Lederle), (2000), that depicts the peak serum concentration as 2 - 5 µg/mL after a single dose of 25mg/kg with the time to peak serum concentration of 2 to 4 hours. The protein binding of ethambutol is low (20 to 30%). Distribution of ethambutol is to most tissues and body fluids, except CSF. Ethambutol does not penetrate intact meninges, but 10 to 50% may penetrate the meninges of patients with tuberculous meningitis.These findings are also in conformity with Venkatesan et al (1998), who reported volum of distribution 1.6 L per kg. Findings of study under discussion are substantiated by the findings of Juan, (2006), who reported daily ethambutol dosage 15–25mg/kg and the maximum administrable per day regimen 2.5g. The main function of ethambutol is to prevent the appearance of resistance. It is

generally well-tolerated and has few toxic effects. There appears to be a possibility of retrobulbar neuritis. It is therefore not recommended for children who are unable to receive frequent ophthalmological check-ups. The findings of this study are in line with the work of WHO/ IUALTD, (2000), who reported 75% resistance at 1stdrug leval. 12.5% resistance at 2 nd drugs level and 12.5% resistance at 4th drug level. These finding are in conformity with the work of AK Praharaj et al., (004) and CCDR, (2004), who reported the resistance of ethambutol resistance *Mycobacterium TB* above than the critical concentration of 2ug/ ml.

5.7.4 Therapeutical interpretation of pyrazinamide against its resistant *Mycobacterium TB*

There were five pyrazinamide levels - 100ug/ml, 200ug/ml, 300ug/ml, 400ug/ml and 500ug/ml prepared to determine the highest possible level of resistance. It was observed that no any pyrazinamide resistant strain inhibited at 1stand 2 nd drug levels100ug/ml and 200ug/ml. 27.66% pyrazinamide resistant *Mycobacterium TB* strains were inhibited at 3 rd pyrazinamide level of 300ug /ml. The maximum plasma concentration than can be maintained in human body reported by different researchers are 9-12ug/ml (Joel et al, 2001), 19ug/ml (Leon et al., 2004), 30-50ug/ml (Bertram, 2004), 37-40ug/ml (Richard et al., 2006). Exceeding of this maximally permitted plasma concentration may introduce sever health hazards. Maintenance of aforesaid plasma concentration is therefore rejected to use in actual chemotherapeutical practice. During the pyrazinamide toxicity the important adverse effect introduced by pyrazinamide are the nausea, hyperuricemia, rashes, joint ache, gout (rare) (Richard et al., 2006), fever, anorexia, malaise, and hepatitis with or without hepatic jaundice, and death can occur (Alfonso et al., 1998). 25.53% resistant *Mycobacterium TB* were inhibited at 4 th pyrazinamide level 400ug/ml, 31.91% resistant stains inhibited at 5 th pyrazinamide level 500ug/ml and 14.89% pyrazinamide resistant stains inhibited at higher than 5 th pyrazinamidelevel 500+ug /ml. The respective plasma concentration at 5 th level of 500ug/ml and higher than 5 th level of 500+ug/ml can not be maintained in tuberculosis patient without the risk of sever patient's health hazards. Therefore regimens are rejected

to use in actual chemotherapeutical practice. The findings of this study are in line with the work of Richard et al., (2006), who reported 18-23ug/ml minimum inhibitory concentration, 37-40ug/ml maximum plasma concentration, 500mg standard therapeutical dose with maximum regimen of 800mg. Our findings are consistent by the work of AK Praharaj et al., (2004), who reported the maximum plasma concentration (Cmax) 40ug/ml, critical drug concentration that incorporated in LJ media to consider as border line for declaration of sensitive or resistant bacteria 100ug/ml, standard dose 500mg and maximally permitted dose 800mg. Our finding are in line with the work of Bertram et al., (2004) who reported the minimum inhibitory concentration 100ug/ml, maximum plasma concentration 30-50ug/ml and standard dose of 25mg/kg/day. These findings are in conformity with Juan, (2006), who reported the usual pyrazinamide dosage 25mg/kg/day with a maximum of 2g per day. Pyrazinamide administered orally and having the bactericidal effect. Its daily dosage for children 20–30 and for adult is 1.5g <50kg 2.0g (51–74kg) 2.5g >75kg. Thrice weekly dose for adult is 2g <50kg 2.5g (51–74kg) 3.0g >75kgl. The action of pyrazinamide is fundamental on slow-multiplying and intercellular bacilli and has a sterilizing effect. It is hepatoxic and interferes with the metabolism of uric acid by increasing its level. The Volum of distribution of pyrazinamide is 0.57 to 0.74 L per kg (Lacroix et al., 1989). These findigns are in line with Ellard, (1987), who reported widely distribution of pyrazinamide to most fluids and tissues, including liver, lungs, kidneys, and bile. Pyrazinamide has excellent penetration into CSF, ranging from 87 to 105% of the corresponding serum concentration. These findigns are in agreement with Stamatakis et al., (1988), who reported the plasma protein binding of pyrazinamide low (10 to 20%), the time to peak serum concentration 1 to 2 hours and the peak serum concentration of approximately 19 mcg/mL after a single dose of 14 mg/kg and approximately 39 mcg/mL after a single dose of 27 mg/kg (Lacroix et al., 1989). The findings of this study are in line with the work of AK Praharaj et al., (2004), who reported the final concentration (100ug/ml) incorporated in LJ media to consider as border line to declare the sensitive or resistant *Mycobacterium tuberculosis* was very much clearly higher than the 100ug/ml. Study by Joel et al., (2001), also reveal a similar findings of 12.5 minimum inhibitory concentration, 9- 12ug/ml maximum plasma concentration following a regimen of 500mg per day.

5.8 Detection of mutation in PncA gene in pyrazinamide resistant *Mycobacterium tuberculosis*:

The *Mycobacterium tuberculosis* Genomic DNA was isolated and amplified. The Single Strand Conformation Polymorphism (SSCP) analysis conducted to elucidate the molecular basis of resistance against the pyrazinamide. It was purified and sequenced by PCR for detection of mutation in PncA gene. Therefore molecular method used to measure the activity of PAZase on basis of the hypothesis thatmutation in the PncA gene is a reliable marker of pyrazinamide resistance. By using the SSCP (Single Strand Conformational Polymorphism), we are able to show most divers pattern. 30 (17.44%) pyrazinamide resistant *Mycobacterium tuberculosis* showed different pattern than sensitive samples. Which indicate the mutation in this domain, while 17 (9.88%) did not show any difference in mobility in comparison to sensitive samples. This indicates that there is no mutation in this region but it should be confirmed by sequencing. These findings are in consistent with the findings of Sreenvatsan et al., (1997) who reported pyrazinamide sensitive strains of *Mycobacterium TB* had normal Pnc A genes, while pyrazinamide resistant strains had PncA mutations. This finding also suggested additional resistancemechanism besides lack of pyrazinamidease activity. Our findings are in line with the work of Raynaud et al., (1999), who investigated uptake of pyrazinamide as a possible resistance mechanism in mycobacteria. They founded an ATP dependent transport system is involved in uptake of pyrazinamide. They investigated four naturally pyrazinamide resistant species of mycbacteria as *Mycobacterium tuberculosis*. Finding of study under discussion are also substantiated by the findings of Stephen, (2002), who reported the mutations in the pyrazinamidase gene and inactivation by IS6110 and PncA gene insertion. Our findings support the work of Lemaitre, (1999), who reported pyrazinamide as necessary to produce the active pyrazinoic acid derivative. Thus; the mutants can't make an active drug. Hirano et al., (1998), reported Pyrazinamidese-negative isolates sequenced, 97% had mutations within the PncA gene. A mutation was seen in various regions throughout the PncA gene. It was surprising that all three strains of in vitro selected pyrazinamide resistant mutants were Pyrazinamidese-positive and showed no

change in the PncA gene. These results indicate that additional mechanisms may be involved in pyrazinamide resistance. No mutations were observed in all of Pyrazinamidese-positive *M. tuberculosis* isolates tested, indicating that mutations in the pncA gene could be involved in the loss of Pyrazinamidese activity. Sequencing analysis of the pncA gene should provide rapid diagnosis of pyrazinamide resistant clinical isolates of *M. tuberculosis*. These findings are similar to earlier study by Scorpio et al., (1997), who reported the gene encoding for *M. tuberculosis* Pyrazinamidase (pncA) cloned, sequenced and unequivocally linked to pyrazinamide resistance. While; 72% and 98% of pyrazinamide resistance clinical isolates were associated with mutations spread all through the the 211 promoter region and 558bp pncA coding region including base substitutions, insertions And deletions. Nadine et al., (1999), reported high incidence of base substitutions resulting in Proline as evidence that these mutations disrupt the alpha-helical structure of the enzyme, thereby accounting for the loss of Pyrazinamidase activity. Study by Shao et al (2000) also reveals similar results, where pyrazinamide resistant *Mycobacterium tuberculosis* has PncA mutations, verifying the PncA mutation as main mechanism of resistance against pyrazinamide. Different diverse and new mutations found in gene PncA. Moreover; multidrug resistant and monoresistant strains isolated from Quebec, Canada hav e the same pyrazinamide resistant mutation profile of 8 nucleotide deletion and an amino acid substitution of Arg 140Ser gene PncA. This information strongly recommend a pyrazinamide monoresistant strain. The Genotypic investigation can confirm the type tuberculosis, pattern of ressitance and respective outbreaks (Mokrousov, et al., 2002), to examine tuberculosis transmission (Van et al., 1999), for recognition of exogenous reinfection patients, to study the strain’s clonal expansion and confirm cross contamination (Jasmer, 2002), and. In some research laboratories the clonal expansion has been investigated extensively. That is also confirmd by a recent analysis of the phylogenetic and epidemiology reconstruction (Bifani, P. J., 2002). Among 8% of pyrazinamide resistant *Mycobacterium tuberculosis* recommended the shortage of PncA mutations, for resistance participation at least one additional gene existed. Thus; PncA structural changes have surprisingly been indentified in more thatn sevetnry percent (70%) of drug resistant isolates. That is supposed because of the structural modifications detrimentally

change the enzyme task, in that way conversion pyrazinamide altered to its bioactive form (Mitchison DA. 19985).

CONCLUSION

CONCLUSIONS

On basis of present study, the following conclusions are drawn,

1. Most tubercular patients founded in wards. They were seriously ill and required special attentions. These indoor departments are also observed as the source of contagious and nosocomial tubercular infection.

2. The sputum is most accepted specimen used to diagnose the tuberculosis. It is also used to determine the sensitivity of pulmonary *Mycobacterium tuberculosis*. Bronchial washing, puss and biopsy fluid also used to evaluate certain clinical tests.

3. Epidemiological comparison of pulmonary and extra-pulmonary tuberculosis pointed out the five time higher prevalence of pulmonary tuberculosis than extra-pulmonary tuberculosis.

4. A significant difference was observed among gender distribution of tuberculosis. Male tubercular patient were four time higher than female patients.

5. The pattern of resistance of indigenous *Mycobacterium tuberculosis* against rifampicin was resolute by drug proportion technique using Lowenstein Jensen medium. Approximately 1/5 of the total collected *Mycobacterium tuberculosis* stains were founded resistant to rifampicin.

6. Approximately 1/6 of total collected *Mycobacterium tuberculosis* strains were resistant to isoniazid. It is also the primary antitubercular drug that contributes to declare as multidrug resistant tuberculosis.

7. Approximately 1/16 of total *Mycobacterium tuberculosis* strains were resistant to ethambutol. It is the minimum number of *Mycobacterium* among all of the four examined antitubercular drugs.

8. Approximately ¼ of total collected *Mycobacterium tuberculosis* strains were resistant to pyrazinamide. It is the maximum resistance of *Mycobacterium*-TB among all of the four examined antitubercular drugs.

9 Maximum monodrug resistance observed against pyrazinamide and minimum resistance against ethambutol concluded by the overall comparison of mono-resistances of *Mycobacterium tuberculosis* strains against all the 1stline anti-tubercular drugs.

10 Three categories of poly-resistant *Mycobacterium tuberculosis* were observed on basis of resistance against two, three and all of the four anti-TB drugs. In two drugs category, isoniazid & ethambutol having the maximum and ethambutol & pyrazinamide having the minimum resistance against *M. tuberculosis*. In three drugs category, the maximum resistance seen against rifampicin, isoniazid & ethambutol and lowest resistance against rifampicin, ethambutol & pyrazinamide.

11 Overall study of trend of resistance prevalence demonstrate the gradually decline in the resistance from November, 2004 to December, 2005. The reduction in resistance against the number of drugs and increase in the sensitivity was fairly observed during aforesaid period. That gave certain lessons to design goals to control resistance in this area. That may also be derived by experiences of successful strategy of rapid implementation of treatment protocol established by DOTS. Moreover; the standardized therapy and continuous follow up is alarming the increase in drug resistan.

12 The ultimate highest level of resistance of rifampicin resistant *Mycobacterium tuberculosis* studied by preparing five gradually increasing concentrations. Maximum resistance observed in 1st& 2 nd drug level and minimum at above than 5 th rifampicin level. Maximum sensitivity observed at above 5 th level and zero sensitivity at 1st& 2 nd drug level.

13 In the case of isoniazid, maximum resistance observed in 1st& 2 nd drug level and minimum at above than 5 th level. Maximum sensitivity seen at above 5 th level and zero sensitivity at 1st& 2 nd level.

14 In case of ethambutol, maximum resistances observed in 1st& 2 nd level and minimum at 5 th level. Maximum sensitivity observed at 5 th level and zero sensitivity at 1st& 2 nd level.

15 Maximum resistances observed in 1st& 2 nd pyrazinamide level and minimum at above than 5 th level. Maximum sensitivity seen at above than 5 th pyrazinamide level and zero sensitivity at 1st& 2 nd level.

16 By the overall comparison of resistance of *Mycobacterium tuberculosis* against 1stline anti-TB drugs, the highest resistance observed at 1st& 2 nd drugs levels and least resistance was founded 3at higher than 5 th drugs level. Most of the resistant *Mycobacterium*s were inhibited at 3 rd drug levels of their respective anti-TB drugs concentrations.

17 First line antituberculisis drugs should therapeutically be interpreted to study pharmacological suitability of dosage and regimen. The pharmacodynamic parameters - Cmax (maximum plasma concentrations), 3therapeutic index and standard dose, maximum regimens, unwanted effects etc considered for accurate scientific outcomes. 13Drug-resistant tuberculosis poses a significant threat to human health and it is important to understand how the resistance emerges if we are to reverse the upward trend. No any rifampicin resistant train inhibited3at 1stand 2 nd drug levels. 40.54% resistant *Mycobacterium* -TB strains inhibited at 3 rd rifampicin leve. Practically it is not feasible to maintain a plasma concentration higher than therapeutic range of 6.5±3.5ug/ml. 35.12% resistant *Mycobacterium TB* were inhibited at 4 th rifampicin leval., 18.91% inhibited at 5 th rifampicin level and 5.40% inhibited at higher than 5 th rifampicin. These final concentrations incorporated in LJ media exceed the therapeutic index, that's why can not be used in actual practice. The possible unwanted effects are rashes, nausea, vomiting, GI upset, hepatitis, fever and flu like syndrome.

18 No any isoniazid resistant strain inhibited at 1st, 2 nd and 3 rd drug levels. 28% resistant *Mycobacterium*-TB strains inhibited at 4 th isoniazid level. Maximum plasma concentration that can be maintained in body is - 4ug/ml. Therefore it can be used in actual practice. The maximum isoniazid regimen is 400mg. 24% resistant *Mycobacterium* These concentrations incorporated in LJ media exceed the therapeutic index. Therefore should not be maintained in actual tubercular treatment. The possible unwanted effects are hypersensitivity, paresthenia, hepatic

enzyme elevation, hepatitis and peripheral neuropathy, nausea, vomiting, epigastric distress, agranulocytosis, thrombocytopenia, fever, various rashes, dermatological and rheumatoid and lupoid syndromes may produce serious health hazards. The adverse effects are related to dosage and duration of administration. Paresthenia is the most common adverse effect, appears to be due to a relative pyridoxine deficiency.

19 No any ethambutol resistant strain inhibited 3at 1stand 2 nd drug levels 2ug/ml and 4ug/ml. 50% resistant *Mycobacterium TB* strains inhibited at 3 rd level of 6ug/ml. The maximum plasma concentration (Cmax) that can be maintained in tuberculosis patient during treatment protocol are described by other researchers as 3-5ug/ml (Bertram G. Katzung, 2004), 2-5ug/ml (Leon et al., 2004) and 4-6ug/ml (Richard et al., 2006). It is very obvious from given data, exceeding the mentioned plasma concentration may introduce toxicity. The most important adverse effect of ethambutol is optic neuritis, diminished visual acuity and red-green color blindness. In addition, urate excretion decreased that may be exacerbated the gout. Drug fever, abdominal pain, headache, dizziness and confusion also observed The respective plasma concentration can not be maintained without the risk of sever patient's health hazards. Therefore regimens are rejected to use in actual chemotherapeutical practice.

20 No any pyrazinamide resistant strain inhibited 3at 1stand 2 nd drug levels. 27.66% pyrazinamide resistant *Mycobacterium TB* strains inhibited at 3 rd pyrazinamide level of 300ug/ml. The maximum plasma concentration than can be maintained in human body reported by different researchers are 9-12ug/ml (Joel et al, 2001), 19ug/ml (Leon et al., 2004), 30-50ug/ml (Bertram, 2004), 37-40ug/ml (Richard et al., 2006). Exceeding of this maximally permitted plasma concentration may introduce sever health hazards i.e. nausea, hyperuricemia, rashes, joint ache, gout (rare), fever, anorexia, malaise, hepatitis with or without hepatic jaundice, and death can occur. Maintenance of aforesaid plasma concentration is therefore rejected to use in actual chemotherapeutical practice.

RECOMMENDATIONS

RECOMMENDATIONS

Based upon the findings of the present study, the recommendations can be divided into following major categories:

General Recommendations for Tuberculosis

1 Implementation of standard infection controlling procedures and guidelines to reduced the spread of tuberculosis pathogenicity in and outside the institutions/ hospitals. ‣ It is highly required to introduce a national mycobacterial infection controlling and molecular resistance surveillance programme that is not currently exist in Pakistan. The computer technology and medical informatics may be used for accurate data collection, efficient characterization and timely analysis to successfully overcome the global tuberculosis burden.

2 The systematic surveillance, quality assurance and digital data support needed for decision making regarding the chemotherapy and/ or prophylaxis purpose of susceptible and resistant *Mycobacterium tuberculosis*. It will be forthcoming to predict emerging resistance among available anti-tubercular drugs, leading to effective treatment that could control dissemination of *Mycobacterium* resistance.

3 Understanding the physiology/ morphology, ecology, genetic and molecular basis of resistance of *Mycobacterium TB* should be increased for successful control of this dangerous microbe.

4 Strengthening the curriculum of human health care professionals in the area of disinfection, house keeping, sterilization and factors contributing to its spread, including inappropriate treatment protocols with ineffective regimens. ‣ Involving the drugs experts (particularly the qualified pharmacists), governmental agencies and welfare organizations to assure the quality medication, therapeutical modifications and implementing the , prophylaxis procedures against tuberculosis.

5 Establishing the laboratory standards for efficient and accurate detection of molecular and genetic basis of resistance of *Mycobacterium tuberculosis*. These standards include

sample collection, transportation, physical examination, molecular identification, genetic characterization and reporting techniques used for molecular epidemiology.

Recommendations to improving the awareness against TB

6 Educating the patients regarding appropriate use of 1stline drugs against susceptible and 2 nd line treatment protocol against resistant *Mycobacterium tuberculosis*.

7 Emphasizing over the research based rapid, reliable diagnostic test and vaccination for prevention and control of mycobacterial infection.

8 Interventioning the health care professionals to reduce the probability to develop resistance to anti-TB drugs. That adversely influences the quality of health care outcomes. A well organized and deep rooted intervention can produce surprising benefits.

9 Establishing effective monitoring of drugs used againt tuberculosis in society.

10 Conducting studies to update the professional knowledge improve the behaviors and inculcate the importance of completion of treatment course to control the tubercular infection in community and hospitals.

11 Increasing the awareness of complications and threats posed by resistant *Mycobacterium tuberculosis* in community.

12 Emphasizing to identifying the potential *Mycobacterium tuberculosis* strains accurately with molecular

13 Ensuring crucially the understanding and participation in efforts to control the spread of resistance against anti-TB agents. Educational programmes describe the complications and provide guidelines for using the antitubculosis drugs in general practice and within institutional health care settings.

Recommendations for Prudential Treatment Practice

14 Selecting carefully the treatment protocol and respective dose of anti-TB drugs. The medication decision is optimized on basis of pattern of sensitivity of patient's infected *Mycobacterium tuberculosis*.

15 A high cure rate without the emergence of resistance can be achieved by adopting thetreatments of internationally approved dose/ regimens. Which also are effective in prevention of the resistance emergence. Moreover; the combination chemotherapies minimize the probabiligy of spontaneous mutant to develop resistance to all of the components of chemotherapy.

16 Establishing the base line of effectiveness of antituberculosis agents, that is compare with: specifications founded in literature, data obtained from therapeutical & resistance surveillance studies and comparative data used to assess the international and/ or local risk factors.

Recommendations related to Epidemiology and resistance prevalence

17 Restricting the over/ under compliance anti-TB drugs usage by offering the qualified pharmacist to play there professional role to develop the successful strategies. Pharmacist audit the prescriptions, evaluate the adherence to pharmacological considerations and formulary constrains to stop mechanism of resistance development.

18 Convincing the drugs experts (pharmacists) and clinicians that anti-tuberculosis drugs therapy should be more closely tailored to the patients requires scientific proof. Prescription practice demands a strong mycobacterial profile, careful laboratory support and confirmed molecular elucidations.

19 Establishing the concurrent morphological, molecular and genetic profile to assess the relationship between *Mycobacterium tuberculosis* emerging in treatment premises and community in various environments.

20 Studying systematically to map the prevalence of bacterial pattern of resistance.

21 Standardizing the methods used for diagnosis and sensitivity evaluation with the reference quality tests, which should closely follow the documents published by World Heath Organization.

22 Interpreting the correct culture and sensitivity results.

23 Getting immunization against tuberculosis by BCG vaccination and improving the socio-economic status.

Therapeutical and *Mycobacterium* resistance management

24 Characterizing and identification of *Mycobacterium tuberculosis* strains at primary health care level to coordinate the TB patient with the genotyping of detected strain. It will help in anti-tubercular drugs usage, monitoring and intervention programs at institution level.

25 Facilitating to prescribe and/ or decide the treatment protocols with respect of mycobacterial clinical elucidations and therapeutical outcomes of pharmacological studies with the aim of improving the rational selection of regimens.

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APPENDICS

APPENDICES

Appendix I. Standard Method of Collecting the Sputum Specimen from TB Suspected Patient.

Following guidelines strictly implemented to assure quality working,

- Be cooperative and kind with patient. Improve his confidence, asking him for having the seat, behaving sympathetically.
- Educate the patient for providing the accurate specimen and importance of pathological evaluations.
- Use open mouth, unbreakable, screw caped and sterilized sputum container to collect the specimen.
- Collect the three specimens 1) first spot specimen: when patient come to you first time, 2) early morning specimen and 3) second spot specimen: when patient bring his early morning specimen.
- Carry the patient in isolation in open air sample collecting place and keep him away from general community to avoid its spreading to other people.
- Collect the sputum of inner lungs instead of saliva or watery/ foamy exudates.
- Provide another sputum collecting bottle to patient to bring the early morning sputum in coming day.
- Guide the patient to wash moth with fresh water, cough to expel forcefully the sputum into the sputum collecting bottle.
- Ask patient to breath deeply and then cough, take a cup of ginger and black pepper extract to enhance the chances the maximum sputum expulsion.
- Always stand at behind of the patient to avoid infection.
- Collect the sputum containing bottle from patient wash with cloth or cotton place at examining place. Then wash hands and other involve material to assure biosafety.
- Mark the bottle, write down the specimen's registration number and enroll the patients in laboratory record.

Appendix II. Reading & Recording the Sensitivity Results of TB Diagnosed Patients

RECORDING THE RESEARCH DATA

"Prevalence, characterization and clinical evaluation of indigenous *Mycobacterium tuberculosis*"

Supervisors:

Dr. Abdul Hameed,
Dept. of microbiology
Faculty of Biological Sciences,
Quaid-e-Azam University, Islamabad

Prof. Dr. Bashir Ahmed
School of Pharmacy
Punjab University, Lahore

DRUG SENSITIVITY REPOT:

No.: ______________________________ Date:______________________________

Culture No. : ______________________ Sample: ___________________________

Source: ___________________________ File # : ____________________________

PATIENT PROFILE:

Patient name:____________________________S/D/O______________________________

Indoor: [ ] Out door: [ ] Male: [ ] Female [ ].

Address: ____

Culture date: __________________________Reading date: ________________________

RESULTS :

Control :10^3 [ ] 10^5 [ ] CS5-10^3 [ ]

CS5-10^5 [ ] NB-10^3 [ ] PNB-10^5 [ ]

SENSITIVITY/ RESISTANCE:

RFP (R/S):-	G:-
RFP (R/S):-	G:-
RFP (R/S):-	G:
RFP (R/S):-	G:

Date: ________________________________

TAHA NAZIR
PhD Scholar (microbiology)
Dept. of biological sciences
Quaid-e-Azam University
Islamabad, Pakistan
, +92-321-6015589

RFP- Rifampicin; PZA- Pyrazinamide; BW- Bronchial Wash; PS- Puss; SPT-Sputum; G – Growth; NG- Not growth; Cont- control; Ctm – Contamination; ISN- Isoniazid; CIP-Ciprofloxacin; ETB- Ethambutol; STP-Streptomycin; PF – Pulmonary fluid

Appendix III. Standard Operating Procedure (SOP) Quality Control Measures.

The laboratories staff, workers and executive staff have to adopt all of the possible safely measures and follow the standard operating procedures to avoid the transmission of this mortal pathogens. The major instructions are as given below,

- Use the mask, overall and gloves to avoid the direct pathogenic contact.
- Be disinfect the premises before and after working/ exposing the *Mycobacterium tuberculosis* in area.
- Always use the laminar flow hood maintained at negative air pressure and comprised of UV light, gas burner and disinfecting chemicals.
- Never eat, drink or smoke in laboratory area.
- Be disinfecting the towel, tools, apparatuses and daily utensils regularly as scheduled in laboratory programme.
- Strictly maintain a high quality house keeping.
- Be sterilize the all involved material and apparatuses by using the autoclave.
- Be assure the proper, smooth and quality supply of water, gas and other material supply in laboratory.

Appendix IV. Definition of the Terms

1 **Tubercular Patient**: An individual with identified isolated of drug susceptible/ resistant *Mycobacterium tuberculosis* referred as TB patient. He may have prior treatment history or documentation of previous TB disease.

2 **Primary Tuberculosis**: 1st exposure/ attack of MTB, characterized by formation of primary complex, parenchymal pulmonary lesion and a small subapical focus.

3 **Secondary Tuberculosis**: It is the active infection in previously sensitized individual. It is Mostly due to reactivation of dormant bacilli from primary lesion. Occasionally the reason observed from an exogenous source.

4 **Extrapulmonary Tuberculosis**: Tubercular Pathogenicity/ infection other than lungs referred as Extrapulmonary tuberculosis.

5 **Drug Resistance**: It is defined tuberculosis caused by *M. tuberculosis* bacilli resistant to one or more antitubercular agent. Drug resistance is further classified into "primary", "initial" or "acquired" according to history of previous tuberculosis treatment.

6 **Initial Drug Resistance:** Includes primary as well as unknown and undiagnosed acquired drug resistance referred as Initial Drug Resistance. (9/I)

7 **Primary Drug Resistance**: Resistance in cultures from patients with no history of previous tuberculosis treatment. 56/ D6 or Resistance in patient who has never taken drugs in the past and gets infected with strain originating from other patient who had acquired resistance.

8 **Acquired Resistance**: Resistance acquired after intake of drugs by patients referred as acquired drug resistance (9/I) or Resistance in cultures from patients with one or more previous tuberculosis treatment episodes (totaling more than one month).

9 **Natural Drug Resistance**: Neither the patient with naturally resistant bacilli nor his source of infection has had chemotherapy in the past. or selective propagation of spontaneously resistant mutants in a wild population. Tubercle bacilli have spontaneous predictable rates of chromosomally borne mutation and propagation of mutants into population leads to resistance. Naturally occurring mutants occur independent of exposure to chemotherapeutic agents and resistance is usually to a single drug.

10 **Multi-Drug Resistant (MDR) Tuberculosis** ***MDR-TB****: Mycobacterium tuberculosis* resistant to Isoniazid and Rifampicm with/without resistance to other drugs. (9/I)

11 **Primary Chemotherapy Failure**: Tuberculosis in newly diagnosed patients who remain smear positive after six months standard chemotherapy.

12 **Relapse Cases**: Recurrence of tuberculosis within one year in a patient who had been declared cured in the past by physician.

13 **Chronic Cases**: Tuberculosis in a patient who remains smear positive after completing retreatment regimen under supervision or the failure of a fully supervised retreatment regimen. A chronic case has received at least two courses of chemotherapy, and sometimes more than two courses (complete or incomplete). Chronic cases are often, but not always, excreters of MDR bacilli. Likewise, patients with retreatment failure are more likely to be harboring multidrug resistant organisms.

14 **Drug Defaulters**: Who consumes less than 80% of the prescribed drugs either in duration or doses or both.

15 **Treatment Failure:** A tuberculosis patient who remains or becomes again smear-positive at five months or later during treatment. is still excreting bacilli at the end of treatment (at five to six months for new cases or seven to eight months for retreatment cases).

16 **Molecular Epidemiology**: The genetic level molecular study of tuberculosis to evaluate the outbreak is referred as molecular epidemiology.

17 **TB diagnosis date**: It can be expressed as the collection date of the first specimen from which an *Mycobacterium tuberculosis* isolate was cultured.

18 **Homelessness:** It is defined as being in a public or private shelter or having no address at the time of the TB diagnosis.

19 **RFLP:** Restriction Fragment Length Polymorphism.

20 **SSCP PCR**: Single Strand Conformation Polymorphism PCR

21 **Luciferase reporter mycobacteriophage test**: MTB infecting virus mycobacteriophage with a cloned gene for production of Luciferase reporter enzyme used to elucidate the sensitivity. The LRM when mixed with culture of bacterial cell result in production of light.

22 **EESBM**: Egg enriched sheep blood media used for rapid growth of MTB.

23 **MGIT Method**: Microbial growth indicator tube method used to identify the susceptible/ resistant MTB.

24 **IID Method**: International Immune diagnosed method used to identify the MTB or its antigens for diagnosis purpose.

25 **Radiometric BACTEC Method**: Becton Dickinson radiometric method of detection of mycobacterial growth by sophisticated gamma camera.

26 **Chemoprophylaxis**: Prophylactic administration of antitubercular agents to reduce the disease risk referred as chemoprophylaxis.

Table 22. The number of samples of MTB strains from TB positive patients and their respective sourced institutions/ hospitals.

	Frequency	Percent	Valid Percent	Cumulative Percent
Mayo Hospital Outdoor	41	23.8	23.8	95.9
Mayo Hospital Indoor	110	64.0	64.0	64.0
Jinnah Hospital	14	8.1	8.1	72.1
DOTS	6	3.5	3.5	99.4
WAPDA Hospital	1	.6	.6	100.0
Total	172	100.0	100.0	

Table 23. The number and types of samples collected from Tuberculosis (AFB) +ive patients.

	Frequency	Percent	Valid Percent	Cumulative Percent
Bronchial Washing	8	4.7	4.7	4.7
Puss	18	10.5	10.5	15.1
Sputum	146	84.9	84.9	100.0
Total	172	100.0	100.0	

Table 24. Comparison of male and female TB positive (AFB positive) patients.

	Frequency	Percent	Valid Percent	Cumulative Percent
Females	50	29.1	29.1	29.1
Males	122	70.9	70.9	100.0
Total	172	100.0	100.0	

Table 25. Distribution of the specimens

	Frequency	Percent	Valid Percent	Cumulative Percent
Pulmonary tuberculosis	145	84.303	84.30	84.30
Extra-pulmonary tuberculosis	27	15.697	15.70	100
Total	172	100.0	100.0	

Table 26. Rifampicin resistance pattern of indigenous *Mycobacterium TB* strains collected from primary culture of TB diagnosed (AFB positive) patients of 14-67 years age group.

	Frequency	Percent	Valid Percent	Cumulative Percent
Resistant	37	21.5	21.5	21.5
Sensitive	135	78.5	78.5	100.0
Total	172	100.0	100.0	

Table 27. Isoniazid resistance pattern of indigenous *Mycobacterium TB* strains collected from primary culture of TB diagnosed (AFB positive) patients of 17-68 years age group.

	Frequency	Percent	Valid Percent	Cumulative Percent
Resistant	25	14.5	14.5	14.5
Sensitive	147	85.5	85.5	100.0
Total	172	100.0	100.0	

Table 28. Ethambutol resistance pattern of indigenous *Mycobacterium TB* strains collected from primary culture of TB diagnosed (AFB positive) patients of 17-68 years age group.

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Resistant	10	5.8	5.8	5.8
	Sensitive	162	94.2	94.2	100.0
	Total	172	100.0	100.0	

Table 29. Pyrazinamide resistance pattern of indigenous *Mycobacterium TB* strains collected from primary culture of TB diagnosed (AFB positive) patients of 17-68 years age group.

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Resistant	47	27.3	27.3	27.3
	Sensitive	125	72.7	72.7	100.0
	Total	172	100.0	100.0	

Table 30. Comparison of resistance percentage and quantity of growth (number of colonies) of indigenous *Mycobacterium TB* in rifampicin incorporated Lowenstein Jensen media, collected from primary culture of TB diagnosed (AFB positive) patients of 17-68 years age group.

Number of colonies	Frequency	Percent	Valid Percent	Cumulative Percent
20	1	2.70	2.70	2.70
30	3	8.11	8.11	10.81
50	6	16.22	16.22	27.03
100	26	70.27	70.27	97.30
200	1	2.70	2.70	100
Total	37	100	100	

Table 31. Comparison of resistance percentage over quantity of growth of indigenous *Mycobacterium TB* in Isoniazid incorporated Lowenstein Jensen media.

Number of colonies	Frequency	Percent	Valid Percent	Cumulative Percent
30	1	4	4	4
50	1	4	4	8
100	21	84	84	92
200	2	8	8	100
Total	25	100	100	

Table 32. Comparison of resistance percentage and quantity of growth (number of colonies) of indigenous *Mycobacterium TB* in ethambutol incorporated Lowenstein Jensen media

Number of colonies	Frequency	Percent	Valid Percent	Cumulative Percent
10	1	10	10	10
100	9	90	90	100
Total	10	100	100	

Table 33. Comparison of resistance pattern (percentage) and quantity of growth of indigenous *Mycobacterium TB* in pyrazinamide incorporated Lowenstein Jensen media

Number of colonies	Frequency	Percent	Valid Percent	Cumulative Percent
30	1	2.13	2.13	2.13
50	4	8.51	8.51	10.64
100	39	82.98	82.98	93.62
200	3	6.38	6.38	100
Total	47	100	100	

Table 34. Over all growth (No. of colonies) based resistance pattern of indigenous pulmonary/ extrapulmonary *Mycobacterium TB* against 1st line antitubercular drugs

Number of colonies	Rifampicin %age	Isoniazid %age	Ethambutol %age	Pyrazinamide %age
10			10	
20	2.70			
30	8.11	4		2.13
50	16.22	4		8.51
100	70.27	84	90	82.98
200	2.70	8		6.38
Total	100	100	100	100

Table 35. Overall mono-resistance pattern of indigenous pulmonary/ extrapulmonary *Mycobacterium TB* against 1st line antitubercular drugs

	Number of MTB Strains	Percent	Valid Percent	Cumulative Percent
MTB resistant to RFP	9	25.71	25.71	25.71
MTB resistant to ISN	3	8.57	8.57	34.28
MTB resistant to ETB	1	2.85	2.85	37.13
MTB resistant to PZA	22	62.85	62.85	100
Total	35	100	100	

Table 36. Poly-resistance profile of indigenous pulmonary/ extrapulmonary *Mycobacterium TB* strains categorized on basis of resistance against one, two, three or all of the four 1st line antitubercular drugs

Anti-tubercular Drugs Combinations	Number of MTB Strains	Percent	Valid Percent	Cumulative Percent
MTB resistant to RFP & ISN	6	19.35	19.35	19.35
MTB resistant to ISN & ETB.	7	22.58	22.58	41.93
MTB resistant to ETB & PZA	1	3.22	3.22	45.15
MTB resistant to ISN & PZA	2	6.45	6.45	51.6
MTB resistant to RFP, ISN & PZA	7	22.58	22.58	74.18
MTB resistant to RFP, ETB & PZA	1	3.22	3.22	77.4
MTB resistant to all of the four Antibiotics	7	22.58	22.58	100
Total	31	100	100	

Table 37. Level of resistance (in % age) of rifampicin resistant *Mycobacterium TB*

RFP level in LJ Media	Rifampicin ug/ ml	No. of MTB Strains	Percent Resistance	Valid Percent	Cumulative Percent
1	40	37	100		
2	80	37	100		
3	120	15	40.541	40.541	40.541
4	160	13	35.135	35.135	75.676
5	200	7	18.919	18.919	94.595
5+	200+	2	5.405	5.405	100
	Total	37	100	100	

Table 38. Level of resistance (in % age) of isoniazid resistant *mycobacterial TB*

ISN Levels in LJ Media	Isoniazid ug/ ml	No of MTB Strains	Percent Resistane	Valid Percent	Cumulative Percent
1	0.2	25	100		
2	0.4	25	100		
3	0.6	9	36	36	36
4	0.8	7	28	28	64
5	1	6	24	24	88
5+	1+	3	12	12	100
	Total	25	100	100	

Table 39. Level of resistance (in % age) of ethambutol resistant *mycobacterial TB*

ETB Levels in LJ Media	Ethambutol ug/ ml	No. of MTB Strains	Percent resistance	Valid Percent	Cumulative Percent
1	2	10	100		
2	4	10	100		
3	6	5	50	50	50
4	8	3	30	30	80
5	10	2	20	20	100
	Total	10	100	100	

Table 40. Level of resistance (in % age) of pyrazinamide resistant *mycobacterial TB*

PZA Levels in LJ Media	Pyrazinmid ug/ ml	No of MTB Strains	Percent Resistane	Valid Percent	Cumulative Percent
1	100	47	100		
2	200	47	100		
3	300	13	27.660	27.660	27.660
4	400	12	25.532	25.532	53.191
5	500	15	31.915	31.915	85.106
5+	500+	7	14.894	14.894	100
	Total	47	100	100	

Table 41. Overall Level of resistance (in % age) of resistant *mycobacterial TB*

Drug Levels	Rifampicin Percent Resistance	Isoniazid Percent Resistance	Ethambutol Percent resistance	Pyrazinamide Percent Resistance
1st Drug level	100	100	100	100
2nd Drug level	100	100	100	100
3rd Drug level	40.541	36	50	27.660
4th Drug level	35.135	28	30	25.532
5th Drug level	18.919	24	20	31.915
5th+ Drug level	5.405	12		14.894