In vitro Molecular analysis and studying the factors affecting subclinical forms of *Mycobacterium*

Thesis submitted in the partial fulfilment of requirements for the degree of Master of Philosophy in 'Biotechnology'.

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بسم الله الرحمن الرحيم

DEDICATION

Dedicated to My Loving Parents

Especially my Father (late)

CERTIFICATE

This thesis submitted by **Noor ul Ain** is accepted in its present form by the Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, as satisfying the thesis requirements for the degree of Master of Philosophy in **Biotechnology.**

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Dr. Syed Waqas Hassan External Examiner

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Prof.Dr.Zabta Khan Shinwari

Dated_____

DECLARATION OF ORIGINALITY

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Noor ul Ain

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LIST OF ABBRIVIATIONS

WHO World Health Organization

BCG Bacille Calmette-Guerin

HIV Human Immunodeficiency Virus

MDT Multi Drug Therapy

DNA Deoxyribonucleic Acid

UV Ultra Violet

CVI Children's Vaccine Initiative

PKG Protein Kinase Gene

PCR Polymerase Chain Reaction

EDTA Ethylenediaminetetraacetic acid

PIMS Pakistan Institute of Medical Sciences

RNA Ribonucleic Acid

TBE Tris-Boric acid-EDTA

MH Military Hospital

MS Microsoft

PAS Para-Aminosalicylate Sodium

ACP Acyl Carrier Protein

rRNA Ribosomal Ribonucleic acid

ORF Open Reading Frame

AIDS Aquired Immuno Defficiency Syndrom

UNISEF The United Nations International Children's Emergency Fund

MDR-TB Multi Drug Resistant Tuberculosis

Abstract

Tuberculosis is the historic but infectious disease which can be curable with little effort. It is affecting the world's population due to the drug resistance that is acquiring by the *Mycobacterium tuberculosis*. However, with the study of the domains those are involved in the drug resistance one can excel and move towards biopharming and drug formation which will be more efficient. Subclinical form of tuberculosis is majorly found in every individual but due to the presence of different environmental factors this form changes into pathogenic form and starts causing disease. In a nut shell one must say that the major factor triggering the subclinical form to become clinical form is smoking, which accounts for 65%, living conditions accounts for 56% and gender of a person accounts for 62% in changing the subclinical form into active TB disease; some how age also have its impact over progression of disease. In our study blood serum of 400 patients were taken for diagnostic analysis using polymerase chain reaction (PCR). From these samples, 300 patients were found to be positive for TB; and from these positive samples 184 (61.3%) found to be drug resistant. Our data suggest that the *Mycobacterium tuberculosis* is becoming resistant towards the major drugs that are currently being used as Isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin due to various factors. To overcome that problem the major genes of *Mycobacterium tuberculosis* that are targeted sites for drug action were analyzed using different databases. Specific domains were found that intract with drugs and mutations in these sites which leads towards the drug resistant.

In this study we also analyzed 11 genes (KatG,inhA, rpoB, pncA, embB, rrs, gidB, tlyA, gyrA, gyrB, thyA) along with their domains were analyzed. Out of these 12 genes eight genes were found to be the targeted sites for drug binding i.e. gyrA, gyrB, tlyA, gidB, rrs, embB,rpoB, inhA.

Chapter 1

Introduction

Tuberculosis is the historic, most prevalent and major cause of disease world wide because of the single bacterium. Although it is curable infectious disease but it is imposing a great impact over world population, researchers and doctors as it is increasing the death toll, infected people due to the acquired resistance against many drugs (Lienhardta *et al*., 2010). Until mid 20 century, tuberculosis was assumed to be deadly particularly in developing countries like Pakistan. Till 2011, 8.7 million people suffered from TB, among which 1.4 million people died and 5.8 million were the recent cases that were diagnosed as TB patients (WHO global TB report, 2012). In 2012, the total of 204 countries worldwide accounts for about 90% of the total reported TB data (WHO global TB report, 2012). Once TB was considered to be non-curable disease due to its high mortality rate but now it can successfully be treated in the limited time span. During 1995 to 2011, 51 million people were successfully treated and mortality rate decreased by 41% since 1990 (WHO global TB report, 2012). Mortality and incidence rate in the top ranked 22 high burden countries also decreased which were considered to account for 80% of the total TB cases (Ren *et al*., 2012).

Globally TB is the one of the major cause of death in females and it accounts for about 500,000(95%) deaths in low to middle income countries , aged between 15-45 till 2012 (WHO global TB report, 2012). Geographically, Asia and Africa is imposing most burdens on TB statistics as only India and China accounts for 40% cases of TB. Pakistan is ranked at 6^{th} position according to the WHO statistics but it is also moving towards the eradication of TB till 2015(Javaid *et al*., 2010). Another problem with this disease is the drug resistance towards many drugs that are used to treat TB. In 2012, 310,000 cases were reported with multi-drug resistance TB (MDR-TB) among these 60% cases was from India, China and Russian Federation (WHO global TB report, 2013).

Mycobacterium causes many diseases in humans most common is tuberculosis. But other species of this bacterium can cause other diseases like for hundreds of years leprosy has been affecting humans as an individual and also as a society. The affected have often been hated and shunned by their communities and families (Britton and Lockwood, 2004). Leprosy has been characterized as a disease responsible for serious deformities and disabilities resulting psychological and social suffering. Today leprosy has been

eradicated form the most parts of the world but in some parts of Asia Africa and South America, particularly in Brazil, Nepal, and Mozambique it is still present (Sinsimer *et al*., 2010).

Leprosy is a dermatological and neurological disease caused by the intracellular infection of *M. leprae* which can cause nerve damage and can lead to severe disabilities. (Monot *et al*., 2010). Despite intensive study, it is still unclear how leprosy is acquired and what elements cause the development of active disease or the level of severity of the disease in different individuals (Sinsimer *et al*., 2010). However, it is clear that the host immune response to *Mycobacterium leprae* determines the clinical manifestation of disease and various severity levels can arise (Ridley *et al*., 1966). Official figures from the World Health Organization (WHO) show that more than 213 000 people mainly in Asia and Africa are infected, with approximately 249 000 new cases reported in 2008. According to reports and statistics from 121 countries the number of new cases detected globally has fallen by 9126 (a 4% decrease) during 2008 compared with 2007 (Accession date, 17 August 2011, Leprosy today, WHO, Geneva, Switzerland. http://www.who.int/lep/en/).

1.1 History

Tuberculosis thought to be an ancient disease which was affecting the people from that time and had high rate of mortality thus considered to be a deadly disease of that time. Historic names that were used to refer this disease were consumption, phthisis, scrofula, pott's disease and white plague. First time word phthisis was appeared in 460 BC, most common cause of illness at that time, mostly affect the people of age between 18-35 (Diego, 2011) and it was considered to be very fetal (Ancell and Henry, 1852). Aristotle believed it might be contagious (Arthur *et al*., 1998). Galen, the Greek physician defined this as the "ulceration of lungs accompanied by cough, fever and pus (Barnes, 1995). About 4000 years ago when human migrated to other parts of world out of Africa, It is also dated that *Mycobacterium bovis* dispersing approximately 6000 years ago and may also be linked with domestic animals and early farming (Arthur *et al*., 1998). Later on ancient bones of humans from Neolithic also showed the presence of bacteria before 18 and 19 century.

Then in 17th century TB epidemic started in Europe which lasted for more than 200 years and this is known as great white plague. In 1650 the principal cause of death was plague. In 2008 evidences were found in Neolithic era mummies which confirmed the presence of TB bacterium 9000 years ago. (Bodington, 1840). In 17 and 18 centuries Francisus sylvius was the first scientist who started to differentiate between different forms of TB (pulmonary, ganglion) (Ancell and Henry, 1852). Mean while another scientist Thomas Willis inferred that all the lung diseases leads to the consumption (Waksman, 1964). He did not know the causative agent but just blamed on sugar or the acidity in blood (Ancell and Henry, 1852).

In 1720 another scientist Benjamin Marten expressed his new views regarding consumption and said that it is due to the presence of some kind of microscopic living thing that can survive inside the human body (Daniel, 2000). In 1768 Robert Whytt gave the first clinical description of TB meningitis (Whytt, 1768). Then another scientist William Stark proposed that even a minor infection with this bacterium can lead to ulcer and cavities; infect its different manifestation of the same diseases (Jules and Dubos, 1987).

Rene Laennec was the physiologist who died of this disease while studying contagious and infected patients via stethoscope (Daniel, 2000). He also wrote the book regarding tuberculosis diagnosis which was translated by John Forbes in 1821(Daniel, 2000). In 1869 another scientist named Jean and Antoine Villemin did several experiments on rabbits to prove that this disease is transmissible or contagious (Barnes, 1995). On 24 March 1882 Robert Koch discovered the causative infections agents which was proved to be a mile stone in the history of tuberculosis (Daniel, 2000). Then in 1895 Wilhelm Roentgen invented X-rays radiations and used them to locate and see the prognosis of the tuberculosis (Shorter, 1991).

Sanatoriums were the medicinal facilities that were provided to the patients at that time and the first such sanatorium was setup in 1854 which was 650 meters above the sea level. The thought which was behind this all was that there will be less atmospheric pressure thus patient's heart could work more effectively but as a matter of fact this all was not proved to be much effective (Jules and Dubos, 1987). Although much of the

advancement has been achieved yet there was no effective medicinal treatment was discovered till 50 years afterwards.

As to talk about 20th century this disease was proved to be fetal and one of the most deadly disease of that time. Then finally in 1902, the international conference on tuberculosis was held in Berlin in which certain steps were taken to eradicate this malady. After passing through all these agonizing conditions the first vaccine was developed in 1906 by Albert Calmette and Camille Guerin who made this vaccine from attenuated borine-strain tuberculosis and it was called as "BCG"(Bacille-Calmette-Guerin) (Beresford and Sadoff, 2010). It was 1921 in which it was used on humans for the very first time. Surely it was a thought provoking malady which impelled Albert Schatz and his co-workers in 1944, to isolate *Streptomyces Griseus* and is was proved to be the first effective antibiotic against *Myobacterium Tuberclosis* (Daniel, 2000). Indeed, streptomycin was not proved to be as effective as it was considered to be. So, in 1952, the first *Mycobacterium* drug; Isoniazid was introduced. After the discovery of Rifampin in 1970 there was a significant decline in the patients of TB. In response to the drugs, bacteria also made its resistant strains against the drugs and as a result drug resistant strains were evolved. Now every year half a million cases were believed to be the multidrug resistant (Beresford and Sadoff, 2010). .

1.2 Risk Factors

The bacterium of TB resides in almost every individual in sub-clinical form which may not harm the body until and unless it is triggered and changed into clinical form which cause disease and harm the body. There are many triggering factors that account for the activation of this bacterium and cause the disease but the major factors that are considered for the activation of this bacterium are as under (Narasimhan *et al*., 2013).

1.2.1 Diabetes

Diabetes is the major factor which is considered to be the risk factor for the TB prognosis. Every year more than 9million peoples fall ill and more than 1.5million die with TB (WHO global TB report, 2012). Diabetes is prevalent in both low income and

high income countries; over 80% deaths occur due to it and it is predicted that its prevalence will increase by 50% till 2013 globally. Chronic diseases like diabetes increases the risk of tuberculosis because it weak the immune system thus increase the risk of TB (WHO global TB report, 2012). Diabetes might also affect the drug effectiveness, treatment and response in TB patients (Kelly and Richard, 2009). Tuberculosis also induces glucose intolerance and severe glycaemic control in people with diabetes (Dooley and Chaisson, 2009). So over all people with diabetes are at high risk of having TB.

1.2.2 Tobacco

Tobacco is another risk factor which also increases the risk by 20% and causalities from TB (WHO global TB report, 2013). Tobacco as a whole is associated with the high risk of having TB as it weak the immune system. Both active and passive smokers are prone to be associated with TB and from being infected to developing infection. Smoking is also found to be associated with the TB mortality and relapse (Slama *et al*., 2007). Passive smoking and consuming other biomass fuel also doubles TB risk, So Patients with TB should receive counseling to stop smoking and to reduce the risk of developing the disease(Lin *et al*., 2008)..

1.2.3 HIV/AIDS

TB is deadly if its not treated and its more deadly if co-infected by HIV. The people who have both HIV and have latent TB infection are more prone to develop active TB than who don't have HIV infection; in 2010 a survey was conducted and more then 10% people were infected with HIV who had active TB. People with advanced infection of HIV have weakened the immune system and thus other infection can easily be developed like TB called "opportunistic infections". Co-infection with HIV also has impact over TB treatment as it makes the treatment more difficult which may lead to multi drug resistance TB (MDR-TB) (Narasimhan *et al*., 2013).

1.2.4 Immunity of the person

Immunity plays an important role in the spread of the disease. As we know that majority of the people have the sub-clinical form of *Mycobacterium* but some will develop the active disease due to their impaired immune system which is caused by infection of HIV, malnutrition or advanced malignancy (Neil and William, 1998). In body traditionally, the immunity is provided by T cells and thus called as T cell mediated immunity, so CD4+ cells are playing a very important role in the immunity regarding TB (Van *et al*., 2002).

1.2.5 Age and sex

Age and gender are also play leading role as a risk factor in TB. It is estimated that it is more prevalent in adult females than adult males (WHO global TB report, 2013). Among the patients of specifically Asia it's more likely to have at the age of 45-64years (Zhang *et al*., 2011).

1.2.6 Dietary conditions

Tuberculosis and malnutrition both are major problem in developing countries of the world and they interact with each other and increase the mortality rate. If we compare the nutritional status of the patient with the healthy one then we will find that nutritional status of TB patients are very low (Narasimhan *et al*., 2013).. Malnutrition basically causes immunodeficiency and increase the host susceptibility to infection. The patients of tuberculosis have low appetite, nutritional malabsorption, micro-nutrient malabsorption thus this all causes deficiencies increase the risk of tuberculosis (Gupta *et al*., 2009).

Another study suggests that malnutrition will cause impaired immune function which will lead to very low or no immune response against pathogen. Due to nutritional deficiencies it will directly affects the depression of lymphocytes and the cell-mediated immunity, where cell-mediated immunity is the principle host defense in host against TB (Cegielski and McMurray, 2004).

1.2.7 Living standards

Living conditions in the house indicates the health conditions of the well-being. Poor living condition and over-crowded homes are associated with the poor living condition and this increase the susceptibility of getting the TB because congested air and poor ventilation condition make the bacterium to spread to other people. Smoke in such environment also contributes towards poor respiratory health; these all condition leads to TB (Larcombe and Orr, 2007).

1.3 Types of tuberculosis

Generally TB is referred as the disease that is localized to the lungs only but it can spread to other parts of the body through blood and lymph fluid to the nearby tissues. Its types are classified on the basis of

1) infectious form of bacterium and;
2) site of infection of bacterium

On the basis of infectious form, TB is classified as

Latent TB

This is the type in which the person has the bacteria in its body but it is in sub-clinical or dormant form and will not cause disease/ infection. But still the person can get disease when the bacteria will get activated due to different risk factors that triggers and activate it and it will start multiplying itself increase in number and cause disease.

Active TB

When the bacteria of TB will get activated due to different factors then it will start multiplying itself and will invades nearby tissues and then will causes disease at primary site that is lungs and thus called as “pulmonary TB” also referred as primary TB; and when it invades the nearby tissues via blood stream or lymph then it is known to be “extra-pulmonary TB”.

(http://www.nationaljewish.org/healthinfo/conditions/tb/types/)

On the basis of site of infection TB is divided into many types and theses all actually comes in active primary TB or extra-pulmonary tuberculosis. These are as under:

1.3.1 Primary Tuberculosis

This is also known as primary TB because it invades the lungs that are first point where the bacterium starts multiplying and invades other parts. It's due to the weaker immune system so it cause disease in older people and children's. It is accompanied with fever and cough (Mazza *et al*., 2012).

Primary tuberculosis causes disease in immune-compromised adult patients, and as it is limited to the lungs thus it accounts for about 80% of all the TB cases and it acquires about 100% transmission rate/probability (Hunter, 2011).

Laryngeal TB is very uncommon form of pulmonary tuberculosis that affects the vocal cords and it's usually mixed with chronic laryngitis and laryngeal carcinoma.

Cavitary TB is the type in which bacterium infects lungs upper lobes and slowly destroys them and then it can spread to other parts of lungs. Its symptoms are cough with sputum and blood, night sweats, fever and weight loss (Converse *et al*., 1996).

Miliary TB is similar to primary TB but the difference is that it is diagnosed with granules that appears in lungs and can be seen in X-ray. It is prevalent in people with weakened immune system and a symptom includes fever, night sweats and weight loss (Ray *et al*., 2013).

TB Pleurisy is bit advanced form of TB. This form is diagnosed quickly as it is accompanied with visible symptoms. In this the bacteria enters and destroy the pleural space or destroy the space between the lungs and chest wall. The patient will feel chest pain and difficulty in breathing (Lin *et al*., 2012).

1.3.2 Extra pulmonary tuberculosis

As we know that primary tuberculosis infects lungs but when it invades nearby tissues it causes extra pulmonary tuberculosis and it is increasing the TB cases for about 20% to 40% of all the TB cases (Mazza *et al*., 2012). So we can say that it is the worst form of

TB as it is invading the other sites of the body after destroying the tissues of primary site of infection.Extra pulmonary tuberculosis can be sub categorize on the basis of region which it is invading and causing diseases. On the basis of region specificity it is more prevalent in the form of lymphadenitis, pleuritis and osteoarticular TB. Where by peritoneal, urogenetial or meningeal TB is less prevalent forms of TB (Neelakantan *et al*., 2013).These sites of infections are difficult to detect because of the difficult methods that are involved in the diagnostics of these forms of TB. As we know that tuberculosis accounts for about 1.4 million deaths per year and it can be spread to nearby tissues causing the TB of that specific region other than lungs. In case of laryngeal TB it affects the larynx and it can leads to laryngeal carcinoma although it is very rare form of TB (Suhail *et al*., 2012). The risk factors that are associated with this type of TB are paan, betal nut, smoking etc

Extra pulmonary tuberculosis tends to develop more in females than males because of the weaker immune system and masses that are of 45 years of their age are more prone to TB (Lin *et al*., 2007). Nasopharyngeal TB is also extra pulmonary form of TB and it is also very rare form and it accounts for about 1% or less than 1% of all the TB cases and it is the TB of upper respiratory track (Patil *et al*., 2013).Adrenal Tuberculosis is extra-pulmonary TB and it affects the adrenal gland and its secretions. Person with this type of TB will feel low and fatigued due to low production of adrenal hormone (Nakaoka *et al*., 2012). Lymph Node Disease is extra-pulmonary TB; it invades the lymph nodes and they become so enlarged that they rupture from the skin if they are not treated (Eshete *et al*., 2011). Osteal Tuberculosis is also extra-pulmonary TB; in this type the bacteria invades the bones leads to the weakening of bones and even causes fractures. It can occur in any bone but the most attacked bone is spine which leads to the compression and fractures and also cause back discomfort (Gur *et al*., 2013). When bacterium infects the outer lining of intestine and cause abdominal pain and discomfort. It can also infect the inner linning of intestine which can cause fluid in small area and this type of TB is called as TB Peritonitis (Sinan *et al*., 2002).

If white blood cells start coming into the urine of any patient then it’s the indication of renal TB; if left untreated then it can spread to the reproductive organs. The person who

have brain tumor or stroke they must be tested for TB bacterium because if present then it is TB meningitis and it is very fatal form of extra-pulmonary tuberculosis (Christensen *et al*., 2011). TB Pericarditisis also extra-pulmonary TB and it affects the function of heart. When excess fluid builds around the heart it will eventually leads to the improper functioning of the heart (Cherian, 2004).

1.4Transmission

As we know that tuberculosis is the bacterial disease that mainly infects the lungs mean while it can be controlled and preventable (WHO global TB report, 2013).

TB can be spread from one to another person through air. The person who have TB (most probably primary TB that mainly affects the lungs), sneeze, cough or spit, the bacterium come into the air from where depending upon the concentration of the expelled bacterium it can be transferred to the other person and can cause disease or even infect the other person(WHO global TB report, 2013). The people who get the bacteria into there body and are just infected, have a 10% chances that they will get the disease in their life time. Other risk factors like malnutrition, poor immune system, poor hygiene, diabetes, HIV, make the person more susceptible of getting this disease.

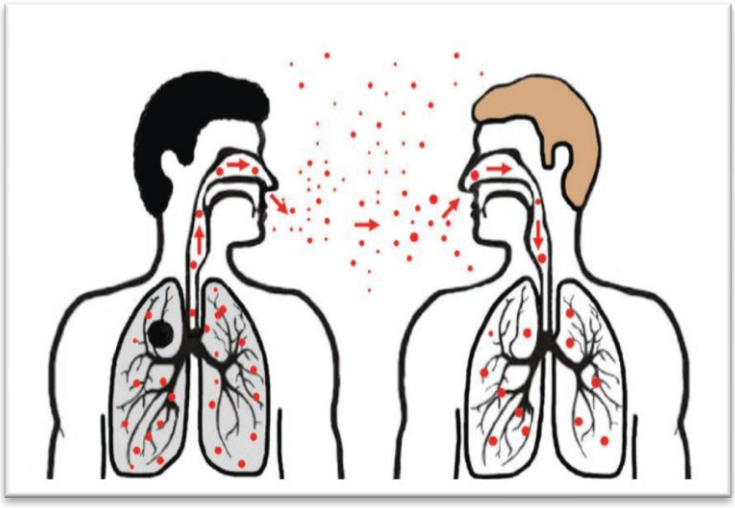

Figure 1.1: TB is spread from person to person through the air. The dots in the air represent droplet nuclei containing *tubercle bacilli*.

(http://www.cdc.gov/tb/education/corecurr/pdf/chapter2.pdf)

Cough is the major source through which this bacterium can be transmitted into other person in the form of droplets (Jones *et al*., 2013). So we can conclude that TB bacterium cannot be transmitted by surface contact. Transmission occurs when a person inhales droplet nuclei containing *M. tuberculosis*, and the droplet nuclei traverse the mouth or nasal passages, upper respiratory tract, and bronchi to reach the alveoli of the lungs.

There are different factors that determine the probability of transmission of *Mycobacterium* from person to person or from the environment to the healthy individual. Susceptibility, Infectiousness, Environment and Exposure to the bacterium determines the transmission of bacterium. These factors altogether accounts for the transmission of pathogen and responsible for illness.

Table 1.1: Factors for transmission

Factors	**Description**
Susceptibility	It refers to the immune status of the individual whether one has good or bad immunity.
Infectiousness	Infectiousness of the person with TB disease is directly related to the number of tubercle bacilli that he or she expels into the air. Persons who expel many tubercle bacilli are more infectious than patients who expel few or no bacilli.
Environment	It refers to all the environmental factors which plays important role in transmission of bacterium.
Exposure	Proximity, frequency, and duration of exposure.

There are different environmental factors that are also responsible or one can say that they enhance the probability of transmission of bacterium and that are Concentration of infectious droplet nuclei, Space, Ventilation, Air circulation, Specimen handling and Air Pressure (Hussain *et al*., 2003).

Table 1.2: Factors that increase the transmission probability of *Mycobacterium*

Factors	Description
Concentration of infections droplet nuclei	The more the number of exhale drops containing bacterium the more will be the chances of getting disease.
Space	It refers to the living standard, the more the conjusted and closed area the more will be the chances of getting this disease.
Ventilation	Inadequate and lack of ventilation will leads towards the transfer of this bacterium from one person to another.
Air circulation	Air plays an important role in transferring the bacterium from one person to another.
Specimen handling	Improper handling of specimen will leads to the transfer of bacterium.
Air pressure	It refers to the pressure that is exerted by air on the bacterium due to which it will be transfer from one to another place.

Another factor also plays its role in transmission of pathogen and that is Proximity and Length of Exposure. Because the person with close proximity and with greater exposure time will have much more chances to get that bacterium than the one with far proximity and with less exposure time. So theses all factors accounts for the transmission of the pathogen (Jones *et al*., 2013).

Table 1.3: factors for transmission

Factors	Description
Duration of exposure to a person with infectious TB	It refers to the exposure time, the more the time of exposure the more the chance of getting disease.
Frequency of exposure to infectious person	The higher the exposure frequency, the more is the probability of getting disease.

Physical proximity to infectious person	The more closer the person with this disease, the more will be the chances of getting the disease.

Young children with pulmonary and laryngeal TB disease are less likely than adults to be infectious. This is because children generally do not produce sputum when they cough. However, transmission from children can occur (Hussain *et al*., 2003). Therefore, children and adolescents with TB disease should be evaluated for infectiousness using the same criteria as adults. These criteria include presence of cough lasting 3 weeks or longer; cavitation on chest radiograph; or respiratory tract disease with involvement of lungs, airways, or larynx.

1.5 Pathogenesis

When the person inhales the droplet nuclei containing the TB bacterium, it reaches the lungs. These bacteria are then ingested by the alveolar macrophages and majority of them are killed or are inhibited. But unfortunately few of them some how manage to survive and may multiply intracellular and when macrophage dies they are released alive and then through blood stream or lymphatic channels they are spread to distant tissues and organs (Smith, 2003). The major factor in dissemination of TB bacterium is the immune system. Pathogenesis of latent TB infection and TB disease are as follows,

1) Droplet nuclei containing *tubercle bacilli* are inhaled, enter the lungs, and travel to the alveoli.

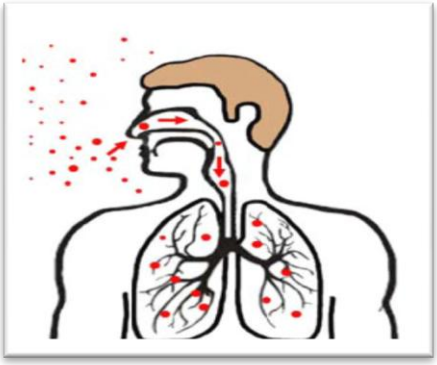

Figure 1.2: Showing the inhalation of bacterium from the environment which is rich in bacterium concentration.

http://www.cdc.gov/tb/education/corecurr/pdf/chapter2.pdf

2) TB bacteria (Tubercle bacilli) multiply in the alveoli and increase their number.

Figure 1.3: When bacterium enters the body and reaches into the alveoli it starts multiplying itself and increases its number.

(http://www.cdc.gov/tb/education/corecurr/pdf/chapter2.pdf)

3) A small number of *tubercle bacilli* enter the bloodstream and spread throughout the body. The tubercle bacilli may reach any part of the body, including areas where TB disease is more likely to develop (such as the brain, larynx, lymph node, lung, spine, bone or kidney.

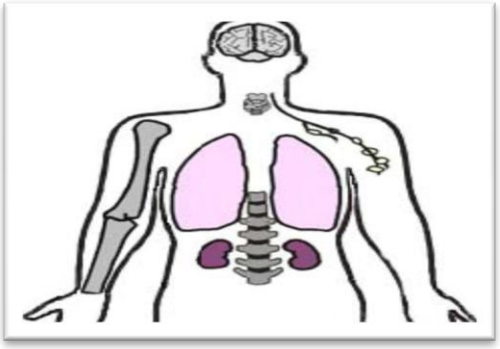

Figure 1.4: When bacterium multiplies itself inside alveoli then after increasing its number this bacterium starts invading the nearby tissues via entering into the blood stream and reaching the specific area causing disease there.

(http://www.cdc.gov/tb/education/corecurr/pdf/chapter2.pdf)

4) Within 2 to 8 weeks, special immune cells called macrophages ingest and surround the tubercle bacilli. The cells form a barrier shell, called a granuloma, that keeps the bacilli contained and under control **(LTBI)** (Smith, 2003)**.**

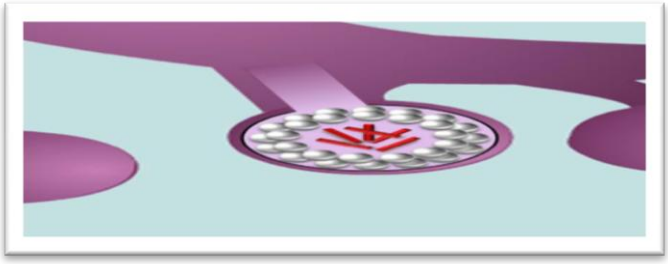

Figure 1.5: When the bacterium is recognized by the body then body starts it defense mechanism by producing macrophages. These special cells surround the bacterium and keep them under control by making shell like structure around them.

(http://www.cdc.gov/tb/education/corecurr/pdf/chapter2.pdf)

5) If the immune system cannot keep the tubercle bacilli under control, the bacilli begin to multiply rapidly (TB disease). This process can occur in different areas in the body, such as the lungs, kidneys, brain, or bone.

Figure 1.6: when macrophages are unable to control the bacterium then break the barrier and move to other distant parts and infect them making disease more worst. (http://www.cdc.gov/tb/education/corecurr/pdf/chapter2.pdf)

1.6 Diagnosis

To confirm the patient have the TB bacterium in its body one must diagnose it from the clinical specimen provided by the patient. But other investigations also play role in accurate diagnosis.

A complete medical evaluation for the diagnosis of tuberculosis must include different factors altogether makes the good diagnosis; those factors includes

- Complete medical history
- Physical examination
- Microbiological examination
- Radiograph examination
- Immunological test

1.6.1Medical history

In medical history the doctor looks for the general symptoms of pulmonary TB that are productive and long-term cough of 3 to 4 weeks, chest pain and hemoptysis. Other systematic symptoms include low grade fever, night sweats, loss of appetite, weight loss, production of sputum with blood (Kumar *et al*., 2007).

Other factors should also be taken in medical history like prior exposure to TB, prior infection or disease, previous treatment; risk factors should also be checked as they increase the risk for TB disease (Burke and Parnell, 1948)

1.6.2 Physical examination

Physical examination is done to access the patient's health in general and to look for the different factors that can be assumed to cause TB and that can affect the TB treatment plan. So after physical examination one can suggest if the person have TB or it can be ruled out.

1.6.3 Microbiological examination

One can only confirm the diagnosis by culturing the *Mycobacterium* on to the culture medium from the sample that is taken from the patient; before culturing one can only assume or make a guess but that is not confirmed (Kumar *et al*., 2007).

1) Sputum test can only be done if the patient is producing sputum (Kumar *et al*., 2007). Then in that case the bacteria can be identified by florescent microscopy that is more accurate than conventional staining procedures (Steingart *et al*., 2006).
2) The person, who is not producing sputum, will go for other alternatives to diagnose bacteria that are swabs, bronchoscopy and may need tissue biopsy.
3) PCR can also be done to check the presence or absence of *Mycobacterium tuberculosis*. As PCR can differentiate this bacterium from other *Mycobacterium*.

1.6.4 Radiograph examination

As we know that in active pulmonary tuberculosis cavities can be seen in the upper lobes of the lungs, however it can appear anywhere on the lungs. So abnormalities on the radiograph can be seen and it can be used to suggest if there is TB but one can't only depend on this (Rossi *et al*., 2005). Cavitations on the upper apex of the lungs can be seen in the chest X-ray of the patient (Kumar *et al*., 2007).

1.6.5 Immunological test

In immunological test we basically look for the antigen antibody interaction like Tuberculin skin test.

1.7 Vaccination

Vaccination has always been an effective tool against viral and bacterial diseases. For many years the only licensed vaccine present against *Mycobacterium leprae* and *Mycobacterium tuberculosis* was Bacille Calmette-Guérin (BCG). BCG was developed in the early 1900s by Albert Calmette and Camille Guèrin. It is made of a live, vitiated strain of *M. bovis.* BCG was first administrated in 1921 to a newly born baby whose mother was a tuberculosis patient. This immunized individual remained free of tuberculosis throughout his life (Rosenthal *et al*., 1945). In 1928 United Nations (The Leauge of Nations) suggested widespread vaccination with BCG. Today about 80% of the world population is vaccinated with BCG. The vaccine is effective against disseminated and meningeal TB in infants and young children, however, unfortunately the protective efficacy of BCG vaccination against *Mycobacterium leprae* has not proved very effective (Fine, 1995).

Due to the poor performance of multidrug therapy (MDT) and BCG vaccination, especially in the developing areas of Asia and Africa, it is necessary to explore new immune-protective vaccines (Andersen and Doherty, 2005) and the identification of protective antigens is particularly important for the subunit vaccine approach (Sanchez and Holmgren, 2008). Immunization with DNA encoding Mycobacterial antigens has already been proven to stimulate successful protective cell mediated immune responses against *Mycobacterium tuberculosis* (Kamath *et al*., 1999) and *M. avium* infection (Faircloth *et al*., 1999) and is a new strategy for leprosy control.

The history of this vaccine is tangled with the vaccine of small pox. In 1854, Jean Antoine Villemin discovered *Mycobacterium* for the first time then Robert Koch differentiated *Mycobacterium Bovis* and *Mycobacterium tuberculosis*. After successful vaccination of small pox scientist starts working on the development of *Mycobacterium*.

A French bacteriologist, Albert Calmette and his colleague, Camille Guerin working at the institute pasteur de lille(France) in 1908; working on virulent strains of tubercle bacillus and made such sort of strains that can be considered for use as a vaccine. This all the research was continued till World War 1 and then they transferred this to Paris Pasteur institute. Finally in 1921, BCG vaccine was used in humans for the first time (Fine *et al*., 1999).

The only problem with this vaccine is that variability efficiency that appears to depend on the geography. First large scale trail of BCG was conducted from 1956 to 1963; this shows 84% efficiency up to 5 years after immunization (Hart and Sutherland, 1977) . Further more when the same trial was conducted in south India it showed no protective effect (Baily *et al*., 1979). BCG has adverse affect in preventing military TB or TB meningitis (Rodriguez *et al*., 2003). So it is still be used on large scale even in those countries where the efficiency of this vaccine is too little or negligible.

There are different factors that can reduce the efficiency of BCG and that are

- Genetic variation in BCG strains (Brosch *et al*.,2007).
- Genetic variation in population can also reduce the efficiency of BCG (Packe *et al*., 1988).
- Interference of other non tuberculosis *Mycobacterium* cause lower efficiency (Brandt *et al*., 2002).
- Interference by concurrent parasitic infection reduces BCG efficiency (Rook *et al*., 2005).
- Exposure to UV light also reduces the performance of BCG (Jeevan *et al*., 2009).

These factors altogether reduce the efficiency of BCG vaccine.

In focus of interest is the *Mycobacterium* pathogens being closely related to each other that justified investing in cross-protective vaccines. In this field tuberculosis (TB) is the widest spread disease, leprosy is one of the cruelest and Buruli ulcer shows an exponential increment in the last decade and

biotechnologists are asked for help (Daar *et al*., 2002). It is an ardent awareness that there has to be done much work to fight these diseases (Jha *et al*., 2002).

TB is a contagious airborne infectious disease. It attacks the respiratory system and is easily spread through coughing and sneezing. 1.86 billion people , one-third of the world population has latent TB infection -that is infection that has not manifested itself as full-blown disease. However, annually 8 million of the infected persons develop severe tuberculosis (WHO in Global Tuberculosis Control 2002). Disease from *Mycobacterium tuberculosis* develops due to exogenous insult, intrinsic genetic susceptibility (Van *et al*., 1999) or both. Similarly leprosy is a communicable disease caused by *Mycobacterium leprae*.

According to the data the existing main tuberculosis vaccine, Bacille Calmette-Guerin (BCG), developed in 1921, based on live, attenuated *Mycobacteria*, has proven inefficient in several recent field trials.Due to its failure to provide adequate protection against pulmonary tuberculosis in adults it is necessary to develop new immuno-protective vaccines (Andersen, 2001) and the identification of protective antigens is particularly important for the subunit vaccine approach (Kaufmann, 2001). TB can be cured by drugs. However, multidrug-resistant strains of TB have already developed.

The first leprosy vaccine commercialised was developed from *M. bovis* strain (BCG) which resulted in immunization and protection from leprosy infection. However, in many parts of the developing world, the expense of immunization programs prohibits the use of the currently available vaccines for large segments of the population. By the WHO ´Leprosy Elimination Group´ a multidrug therapy is provided in health centres to cure the disease. However, antibiotic resistances are emerging rapidly (Daar *et al*., 2002).

Also for Buruli ulcer at the present time, BCG vaccination is the only biomedical intervention that may help control the disease in the highly affected areas (Weir, 2002). However, also for this disease BCG vaccination appears to offer only

some short-term protection. Treatment of Buruli ulcer with antibiotics has been unsuccessful to date. At the present time, the only treatment available is surgery to remove the lesion followed by a skin graft if necessary. This can lead to permanent disability (Werf *et al*., 1999). Summarizing the BCG vaccine is of insufficient efficacy for protection and the therapeutically costs still exceed the financial power of affected regions.

The World Health Organization (WHO) and the CVI (Mitchell *et al*., 1993) have challenged researchers to discover less expensive methods to deliver vaccines to developing countries. Unless the price for the vaccines falls below one US-Dollar a dose, the rest of the developing world cannot access it (Fox, 1996). This limitation has led researchers to express antigens in plants as a means of developing a less expensively produced vaccine that would not require refrigeration during distribution. Oral vaccines are desirable for many reasons, including simplicity of use, security, without the HIV-risk through syringes (Kane *et al*., 1999, Shiao *et al*., 2002) and enhanced immune response (Jodar *et al*. 2001).

1.8 Virulence factors of *Mycobacterium* that are involved in pathogenesis

As we know that *Mycobacterium* cause severe infection in the body of an organism so there must be an efficient system for stability of bacterium inside the human body so that it can cause infection and will lead towards the tuberculosis. *Mycobacterium* is divided into two major groups on the basis of their growth pattern (Kamalakannan *et al*., 2007).

1. It contains slow growing species and they are well known pathogens of humans and these include *Mycobacterium tuberculosis, Mycobacterium bovis and Mycobacterium leprae.*
2. This group includes fast growing group of *Mycobacterium* and these are non pathogenic bacteria and this include *Mycobacterium smegmattis.*

As we know that *M. tuberculosis* is highly pathogenic so its pathogenicity is controlled by its many genes that alter the host normal pathways which will leads towards the abnormalities and cause disease. Once a gene of *Mycobacterium* plays an important role in virulence and that is 'nuoG'. This gene has ability to inhibit the death of infected host cell via apoptosis thus this all will leads to the pathogenecity of bacteria (Kamalakannan *et al*., 2007).

Another factor of *Mycobacterium* play an important role in pathogenecity and that is mycolic acid. It plays important role in growth, pathogenecity, survival and infectivity of bacteria inside the human body. Mycolic acid is present on the cell wall of the bacterium and its amount varies from specie from specie (Vander *et al*., 2011).

After getting into the host cell it is very important for *Mycobacterium* to protect itself and survive inside it. For that purpose *Mycobacterium* has a gene that is protein kinase gene (PkG) that increases the ability of *Mycobacterium* to survive inside the host cell. Basically, it inhibits the intracellular degradation of *Mycobacterium* in lysosomes thus increasing their chance of survival to cause disease (Nicole *et al*., 2007).

Some bacterial proteins also play a very important role in the infection and protection of bacteria. Bacterial proteins ESX-1 plays important role in causing disease as this protein is secreted by bacteria and transferred into the host cell during infection. These proteins are highly pathogenic/ antigenic so its secretion leads to successful infection (Sridharan *et al*., 2008).

Aim and focus of the work

Due to increasing number of patients suffering from different contagious diseases especially in resource poor countries it is important to produce efficient and cost effective alternative vaccines. It is also very important to study the different environmental factors which are affecting the subclinical form of TB and making it drug resistant.

Plants provide us with a unique system of foreign protein production which has potential to address the problems mentioned above. Among bio-pharming, chloroplast transformation system has great advantages. Besides its eco-friendliness and high containment due to maternal inheritance, it offers the opportunity to use multiple copies of plastid genome per cell resulting in high level of protein expression with minimum or no risk of epigenetic effects.

The following list gives the objectives addressed in the current study.

- Molecular study to determine the importance of different environmental factors which are responsible for disease development.
- Insilico analysis of the factors responsible for the drug resistance.
- Proof of TB with regard to different factors.
- Statistical analysis of the different factors with TB.
- Insilico study of the genes that are involved in development of drug resistance TB.
- Evolutionary study of *Mycobacterium* with reference to domain that is involved in drug resistance.

Thus the aim of this study is the evaluation of the potential factors that affects the subclinical form of TB, and makes it active. It is also aimed to study the factors that are responsible for the drug resistant TB. So that one can overcome that problem and this all will leads to the drug development which will have greater efficiency with high rate of action.

Chapter 2

Materials & Methods

2.1 Material and chemicals

DNA isolation reagents

GF-1 bacterial DNA extraction kit vivantis

PCR Consumables

Magnesium chloride	Fermantas
Taq polymerase	Biotools
10X Buffer	Fermantas
dNTP's	Fermantas
Primers	Alpha DNA

Electrophoresis Consumables

Agarose	Vivantis
Tris	Sigma
EDTA	Sigma
Ethidium bromide	Sigma

Equipment and supplies used

Table 2.1: Equipment used in research

Sr. No	Instrument Name	Company
1	Thermal cycler	Applied Biosystem
2	Bio Doc Analyzer	Biometra
3	Centrifuge Machine	Eppendorf

4	Horizontal Gel Tank	Thermo
5	Vertical Gel Tank	Whatman Biometra
6	Oven	Dawlance
7	UV Lamp	Vilber Lourmat
8	Autoclave Machine	Hiclave
9	Thermostat Plus	Eppendorf
10	Power Supply	Thermo
11	Weighing Balance	Sartorius
12	Power Supply	Thermo Electron Corporation
13	Ice Machine	Scotsman

2.2Identification of Patients

The present study was based on the screening of tuberculosis as well as studying the factors that affects the subclinical forms of tuberculosis among Pakistani population. For this purpose,samples were collected from different hospitals located in Islamabad, Rawalpindi and Faisalabad.These included Allied Hospital Faisalabad,TB hospital Faisalabad,TB hospital Muree, PIMS/POLY CLINIC Islamabad and Military hospital Rawalpindi.

2.3 DNA Extraction from bacterial cell for *Mycobacterium tuberculosis* Screening

The following protocol is one of the established methods of DNA extraction and works well with a wide range of bacterial cells. Proteins are digested with proteinase K. DNA is then precipitated with ethanol .The resultant DNA (10-20µg) is of high molecular weight and is a suitable template for polymerase chain reaction.

Procedure

For the extraction of DNA of *Mycobacterium tuberculosis* I took the sample first in the form of any fluid i.e. serum, plasma, blood or any body fluid etc and by using GF-1 bacterial DNA extraction kit the following protocol was used to extract the DNA from bacterial cell. .

i. First of all put the fluid 100 µl in the tube along with the positive and negative control samples.
ii. Then put Pk= 20µl , lysis buffer= 100µl , Buffer R2 = 180µl in all the samples.
iii. Vertex the tubes.
iv. Incubate the tubes for 20 min at 65°C.
v. Short spin the tubes
vi. After this add 400µl of buffer BG and invert for 40 to 50 times.
vii. Then add 200µl of ethanol(99.9%) at room temperature.
viii. Again invert for 40 to 50 times.
ix. Short spin .
x. After short spin shift the material to the column and centrifuge for 1mint at 10,000rpm and discard the flow through.
xi. Add 500µl wash buffer and centrifuge for 1mint at 10,000rpm, discard flow through.
xii. Then centrifuge the column at 10,000 rpm for 1 min to remove residual ethanol.
xiii. Place the column into clean micro-centrifuge tube. Add 50µl of pre-heated elution buffer. Centrifuge at 10,000rpm for 1 min to elute DNA. Store DNA at 4°C.

2.4 Quantification of Extracted DNA

The quantity of the extracted DNA in the T.E (10 mM Tris, pH 8.0 , 1 mM EDTA) solution was estimated by using two methods; spectrophotometry and Yield Gel Electrophoresis.

2.4.1 Spectrophotometry

Extracted DNA was quantified using ddH_20 on spectrophotometer"GENESYS". Firstly 400µl of autoclaved distilled water was dispensed into the cuvette as a reference and then sample DNA was poured into cuvette and placed in spectrophotometer, DNA was scanned for a range of UV radiation (260nm-280nm). UV absorbancy value at 260nm and 280nm of wavelength was noted and a ratio of 260:280 was calculated.

The quantity of DNA in dilution was calculated as follows:

$$\text{Amount of DNA (ng/µL)} = \text{Absorption at 260} \times 50 \times \text{Dilution Factor (DF)}$$

$$\text{DF} = \frac{\text{Total volume of dilution}}{\text{Volume of stock DNA solution in the dilution}}$$

From the calculated amount of DNA in UV spectrophotometery, 5ng/µl dilution was prepared in 100µl volume. The dilution was prepared as follows:

$$V1 = \frac{X2 \times V2}{V1}$$

Where,

X1 = Amount of DNA in stock solution.

V1 = Volume of DNA of stock solution to be diluted.

X2 = Amount of DNA in dilution to be prepared (5ng/µl).

V2 = Volume of DNA dilution to be prepared (100mL).

All the dilutions made were kept at 4°C for further use.

2.4.2 Gel Electrophoresis

Electrophoresis is a technique that separates molecules on the basis of their different rates of movement in an applied electric field through a porous semisolid matrix. Agarose is a polysaccharide consisting of a linear polymer (repeating units) of D-galactose and 3, 6 anhydro L-galactose. The movement of molecules through an agarose gel is dependent on the size, charge of molecules and the pore sizes present in the agarose gel. Higher concentrations of agarose facilitate separation of small DNAs, while low agarose concentrations allow resolution of larger DNAs At neutral pH, DNA, RNA, and proteins migrate toward the anode (positive electrode) when an electric field is applied across the gel. Genomic DNAs usually run as a "smear" due to the large number of fragments with only small differences in mass

For DNA and RNA 1%agarose is used,which was prepared by heating 1.0g of agarose powder in 100mL of 1X TBE buffer (Tris-Borate electrophoresis buffer).When the agarose completely dissolved in buffer, 5µl of 5mg/mL of ethidium bromide was mixed into the gel solution and it was poured into dual comb 16 teethed caster (Hoefer scientific Inc.) and allowed to solidify.

- Dilutions of the samples used for loading are mentioned below.

Stock DNA	=	2µl
10X loading dye	=	2µl
Distilled Water	=	6µl
Total Volume =		10µl

From standard DNA having 15ng/µl conc. the following loadings were made as,

Standard DNA (15ng/µl)	=	1µl
10X loading dye	=	2µl
Distilled Water	=	7µl
Total Volume	=	10µl

Agarose gel was run at 120V/cm and 47mA for 1hour.Amount of DNA was estimated from yield on gel electrophoresis, in each of 5ng/µl dilutions.

2.5 Amplification of DNA

Primers pairs for analysis were designed against exon of *Mycobacterium tuberculosis* gene using Primer blast software available online (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Both primers were approximately of 18-20 bp size. GC content varied from 50-60%. Primer sequences of exon of *Mycobacterium tuberculosis* are shown.

After extracting DNA, it is amplified by using the set of primers

Forward Primer (5'-3')

T1= GGCTGTGGGTAGCAGACC

Reverse primer (3'-5')

T2= CGGGTCCAGATGGCTTGC

2.6 Polymerase Chain Reaction (PCR) for amplification of target exon

Developed in 1984 by Kary Mullis, PCR is now a common and often indispensable technique used in medical and biological research laboratories for a variety of tasks. PCR is used to amplify specific regions of DNA strand. This can be a single gene, a part of a gene, or non-coding sequence. Most PCR methods typically amplify DNA fragments of up to10 kilo base pairs (Joshi and Deshpande, 2011).

2.6.1 Amplification of Sample and Control DNA

Amplification of patients DNA TB samples along with their adjacent normal control samples (10ng/µL dilution) was carried out for *Mycobacterium* gene using region specific oligonucleotides mentioned earlier.

2.7 Optimization of *Mycobacterium tuberculosis* Primer

Reaction conditions for PCR amplification of *Mycobacterium tuberculosis* gene were optimized to conduct the expression analysis of tuberculosis gene in TB patients of Pakistani population. For this purpose PCR reactions were set up with different primers concentrations and annealing temperatures. After an extensive experimentation the reactions conditions were optimized which are given in table below:

Table 2.2: showing the amount of consumables of PCR reaction mixture

Reagent	volume
DNA	5 µL
Taq buffer	2.5 µL
MgCl2	2 µL
DNTP's	2 µL
TB1	1 µL
TB2	1 µL
Taq polymerase	2 µL
Water	9.5 µL

PCR Program Profile

Table 2.3: PCR program profile

Steps	Temperatures	Time	No. of Cycles
Denaturation	94˚C	2min	1
Denaturation Anealing Extension	94˚C 52˚C 72˚C	15sec 30sec 1min	50
Final extension	72˚C 4˚C	2min ∞	1

2.8 Horizontal Gel Electrophoresis

Amplified PCR products were analyzed on 2 % agarose gel, prepared by melting 2 g of agarose in 100 ml 1X TBE buffer (0.89 M Tris-Borate, 0.032 M EDTA, pH 8.3) in a microwave oven for one minute. Ethidium bromide (5 µL) as 0.5 µg/ml final concentrations was added for staining DNA.

Amplified PCR product of each sample was mixed with loading dye (0.25 % bromophenol blue, 40 % sucrose) and loaded into the well. Electrophoresis was performed at 100 volts (80 mA) for half an hour in 1X TBE buffer. Amplified products were visualized by placing the gel in UV transilluminator (Life Technology, USA) or Gel Doc apparatus (Bio Rad). PCR bands were analyzed by the help of the installed software.

Chapter 3

Results

The present work was aimed to screen the tuberculosis patients and expression analysis of *Mycobacterium tuberculosis* in TB patients among Pakistani population. It is also aimed to figure out the role of different factors affecting the sub clinical form of tuberculosis.

3.1 TB blood/ serum sampling

Samples were collected after informed consent was obtained from tuberculosis patients. Sampling was carried out with approval of ethical committee of Department of Biotechnology, Quaid i Azam University Islamabad, PIMS (Pakistan Institute of Medical Sciences) Islamabad, Military Hospital (MH) Rawalpindi and Allied Hospital Faisalabad. Samples from TB patients, comprising of blood / serum sample was collected from each individual to check the presence of *Mycobacterium* in these patients.

The TB patients were classified on the basis of their age, smoking, vitamin D deficiency, living standards etc. And clinic pathological parameters such as stage of TB, type of TB, TB site etc were collected from reports.

3.2 Data Analysis

Table 3.1: Clinical characteristics of tuberculosis patients

Variables	Number of patients (300)	Percentage
Age (years)		
<=25	40	13%
>=50	180	60%
<=75	80	26%
Gender		
Male	186	62%
Female	114	38%
Site of infection		
Pulmonary	235	78%
Extra-pulmonary	65	22%
Smoking history		
Smokers	196	65%
Non smokers	104	35%
Living standard		
Good	130	43%
Poor	170	57%
Stage of tuberculosis		
Initial stage	123	41%
Well developed	177	59%
Vitamin D concentration in blood		
Deficiency	132	44%
Normal	168	56%
Marital status		
Married	230	77%
Unmarried	70	23%

The results were statistically analyzed with MS excel. The data analysis of 300 tuberculosis patients was carried out in correlation to different parameters e.g. age, gender, site of infection, smoking, living standard and other factors. The data obtained from patients is summarized in table above.

3.3 Correlation of tuberculosis with Patient's clinic-pathological parameters

3.3.1 Age

All 300 patients included both females and male encountered during the sample collection. The age of TB patients ranged from 5 to 85 years. Less than or equal to 25 years with 30 case (10%), followed by 25-50 years with 180 cases (60%) and 90 cases with 50-85years (30%). Most of the patients had their age between 35 to 60 years and account for 56% of total number of patients.

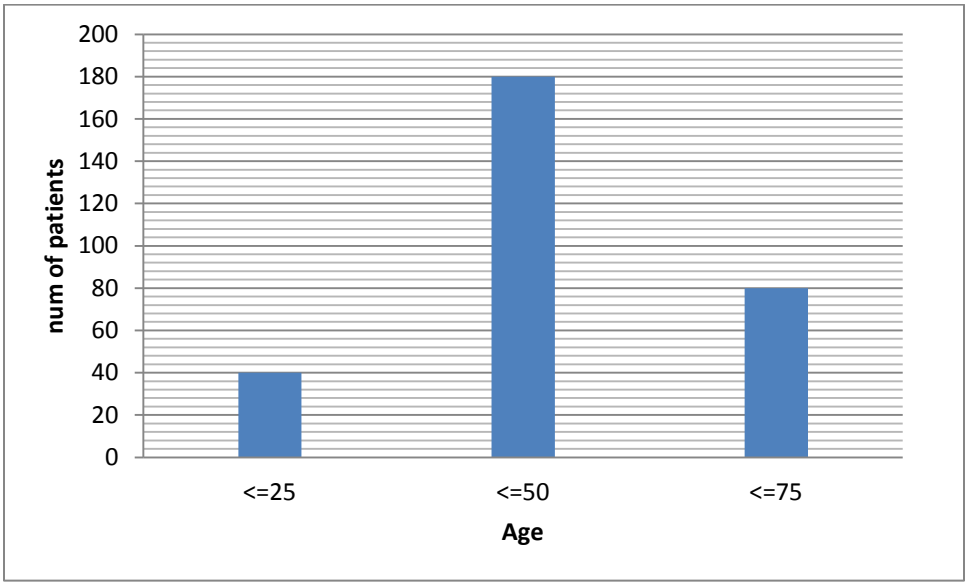

Figure 3.1: Bar chart showing the number of patients in correlation to age

3.3.2 Stage of tuberculosis

As mentioned in table above, 123 out of 300 (41%) patients were at initial stage and 177 out of 300(51%) were at the stage of well developed TB. Distribution of patients according to their age compared with diagnosed stage of Tuberculosis is mentioned in table below. There was significant correlation found between age and stage of tuberculosis.

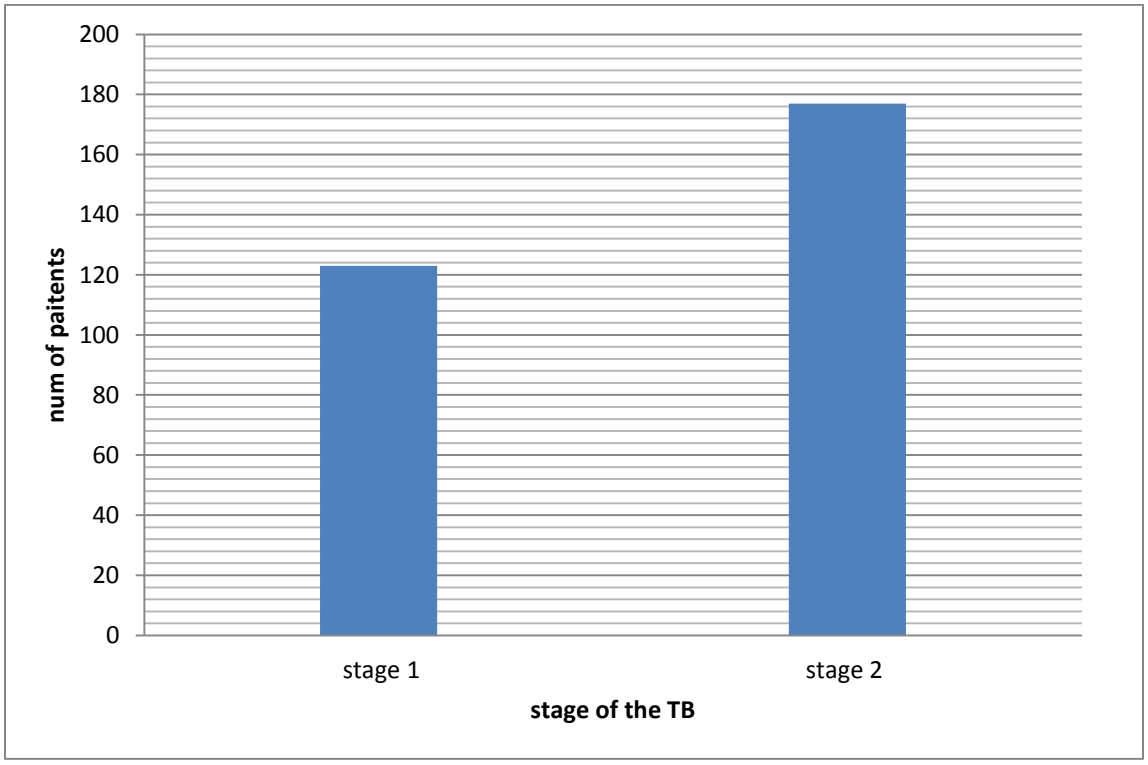

Figure 3.2: Bar chart showing the number of patients in correlation to stages of patients

3.3.3 Gender and tuberculosis

All 300 patients included both females and male encountered during the sample collection. As mentioned in table above, 114 out of 300 (38%) patients were male and 186 out of 300(62%)were female . Distribution of patients according to their gender is compared and is mentioned in table below. There was significant correlation found between gender and tuberculosis.

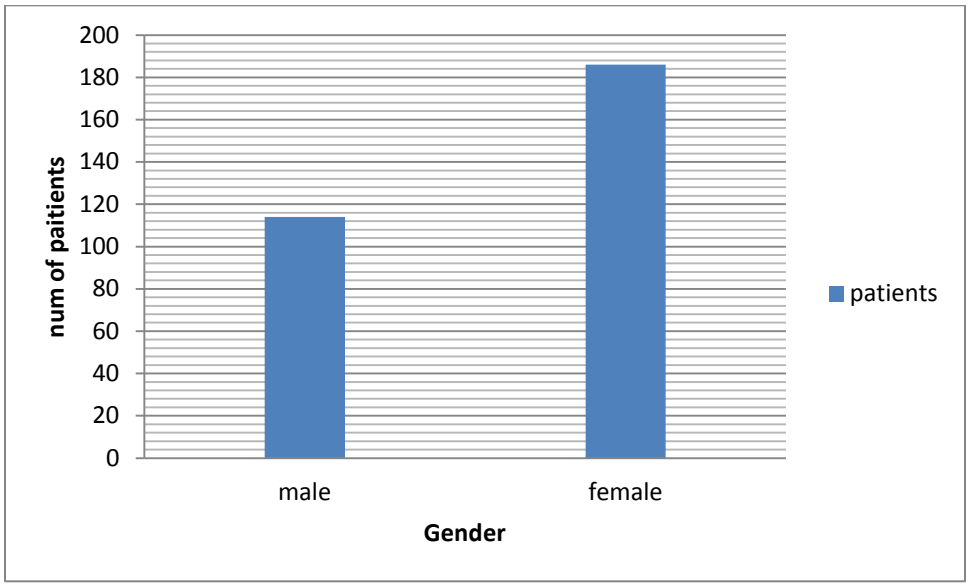

Figure 3.3: Bar chart showing the number of patients in correlation to gender

3.3.4 Site of infection and TB

As mentioned in table above, 235 out of 300 (78%) patients were having pulmonary TB only as it is the primary site of infection for this bacterium and 65 out of 300(22%) were having extra pulmonary TB as it invaded nearby tissues via lymph fluid and blood and is well developed TB. Distribution of patients according to their site of infection of Tuberculosis is mentioned in table below. There was significant correlation found between age and site of infection of tuberculosis. And also it can be inferred that primary/pulmonary infection in more common than extra-pulmonary TB in Pakistani population.

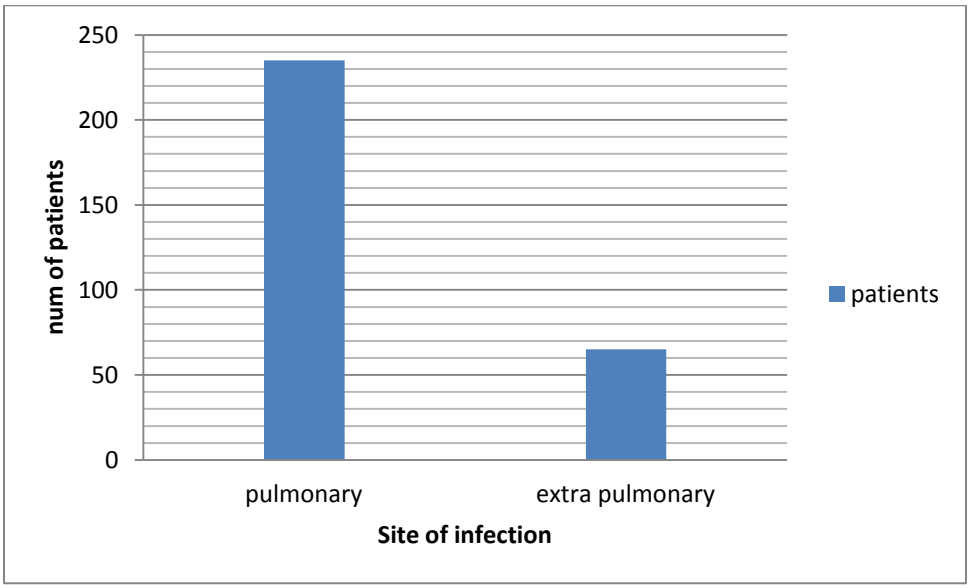

Figure 3.4: Bar chart showing the number of patients in correlation to site of infection

3.3.5 Smoking and tuberculosis

Smoking is considered to be the most important factors that contributes in the progression of TB and it can also be figured out as mentioned in the above table that 196 out of 300(65%) patients were smoker and 104 out of 300(34%) were non-smokers. From this we can infer that there is significant correlation between smoking progression and TB prognosis, this can also be seen from below graph.

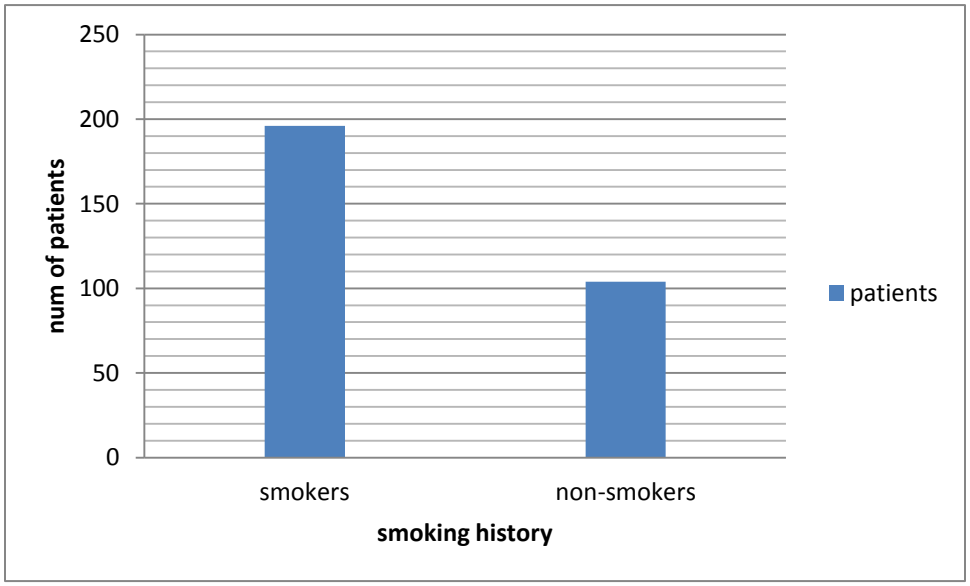

Figure 3.5: Bar chart showing the number of patients in correlation to smoking

3.3.6 Living standard and TB

Living standard also proved to have affected on the TB prognosis. As the people living in a closed, poor and close connection with TB patients will have more chance to get TB as compared with the person who lives in open, clean and airy environment. As it can also be inferred that 170 out of 300(56%) patients were living in poor conditions and 130 out of 300(43%) patients were living in good conditions . so from this we can find a significant relation between living standard and TB.

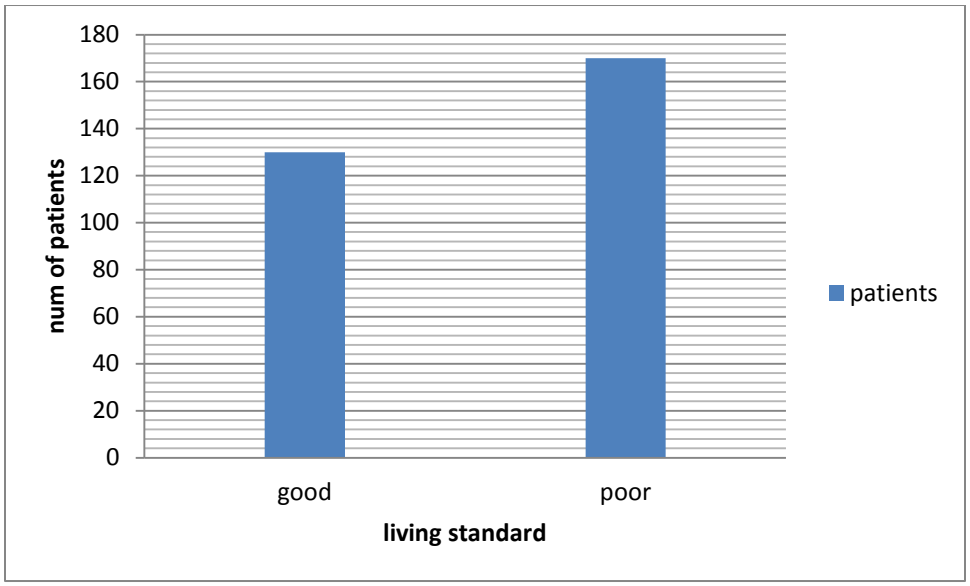

Figure 3.6: Bar chart showing the number of patients in correlation to living conditions.

3.3.7 Marital status and TB

Marital status also contributes in spreading the TB. As mentioned in above table, 230 out of 300(76%) were married and 70 out of 300(23%) were un-married. So we can some-how compare the marital status with the spread of TB. As mentioned below

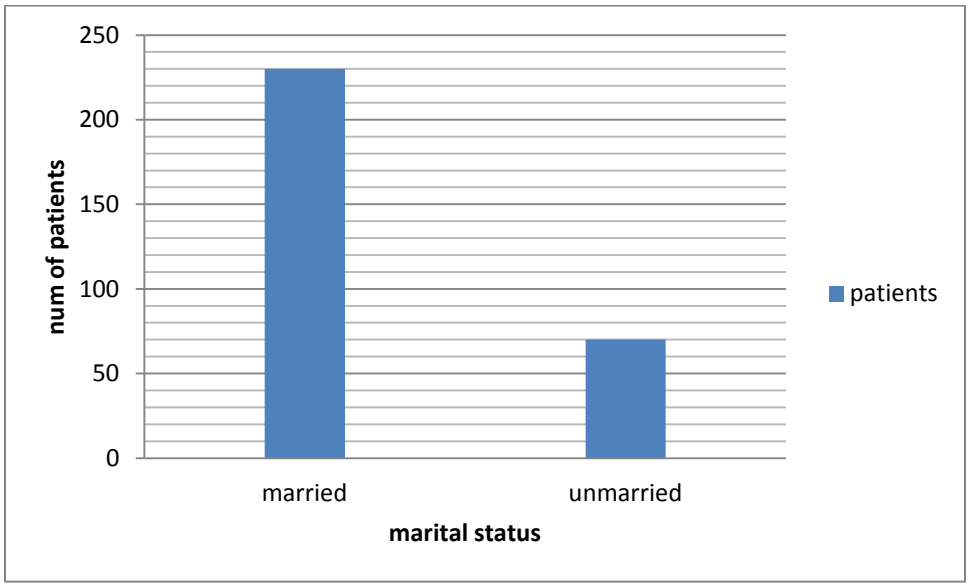

Figure 3.7: Bar chart showing the number of patients in correlation to marital status.

3.3.8 Vitamin D and TB

Vitamin D concentration in blood also plays an important role in the prognosis of TB. Lower the concentration of vitamin D in blood will have more chance to get TB as compared to the people who have normal concentration of vitamin D concentration in blood as vitamin D plays important role in preventing the TB prognosis. This can also be inferred from the data collected that 163 out of 300(54%) were the patients with low level of vitamin D in their blood and 137 out of 300(46%) were the patients with normal vitamin D concentration in their blood as mentioned below.

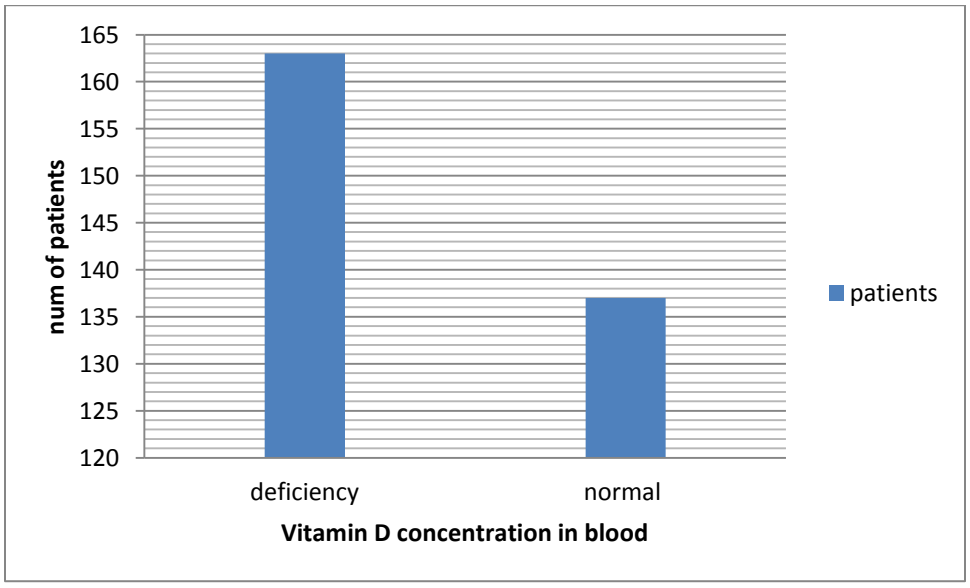

Figure 3.8: Bar chart showing the number of patients in correlation to vitamin D concentration in blood.

3.4: DNA Extraction for *Mycobacterium tuberculosis* screening

DNA extraction of 300 blood/ serum samples along with some adjacent normal control blood/ serum samples of patients was carried out by using GF-1 bacterial extraction kit. Extracted DNA was diluted to 50ng /µl. banding patterns of extracted DNA of diseased (TB) and normal samples on 1% agarose is shown in figure below

Figure 3.9: Ethidium bromide stained 1% agarose gel Electropherogram showing extracted DNA from TB samples.

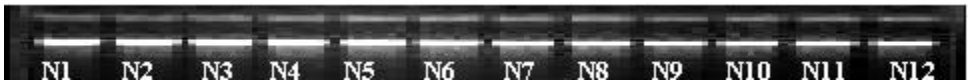

Figure 3.10: Electropherogram showing ethidium bromide stained 1% agarose gel showing extracted DNA from TB samples.

3.5: PCR amplification for diagnosis of TB

Polymerase chain reaction was performed to amplify the desired target regions of the *Mycobacterium tuberculosis* so that one can identify and diagnose the disease. To confirm the presence or absence of pathogen, amplified products were observed on 2% agarose gel. The DNA was amplified using a designed set of primers. Banding patterns of all these exon were observed on 2% agarose gels as shown in figures.

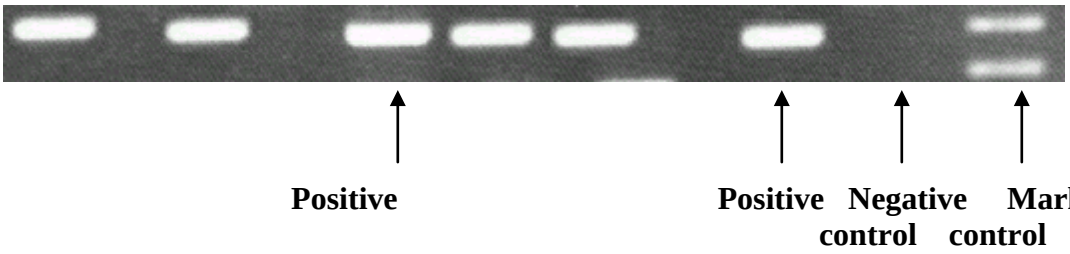

Figure 3.11: Showing the positive diagnosed patients of TB

The 2%gel picture is showing the positive control sample, negative control sample along with the patients PCR samples, and a marker which is used to confirm the presence of the *Mycobacterium tuberculosis* in the sample. In the above gel 8 samples were loaded onto the gel and after electrophoresis we have got the results in which 5 samples were positive with TB whereby 3 samples were negative; as shown above.

Figure 3.12: Showing the negative diagnosed patients of TB

In this above gel electrograph there are 5 people with positive results and confirm the presence of TB. On the contrary only 2 people are negative for the test of tuberculosis which confirms the absence of *Mycobacterium tuberculosis* in them.

3.6 *Insilico* study of the genes of *Mycobacterium tuberculosis* involved in the drug resistance

There are many genes of *Mycobacterium* which are involved in pathogenesis of the disease but some genes are very critical as they are targeted action sites for some drugs which are used to treat this disease. Somehow mutations in these pockets (targeted drug binding sites) caused bacteria to attain the resistance drug to which the drug becomes ineffective and a bacterium attains drug resistance.

So to make drug effective here I have studies the genes that are crucially important in drug resistance against certain important drugs like isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin etc. these drugs have potential binding sites on the genes due to which they adhere and perform its function by blocking the function of the gene itself. But if the bacterium mutates its sequence of genes due to certain environmental factors then the drug will be no more effective against the disease as there will be no binding site for the drug due to the mutation in the binding site.

There are many genes that are considered to be potential but here we are mentioning some most important gene with reference to the drug action as these genes serves the binding site for the drugs.

Table 3.2: Genes involved in drug resistance

Gene	Drug	Gene function	Role
KatG	Isoniazid	Catalaste-peroxidase,	Pro-drug conversation
inhA	isoniazid	ACP reductase	Drug target
rpoB	rifampicin	Sub unit of RNA polymerase	Drug target
pncA	pyrazinamide	pyrazinamidase	Pro-drug converstion
embB	ethambutol	Arabinosyl transferase	Drug target
rrs	streptomycin	16s ribosomal RNA	Drug target
gidB	streptomycin	rRna methyl transferase	Drug target
tlyA	capreomycin	methyltransferase	Drug target
gyrA/gyrB	quinolones	DNA gyrase subunits	Drug target
thyA	PAS	Thymidylate synthase	Drug activation

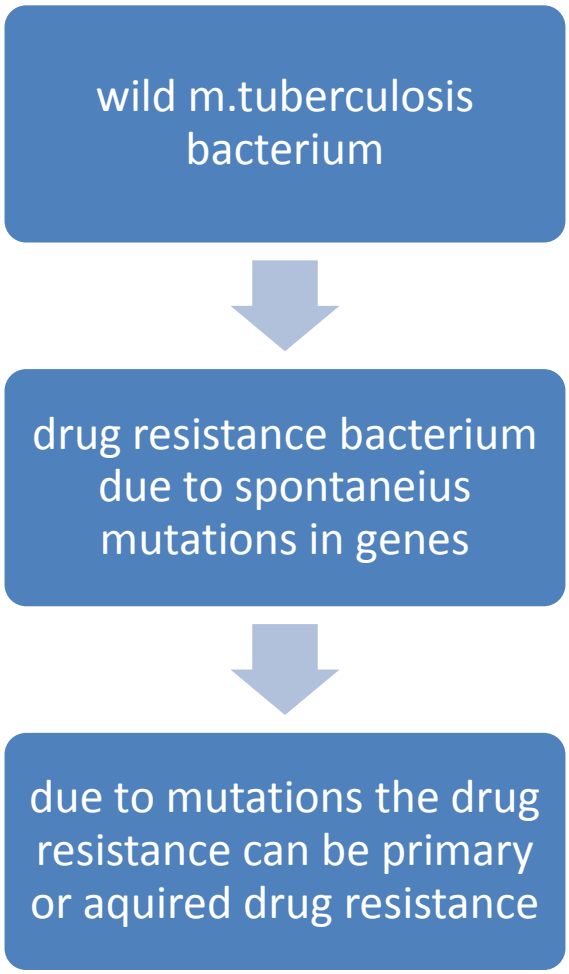

Figure 3.13: Flowchart showing that how wild type *M.tuberculosis* changed into resistant type (Zhang and Yew, 2009).

Some of the genes are discussed here

3.6.1 Kat G

3.6.1.1 DNA sequence

>gi|126256233|gb|EF421712.1| *Mycobacterium* sp. DSM 3803 FurA (furA) and KatG (katG) genes, complete cds

3.6.1.2 Protein sequence

>gi|126256235|gb|ABO09797.1| KatG [*Mycobacterium* sp. DSM 3803]
MSSDTSDSRPPYPNEATASRSESENPAIASPTPKAHAPLTNQDWWPDQIDVSRLH
PHSEQANPLGADFDYAAEFAKLDVDALKADLLALMTQSQDWWPADYGHYGGL
FIRMSWHAAGTYRIFDGRGGGGQGMQRFAPLNSWPDNANLDKARRLLWPVKQ
KYGNKISWADLLVFAGNVALESMGFKTFGFGFGRPDVWEPEEILFGEEDTWLGT
DKRYAGRRELAQPYGATTMGLIYVNPEGPEGQPDPVAAAHDIRETFGRMAMND
EETAALIVGGHTFGKTHGAGDADLVGPEPEAAPIEQQGLGWKSSYGTGKGKDAI
TSGLEVVWTPTPTAWDNSFLETLYGYEWELTKSPAGAWQFTAKDGAGAGTIPD
PFDGPGRAPTMLVTDISMRVDPIYGPITRRWLDHPDELADAFAKAWYKLLHRD
MGPISRYLGPWVAEPQLWQDPVPPVDHELVDDRDIAALKRQVLESGLSVPQLVK
TAWAAAASYRNTDKRGGANGARIRLEPQKNWEVNEPAELAKALPVLEQIQQDF
NASAPGGKKISLADLIVLAGAAAVEKAAGDAGHDVTVPFTPGRTDATQENTDVE
SFAVLEPRADGFRNYVRPGEKTPLEKLLLERAYFLGVTAPELTVLIGGLRAMGA
NHGGSKHGVFTDRPGTLTTDFFRNVVDMGIEWKASETSENVYEGHDRASGTPK
WTATANDLVFGSHSVLRALAEVYAQSDAEDRFVRDFVRAWDKVMNNDRFDLK

3.6.1.3 Domain involved

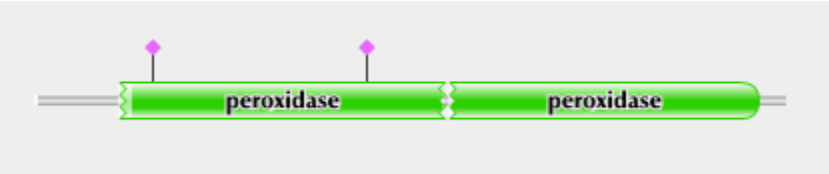

Figure 3.14: Showing domain of katG gene that is involved in drug resistance.

3.6.2 inhA

3.4.2.1 DNA sequence

>gi|66737266|gb|DQ056349.1| *Mycobacterium tuberculosis* strain CNFD04010 InhA (inhA) gene, complete cds

3.6.2.2 Protein sequence

>gi|66737267|gb|AAY54545.1| InhA [*Mycobacterium tuberculosis*]
MTGLLDGKRILVSGIITDSSIAFHIARVAQEQGAQLVLTGFDRLRLIQRITDRLPAK
APLLELDVQNEEHLASLAGRVTEAIGAGNKLDGVVHSIGFMPQTGMGINPFFDA
PYADVSKGIHISAYSYASMAKALLPIMNPGGSIVGMDFDPSRAMPAYNWMTVA
KSALESVNRFVAREAGKYGVRSNLVAAGPIRTLAMSAIVGGALGEEAGAQIQLL
EEGWDQRAPIGWNMKDATPVAKTVCALLSDWLPATTGDILYADGGAHTQLL

3.6.2.3 Domain involved

Figure 3.15: Showing domain of inh A gene that is involved in drug resistance.

3.6.3 rpoB

3.6.3.1 DNA sequence

>gi|468333|gb|L27989.1|MSGRPOB *Mycobacterium tuberculosis* RNA polymerase beta-subunit (rpoB) gene, complete cds and RNA polymerase beta'-subunit rpoC gene, partial cds

3.6.3.2 Protein sequence

>gi|468334|gb|AAA21416.1| RNA polymerase beta-subunit [*Mycobacterium tuberculosis*]
MLEGCILADSRQSKTAASPSPSRPQSSSNNSVPGAPNRVSFAKLREPLEVPGLLDV
QTDSFEWLIGSPRWRESAAERGDVNPVGGLEEVLYELSPIEDFSGSMSLSFSDPRF

DDVKAPVDECKDKDMTYAAPLFVTAEFINNNTGEIKSQTVFMGDFPMMTEKGT
FIINGTERVVVSQLVRSPGVYFDETIDKSTDKTLHSVKVIPSRGAWLEFDVDKRD
TVGVRIDRKRRQPVTVLLKALGWTSEQIVERFGFSEIMRSTLEKDNTVGTDEALL
DIYRKLRPGEPPTKESAQTLLENLFFKEKRYDLARVGRYKVNKKLGLHVGEPITS
STLTEEDVVATIEYLVRLHEGQTTMTVPGGVEVPVETDDIDHFGNRRLRTVGELI
QNQIRVGMSRMERVVRERMTTQDVEAITPQTLINIRPVVAAIKEFFGTSQLSQFM
DQNNPLSGLTHKRRLSALGPGGLSRERAGLEVRDVHPSHYGRMCPIETPEGPNIG
LIGSLSVYARVNPFGFIETPYRKVVDGVVSDEIVYLTADEEDRHVVAQANSPIDA
DGRFVEPRVLVRRKAGEVEYVPSSEVDYMDVSPRQMVSVATAMIPFLEHDDAN
RALMGANMQRQAVPLVRSEAPLVGTGMELRAAIDAATSSSQESGVIEEVSADYI
TVMHDNGTRRTYRMRKFARSNHGTCANQCPIVDAGDRVEAGQVIADGPCTDD
GEMALGKNLLVAIMPWEGHNYEDAIILSNRLVEEDVLTSIHIEEHEIDARDTKLG
AEEITRDIPNISDEVLADLDERGIVRIGAEVRDGDILVGKVTPKGETELTPEERLLR
AIFGEKAREVRDTSLKVPHGESGKVIGIRVFSREDEDELPAGVNELVRVYVAQKR
KISDGDKLAGRHGNKGVIGKILPVEDMPFLADGTPVDIILNTHGVPRRMNIGQILE
THLGWCAHSGWKVDAAKGVPDWAARLPDELLEAHANAIVSTPVFDGAQEAEL
QGLLSCTLPNRDGDVLVDADGKAMLFDGRSGEPFPYPVTVGYMYIMKLHHLVD
DKIHARSTGPYSMITQQPLGGKAQFGGQRFGEMECWAMQAYGAAYTLQELLTI
KSDDTVGRVKVYEAIVKGENIPEPGIPESFKVLLKELQSLCLNVEVLSSDGAAIEL
REGEDEDLERAAANLGINLSRNESASFEDLA

3.6.3.3 Domain involved

Figure 3.16: Showing domain of rpoB gene that is involved in drug resistance.

3.6.4 pnc A

3.6.4.1 DNA sequence

>gi|367464928|gb|JN416585.1| *Mycobacterium tuberculosis* strain 0902 pyrazinamidase (pncA) gene, complete cds

3.6.4.2 Protein sequence

>gi|367464929|gb|AEX15262.1| pyrazinamidase [*Mycobacterium tuberculosis*]
MRALIIVDVQNDFCEGGSLAVTGGAALARAISDYLAEAADYHHVVATKDFHIDP
GDHFSGTPDYSSSWPPHCVSGTPGADFHPSLDTSAIEAVFYKGAYTGAYSGFEGV
DENGTPLLNWLRQRGVDEVDVVGIATDHCVRQTAEDAVRNGLATRVLVDLTA
GVSADTTVAALEEMRTACVELVCSS

3.6.4.3 Domain involved

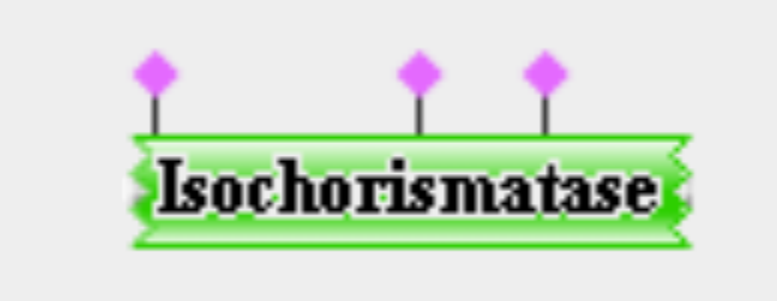

Figure 3.17: Showing domain of pncA gene that is involved in drug resistance.

3.6.5 embB

3.6.5.1 DNA sequence

>gi|148562394|gb|EF434318.1| *Mycobacterium tuberculosis* isolate MTB-21 EmbB (embB) gene, partial cds

3.6.5.2 Protein sequence

>gi|148562395|gb|ABQ88328.1| EmbB *[Mycobacterium tuberculosis*]

AGYMSKYFRWFGSPEDPFGWYYNLLALMTHVSDASLWMRLPDLAAGLVCWLL
LSRAVLPRLGPAVEARKPAYWAAAMVLLTAWMQFNNGLRPEGIIALGSLVTYV
LIERSMRYSRLTPAALAVVTAAFTLGVQPTGLIAVAALVAGACPMLRIL

3.6.5.3 Domain involved

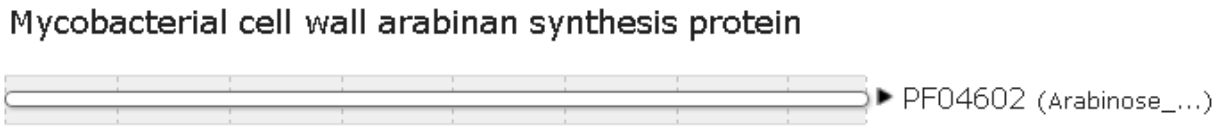

Figure 3.18: Showing domain of embB gene that is involved in drug resistance.

3.6.6 Rrs

3.6.6.1 DNA sequence

>gi|401871226|gb|JX289820.1| *Mycobacterium tuberculosis* strain 46 16S ribosomal RNA (rrs) gene, partial sequence

3.6.6.2 Domain involved

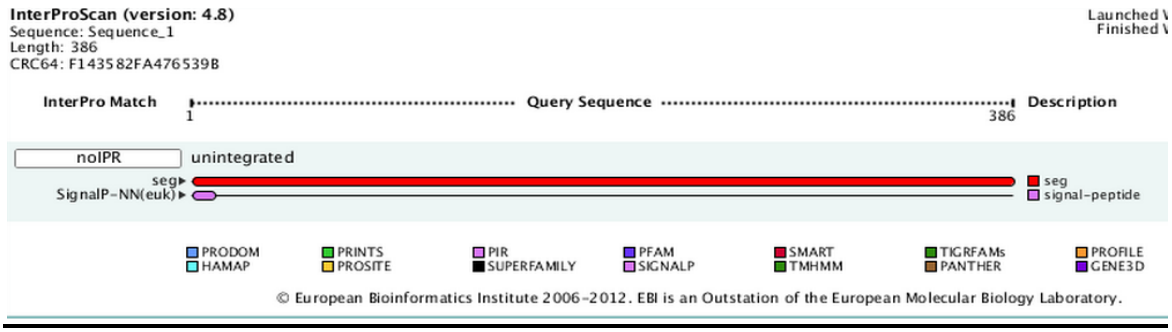

Figure 3.19: Showing domain of Rrs gene that is involved in drug resistance.

3.6.7 gidB

3.6.7.1 DNA sequence

>gi|315420684|gb|HQ611146.1| *Mycobacterium tuberculosis* strain MP 9 7-methylguanosine methyltransferase (gidB) gene, complete cds

3.6.7.2 Protein sequence

>gi|315420685|gb|ADU15875.1| 7-methylguanosine methyltransferase [*Mycobacterium tuberculosis*]

MSPIEPAASAIFGPRLGLARRYAEALAGPGVERGLVGPREVGRLWDRHLLNCAVI
GELLERGDRVVDIGRGAGLPGVPLAIARPDLQVVLLEPLLRRTEFLREMVTDLGV
AVEIVRGRAEESWVQDQLGGSDAAVSRAVAALDKLTKWSMPLIRPNGRMLAIK
GERAHDEVREHRRVMIASGAVDVRVVTCGANYLRPPATVVFARRGKQIARGSA
RMASGGTA

3.6.7.3 Domain involved

Figure 3.20: Showing domain of gidB gene that is involved in drug resistance.

3.6.8 TlyA

3.6.8.1 DNA sequence

>gi|2370316|emb|X98295.1| M.tuberculosis TlyA gene

3.6.8.2 Protein sequence

>gi|2370317|emb|CAA66941.1| cytotoxin /haemolysin homologue [*Mycobacterium tuberculosis*]

MARRARVDAELVRRGLARSRQQAAELIGAGKVRIDGLPAVKPATAVSDTTALT
VVTDSERAWVSRGAHKLVGALEAFAIAVAGRRCLDAGASTGGFTEVLLDRGAA
HVVAADVGYGQLAWSLRNDPRVVVLERTNARGLTPEAIGGRVDLVVADLSFISL
ATVLPALVGCASRDADIVPLVKPQFEVGKGQVGPGGVVHDPQLRARSVLAVAR
RAQELGWHSVGVKASPLPGPSGNVEYFLWLRTQTDRALSAKGLEDAVHRAISE
GP

3.6.8.3 Domain involved

Figure 3.21: Showing domain of tlyA gene that is involved in drug resistance.

3.6.9 gyrA

3.6.9.1 DNA sequence

>gi|378787159|gb|JQ699172.1| *Mycobacterium tuberculosis* clone 829A GyrA (gyrA) gene, complete cds

3.6.9.2 Protein sequence

>gi|378787160|gb|AFC39819.1| GyrA [*Mycobacterium tuberculosis*]
MTDTTLPPDDSLDRIEPVDIQQEMQRSYIDYAMSVIVGRALPEVRDGLKPVHRRV
LYAMFDSGFRPDRSHAKSARSVAETMGNYHPHGDASIYDTLVRMAQPWSLRYP
LVDGQGNFGSPGNDPPAAMRYTEARLTPLAMEMLREIDEETVDFIPNYDGRVQE
PTVLPSRFPNLLANGSGGIAVGMATNIPPHNLRELADAVFWALENHDADEEETL
AAVMGRVKGPDFPTAGLIVGSQGTADAYKTGRGSIRMRGVVEVEEDSRGRTSL
VITELPYQVNHDNFITSIAEQVRDGKLAGISNIEDQSSDRVGLRIVIEIKRDAVAKV
VINNLYKHTQLQTSFGANMLAIVDGVPRTLRLDQLIRYYVDHQLDVIVRRTTYR

LRKANERAHILRGLVKALDALDEVIALIRASETVDIARAGLIELLDIDEIQAQAILD
MQLRRLAALERQRIIDDLAKIEAEIADLEDILAKPERQRGIVRDELAEIVDRHGDD
RRTRIIAADGDVSDEDLIAREDVVVTITETGYAKRTKTDLYRSQKRGGKGVQGA
GLKQDDIVAHFFVCSTHDLILFFTTQGRVYRAKAYDLPEASRTARGQHVANLLA
FQPEERIAQVIQIRGYTDAPYLVLATRNGLVKKSKLTDFDSNRSGGIVAVNLRDN
DELVGAVLCSADDDLLLVSANGQSIRFSATDEALRPMGRATSGVQGMRFNIDDR
LLSLNVVREGTYLLVATSGGYAKRTAIEEYPVQGRGGKGVLTVMYDRRRGRLV
GALIVDDDSELYAVTSGGGVIRTAARQVRKAGRQTKGVRLMNLGEGDTLLAIAR
NAEESGDDNAVDANGADQTGN

3.6.9.3 Domain involved

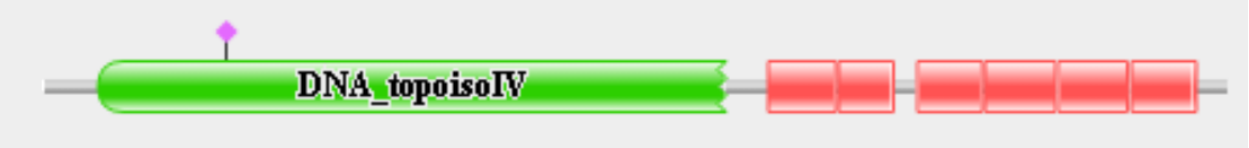

Figure 3.22: Showing domain of GyrA gene that is involved in drug resistance.

3.6.10 gyrB

3.6.10.1 DNA sequence

>gi|474434|emb|X78888.1| M.tuberculosis gyrB gene

3.6.10.2 Protein sequence

>gi|474435|emb|CAA55486.1| DNA gyrase subunit B [*Mycobacterium tuberculosis* H37Ra]
MGKNEARRSALAPDHGTVVCDPLRRLNRMHATPEESIRIVAAQKKKAQDEYGA
ASITILEGLEAVRKRPGMYIGSTGERGLHHLIWEVVDNAVDEAMAGYATTVNVV
LLEDGGVEVADDGRGIPVATHASGIPTVDVVMTQLHAGGKFDSDAYAISGGLHG

VGVSVVNALSTRLEVEIKRDGYEWSQVYEKSEPLGLKQGAPTKKTGSTVRFWA
DPAVFETTEYDFETVARRLQEMAFLNKGLTINLTDERVTQDEVVDEVVSDVAEA
PKSASERAAESTAPHKVKSRTFHYPGGLVDFVKHINRTKNAIHSSIVDFSGKGTG
HEVEIAMQWNAGYSESVHTFANTINTH
EGGTHEEGFRSALTSVVNKYAKDRKLLKDKDPNLTGDDIREGLAAVISVKVSEP
QFEGQTKTKLGNTEVKSFVQKVCNEQLTHWFEANPTDAKVVVNKAVSSAQARI
AARKARELVRRKSATDIGGLPGKLADCRSTDPRKSELYVVEGDSAGGSAKSGRD
SMFQAILPLRGKIINVEKARIDRVLKNTEVQAIITALGTGIHDEFDIGKLRYHKIVL
MADADVDGQHISTLLLTLLFRFMRPLIENGHVFLAQPPLYKLKWQRSDPEFAYS
DRERDGLLEAGLKAGKKINKEDGIQRYKGLGEMDAKELWETTMDPSVRVLRQV
TLDDAAAADELFSILMGEDVDARRSFITRNAKDVRFLDV

3.6.10.3 Domain involved

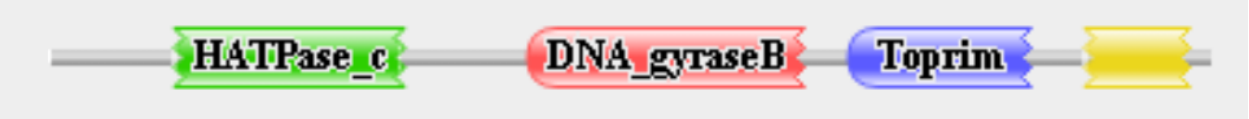

Figure 3.23: Showing domain of gyrB gene that is involved in drug resistance.

3.6.11 ethA

3.6.11.1 DNA sequence

>gi|317457547|gb|HM587471.1| *Mycobacterium tuberculosis* strain G124D-A199V monooxygenase EthA (ethA) gene, complete cds

3.6.11.2 Protein sequence

>gi|317457548|gb|ADV29792.1| monooxygenase EthA [*Mycobacterium tuberculosis*]
MTEHLDVVIVGAGISGVSAAWHLQDRCPTKSYAILEKRESMGGTWDLFRYPGIR
SDSDMYTLGFRFRPWTGRQAIADGKPILEYVKSTAAMYGIDRHIRFHHKVISAD
WSTAENRWTVHIQSHDTLSALTCEFLFLCSGYYNYDEGYSPRFAGSEDFVGPIIH

PQHWPEDLDYDAKNIVVIGSGATAVTLVPALADSGVKHVTMLQRSPTYIVSQPD
RDGIAEKLNRWLPETMAYTAVRWKNVLRQAAVYSACQKWPRRMRKMFLSLIQ
RQLPEGYDVRKHFGPHYNPWDQRLCLVPNGDLFRAIRHGKVEVVTDTIERFTAT
GIRLNSGRELPADIIITATGLNLQLFGGATATIDGQQVDITTTMAYKGMMLSGIPN
MAYTVGYTNASWTLKADLVSEFVCRLLNYMDDNGFDTVVVERPGSDVEERPF
MEFTPGYVLRSLDELPKQGSRTPWRLNQNYLRDIRLIRRGKIDDEGLRFAKRPAP
VGV

3.6.11.3 Domain involved

Figure 3.24: Showing domain of ethA gene that is involved in drug resistance.

3.6.12 thyA

3.6.12.1 DNA sequence

>gi|44681|emb|X59273.1| *Mycobacterium tuberculosis* thyA gene (putative) for thymidilate synthase, and ORF (homologous to unidentified ORF adjacent to dnaB in Bacillus subtilus [BSDNAB2])

3.6.12.2 Protein sequence

>gi|581380|emb|CAA41963.1| thymidylate synthase [*Mycobacterium tuberculosis*]
MSAGGVTKDVNIVFRLASLPMGSEAMALLRLPLVLPAVQIAGRIVGQGHRYHQL
GARPAQCAAHFGRPARADGFCGVRPLRQWWTGGGRWSPCWTDAISTEAIPVQII
WGTKDVVLPVRHAHMALPPSGLAIGDFRGLGTFPVSRRPCALHRHRRTLHGHTE
PAEYDQAALRCASPGWRRTTVTGSADTRVAVLNAIGSNERSATLITTGSVRALP

QVVQSGHGIEVSVLRVFTDSDGNFGNPLGVINASKVEHRDRQQLAAQSGYSETIF
VDLPSPGSTTAHATIHTPRTEIPFAGHPTVGASWWLRERGTPINTLQVPAGIVQVS
YHGDLTAISARSEWAPEFAIHDLDSLDALAAADPADFPDDIAHYLWTWTDRSAG
SLRARMFAANLGVTEDEATGAAAIRITDYLSRDLTITQGKGSLIHTTWSPEGWVR
VAGRVVSDGVAQLD

3.6.12.3 Domain involved

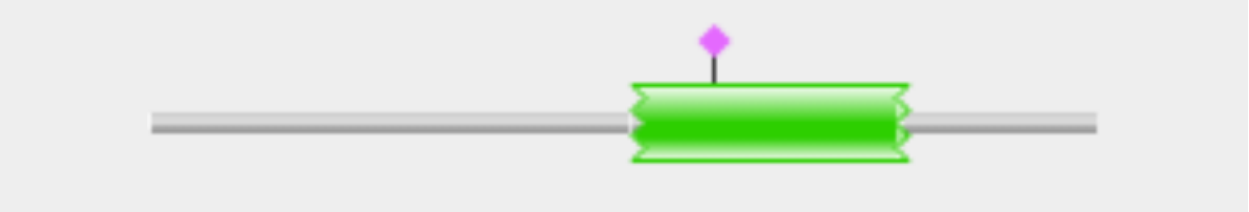

Figure 3.25: Showing domain of thyA gene that is involved in drug resistance.

Chapter 4

Discussion

Tuberculosis thought to be the historical infectious disease which can be spread easily but can be prevented easily by using simple preventive methods and with the use of proper medicine at regular basis. In early era it was considered to be the deadly disease due to its high mortality rate (Lienhardta *et al*., 2010). Geographically Asia is imposing most of the burden on all the TB cases in the world as only India and China accounts for about 40% of the entire TB cases world wide. Pakistan is ranked at 6^{th} position according to the survey of World Health Organization (Javaid *et al*., 2010). Doctors and researching are finding the ways to eradicate this disease but the major problem which they are facing is the drug resistance due to which there is 60% increase in the TB cases. Mortality and incidence rate in the top ranked 22 high burden countries also decreased which were considered to account for 80% of the total TB cases (Ren *et al*., 2012).

Basically *Mycobacterium* is divided into two major groups i.e. slow growing species of *Mycobacterium* these are well known pathogens of humans(*Mycobacterium tuberculosis, Mycobacterium leprae, Mycobacterium bovis*) where by other group of *Mycobacterium* is fast growing these are non pathogenic (*Mycobacterium smegmattis*) . There are many species of *Mycobacterium* which cause disease but if we talk about humans then most common is tuberculosis but other disease can also prevailed as leprosy (Britton & Lockwood, 2004).

There are many factors that act as risk factors and increase the probability of having TB like diabetes, vitamin D deficiency, living conditions, tobacco, HIV/AIDS, immunity of person, age and sex and many other factors (Narasimhan *et al*., 2013). But the most important factors that increase the chances of getting TB are the use of tobacco, immunity of the person and the living conditions; which all depends on age and sex of the individual. Tobacco use is one of the major causes of TB which can also leads to the lung cancer (Slama *et al*., 2007). Tobacco in active and even in passive smoking is very harmful and can leads to TB. Diabetes also plays some role in the TB prognosis as it affects the treatment and cause severe glycaemia (Kelly and Richard, 2009).

Tuberculosis is the disease which is generally referred to the disease of lungs or the disease that is just localized to lungs; but tuberculosis is classified into two groups that is the primary/ pulmonary tuberculosis which is just localized to the lungs and disrupts the

alveoli and cause disease it accounts for about 80% of all the TB cases (Hunter, 2011). Whereby the other type of tuberculosis is extra pulmonary tuberculosis in this type of TB the bacterium overcomes the macrophages and spread to the nearby tissues via lymph nodes and blood stream and invades the distant tissue. It accounts for 40% of all the TB cases (Mazza, 2012).Extrapulmonary tuberculosis can be sub categorize on the basis of region which it is invading and causing diseases. On the basis of region specificity it is more prevalent in the form of lymphadenitis, pleuritis and osteoarticular TB. Where by peritoneal, urogenetial or meningeal TB are less prevalent forms of TB (Neelakantan *et al*., 2013).These site of infections are difficult to detect because of the difficult methods that are involved in the diagnostics of these forms of TB.

If we talk about the diagnostic conditions of the disease in Pakistan then the most common diagnostic method that is conducted is the sputum test in which the sputum is cultured and checked for the bacterium (Kumar *et al*., 2007). After the sputum test another important test is micro-dot test in which we look for the antigen and antibody interaction. For molecular analysis PCR (polymerase chain reaction) is done which is the confirm proof for the absence and presence of the bacterium. And then depending upon the qualitative analysis of the sample the patient is given a certain set of treatment.

There are different drugs that are used to treat tuberculosis but some how due to the presence of different environmental conditions the drug became ineffective and bacterium becomes drug- resistant towards many drugs (Seung and Linton, 2013). And this resistance towards single drug or can be towards multiple drugs of the tuberculosis leads to the multi-drug resistant bacteria. Major drugs that are currently being used are Isoniazid, rifampicin, pyrazinamide, ethambutol, streptomycin, capreomycin, quinolones and PAS. Bacteria is also getting resistance due to different factors for which they leads to the spontaneous mutations in the drug targeted genes and the outcome of that mutation is the multi drug resistance (Zhang and Yew, 2009). Due to which the patient doesn't recovers from this disease and finally dies.

Another important factor in the disease is vaccination. Due to the inefficient performance gained by the drug therapy; researchers, scientist and doctors start looking at an alternative to stop the huge effect of *Mycobacterium* tuberculosis.Bacille Calmette Guerin (BCG) is the current vaccine that is used for the protection against TB (Fine *et al*.,

1999). A French scientist albert Calmette along with his colleagues working at institute of Pasteur de lille in 1908; For the very first time it was used in 1921. In children's its one and only most effective shield against this *Mycobacterium* (WHO) Pakistan introduced BCG immunization in 1948 (Javaid *et al*., 2010). But there are again many factors which in turn reduce the efficiency of vaccine like genetic variation in BCG vaccine or population is the major hindrance that is limiting the affect of vaccine.

With all these things keeping in mind if we want to make revolution in terms of drugs to be effective on an individual then we must have and adopt for alternative and that is to make our drugs to be more powerful so that it can stop the growth of bacterium inside the human body. For this purpose in this thesis I have done the Insilco analysis of the genes that are targeted for the drug action. Major genes that are chosen for this analysis are the targeted sites for the drugs; but mutation due to any environmental factors leads towards the drug resistance and the drug becomes ineffective. So to overcome this problem the analysis is done in which I have found the domains that are targeted for the drug action and major of them are drug binding sites. If there is mutation in these sites then there will be a change due to which the drug will not bind with that domain and there will be no effect of that drug to be seen. But on the other side if there is no problem/ mutation in the targeted site then the drug will bind there efficiently and will perform its function. So if we want to overcome that problem then we have to insert some changes in the drug so that it can bind efficiently and perform its function.

Chapter 5

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