Dedications

To my parents
Who surrounded me by their warm heart and care, for their continuous giving

To my dear brother and sisters
Whom I love, always thanks God for giving me them, for their kind and real love

To my reverend Dr. M.E. Hamid
Who taught us how the man can be human and how the teacher can be prophet

To all my dear friends
For sharing me the worst moments and for extending hand of help to me
Special Dedication

To

My lovely rose

Rawan

Asking God to bless her
Acknowledgements

First of all, all praise be to Allah the most merciful, who gave me the ability and strength to complete this work.

I am grateful to my supervisor Dr. Nageeb Suliman Seed, Department of Microbiology, Faculty of Medicine, University of Khartoum, for his valuable guidance and his grateful help and without his advices this work would have been difficult to do.

I deem it a privilege in expressing my profound and cardinal gratitude to Dr. Mohamed Elamin Hamid, Department of Preventive Medicine, Faculty of Veterinary Medicine, university of Khartoum, for his kind help, precious devotion, encouraging suggestions, for patient and for valuable recommendations throughout this work.

Also I would like to thanks faculty of pharmacy university of Khartoum, first to give me the chance to register for MSc degree and to give me it's name. Thanks extended to Dr. El Amin E. El Neama the dean of the faculty of pharmacy university of Khartoum for his great help and for extending hand for help not only for me but also for all.

I would like to thank Professor M. Goodfellow of the University of Newcastle for help with sequencing nocardia strains.

Deep appreciation is extended to my family and to mama Khawla to my faithful friends Manahil, Nawal, Amani, Zinab, Safa, Khalid, Adil, Momen, and to the memory of my dear friend Hussam El-Mahdi and to many other friends who surrounded me by their real care and love, and always supported me to complete this work.

I am also very thankful to the International Atomic Energy Agency for supporting this work and providing the reagents and training (RAF 017), especially Baldip Khan, IAEA technical officer for giving me chance to do some of this work in Thailand. Thanks also go to Alexander Von Humbols for supporting our molecular laboratory with equipment.

I deeply thank the staff of the National Health Laboratory, especially members of the Sudan Molecular Tuberculosis Research Unit namely; Tarig, Ghada and Enaam for their help. I also thank Sudan Tuberculosis Reference laboratory staff, Asrar, Noha, Yasir, Aymin and all the rest for their great help in staining and culture and for allowing me to use their equipment and reagents.
Abstract

In Sudan as in many other countries the emerging drug resistant tuberculosis is causing a major public health threat. In the present study 250 sputum samples randomly collected from new tuberculosis patients were collected before administration of drugs from patients of different age groups of both sexes. The patients were from Khartoum Teaching Chest Hospital and Hamed El-Nile Hospital. The samples were collected during the period July 99 to May 2000. All studied patients were clinically diagnosed as tuberculosis cases. The samples were collected to study the primary drug resistance mutation in \textit{M. tuberculosis} complex.

201 out of the 250 samples (80.5\%) were found to have acid fast bacilli (AFB) on microscopy. 147 out of the 201 AFB positive (73.1\%) gave growth of \textit{M. tuberculosis} complex on Löwenstein Jensen medium (LJ) and three (6.1\%) out of 41 which were not (AFB). 17 of the isolates (8.5\%) were rapid grower mycobacteria other than tuberculosis (MOTT). Seven (3.5 \%) were \textit{Nocardia} sp. which were fully identified using biochemical test and molecular methods such as sequencing. The species was discovered to be a new entity and given a new name (\textit{Nocardia africana}) by the International Taxonomy for bacteria, and 14 (6.9\%) of the samples shown were contaminated cultures and 16 (7.9\%) of the samples did not show any growth.

The polymerase chain reaction (PCR), amplification of \textit{IS6110} and the Restriction Fragment Length polymorphism (RFLP)-fingerprinting using \textit{PVUII} enzyme was used to confirm the above phenotypic identify of the mycobacteria, using these techniques, 150 isolates were found to be \textit{M. tuberculosis} complex.

DNA from all the 150 \textit{M. tuberculosis} isolates was extracted and then amplified by PCR using specific primers (TR8, TR9) to amplify \textit{rpoB} gene around codon 43. Mutation in this gene is known to confer resistance to rifampicin. Single Strand Conformation Polymorphism (SSCP) technique was used to detect the presence of mutations among the 150 \textit{M. tuberculosis} strains. Only four strains showed mobility shift due to \textit{rpoB} mutant gene. This is indicative of primary drug resistance to rifampicin.

The analysis of \textit{rpsL} gene around codon 43 by PCR- RFLP to the 150 \textit{M. tuberculosis} strains revealed that only two isolates of \textit{M. tuberculosis} did not cut with the restriction enzyme \textit{MboII}. This indicates that these two strains have mutation at the \textit{rpsL} gene which is responsible for resistance to streptomycin.
The rate of primary mutation among the 150 *M. tuberculosis* strains isolated from new tuberculosis (received no treatment) was thus found to be 2.7 %, 1.33 % for *rpoB* and *rpsL* genes, respectively. And the rate of both genes was found to be (0.7%).
الملخص

في السودان كما في العديد من الدول الأخرى أصبحت مشكلة مقاومة

عصيات السل (M. tuberculosis) للعقاقير المستخدمة في علاج المرض تشكل تهديداً رئيسياً للصحة العامة.

في هذه الدراسة تم أخذ 250 عينة بلغم عشوائياً من حالات حديثة لمرضى مصابين بالتدرن الرئوي وذلك قبل البدء في تعاطي العلاج، اخذت العينات من اعمار مختلفه بغض النظر عن الجنس. تم جمع العينات من المرضى المترددين في مستشفى الخرطوم التعليمي ومستشفى جمالي للصرد وذلك في الفترة من يوليو 1999 وحتى مايو 2000م وذلك بغرض دراسة مقاومة عصيات السل الرئوي للعلاج.

من مجمل 250 عينة وجد ان 201 منها موجبة اللطاخة للعصيات الدرنية، وبعد ذلك تم الاستنباط الزراعي القياسي لها في مستشفى لونستن جونسون حيث وجد من مجمل للطاخات الموجبة للعصيات الدرنية ان 147 عثره متفطرة سليمة (73.1%) بالإضافة إلى ثلاث عثرات من الع hát السائبة اللطاخة للعصيات الدرنية و17 عثره سريعه النمو متفطرة غير متدربة (8.5%) و7 عينات (3.5%) ووجد انها من جنس النوكارديا وباشراء مزيد من التحليل الميكروبيه التميزيه وجد انه فصيل جديد وتمت اضافته بواسطة التصنيف العالمي للبكتيريا إلى عائلة النوكارديا وسمي بنوكارديا افريكانا و14% (6.9) عينه حدث لها تلوث بينما 16 (7.9) عينة كانت سلبية النمو. تم استخدام طريقه التفاعل السلسلي المتبلمر (PCR) – التحوير الترميمي الطولي للقطع (البصمه) باستخدام الانزيم (PVU II) والعنصر المدخل (16S110) وذلك لتأكيد نتائج اختبارات النمط الظاهري لل150 عينة التي وجدت متفطرة سليمة ولقد
تظهر التحاليل الجينية (PCR) لجين rpoB و rpsL على التفاعل السلسلي المتبلمر باستخدام البادي الخاص (primer) TR8 و TR9 حول الكودون 43 و تقنية التحوير التأكيدى للخط السلف (SSCP) أربع عترات فقط (2.7%) من جملة العترات المتطرفة السليحة اظهرت الحركة التبادلية للطافر (rpoB) وهذا يعني أنها مقاومةً بدائياً لعقار الريفاميسين.

التحليل الجيني للجين rpsL (المسؤول عن المقايضه لعقار الاستربتوماسين) تم التحليل باستخدام تقنية التميم القطعي للتفاعل السلسلي المتعدد (RFLP) وجد أن هنالك عترتين فقط (1.3%) من جملة العترات المتطرفة السليحة لم يحدث لها قطع انزيمي وبالتالي فهي تحوى الطافر الجيني (rpsL) المقايوم لعقار الاستربتوماسين. معدل الطفرة الابتدائية بين ال150 عترة متطرفة سليحة وجد 2.7% و 1.33% للطافرين على التوالى. كما وجد ان المعدل للطافرين معاً 0.7%.
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CHAPTER ONE
INTRODUCTION AND OBJECTIVES

Today tuberculosis is a major cause of morbidity and mortality (8 millions new cases annually) in the world particularly in developing countries. More than one billion individuals are infected with \textit{M. tuberculosis} and more than 3.5 million people die per annum worldwide (Avachee \textit{et al.} 1999; Hass and Des Prez 1995). The epidemiology of the illness is changing in relation to HIV infection epidemic, socioeconomic problems and emergence of drug-resistant strains (Sudre \textit{et al.}, 1992; Centre for Disease Control, 1993; Cohn \textit{et al.}, 1997). Despite the availability of effective chemotherapies, tuberculosis is responsible for one in four avoidable adult deaths in developing countries (Nolte and Mitchock, 1995).

There is an increasing number of tuberculosis cases and outbreaks of multidrug-resistant tuberculosis strains (MDR) over the past few years, Therefore has been a resurgence of interest in rapid diagnostic of \textit{M. tuberculosis} infection (Nolte and Mitchock, 1995). The delay of the diagnosis of the tuberculosis not only delays the treatment of the patients but may lead to increasing the spread of disease and MDR strains (Musser, 1995). The worldwide resurgence of tuberculosis has prompted the development of new (molecular) techniques to classify the bacillus, which have advanced the understanding of the transmission of the disease (Musser, 1995).

Infection with drug sensitive strains of \textit{M. tuberculosis} can be effectively cured with combination of isoniazid (INH), rifampicin (RIF) and pyrazinamide (Combs, \textit{et al.}, 1990). However, the particular susceptibility and increased mortality of the disease among individuals infected with human immunodeficiency virus (HIV) pose a serious threat to tuberculosis control programs (Perrins, 1991). Moreover, the emergence of multi-drug resistant strains of \textit{M. tuberculosis} (MDR-TB) has resulted in fatal outbreaks in many countries, including the Unite States (Snider and Robert, 1992).

According to WHO/IUALTD, 63 survey of resistance to antituberculous drugs have been performed between 1985 and 1994. Rates of primary resistance to isoniazid administered as single agent ranged from 0 to 16.9\% (median rate, 4.1\%) to streptomycin 0.1-23.5\% (median rate, 3.5\%) to rifampicin 0.-3.0\% (median rate, 0.2\%) and to ethambutol 0. –4.2\% (median rate, 0.1\%). The rates of acquired resistance were higher than those of primary resistance, which were as follows: isoniazid 4.0-53.7\% (median rate, 10.6\%) to streptomycin 0. –19.4\% (median rate, 4.9\%) to rifampicin 0.-14.5\% (median rate, 2.4\%) and to ethambutol 0. –13.7\% (median rate, 1.8\%). The highest rates of MDR-TB have been
reported in Nepal (48.0%), Gujarat, India (33.8 %), New York City (30.1 %), Bolivia (15.3 %) and Korea (14.5 %).

According to WHO/IUALTD a previous survey of drug resistance from 35 countries, the weighed mean of primary resistance to any drug ranged from 2 % to 41 % and the acquired resistance to any drug from 5.3 % to 100 % (WHO, 1997).

By the end of second quarter of the 1998 the total number of tuberculosis cases in the Sudan was 82860, according to the Sudan National Tuberculosis Program (Elsony et al, 2000). Sharaf Eldin (2001) found that the acquired resistance to streptomycin was 30 % (20 % rpsL gene and 10 % rrs gene) and to rifampicin was 8% (rpoB gene) and to isoniazid was 12 % (Kat G gene) and for ethambutol was 4 % (embB gene) and 4 % had mutations in genes associated to both INH and RIF in Khartoum state.

Objectives

**The main objective**
To detect the primary drug resistance in rpoB gene associated with rifampicin and in rpsL gene associated with streptomycin among new tuberculosis patients in Khartoum State.

**Other objectives of this study**
To use molecular methods as rapid and reliably to detecting of *M. tuberculosis*. 
To estimate the rate of resistant strains of *M. tuberculosis* in Khartoum State.
CHAPTER TWO

LITERATURE REVIEW

1. Tuberculosis

1.1 Tuberculosis; The early days

Tuberculosis is one of the most prevalent infectious diseases of mankind, and is a leading cause of mortality as a single infectious agent worldwide (Bloom, 1992). Tuberculosis remains one of the most important infectious diseases in the world socio-economically, particularly in parts of Asia and India and Africa (Bloom, 1992).

Tuberculosis was known in antiquity, for it is ravages can be recognized in lesions of bones that have survived. Probably the first known example of disease of the vertebral column dates for the Neolithic period (about 5000 BC). There is evidence of bony lesions in Egyptian mummies and the disease may have been common in the predynastic period of Egypt (Florey, 1961). A clay statuette dating from about 4000 BC shows the distortion of the spine often associated with tuberculosis (Sigerist, 1951). It does not seem that tuberculosis of the lungs was common in Egypt, for no description of this form of the disease exists, but Phthisis was recognized by the Greeks, and described in the Hippocratic writing (ca.400 BC). Tuberculosis of lungs was also mentioned in the Indian Rig-Veda (about 1500 BC), and there were a number of representation of the disease among the primitive statuettes of the New World. The idea of tuberculosis as a separate disease did not emerge until 1671 when Francis Sylvius (De le Boe) used the term tubercular minoravel majora to describe the appearances of lesions of the lungs. Very accurate description of the macroscopic appearances of tubercles lesions were given by William Stark (1740-1770), whose work appeared in 1788, and by Matthew Baillie (1761-1823), who published his observations in 1793 (Florey, 1961).

*Mycobacterium* has caused disease in humans since recorded history began; lesions clearly caused by mycobacteria have been identified in Egyptian mummies related to the *Corynebacteria* and to *Nocardia* species. The mycobacteria contain several pathogenic species to humans as well as those pathogenic to animals and fish. *Mycobacterium tuberculosis* is found only in human, its infectious nature was suspected by Fracastorius in the early part of the sixteenth century.
In 1865 Villimen showed that the disease could be transmitted by the inoculation of tubercles material. It was in 1882 that Koch demonstrated the tubercle bacillus by special staining isolated and grew it in pure culture, and reproduced the disease by the inoculation with the bacilli (Burrow, et al, 1995).

Robert Koch was the first to establish the causal relationship between the tubercle and tuberculosis. The organism was named *Mycobacterium tuberculosis* in 1886, presumably because it resembled a fungus in its slow growth and colony morphology (Patrick and Ellen., 1995).

One hundred years after Robert Koch's discovery of the tubercle bacillus, tuberculosis is still the most important specific communicable disease in the world. It is estimated that eight million new cases of tuberculosis appear each year in developing countries and at least three million persons die of the disease, in the technically advanced countries the incidence much less than this (WHO 1996).

1.2 Definition

Tuberculosis is a chronic infectious disease of lungs and it can affect every organ system, it is clinical manifestations are fatigue, weakness, weight loss and fever. Pulmonary involvement giving rise to chronic cough and spitting of blood is usually associated with far-advance lesion, Meningitis or urinary tract involvement can occur in the absence of other signs of tuberculosis. Blood stream dissemination leads to miliary tuberculosis with lesion in many organs and high mortality rate ((Walter and Israel, 1987)

Tuberculosis affects mammalian, avian, and cold-blooded animals. Mammalian tuberculosis is divided into human, bovine and vole types, the human variety is partially always responsible for pulmonary tuberculosis in adult, and usually the bovine variety may occur occasionally in the pulmonary form (Powel, et al. 1980).

Tuberculosis in human caused by *Mycobacterium tuberculosis* complex, which includes *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*.

1.3 Methods of the diagnosis of TB

There are many methods to diagnose a patient as TB patient and it s usually done as follow;

1.3.1 Clinical diagnosis

Clinically TB diagnosis is based on characteristic symptom such as; fatigue, weakness, weight loss and fever may be signs of tuberculosis disease. Pulmonary involvement giving rise to chronic cough and spitting of blood usually is associated with far-advance lesion (WHO, 1998).
1.3.2 X - Rays
Chest x-ray it is one of the most important methods of diagnosis of pulmonary tuberculosis (WHO, 1998).

1.3.3 Tuberculin test
Tuberculin skin test which consists of injection of purified protein derivative intra dermal, is used to identify individuals with tuberculosis disease (WHO, 1998).

1.3.4 Microscopic examination
Ziehl Neelson (ZN) or Kinyoun or flurochrome dyes are used to stain the *Mycobacterium* and then examined under microscopy, ZN stain is rapid and low cost, but low sensitivity (Yeager et al, 1967). ZN stain recommended by WHO to be used for tuberculosis microscopy is use to diagnosis TB (WHO, 1998)

1.3.5 Culture methods
Löwenstein-Jensen medium (LJ) is used for tuberculosis culture, LJ with glycerol used for the growth of *M. tuberculosis* and with pyruvate for *M. bovis* (WHO, 1998)

1.3.6 Serological test
The Serological test method is used to detect *M. tuberculosis* antigens, this test depend on antigens and antibodies reaction, It is measurement of IgG antibody, with specific antigens in an ELISA test (WHO, 1998)

1.3.7 Molecular methods
There are many molecular methods to diagnosis of TB targeting the nucleic acids such as; PCR and sequencing.

1.3.7.1 Polymerase Chain Reaction (PCR)
PCR is an in vitro nucleic acid amplification method. The reaction comprises repeated thermal cycling of the reaction mixture .the thermal cycling serves to dissociate the products of the previous thermal
cycle then allow the association of these dissociated oligonucleotide strand from the previous thermal cycle, products with further reaction starting materials for another phase of synthesis. (Saiki et al, 1985 and 1988). Specifically, substrate DNA is denatured at a high temperature to give single-stranded (template) molecules. This is followed by short oligonucleotide primers (amplimers) annealing to specific, nucleotide-sequence-defined regions of the template at a lower temperature. The positions where the amplimers anneal to the template define the (target) the thermal cycle is concluded by the amplimers being enzymatically extended by the coupling of appropriately base-paired deoxynucleoside triphosphates (dNTPs) on the template at an intermediate temperature, thus producing another double-stranded DNA (ds DNA) copy of the original target. (Saiki et al, 1985).

1.3.7.2 PCR-Gene specific

PCR-Gene specific it is specific sequences; dnaJ gene (Takewaki et al., 1993 and 1994) Genus-specific targets such as 65-Kda heat shock protein gene (Telenti et al., 1993) and ribosomal DNA genes have been used for this purpose (Vaneechoutte et al. 1993). The PCR- dnaJ gene recognizes the broad spectrum of mycobacterial species (Syun et al., 1993)

1.3.7.3 DNA fingerprinting designated 1S6110

It was first reported by Thierry et al., (1990) is usually used to identify M. tuberculosis complex. This ability to accurately identify related strains of M. tuberculosis complex can help to define the people and places involved in M. tuberculosis transmission. Tuberculosis control personnel can use this information to design and implement more effective TB control measures. M. tuberculosis DNA fingerprinting is based on small piece of repitive DNA, named 1S6110, that may be present from 1 to 20 or more times in the M. tuberculosis genome

1.3.7.4 Sequencing

The 16S-rDNA is the target used to this method (Jonas et al., 1993; Van der Vliet et al., 1993), the attractive feature of this molecule is that contains conserved gene specific as well as various specific regions. Therefore, it was straight forward to use this molecule or the gene coding 16S rRNA as a powerful target for amplification of mycobacteria to both genus and species level (Kox et al., 1995; Van der Vliet et al., 1993), this strategy is applicable to an other target in the RNA, particulary the 23S RNA (Shah et al, 1995) or 23S-16SrDNA spacer (Frothingham and Wilson, 1993; Glennon et al, 1994)
1.4 Pathology

1.4.1 Pathogenesis of the tubercles lesion

The following sequence of events occur when tubercle bacilli are introduced into the tissue. Transient acute inflammatory reaction with an infiltration of polymorphs. These cells are rapidly destroyed by the organisms. A progressive infiltration of macrophages derived mostly from the monocytes of the blood. The macrophages are responsible for the destruction of the organisms. The macrophages phagocytose the bacilli. In a short time their character changes: their cytoplasm become pale and eosinophilic and their nuclei elongated and vesicular. Their appearance bears a resemblance to the epithelial cell, and they are called epithelioid cells. Some macrophages, instead of becoming epithelioid cells fuse to form langhan's giant cells. surrounding this mass of altered macrophages there is a wide zone of small round cells, mostly lymphocytes and fibroblasts. (Walter and Israel, 1987)

Within 10 to 14 days necrosis begins in the center of this mass, which consists of altered macrophages and cells, peculiar to the tissue of the part. This is a firm, coagulative necrosis called caseation, and it is characterized by a high content of lipid and a tendency to resist autolysis and demolition. Histologically the necrosis is less structure and eosinophilic, but with silver stains reticulum fibers can sometimes be demonstrated. (Walter and Israel, 1987)

The caseation is due primarily to hypersensitivity to products of the bacilli and notable tuberculo-protein. Ischaemia plays some part also, in as much as the blood supply become inadequate for the heavy infiltration of cells into the affected area, this produced the tubercle follicle which consist of central mass of caseation surrounded by epithelioid and giant cell, which in turn are enclosed in wide zone of small round cells. The appearance is characteristic of tuberculosis, but a rather similar picture is seen in some of the deep-seated mycoses. This is a common type of lesion of tuberculosis and is called productive or proliferative, because its main components are cells rather than fluid exudates. Another type of lesion is the exudative form of tuberculosis which is characterized by the out-pouring of an inflammatory exudate rich in fiber. There is a considerable infiltration of lymphocytes, and often polymorphs are present, but epithelioid and giant cells are rather scanty. Exudative lesion is typical of tuberculosis of serous cavities. (Walter and Israel, 1987).

1.4.2 Distribution of lesions in the body

The principles differ in no significant way from those of other infections. Local spread by macrophages has been as fellows, progressive infiltration of macrophages derived mostly from the monocytes of the
blood. The macrophages are responsible for the destruction of the organisms. (Walter & Israel, 1987). Lymphatic spread is a continuation of local spread. The result is a regional tubercles lymphadenitis which is typical of childhood infection. The primary focus with its attendant lymphangitis is called the primary complex. Now a day nearly all primary complexes are pulmonary (Walter & Israel, 1987).

Blood spread occurs as an extension of lymphatic involvement, in an overwhelming infection. The organisms escapes from the lymph nodes and enters the blood stream to produce miliary tuberculosis, which in extreme cases is of the non-reactive type. The lungs, spleen, liver, kidney, and to a lesser extent other organs, are seeded with tubercle bacilli which produce numerous follicles of millet-seed size lesions. Blood spread also occurs when caseous hilar nodes directly implicate an adjacent pulmonary vein. If there is a discharge of large numbers of organisms into the blood stream, miliary tuberculosis occurs, but the lungs are relatively spared. Spread in serous cavities is seen in the pleurisy that may complicate lung lesion, the localized peritonitis found around tubercles salpingitis, and in tubercles meningitis. (Walter & Israel, 1987)

Spread along epithelial-lined surfaces is typified by the inter-bronchial spread of tuberculosis that occurs when sputum inhaled into adjacent lung segments. If the sputum is coughed up it can produce tubercles laryngitis, and when swallowed, the bacilli may infect the ileo-caecal area of the bowel and lead to tubercles enteritis. Rectal involvement is manifested by ischiorectal abscesses. Like-wise tuberculosis of the kid-ney can spread down the ureter to implicate the trigone of the bladder. Tuberculosis of the skin and bone e.g. Pot's disease. (Walter & Israel, 1987)

1.5 Epidemiological aspects:

1.5.1. Transmission and Sources of infection
Pulmonary tuberculosis infection is usually by respiratory droplets, discharged on coughing by person with infectious pulmonary lesion. It may also result from the swallowing of organisms from hands or feeding utensils contaminated by infected person. Indiscriminate spitting can contribute to transmission since sputum from infected person often contains infectious bacilli (Sepkowitz et al, 1995). Non-pulmonary infection caused by *M. bovis* usually resulted from the drinking of milk from infected cows or through contact with infected cattle, but in the tropics most non-pulmonary tuberculosis is caused by *M. tuberculosis* (Woods, 1993)

1.5.2. Distribution of tuberculosis
Tuberculosis (TB) called the captain of all the infectious diseases that have plagued man (Doubs, et al.1952). Tuberculosis is the most significant single infectious cause of morbidity and mortality,
producing eight million new cases and killing three million people annually. It causes approximately one quarter of all avoidable adult death from infection. (Raviglione et al. 1995, Murray et al 1990 and WHO 1996)

1.5.2.1. Distribution of tuberculosis in the world:

During this period (1990-2000) it was estimated that 88.2 million people will develop tuberculosis, 8.0 million of which will be attributable to HIV infection. In South East Asia, 35.1 million new cases of tuberculosis were expected during the decade, including 2.8 million (8%) HIV - attributable cases. Around 20.5 million new cases were predicted for the Western Pacific region, including 0.4 million (2%) HIV attributable cases, while 15.0 million cases were expected in Sub-Saharan Africa during the decade, including 3.9 million (25%) cases attributable to HIV infection. During these ten years it was estimated that 30.0 million people will die of tuberculosis; 2.9 million of these deaths (9.7%) will be attributable to HIV infection. In South-East, 12.3 million tuberculosis deaths may occur during the decade, including 1.0 million HIV attributable deaths. Around 6.0 million tuberculosis deaths were expected in Sub-Saharan Africa, 1.5 million (25%) of which will be attributable to HIV infection (Fig. 1) (WHO, 1997).

The WHO global tuberculosis programme joined forces with International Union Against Tuberculosis and Lung Disease (IUATLD) and started the global project on anti-tuberculosis drug resistance surveillance including 35 countries and approximately 50,000 tuberculosis cases. Testing for resistance to EMB, STR and INH, the mean findings; the prevalence of primary drug resistance to any drug ranged from 2% (Czech Republic) to 41% (Dominican Republic), with a medium value of 10.4%.

Primary resistance to all 4 drugs tested was found in a medium of 0.2% of the case (rang 0 to 4.6%). Primary MDR-TB was found in every country surveyed except Kenya, with medium prevalence of 1.4% (rang 0 Kenya to 14.4% Latvia).

The prevalence of acquired resistance to any drug ranged from 5.3% (New Zealand) to 100% (Ivanovo Oblast, Russia) with median of 4.4% of cases rang (from 0 to 17%). The median prevalence of acquired MDR-TB was 13% with a range from 0% (Kenya) to 54% (Latvia) with median of 4.4% of cases rang (from 0 to 17%). MDR-TB is present in five countries surveyed had 5% of TB patients with MDR-TB. In the Dominican Republic, 10% of TB patients had MDR-TB (WHO 1997).
1.5.2.2 Distribution of tuberculosis in Sudan

The best indicator of the extent of the tuberculosis problem in Sudan is the average annual risk of infection (ARI), which is the proportion of the population that is likely to be newly infected over a period of one year. The ARI is derived from studies of the prevalence of tuberculin positively. An ARI of 1% corresponds to an approximate incidence of 50 cases of pulmonary smear-positive tuberculosis per 100,000 populations. The incidence of all forms of tuberculosis is estimated at twice that rate. The prevalence of tuberculosis infection is probably very different in different regions of Sudan. The reported ARI of 1.8% represents an average that can be used at the central level for program planning and management (SNTP, 1994). The reported pulmonary tuberculosis cases by hospitals during 1987 and 1988 tuberculosis cases were 3749 and 3983 respectively (SNTP, 1994).

The estimated average incidence of all forms of tuberculosis is twice the incidence of pulmonary smear-positive cases, or 180 per 100,000. In the population of 24,400,000 in 1987 the total new cases was estimated to be 43,000. Estimating the population of reporting areas to be 15,000,000 (Khartoum, Central, Northern, Eastern, Kordofan region), and the number of cases of all forms of tuberculosis to be 27,000, the coverage of case-finding is 67%. There are no official data on tuberculosis among displaced populations, but the average incidence can be considered to be close to that of settled population. In overcrowded camps it is likely that sputum-positive person will transmit the infection to more people than in the settled population. Malnutrition, stress and other problems may cause more breakdowns from infection to active disease among displaced populations than in the average Sudanese population.

The only available data on displaced people were provided by the Sudan Council of Churches. Data on tuberculosis drug resistance patterns in the population and on the extend of the HIV epidemic among TB patients are not available in either displaced or settled populations (SNTP, 1994).

1.2 Mycobacteriology

1.2.1. Taxonomy of the genus *Mycobacterium*

The genus *Mycobacterium* is the only genus in the family Mycobacteriaceae. The high G + C content of the DNA of Mycobacteria (62 to 70 %) is similar to that of the other mycolic acid-producing bacteria, *Nocardia* (60-69 %), *Rhodococcus* (59-69 %), *Corynebacterium* suborder Corynebacterineae (Stackebrandt *et al.*, 1997) *Gordonia*, *Skermania* and *Tsukamurella* (51-59 %), (Chun *et al.*, 1996;
Goodfellow et al., 1998). This similarity may support the consolidation of these genera into a single family. (Wayne and Kubica 1986).

A natural division occurs between slowly and relatively rapidly growing species of mycobacteria. Slow growers require more than 7 days producing easily seen colonies on solid media from a dilute inoculum under ideal culture conditions. Rapid growers require less than 7 days under comparable conditions. In practice, different sets of biochemical tests are necessary to characterize slowly growing and rapidly growing species. (Wayne and Kubica, 1986).

*M. kansasii* is the name currently given to photo-chromogenic (yellow bacillus) originally described in 1953. Studies of the base sequences of the 16S r RNA and of catalase serology suggest that *M. kansasii* is phylogenetically very closely related to a non-pathogenic, slowly growing, non-pigmented species, *M. gastri* (Wayne and Diaz, 1982; Rogall et al., 1990).

1.2.2. **Description of the genus *Mycobacterium***

The mycobacteria are slightly curved or straight bacilli, 0.2 to 0.6 by 1.0 to 10um in size, sometimes with branching. Filamentous or mycelium-like growth may occur, but it easily fragments into rods or coccid elements. Mycobacteria have cell walls with a high lipid contents that include waxes having characteristic mycolic acids with long, branched chains (Wayne and Kubica, 1986).

The high lipid content of the cell wall excludes the usual aniline dyes. Mycobacteria are not readily stained by the Gram methods but are considered Gram positive. Special staining procedures are used to promote the uptake of dye, and once stained; mycobacteria are not easily decolorized even with acid-alcohol. This resistance to decolourization partly or completely lost at some stage of growth by some proportion of the cells of some species. Cells of rapidly growing mycobacteria may be less than 10% acid fast. (Wayne and Kubica, 1986)

The mycobacteria are aerobic, non-spore-forming, non-motile bacilli. Colony morphology varies among the species. *M. tuberculosis* forms rough colonies with the bacilli compacted into curving strands (cords). In contrast, MAC usually forms smooth transparent colonies with the bacilli arranged in no definite pattern on primary cultures. *M. kansasii* is intermediate between the previous examples, forming slightly rough colonies. Diffusible pigment is rare, but colonies of some species are regularly or variably yellow, orange, or rarely, pink. Some species require light to form pigment (photochromogens), and others form pigment in either light or dark (scotochromogens). Aerial filaments are rarely formed and are never visible without magnification. (Patrick, 1995).
Growth rates for mycobacteria are slow to very slow, with generation times varying species and ranging from 2 to 20 hrs. Easily visible colonies may be produced after two days to eight weeks of incubation under optimal conditions, depending on the species. Optimal temperatures for growth vary widely among species, ranging from 30 to almost 45°C, most species adapt readily to growth on simple substrates, using ammonia or amino acids as nitrogen sources; and glycerol as a carbon source in the presence of mineral salts. Some species require medium supplements such as hemin, mycobactins, or other irons transports compounds. *M. leprae* and *M. intermedium* Which isolated from AIDS patients in Germany have not been cultured outside of living cells (Patrick, 1995).

A grouping system of Non-tubercle mycobacteria (NTM) based on pigmentation, colonial morphology and growth rate was established by Runyon, (1959). It soon became apparent that all species of mycobacteria did not fit within the four groups of the Runyon scheme. Clinical laboratories have abandoned the Runyon groups in favour of identification to species level. The term atypical mycobacteria is of historical interest but should not be used by clinical laboratories when referring to non- *M. Tuberculosis* mycobacteria. When this species were first described, they were grouped together as atypical mycobacteria because they were not typical of *M. tuberculosis*. Obviously, since these mycobacteria are not atypical but are characteristic of their own species, the term NTM is preferable when referring to these species as a group. Whenever possible, species names should be used (Patrick, 1995).

### 1.2.3. Clinically significant mycobacteria:

The genus *Mycobacterium* includes obligate parasites, saprophytes, and opportunistic pathogens. Most species are free living in soil and water, but the major ecological niche for other such as *M. tuberculosis* complex and *M. leprae* is diseased tissue of human and other warm-blooded animals (Wayne *et al*, 1992).

Descriptions of the diseases associated with the clinically important and potential pathogenic species of mycobacteria is shown in (table 1). (Lawrence *et al*, 1992).

### 1.2.4 Biochemical methods of distinguishing between species of genus *Mycobacterium*

Mycobacteria should always be identified to the species level if possible. According to the traditional methods, mycobacteria are usually identified by traits such as rate of growth, colonial morphology, pigmentation and biochemical test (table 2) (Patrick, 1995). And molecular methods were used to distinguish between species of genus *Mycobacterium*, such as Finger printing, and sequencing. *M. tuberculosis* complex includes the species *M. bovis*, *M. microti* and *M. africanum*. *M. bovis* was the
name given to the bovine tubercle bacillus in 1896. The bacillus of Calmette-Guerin (BCG), which is used as a vaccine against tuberculosis in many parts of the world, conforms to the properties described for *M. bovis* except that its pathogenicity is more attenuated, *M. africanum* discover by Castets 1969 and its type strain is American Type Culture Collection (ATCC 25420) it is a group of strains causing human tuberculosis and taxonomically were assigned to two cluster; strains from West Africa (similar in many respects to the human type) and strains from Rwanda and Burundi (closely resembled the bovine type).its mycolic acid pattern type α- keto- methoxy, (Goodfellow and Magee, 1998) M. microti also occupies a position along the phenotypic continuum between *M. tuberculosis* and *M. bovis* (Patrick, 1995)

*Mycobacterium avium* Complex (MAC) consists of 28 serotypes of two distinct species *M. avium* and *M. intracellulare*, and has in the past also included three additional serovars of *M. scrofulaceum* (*M. avium, M. intracellulare* and *M. scrofulaceum complex*). Inclusion of *M. scrofulaceum* in the MAC is no longer appropriate, given recent advances in mycobacterial systematics (Wayne and Sramek, 1992).

The criteria used to distinguish *M. avium* from *M. intracellulare* are now well established. There subspecies of *M. avium* were proposed on the basis of phenotypic characteristics and genetic studies: *M. avium* subspecies *avium, M. avium* subspecies paratuberculosis, and *M. avium* subspecies. *silvaticum* (Thorel, et al., 1990) The International Working Group on Mycobacterial taxonomy has suggested that there is taxonomic evidence for a third species within the MAC (Wayne et al, 1991).

**1.2.4.1 Ziehl Neelsen microscopy for Acid-Fast Bacilli**

The "acid-fast" staining reaction of mycobacteria, along with their beaded and slightly curved shape, is a valuable aid in the early detection of infection and in the monitoring of therapy (Cowan and Steel, 1995). The finding of acid-fast bacilli in the sputum, is often considered presumptive evidence of active tuberculosis and sufficient causes to initiate therapy. It has been estimated that there must be 10,000 acid-fast bacilli per ml of sputum to be detected by microscopy. With continued drug treatment, more organisms are killed and fewer shed, so that following the number of stainable organisms in the sputum during treatment can provide an objective measure of response before the results of cultures is a viable from 3 to 6 weeks later (Hobby et al, 1973). Reports from examination of stained smears from clinical specimens should provide some quantitation of the number of acid-fast bacilli present and the degree of activity of the disease (David, 1976).

**1.2.4.2 Isolation of *Mycobacterium tuberculosis***

Specimens from patients with tuberculosis usually contain mixed bacterial flora. Methods for the collection of specimens should be directed toward minimizing the number of contaminating bacteria (Krasnow et al, 1969).
*M. tuberculosis* show faster growth with fewer contaminants when collected early in the morning. Although it has been noticed that 24-hours sputum collections will ultimately yield more positive cultures than early morning specimens (Krasnow *et al*, 1969), growth is usually slower and contamination rate is significantly higher with the sputum pools (Kestle, *et al*, 1967). Three early morning specimens will usually be sufficient to identify the patient with active disease (Krasnow *et al*, 1969).

Specimens submitted for recovery of mycobacteria were first treated with an alkaline or acid decontamination agent to reduce bacterial overgrowth and liquefy any mucus present to promote concentration of the organisms by centrifugation. After a carefully timed exposure of the specimen, while being mechanically shaken, the acid or alkaline solution was neutralized and then centrifuged at high speed to concentrate the mycobacteria. The centrifugal force should be as high as possible because the lipids content of the mycobacterial cell wall provides a buoyant effect and makes their specific gravity close to unity. For this reason the selective sedimentation of mycobacteria in a thick, viscous sputum specimen was difficult. Exposure of specimens to strong decontaminating agents such as 3 or 4 percent NaOHL, or 5 percent Oxalic acid, must be carefully timed to prevent excessive chemical injury. Neutralization of a strong decontaminating solution requires an equally strong acid or alkali, and often titration to a neutral end point might be incomplete with the specimen remaining either strongly alkaline or acid. Benzalkonium chloride should be inoculated to egg-base culture media in order to neutralize the growth inhibition characteristic of benzalkonium chloride. The concentrate should be neutralized by adding lecithin if it is to be inoculated to agar base media (Runyon *et al*, 1974). In response to the increased need to recover mycobacteria for susceptibility testing Kubica, *et al*.(1975) Contaminating bacteria, particularly gram-positive bacteria, are controlled in part by the addition to the medium of aniline dyes such as crystal violet and malachite green. The concentration of aniline dye in a medium is an important variable as light increases over the specified amount can result in significant inhibition of mycobacterial growth as well as growth of contaminants. Mycobacterial culture media contains anti-microbial agents to suppress bacterial and fungal contamination been used for many years. One of the more commonly used selective culture media was developed by Gruft, who added penicillin, nalidixic acid, and RNA to Löwenstein-Jenesen medium (Gruft,1971). A medium containing cyclohexamine, lincomycin, and nalidixic acid was also effective in the control of fungal and bacterial contaminants (Petran and Vera, 1971).
1.2.4.3 Molecular methods

1.2.4.3.1 The Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a rapid and powerful procedure for the in vitro generation of millions of copies of a specific deoxy ribonucleic acid (DNA) segment. This technique first introduced in 1985 by Sacki et al., as the worldwide resurgence of tuberculosis has prompted the development of new (molecular) techniques to classify the bacillus, have advantages in help understanding the transmission of the disease.

PCR is an in vitro nucleic acid amplification method. The reaction comprises repeated thermal cycling of the reaction mixture. The thermal cycling serves to dissociate the products of the previous thermal cycle then allow the association of these dissociated products with further reaction starting materials for another phase of synthesis. (Saiki, et al, 1985 and 1988). Specifically, substrate DNA is denatured at a high temperature to give single-stranded (template) molecules. This is followed by short oligonucleotide primers (amplimers) annealing to specific, nucleotide-sequence-defined regions of the template at a lower temperature. The positions where the amplimers anneal to the template define the (target) the thermal cycle is concluded by the amplimers being enzymatically extended by the coupling of appropriately base-paired deoxynucleoside triphosphates (dNTPs) on the template at an intermediate temperature, thus producing another double-stranded DNA (ds DNA) copy of the original target.

Each PCR cycle comprises a set of time and temperature-controlled incubation. The function of each incubation is to:

First: Denature the target nucleic acid at temperature in the region of 94°C. Second: Annealing amplimers at a temperature dependent on their calculated annealing temperature (usually in the rage 30-60°C).

Third: Extend the primers by the thermostable DNA polymerase catalyzed addition of nucleotides to the 3’ end of each primer at a temperature of about 72°C.

If each PCR cycle were 100% efficient, each cycle would give rise to a doubling of the number of copies of the original target sequence, since the product of one PCR cycle becomes additional substrate for the next. The PCR process can therefore give rise to millions of copies of the target sequence,
enabling trace amounts to be detected against a background of a complex mixture of sequences. PCR may be used to analyze or determine the presence of specific DNA targets or, after a reverse transcription step. RNA sequences can also be amplified as DNA copies. (Saiki, et al., 1995 and 1988).

1.2.4.3.2 Mutational analysis

Several groups of investigators have developed Polymerase Chain Reaction (PCR) assays for direct detection and identification of M. tuberculosis in clinical specimens (Brisson et al., 1991; Eisenach et al., 1991; Kolk et al., 1992; Shawar et al., 1993). Also many PCR-based strategies have been used to detect mutations in genes of interest which include DNA sequencing (Musser et al., 1995) and Dideoxy fingerprinting (Felmlee et al., 1995), heteroduplex analysis (Williams et al., 1994). Restriction Enzyme Digest, (Wegenack et al., 1997) and Single Strand Conformation Polymorphism, (De Beenhouwer et al., 1995)

1.2.4.3.2.1 Restriction fragment length polymorphism (RFLP)

The main idea of this method is to amplify the target gene by the specific primer and cut the Wilde types of the gene with specific enzyme and the mutant type were not cut. (Victor et al., 1997 and Wegenack et al., 1997)

1.2.4.3.2.2 Single Strand Conformation Polymorphism (SSCP)

This method depends on the mobility shift of the mutant gene (Victor et al., 1997; Telenti et al. 1993) and the use of probes (De Beenhouwer et al., 1995; Rossau et al., 1997).

1.3 Chemotherapy:

1.3.1 Chemotherapy - the early days

As mankind has been affected by tuberculosis for at least 5,000 years, a plethora of treatments, ranging from traditional healing to surgery, has been described. It was not until 1943, however, when Selman Waksman discovered streptomycin that physicians had at their disposal a powerful tuberculocidal agent with scientifically proven activity. Shortly after wards, in 1946 para-amino-salicylic acid was shown by Jorgen Lehman to be active against M. tuberculosis and in 1952 three pharmaceutical companies simultaneously announced the discovery of the exquisitely potent anti-tuberculosis drug isoniazid. In the early days of tuberculosis chemotherapy, these compounds were used individually and in many
cases, despite making a good initial recovery, the patients relapsed with a drug-resistant form of the disease. It is easy to understand why, as frequency of mutation to streptomycin resistance is now known to be $10^{-6}$ and it is not uncommon for patients with pulmonary disease to harbour such numbers of tubercle bacilli in a single lesion (Canetti, 1965). Consequently, regimens were developed in which streptomycin was associated with another drug, as it was correctly reasoned that the streptomycin resistant mutants present in the population would remain susceptible to the second drug (Medical research council, 1962).

This work culminated in the first generation of multi-drug treatment regimens for tuberculosis. These were subsequently improved and strengthened as newer more powerful and better tolerated drugs. Such as ethambutol and rifampicin were introduced in 1962 and 1967, respectively. Pyrazinamide, a compound that is bactericidal at acid pH and acts on intracellular bacteria, was also found to be of great value. No new anti-tuberculosis compounds have been developed by the broad spectrum fluoroquinolone, sparflloxacin, has been shown to be highly active against *M. tuberculosis* in experimentally infected mice (Lalande *et al.*, 1993).

### 1.3.2 Short course chemotherapy

As a result of the research and many controlled clinical trials, the current standard regimen for chemotherapy was established (WHO, 1983).

The short course chemotherapy treatment lasts 8 months and consists of two phases. During the initial intensive phase, which consists of 2 months, four drugs (isoniazid, rifampicin, pyrazinamide or ethambutol and streptomycin) are given daily; whereas, during the continuation phase, lasting 6 months, isoniazid and rifampicin are administered at best daily and at worst twice weekly. When pyrazinamide is not employed during the intensive phase, the treatment duration is increased to 9 months (WHO, 1997; American thoracic society, 1986). Numerous studies have shown that success rate for the treatment of drug sensitive tuberculosis by short course chemotherapy was close to 100 % provided that the regimen was adhered to rigorously both by the patient and the clinician (Grosset, 1989, and Iseman, 1993)

Although it is strongly recommended that short course chemotherapy be employed for treating tuberculosis, this is not always possible. In many developing countries, some of the more expensive bactericidal drugs, such as rifampicin, are not available. This often results in their replacement by second-line drugs, such as thiacetazone. In much of Africa, it is not uncommon to treat patients for 12
months with a regimen including isoniazid and thiacetzone, supplemented with streptomycin in the initial 2 months (Cole and Telenti, 1995)

1.4 Drug resistance:

Drug resistance is defined as a decrease in the vitro susceptibility of \textit{M. tuberculosis} of sufficient degree to be reasonably certain that the strain concerned is different from a wild strain that has never come into contact with the drug.

Primary resistance refers to the resistance observed following infection with an isolate of \textit{M. tuberculosis} that is already resistant to a given anti-tuberculous agent; whereas, secondary, or acquired resistance corresponds to drug resistance, which emerges during treatment (Bradford et al., 1996). The latter is generally the result of poor compliance on the part of the patient, or poor conception of the regimen and inadequate supervision on the part of the physician (Iseman, 1993; Vareldzis et al., 1994).

Primary drug resistance in industrialized countries has been periodically survey since the introduction of short course chemotherapy, and generally found to be at a low level (Canetti, et al, 1972).

1.4.1 Origin of drug resistance

Drug resistance can emerge to all of the antibacterial drugs used in the treatment of mycobacterial disease. It occurs most commonly when a single drug is given alone and when the viable bacterial population in the lesion is large. In tuberculosis, it first appears after an interval of at least two weeks and more usually one to four months after the start of chemotherapy (David III, 1970).

The occurrence of drug resistance is widely thought to be due to the overgrowth of sensitive organisms by mutant resistant bacilli present in wild strains before they were ever in contact with drug concerned (Canetti,1961) performance of the fluctuation test of Luria and Delbruck indicated mutation rates in \textit{Mycobacterium tuberculosis} of about $2 \times 10^{-8}$ for resistance to isoniazid and streptomycin,$2 \times 10^{-10}$ for rifampcin and $1 \times 10^{-7}$ for ethambutol (David iii, 1970).The proportion of resistant mutants is variable from one culture to another and for most drugs, is much greater for mutants with low minimal inhibitory concentration (MICs) that for those with higher (MICs) (Cantetti and Grossel,1961; Mitchison,1961)

In every approximate term, the ratio of mutants able to grow during treatment to sensitive organism in wild strains is about $1:10^6$ for isoniazid and streptomycin and $1:10^7$ for rifampcin (David, 1970; Canetti, 1961).
Mutation to drug resistance is usually though to occur in bacterial chromosomes. The isolation of plasmids from opportunist or saprophytic mycobacteria has been described (Crawfordeta, 1979; Mizuguchi et al, 1981 -Jones et al, 1972). Though not from *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium leprae*. Although it has been claimed that these plasmid, associated with aminoglycoside resistance. The issue is still uncertain, it seems reasonable to assume that resistance to isoniazid rifampicin, pyrazinamide, dapsone and other non-aminoglycosides in tubercle bacilli is probably chromosomal. In the treatment of tuberculosis, the pattern of the emergence of resistance suggests little linkage between unrelated drugs, even among the aminoglycosides, though there are several well established patterns of cross resistance between closely related drugs (Mitchison et al, 1984). The species of Mycobacteria that are well adapted to parasitic role in man, *Mycobacterium tuberculosis* *Mycobacterium africanum* and *Mycobacterium bovis* all exist within closed lesions in human or animal hosts and do not come into contact with other bacteria, including other strains of mycobacteria, which might transmit plasmid-mediated resistance system could not readily be exploited by them to increase their genetic variability.

1.4.2 Mutation structure and response to chemotherapy

The relationship between mutant structure, drug dosage and the response of newly diagnosed patients with pulmonary tuberculosis to treatment with isoniazid was thoroughly explored during the period when this drug was being used alone in clinical studies (WHO, 1960 and 1963; Mitchison, 1973). The rate at which Isoniazid is a acetylated to microbiologically inactive acetyl isoniazid in man is genetically controlled by a single gene, about 40 % of most populations (including Indians) being virtually indistinguishable homozygous or heterozygous rapid in activators and the remainder slow in activators. In a comparison of the plasma Isoniazid concentration time curves for rapid and slow in activators, rapid in activators had only a slightly lower peak concentration than slow in activators, but had much smaller areas under the curve (exposure) and much shorter duration of coverage during which bacteriostatic concentration is maintained according to the mechanism suggested to explain the association, Isoniazid resistant mutants with the increasingly high degree of resistance were inhibited by the concentrations reached near the peak as the size of the dose of Isoniazid was raised (Selkon et al., 1964) It is of interest that the improvement in response with increasing dose size appeared to flatten off at about 8.7 mg/kg b/w. in slow in activators, when the peak concentration was about 6mg/l.(Canetti G et al;1961) found that mutants capable of growth on 5mg/l or more in vitro were all highly resistant, so that one would expect no increase in the number of mutants inhibited in the lesion when patients were treated with doses higher than about 8.7 mg/kg.
1.4.3 Nomenclature

A specialized nomenclature is used to describe the isolation of resistant organisms from patients in relation to their history of previous chemotherapy (Mitchison, et al, 1984).

Primary resistance is the term used when the strains are either naturally resistant to the drug (*Mycobacterium bovis* resistance to pyrazinamide) or have acquired resistance from another person resulted to in effective chemotherapy. Initial resistance is the isolation of resistant strains from patient who claims never to have had chemotherapy, it includes primary and undisclosed acquired resistance which is due to insufficient patient who never trust between him and his new medical advisers, patients may be give a frank history of previous successful chemotherapy and then said to have disclosed acquired resistance (Mitchison, et al 1984).

1.4.4 Factors influencing the emergence of resistance

The theory, giving two drugs together should eliminate the emergence of resistance organisms since one drug should prevent the growth of mutants resistant to the other drug. As mutants comprise about $1:10^6$ of the bacterial population in tuberculosis, adoubly resistant mutant should only occur once if the total population were about $10^{12}$, perhaps 100 times higher than the occurrence rate even in the extensive pulmonary tuberculosis. (Mutchision, 1984).

1.4.4.1 Cross resistance

There may be cross-resistance between the two drugs, so that inhibition of one or both sets of mutants is absent or incomplete, for examples in *M. tuberculosis* are partial cross resistance between thiacetazone and ethionamide (Bartman et al 1960) and capreomycin, viomycin and kanamycin (Tsukamura *et al*, 1975; Clatchy *et al*, 1977) while complete cross resistance occurs between kanamycin and amikacin (Allen *et al*, 1983). In practice, cross-resistance is a rare cause emergence of resistance (Mutchision, 1984).

1.4.4.2 Initial resistance

Patients may be started on treatment with two drugs, but have initial resistance to one of them, so that there is little or no inhibition of the mutants resistant to the other drug. However it should be noted that 10 of 20 patients with severe pulmonary tuberculosis and primary isoniazid resistance showed a
definite initial bacteriological response to treatment with isoniazid alone or isoniazid and para-amino salicylic acid (PAS), (Devadattas et al, 1961) and four out of ten patients with strains initially resistance to para-amino salicylic acid. Streptomycin and isoniazid nevertheless attained quiescent disease when treated with these three drugs (British Medical Research Council, 1972).

1.4.4.3. Drug potency

Drugs vary in their ability to suppress the emergence of resistance to another drug. A highly potent drug is able to prevent the growth of -and usually kills- all the organisms in the lesion and continue to do so despite minor irregularity in drug taking by the patient. This implies that drug concentrations considerably higher than drug (MIC) are present for some of the time that drug action is not reduced by pH or environmental factors, and that exposure to the drug is followed by a period of several days (in tuberculosis), in which the organisms do not began to grow again (Dickinson, et al,1973). Example of less potent drugs are ;streptomycin because it dose not act on bacilli in an acid environmental many occur inside macro phases or extra cellular in areas of acute inflammation. Pyrazinamide because it only acts on bacilli at pH values of less than 5.6, thiacetazone because it's only bacteriostatic and bacilli will start to grow again if a dose is missed .Other drugs such as ethambutol, kanamycin and ethionamid can only be given in sub-optimal dosages because of the risk of toxicity (Mitchison et al, 1984)

1.4.4.4 Number of viable bacilli

The probability of resistance emerging is clearly greatly influenced by the number of viable bacilli in the lesions at the time that chemotherapy is started or a regimen changed. In tuberculosis, the risk is greatest in cavitated, pulmonary lesions with bacillary populations of perhaps 10^8-10^9 colony forming units (c.f.u.) which are usually treated with three drugs initially (East and Central African and British Medical Research Council, 1983). However, the risk is so small in closed tuberculomata; with populations usually of less than 100c.f.u. that monotherapy is justified. Lesions with fairly small bacterial populations, such as bone and joint tuberculosis and tuberculosis meningitis, could probably be safely treated with a two-drug regimen of isoniazid and rifampicin, since even if there was initial resistance to isoniazid, the probability of there being sufficient organisms to allow rifampicin resistance to emerge would be small. Again, monotherapy with isonaizid or, an experimental basis, with rifampcin is to be recommended for chemoprophylaxis in contact or patient with minimal inactive lesion. While chemotherapy for pulmonary lesions is usually started with two or three drugs given together for the first 2-3 months continuation can safely be with isoniazid alone because the number of
viable bacilli at risk from which mutants might grow has by then been reduced to a very low level. (East and Central Africa and British Medical Research, 1983)

1.4.5 Prognostic importance of resistance

Data on the prognostic influence of initial drug resistance in tuberculosis have been drawn entirely from studies carried out under the auspices of the Medical Research Council, since it is only in these studies that treatment was continued without alteration despite a finding of initial resistance, and the methods of sensitivity test were also the same.

The influence of primary or initial resistance, and on the results of the chemotherapy of newly diagnosed, severe, pulmonary tuberculosis with isoniazid alone or with another weak second drug such as PAS or thiacetazone (the prognostic effect was considerable. Thus, in the pooled result of four controlled clinical trials carried out at the Tuberculosis Chemotherapy Centre, Madras).

15 (71%) out of 21 patients with primary isoniazid resistance had an unfavourable response with bacteriologically active disease at the end of treatment, as compared with only 14 % of patients with sensitive organisms on admission to the study .Similar results were obtained in four co-operative studies in East African centres in which patients were treated either with isoniazid and PAS or with isoniazid and thiacetazone, these results were obtained in the 1960s when there was little alternative to PAS or thiacetazoneas companion drugs to be given to prevent the emergence of resistance drugs to isoniazid, particularly in developing countries.

The position has, however, completely changed as the result of introduction of newer and more potent drugs and the development during the last decade of short course regimens which greatly reduce the period of treatment necessary to obtain a bacteriologically quiescent status (Fox, 1981). The most important of these drugs, whose action has been reviewed elsewhere, (Mitchison, 1979) is rifampicin excellent both as a sterilizing drug which kills the last few semi dormant organisms in the lesion and also, as we have seen, in preventing growth of organisms throughout the lesion and therefore the emergence of resistance to a companion drug.

Pyrazinamide is almost as good as a sterilizing drug ,but is much less effective than rifampicin in preventing the emergence of resistance Ethambutol appears to be of no value as a sterilizing drug, but is moderately effective in preventing resistance.

The prognostic influence of initial resistance to isoniazid alone or isoniazid and streptomycin in the pooled results from a series of studies of short course regiment including these drugs,(Mitchison, 1982)
The results obtained in patients with initially sensitive organisms are not included since they are, with very few exceptions, uniformly favourable, irrespective of the regimen. Results in patients with initial resistance to streptomycin only are also omitted, since this resistance did not appear to influence the response to treatment. (Mitchison, 1984).

1.4.6 Molecular basis for drug resistance

*M. tuberculosis* is naturally resistant to many antibiotics, particularly those belonging to the β-lactam, macrolide or tetracycline families, and this may be a result of its highly lipophilic cell envelope acting as an efficient permeability barrier (Cole and Telenti, 1995). There are many mycobacterial genes with mutations associated with anti-tuberculosis resistance (table 3).

1.4.6.1 Streptomycin

Streptomycin was first shown to be an effective antitubercular drug in 1944 (Schatz and Waksman, 1944). The liquid MIC (Minimum Inhibitory Concentration) of streptomycin against *M. tuberculosis* has been reported to be 0.4-1.5 ug/ml (Heifets, 1994) making it one of the most effective early antitubercular drugs. The antibacterial activities of streptomycin, and related aminoglycosides, are due to the inhibition of prokaryotic protein translation. Specifically, initiation of mRNA translation appears to be inhibited, although translational accuracy is also affected (Benveniste and Davies, 1973). Mutation associated with streptomycin (STR) resistance in *M. tuberculosis* have been identified in two targets, the 16S rRNA gene (*rrs*) and the gene (*rpsl*) encoding ribosomal protein S12 (Finken et al., 1993 and Nair et al., 1993). A common mechanism of resistance to aminoglycosides antibiotics in other bacteria is drug inactivation via acetylation (Benveniste and Davies, 1973). However, this mechanism of resistance has not been reported in *M. tuberculosis*. Instead two classes of mutations account for some 80% of the high level streptomycin resistance in *M. tuberculosis* (Honore and Cole, 1994). The first consists of point mutations in the ribosomal S12 protein (Douglass et al., 1993 and Yamada et al., 1985) encoded by the *rpsl* gene, resulting in single amino acid replacements (Finken et al., 1993 and Nair et al, 1993). These mutants account for two thirds of the resistant mutations. Mapping of these mutations revealed that all mutations occurred in highly conserved region of the gene encoding one of two critical lysine residues (K43 and K88). In all cases, either K88 was converted to an arginine residue or K43 was converted to either an arginine or threonine residue (Meier et al, 1994).

1.4.6.2 Rifampicin

Rifampicin a semi-synthetic derivative of the natural product rifampicin, obtained from culture filterates of *Streptomyces mediterranei*, was introduced in 1972 as an anti tubercular drug (Woodley et
Rifampicin is extremely effective against *M. tuberculosis* (MIC. 1-0.2 ug/ml) and its rapid bactericidal activity (Mitchison. 1985) helped to shorten the course of treatment against drug-susceptible infections. For more than 20 years the target of rifampicin action in *M. tuberculosis* has been assumed to be the mycobacterial RNA polymerase (Siddiqi *et al.* 1981).

Resistance to rifampicin in many *E. coli* strains was known to arise as a result of missense and other mutations occurring in a discrete region of the *rpoB* gene (Jin and Grosst, 1989; Ovchinnikov, *et al.*, 1981 and 1983).

Resistance to rifampicin is increasing rapidly as a result of its widespread use. Rifampicin-resistant tuberculosis, often observed in conjunction with isoniazid resistance, leads to a longer- treatment period and significantly poorer chemo-therapeutic outcomes. Resistance has been observed in 3.9% of all cases nationally, but in 9.0% of patients who have been previously treated for tuberculosis (Bloch, *et al.*, 1994). Resistance is acquired at a rate of 18-8 in cultures of *M. tuberculosis*, evidence of a single-step mutational event (Tsukamura, 1972). The genes encoding the β subunit of the mycobacterial RNA polymerase, *rpoB*, have been sequenced from *M. tuberculosis* (Miller *et al.*, 1994).

The vast majority of rifampicin resistance-conferring mutations in the mycobacterial *rpoB* encoded RNA polymerase are single nucleotide changes that result in single amino acid substitutions (93%) (Honore and Cole, 1993). The remaining mutations are insertions (3%) and deletions (4%), but all mutations map to the presumed rifampicin binding site between amino acid positions 511 and 533. In two recent studies, the most commonly encountered amino acid substitutions occurred at Histadine526 and Serinine531 (60% of all mutations (Williams, *et al*, 1994) and additional mutations have been mapped to positions 512, 513 and 516 (20%) (Donnabella *et al.*, 1994). A very small percentage of rifampicin resistant clinical *M. tuberculosis* isolates do not map to the 511-533 region of RNA polymerase, but may be preset in the carboxy terminal region of the protein (Blanchard, 1996).

Other species of the genus *Mycobacterium* where found to have the same mutations in the same gene but in different locations, as *M. leprae* (Honore, *et al*, 1993; Guerrero, *et al*, 1994.), *M. avium* (Williams, *et al*, 1994) and *M. africanum* (Williams, *et al*, 1994;, Vareldzis,*et al*, 1994)

### 1.4.6.3 Isoniazid (INH)

Primary resistance of *M. tuberculosis* to isoniazid (INH) arises in the laboratory at an estimated frequency of $10^{-5}$ to $10^{-7}$ (David, *et al*, 1971), which is several orders of magnitude greater than that reported for RIF (Zhang ,*et al*, 1993). In an effort to elucidate the molecular mechanism responsible for
the observed decreased catalase activity associated with INH-resistant organisms, (Zhang, et al, 1992). Cloned and Heym et al. (Heym, et al, 1993) characterized the gene (KatG) coding for the M. tuberculosis catalase – peroxidase. The M. tuberculosis enzyme is an 80000-Da protein with substantial homology to hydroperoxidase I from E.Coli, catalase-peroxidase from M. intracellulare (Morris, et al, 1992), and other bacterial catalase – peroxidases. Transformation of M. segmatis and M. tuberculosis with wild-type katG restored INH susceptibility to resistant isolates (Zhang, et al, 1993) a result confirming that the protein product of katG (katG) participated in INH action. In addition, it was observed that in two of three high-level (MIC >50µg/ml )resistant patient isolates katG was deleted from the chromosome ((Zhang ,et al, 1992). These investigators concluded that resistance in a subset of M. tuberculosis strains is due to loss of the complete katG gene . However, they also observed by Southern analysis that most INH-resistant strains had an apparently intact katG gene, although the levels of catalase-peroxidase activity recorded for these organisms were greatly decreased. In addition. Stoeckle, et al . (Stoeckle, et al, 1993) studied by PCR 80 randomly selected isolates recovered from patients in New York City and found that 35 (90%) of 39 INH-susceptible and 31 (76%) of 41 INH – resistant strains contained apparently intact katG sequences and therefore did not have a gross deletion of this gene . Moreover, there was no simple relationship between absence of katG and occurrence of high – level (>1µg/ml) INH resistance. These observations suggested that for most strains either simple base-pair (bp) changes resulting in (for example) missense or stop mutations or small deletions were associated with resistance.

The observation that most INH-resistant M. tuberculosis strain did not have gross katG deletion suggested the need to more precisely analyze the structure of katG present in resistant organism. Several groups have now reported that many INH-resistant strains contain missense and other types of mutations organisms. For example, Altamirano et al (1994) studied nine INH – resistant isolates and one INH – susceptible patient isolate and found that a 237 bp fragment of katG could be amplified from eight of nine INH-resistant isolates. These eight INH –resistant organisms had missense mutations, deletions of 1 base, or insertions of up to 3 bases in the region studied (nucleotides 3 through 239 located at the 5 end of the gene).

Heym et al (1994) recently reported the results of characterization of the katG gene in 20 INH- resistant organisms and 16 strains that were resistant to both INH and ethionamide (ETH). For these organisms, the MICs of INH ranged from 0.2 to >10 µg/ml. These investigators found that 23 of 36 (64%) organisms had mutations in katG, as defined by SSCP analysis.
Characterization of \textit{katG} sequences in 18 INH-resistant organisms with altered SSCP patterns found several types of mutation located predominantly in the region encoding the amino-terminal one-half of the protein (Heym, et al, 1995) The most common mutation (n=7 strains) was a CGG$\rightarrow$CTG change resulting in an Arg$\rightarrow$Leu substitution at amino acid position 463. Four additional missense mutations were identified ,including GTG$\rightarrow$GCG (f-met$\rightarrow$Ala, position 1)in one strain ,GAC$\rightarrow$GCC (Thr-275 pro) in one strain, AGC$\rightarrow$ACC (Ser-313 Thr) in five strains, and CTG$\rightarrow$ATG (Leu-587Met) in one strain. Tow strains that had a deletion of 12 bp resulting in absence of amino acids 120 to 123 were identified, and one strain had an insertion of 3 bp (CAT, Ile) between codons 125 and 126.

The availability of the crystal structures for related enzymes, including yeast cytochrome c peroxidase (Finzel \textit{et al}, 1984), permitted several inferences to be made about structure-function activities of the \textit{katG} product. The deletion reduce residues 120-123 and the insertion of Ile between amino-acids 125 and 126 were predicted to result in diminution of catalase activity, and it was shown that organisms bearing these mutations had only approximately 10\% residual catalase-specify activity related to strains with wild-type \textit{katG} alleles. The amino-acid replacements at position 275 ( Thr$\rightarrow$Pro) and 315 (Ser$\rightarrow$Thr) were also predicted to result in decreased catalase activity because amino-acid residues located close to the equivalent positions in yeast cytochrome C peroxidase and \textit{E.coli} hydroperoxidase are functionally important (Loemen \textit{et al}, 1990; Pelletier \textit{et al}, 1992; Sivaraja \textit{et al},1989) theses predictions were born out by enzymatic studies demonstrated \textit{M. tuberculosis} strains with the position 275 or 315 substitutions have virtually no detected catalase activity (Heym \textit{et al}, 1994). In contrast to the diminished catalase activity observed among organisms with the mutations described above, strains with the Arg-463$\rightarrow$Leu substitution had essentially wild-type levels of enzymes activity.

\textit{KatG} gene characterized also by Cockerill \textit{et al} (Cockerill ,\textit{et al}, 1995) in 15 strains of \textit{M. Tuberculosis} For which INH MICs ranged from < 0.12 to > 32 µg/ml, including 9 resistant and susceptible strains. Sequencing of \textit{katG} from all organisms 6 of 9 INH-resistant strains had one or more missense-mutations, one strain had a non-sense mutation, one had an 8-bp deletion, and one had no mutations in the coding sequence. In Striking contrast to the data presented by Heym et al(Heym ,et al,1994), in which virtually all strains had single amino-acid substitutions, 9 of the strains had multiple missense-mutations, including one organisms . Five of six INH – resistant strain with missense mutation had, in addition to other change, a G$\rightarrow$T transversion in codon 463, resulting in the substitution of Leu for Arg. The six INH- susceptible strains had none to 11 amino acid substitutions compared with a consensus sequence formulated from the data available for all 15 organisms; however, nine of the mutations affected Arg-463. The G$\rightarrow$T change in codon 463 results in loss of a restriction enzyme site
recognized by NciI and MspI and thereby generates a convenient strategy to rapidly screen large collections of organisms for this polymorphism. Analysis of 32 INH-susceptible and 43 INH-resistant strains demonstrated that 19 of 43 (44%) of resistant strains, but only 1 of 32 (3%) of INH-susceptible strains, had lost this restriction site and were presumed to contain the G→T mutation resulting in an Arg → Leu substitution.

Moreover, sequence analysis of a region of *katG* encoding residues Ser-315 and Arg-463, and the inhA locus, in 10 susceptible and 51 INH-resistant bacteria had mutations in *katG* or the inhA locus. Interestingly, all 16 strains of *M. bovis* and *M. microti* had Leu-463 rather than Arg-463 is the ancestral condition in *M. tuberculosis*. Taken together, the data are consistent with results showing that very little *katG* allelic variation occurs in INH-susceptible strains, and a restricted subset of missense changes are repeatedly associated with INH resistance. Although there is evidence to suggest that one or more of the variant *katG* alleles confers INH resistance, there is clearly a need for additional molecular genetic data addressing the exact role of some of the non-synonymous substitutions described by several investigators.
Table 3. Mycobacterial genes with mutations associated with anti-microbial resistance *

<table>
<thead>
<tr>
<th>Anti-microbial agent</th>
<th>Species</th>
<th>Gene</th>
<th>Size(bp)</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td><em>M. tuberculosis</em></td>
<td></td>
<td>157</td>
<td><strong>β-</strong> sub-unit of RNA polymerase</td>
</tr>
<tr>
<td></td>
<td><em>M. africanum</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>M. leprae</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>M. avium</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoniazid</td>
<td><em>M. tuberculosis</em></td>
<td></td>
<td>2,205</td>
<td>Catalase-peroxidase</td>
</tr>
<tr>
<td></td>
<td><em>M. tuberculosis</em></td>
<td></td>
<td>810</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>katG</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>inhA</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>kas A</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptomycin</td>
<td><em>M. tuberculosis</em></td>
<td></td>
<td>1,464</td>
<td>16S r RNA</td>
</tr>
<tr>
<td></td>
<td><em>smegmatis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rpsL</td>
<td></td>
<td>272</td>
<td>Ribosomal protein S12</td>
</tr>
</tbody>
</table>

* Adapted from Muser (1995)
2.1 Area and period of investigation

Two hospitals were selected for the collected of samples, namely: El Shaab Teaching Hospital at Khartoum and Hamed EL-Neel hospital at Omdurman, the hospitals were chosen because of the high number of tuberculosis patients attending these centres. The collection was undertaken in the period between July 1999 to May 2000.

2.2 The clinical specimens

2.2.1 Patients

250 sputum specimens were collected from new tuberculosis cases before they were given anti-tubercle drugs. The disease was suspected according to the characteristic TB symptoms notably fever, productive sputum and weight loss (WHO, 1996). The patient samples were selected randomly and were of different age groups and of both sexes, some of them were BCG vaccinated and others were not. They came from different states of Sudan and represent different tripes.

2.2.2 Samples collection

Sputum specimens from the first productive deep cough of the morning were obtained from each patient in a sterile screw top plastic disposable cup container (Vderman International). Care was taken to avoid contamination of saliva or nasal secretions. Before sampling, patient was asked to rinse his mouth with water.

2.3 Microscopy and culture methods

2.3.1 Ziehl Neelsen stain procedure (ZN)

Slides for staining were placed on a staining slide-rack over the sink with smeared side uppermost, their edges separated about one cm. Thin smear from each sample was made, fixed with heat then stained with ZN method as fellows: a strongly acting dye with a mordant, was needed, carbol fuchsin,
was poured on slide with the application of heat to facilitate impregnation; this was followed by the
decolourization with 20% sulphuric acid and 95% alcohol separately or 3% hydrochloric acid in 95%
alcohol and then counter stained with methylene blue. Acid fast bacteria appear red in a blue
background (Gowan and Steel, 1995).

2.3.2 Culture of *M. tuberculosis*

Sputum sample was transferred to sterile, graduated, 50 ml plastic conical centrifuge tube, an equal
volume of 4%NaOH solution was added, and the tube capped and then the contents mixed for
approximately 20s on a vortex mixer until they were liquefied. The mixture was swirled by shaker for
20 min to further mix the contents, then the solution was centrifuged for 20 min at > 3000xg. The
supernatant was carefully decanted into splash-proof discard container containing phenol disinfectant.
The sediment was washed by sterile distil water, and the supernatant was carefully decanted into
splash-proof discard container containing phenol disinfectant. Sterile pipette was used for each tube, a
few amount of sediment was took using separate sterile pipette for each tube, The sputum sediment was
divided into two aliquots: the first part was used to prepare the smear for microscopic examination and
from the second part 2-3 drops were inoculated on the surface of two solid culture media (Löwenstein-
Jensen medium). The specimen was incubated at 37°C for at least 8 weeks.

2.4 Molecular detection of drug resistance

There are many molecular methods to detect drug resistance; two of them were used to detect
rifampicin (SSCP) and streptomycin (RFLP) resistant.

2.4.1 Precautions to prevent contamination of molecular methods

Prevention of contamination was accomplished by physical separation of different steps in the PCR
procedure, using different pipettes, and wearing separated coats and gloves in each laboratory. The
PCR mix was prepared in the PCR room, where work was conducted in a safety cabinet to prevent
contamination to the PCR reagents. Chemicals used in the PCR and for the treatment of clinical
samples were weighed in this room on a balance, which was used only for this purpose. Ultra-pure
water was used to make the solutions, which were supplied kindly by Dr. M.E. Hamid, Faculty of
Veterinary Medicine. In the Pre-PCR room, clinical samples were treated in a safety cabinet and added
to the PCR mix. Chemical decontamination of surfaces and equipment was done weekly in both rooms
with sodium hypochlorite (Prince and Andrus, 1992). The detection of PCR products by agarose gel
electrophoresis was done in a third room (Post-PCR room).
2.4.2 DNA extraction

Approximately five colonies of *M. tuberculosis* were scraped from the L J slant and transferred to 1.5 micro-centrifuge tube containing 100 µL of sterile distilled water. The preparation was vortex for 3-5 min then heated in a heating block at 80 °C for 30 min to kill *M. tuberculosis* and to release the DNA. Five microlitres were used for each PCR reaction.

2.4.3 Molecular identification of acid fast isolates

Two molecular methods were used to identify of acid fast isolates; PCR and RFLP- fingerprinting

2.4.3.1 DNA amplification of *M. tuberculosis* mutant genes

PCR was performed in a total volume of 100µl reaction mixture containing 10X PCR buffer (10µl) , 25 mM magnesium chloride (6µl), 10 mM dNTPs mixture (8µl), each set of primers (2µl), 5 U/µl of *Thermus aquaticus* DNA polymerase (0.3µl) (supplied by Promega). The reaction mixture was overlaid with 1-2 drops mineral oil to avoid evaporation. The tubes were then subjected to 37 thermal cycles in a programmable thermocycler (Biometra) as follows: DNA was denatured at 94 °C for 3 minutes for one cycle followed by 94 °C for one min as secondary denatured and annealed at rang 50- 64 °C (The annealing temperature depend on the specific primer set – Tm = 4 (G+C) + 2 (A+T) (table 4) followed by extension at72°C for one min for 35 cycles and a final extension at 72 °C for 10 minutes for one cycle.

2.4.3.2 RFLP – Fingerprinting

RFLP – Fingerprinting, using Van Soolingen method was done to identify *M. tuberculosis* complex by using *IS6110* and *Mob II* restriction enzyme as follow;

2.4.3.2.1 Isolation of Genomic DNA from mycobacteria

Several loops of colonies were transferred into a microcentrifuge tube containing 400 ul 1X TE, and were heated at 80 °C for 20-60 min to kill the cells.50 ul of 10 mg /ml lysozyme were added and the samples were well vortexed and spined down, then incubated for an hour at 37 °C .75 ul of 10% SDS (10gm / 100ml ddH2O) /proteinaseK (10mg / 1ml ddH2O) were added, shortly vortexed and incubated at 65 °C for 10 min.100ul of 5M NaCl (29.2 gm / 100ml dH2O ) and another 100ul of CTAB / NaCl solution (4.1 gm NaCl / log CTAB up to 100ml dH2O ) which was pre-warmed at 65°C , were added, and the mixture was vortexed until the liquid has become milky solution, then incubated at 65°C for 10
min. 750 ul of chloroform / isoamly alcohol (24 / 1 volume) were added, vortexed for at least 10 sec, and centrifuged at room temp. for 5 min at 12,000g.

All of the aqueous supernatant was transferred into a fresh microcentrifuge tube (180 ul), and carefully 0.6 volume (450ul) isopropanol were added to precipitate the nucleic acids, and the tubes were inverted up side down several times by hand. The amount of 1X TE in which the DNA should be dissolved, was estimated according to the size of the precipitate and was found to be 20ul. The samples were placed at -20°C for 30 min, then spined for 15 min at room temp. in a microcentrifuge at 12,000g. Most of the supernatant was taken out, and 1ml of 70% ethanol was added and the tube was turned slowly up side down a few times, and spined for 5 min at 4 °C in a microcentrifuge, and the supernatant was discarded as much as possible. The tube was spined 1 min in a microcentrifuge and the last leftover of supernatant was cautiously discarded to make sure that all traces of ethanol were removed. The pellet was permitted to dry at room temp for 10 min, and re-dissolved in 1X TE, using 25ul TE. Spectrophotometer was used to estimate the concentration of DNA of each sample.

### 2.4.3.2.2 Digestion of chromosomal DNA by Pvu II

About 4.5 ug of genomic DNA samples were digested in a final volume of 20ul in a microcentrifuge tube, and at the same time 4.5 ug of the reference strain H37Rv were digested. The volume of enzyme was less than 10% of the total reaction volume.

| 9 ul DNA ( 4.5 ug )       |
| 2 ul 10X digestion buffer |
| 1 ul PvuII (10U / ul )   |
| 8 ul ddH2O                |

20 ul ( Total volume )

The sample was mixed and briefly centrifuged for 5 sec at 12,000g, then incubated for 1 hour at 37°C.

### 2.4.3.2.3 Confirmation of DNA concentration and enzyme digestion

0.8% agarose gel in 1X TBE buffer was made for a small gel, and was swirled and cooled down to 65°C. The gel was poured in a tray without making air bubbles in the gel, and was left to solidify. The buffer vessel was filled with the 1X TBE. 5 ul of 5X DNA sample buffer with RNase was added to 20
ul of the DNA samples, then mixed and spined down for 5 sec. 5 ul of the lambda-Hind III mix were loaded in the first slot. Each DNA samples was loaded and electrophoresised for 20 min at 100V. A gel was stained in a EtBr solution for 5 min and destained in water for 20 min, and then viewed on an UV transilluminator using short waves. A picture of the gel was taken.

2.4.3.2.4 Separation of DNA fragments by electrophoresis for southern blotting

A large (16X24 Cm ) 0.8% agrose gel in1X TBE buffer was made. An EtBr was added to the dissolved agrose to a final concentration of 0.5 ug / ml, and was poured in the gel-former and left to solidify. The buffer vessel was filled with the 1X TBE containing 0.5 ug / ml EtBr, and the gel was covered to a depth of about 2 mm. 5 ul of the lambda-HindIII was loaded in the first slot. Also equal amounts of the genomic DNA samples were loaded in all slots. The samples were electrophoresised for 10 min at 100V and the voltage was decreased to 30V and run overnight. The gel was checked on an UV transilluminator and electrophoresised further until the 2 kb lambda-Hind III band has reached a distance of 7 Cm from the wells.

2.4.3.2.5 Blotting by the capillary method

A gel–holder was placed up side down in a big tray and covered with a piece of Whatman paper that was wider than the gel-holder, so that the paper could touch the bottom of the tray, and absorb the buffer in the tray. It was carefully soaked with 10X SSPE. The inverted gel was placed on the Whatman paper, avoiding the air bubbles between the gel and the paper. A Hybond N-plus membrane was cut to the same size as the gel, one edge of the membrane was cut for marking the membrane. The 4 side of the gel were surrounded with Parafilm. 2 pieces of 3MM paper were wetted in 2X SSC and placed on top of nitrocellulose membrane. Finally a pile of paper towels, just smaller than the 3MM papers was placed, and a glass plate was placed on top of the stack, and was weighted down with 500g weight on a glass plate on top of the blot-tower. The transfer of DNA was allowed to proceed for 20 hours. The paper towels and the 3MM papers above the gel were removed. The gel side up was laid on a dry sheet of 3MM paper. The positions of the gel slots on the filter were marked with a pencil. The gel was peeled.
### Table 4. Primers and specifications of *rpoB*, *rpsL* genes and insertion element *IS6110*

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Tm</th>
<th>Fragment length</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS6110 INS1</td>
<td>CGTGAGGGCATCGAGGTGGC</td>
<td>65°C</td>
</tr>
<tr>
<td>IS6110 INS2</td>
<td>GCGTAGGCGTCGGTGACAAA</td>
<td></td>
</tr>
<tr>
<td><em>rpoB</em> gene TR 8</td>
<td>TGCACGTCGCGGCCCTCCA</td>
<td>58°C</td>
</tr>
<tr>
<td><em>rpoB</em> gene TR 9</td>
<td>TCGCCCGCGATCAAGGAGT</td>
<td></td>
</tr>
<tr>
<td><em>rpsL</em> gene STR 52</td>
<td>GTCAAGACCGCGGCTCTGAA</td>
<td>60°C</td>
</tr>
<tr>
<td><em>rpsL</em> gene STR 34</td>
<td>TTCTTGACACCCTGCGTATC</td>
<td></td>
</tr>
</tbody>
</table>
from the filter and stained for 10 min in a solution of EtBr (0.5 ug/ml) in water, destained with water and examined by UV transilluminator for complete transfer of the DNA from the gel. The filter was soaked in 6X SSC for 5 min at room temp. to remove any piece of agarose sticking to the filter. Then the filter was removed from the 6X SSC and excess fluid was allowed to drain away. The filter was placed flat on a paper towel to dry for 30 min. The filter was sandwiched between two sheets of dry 3MM paper, and the DNA was fixed to the filter by baking for 1 hour at 80°C in a vacuum oven.

2.4.3.2.6 Preparation of DNA probe by PCR

The PCR mixture was prepared using 2 ul of genomic DNA M. bovis BCG and INS1 and INS2. The amplified PCR products were checked by running on an agarose gel.

2.4.3.2.7 Purification of the PCR product using geneclean-kit

The PCR product was run on an 1% agarose gel in 1X TAE buffer. The band of the PCR product was excised, and weighted. 5 ul NaI were added to a solution of DNA, the NaI-agarose solution was incubated at 55°C to melt gel for 5 min with occasional mixing by tapping. 5 ul of GLASSMILK suspension were added for the 5ug DNA purification. The DNA solution was incubated at room temp for 5 min to allow binding of the DNA to the silica matrix and was mixed every 2 min to ensure that GLASSMILK stayed suspended. The GLASSMILK /DNA complex was pelleted by spin in a microcentrifuge for 5 sec at full speed. The pellet was washed 3 times with new wash (40 volumes of the wash were added), and the complex was resuspended by pipetting back and forth, after suspension, it was spun for 5 sec in the centrifuge, and the supernatant was discarded, the wash procedure was repeated two more times. The pellet was dried by placing the tube under vacuum for 3 min. The then washed, white pellet was resuspended with water, TE and left for 5 min at room temperature, then
centrifuged for 3 min and carefully the supernatant containing the eluted DNA was removed and placed in a new tube.

2.4.3.2.8 Labeling the probe

The probe was diluted with water from the ECL-kit (Amersham) to a concentration of 10 ng/µl. The probe was then denatured for 5 min by placing it in a boiling water bath, and chilled on ice immediately for 1 min. The probe mixture was spined a few seconds in a microcentrifuge at 12,000g, and an equal volume DNA labelling reagent was added, mixed well by pipetting. The same volume glutaraldehyde was added, mixed and carefully vortexed for 1 second. The solution was spined a few seconds in a microcentrifuge at 12,000g and incubated for 10 min at 37 °C. The probe was then ready to use in a hybridization experiment.

2.4.3.2.9 Hybridization

The filter was prehybridized in a sealed plastic bag for 15 min at 42 °C. in hybridization buffer, which was poured in a bottle and the labeled probe was added, mixed well and poured back on the filter. That was hybridized overnight at 42 °C. under shaking.

2.4.3.2.10 Washing the filter after Hybridization

The primary wash buffer was pre-warmed to 42 °C. The filter was placed in a clean plastic box and washed twice with primary wash buffer for 20 min at 42 °C. 500 ml secondary wash buffer were added to the filter, the filter was washed for 5 min at room temperature on a shaking platform, that wash was repeated twice.

2.4.3.2.11 Detection

Equal volumes of ECL detection reagent 1 and 2 were mixed in a bottle. The red light was turned on and the light was turned off. The filter was taken out of the secondary wash buffer, and excess fluid was drained, and moved to a clean plastic box. The detection mixture was poured directly on the blot on the DNA-side, and was evenly separated by rotating the box for exactly 1 min. the filter was taken from the box, drained and but onto a carrier and then warped in Saran warp and placed in a cassette with DNA side on top. The film twas place on the filter with the folded corner on the right top of the
filter. The cassette close one min, then the film replaced for a new exposure. The film was develop by the developer fot 5min and fixed.

2.4.3.3 Gel electrophoresis

2.4.3.3.1 Agarose gel electrophoresis

The standard method for agarose gel electrophoresis 1.8% was described by Kolk et al (1992). In this method 1.8% 1XTBE (Tris boric EDTA) agarose gel (1.8 agarose, 100ml of 1XTBE buffer) was poured in a horizontal electrophoresis apparatus. Ethidium bromide (EtBr) was added to the gel (5ul of 10mg/ml EtBr per 100ml gel solution). The gel was covered with 1X TBE.

Five microlitres of each PCR product were mixed with 3ul of loading buffer (glycerol, bromphenol blue and water ratio 1:1:8). A size marker DNA in the range 100-1000 bp (Boehringer Mannheim) was loaded for the determination of the amplified DNA size. The apparatus was connected to a power supply of a 80mA current for 30-45 mins. After completion of the run, the gel was observed under ultraviolet light to determine if PCR products have been successfully amplified. The bands size were estimated by base pair were depending on positive controls and the DNA marker. Negative controls were used to avoid contamination, sensitive controls used to avoid false positive results and positive controls consisted of resistant \textit{M. tuberculosis} which were used to avoid false negative results and to compare them with the positive results. Photography of the gel was performed by a Polaroid camera (Sigma) when needed.

Controls:

\textit{Mycobacterium tuberculosis} strain H37Rv was kindly supplied by Dr. M. Hamid, Faculty of Veterinary Medicine, University of Khartoum and used as a sensitive positive control. Positive and negative controls of \textit{rpsL} and \textit{rpoB} were obtained from Stellenbosch University R.S.A. Negative control which included PCR mix without DNA, and DNA marker, were included in each run.

2.4.3.3.2 Polyacrylamide gel electrophoresis (PAGE)

The PAGE was prepared according to the procedure described by the IAEA protocol, (1999). 12% PAGE solution contains 30% acrylamide; 29g acrylamide, 1g N,N,-methylene Bis-acrylamid, water to 100ml), 3% ammonium persulphate; ( 0.3g Ammonium Persulphate, 10ml
water), 10X TBE; (108 g Tris, 55g boric acid, 7.4g EDTA), and 1000ml Water. PH8.30). Loading Buffer; (0.25% xylene cyanol, 30% glycerol) was used to visualize the DNA of *M. tuberculosis* complex. PAGE gel was stained with ethidium bromide (EtBr) for visualizing the amplified DNA fragments. The gel was left for one hour to set, then the molecular marker and the PCR product were loaded, 15 µl from each PCR product mixed with 3µl loading buffer and ran on PAGE gel for approximately 2 hour at 80 W in 1X TBE buffer, on vertical mini-gel apparatus. The results were viewed on transilluminator.

2.5 Detection of mutations

2.5.1 Single Strand Confirmation Polymorphism (SSCP)

The SSCP technique which depends on mobility shift was used to detect *rpoB* mutant genes. This process involves 6% PAGE (40ml from 30% acrylamid,4.2ml from 3% ammonium persulphate, 20ml from 6X TBE, 135ml water, 10ml glycerol,100µl TEMED) on vertical electrophoresis. The wells were cleaned by using a syringe and needle, pre-run for 10 min at 50W was done on a cold place. 5µl of the PCR product was mixed with 5µl stop solution (95% formamide, 0.005% brom-phenol blue, 0.005% xylene cyanol, 20mM EDTA). The mixture was denatured at 95°C for 2 min, and immediately kept on ice, 10µl of samples and 5µl of marker was loaded onto gel, then electrophoresed at 50W in 0.6X TBE-buffer for 3 hours. The plate was then detached from the electrophoresis unit.

The PAGE gel stained by silver stain as follows; the gel was fixed and shook gently for 8 min in solution 1( 0.5% acetic acid (5 ml), 10% ethanol (100ml), 895ml water) poured off and washed with water, then incubated for 10 min in salutation 2 (1g silver nitrate, 1000ml water) poured off and washed twice with water then incubated and shook gently in solution 3 (60ml sodium hydroxide; 45g sodium hydroxide, 300ml water ), 60ml sodium borohydrate; (0.3g sodium borohydrate, 300ml water), 2.4ml formaldehyde, water up to 600), after that the reaction quenched by washing the gel in solution 4 ( 7.5g sodium carbonate, 1000ml water) for 10 min.,Then the gel took to trnsimilator to looked for mobility shifts of bands in comparison to control samples and the marker.

2.5.2 Restriction fragment length polymorphism (RFLP)

RFLP was used to detect polymorphisms in *rpsL* gene using the restriction endonuclease *Mob II*. The set of primer *STR52* and *STR34* were used to amplify a 272 bp region of the *rpsL* gene (around codon
43). 10 µl PCR product was added to mixer content (*Mob II* enzyme 1µl (10U), 10X enzyme buffer 2µl, water 7µl). The mixture was incubated at 37°C for 2 hours to activate the enzyme reaction, then the activity of the enzyme was stopped by incubating the mixer at 72°C for 10 min, after this steps 3µl loading buffer was added to each restricted product. The result was confirmed on 12% PAGE on mini-gel and visualized after staining with EtBr. In this particular case a mutation at codon 43 of the *rpsL* gene removes the *Mob II* cleavage site and isolates carrying the mutation at this position therefore remain undigested (normal gene must cut- but mutant gene dose not cut).

**CHAPTER THREE**

**RESULTS**

3.1 Microscopic examination

out of 250 suspected tuberculosis sputum samples 201 (80.4%) were found to be AFB following Ziehl Neelsen (ZN) stain and microscopic examination. Samples were taken from suspected tuberculosis cases but these received on previous treatment (primary cases)

3.2 Culture characterization

All the 250 specimens were cultured on Löwinstein Jensen media at 37°C for at least 8 weeks using the pre-treatment methods. 147 (73.1%) out of the 201 specimens which were AFB positive, and three out of 49 which were negative AFB revealed growth of *M. tuberculosis*, the growth was obtained in more than two weeks with rough, dry colonies and cream colour without pigmentation. 17 (8.5%) were rapid grower mycobacteria (MOTT) which were not fully identified to species level. Four out of them were with yellow pigmentation. Seven of the strain revealed growth similar to *Nocardia* spp., whereas 14 (6.9%) and 21(8.4 %) from which were AFB negative was contaminated plates and 16 (7.9%) from AFB positive and 17 (6.8 %) which were AFB negative specimens did not show growth on LJ medium. These results are summarised in, table 5 and Fig. 2 exhibits the correlation of tuberculosis and other organisms among male (n=96= 38.4%) comparable to female (n=54=21.6%). Also the correlation of
tuberculosis and other organisms isolated from tuberculosis cases to BCG vaccination; 65 (26%) were vaccinated whereas 185 (74%) were not vaccinated, exhibits in (table 5 and Fig. 3).

Table 6 and figure 4. Illustrates the comparison between microscopic and culture results

3.3 DNA fingerprinting of IS6110

Results of Fingerprinting of IS6110 analysis performed on culture is shown in (Fig. 5). 150 (60 %) IS6110- culture samples out of 250 culture samples were classified as M. tuberculosis complex. The resistant strains to rifamipcin gave exactly match patterns and also five of the sensitive strains gave similar patterns too. While two sensitive strains revealed patterns with an extra band in one of them.

Table 5.
Correlation of tuberculosis and other organisms isolated from tuberculosis cases to BCG vaccination and sex.

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>Species</th>
<th>BCG</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>vaccinated</td>
<td>Not</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>vaccinated</td>
</tr>
<tr>
<td>150 (60 %)</td>
<td>M. tuberculosis</td>
<td>9 (3.6%)</td>
<td>141(56.4%)</td>
</tr>
<tr>
<td>17 (6.8 %)</td>
<td>Rapid grower</td>
<td>12(4.8%)</td>
<td>5 (2%)</td>
</tr>
<tr>
<td>7 (2.8 %)</td>
<td>Nocardia sp.</td>
<td>3 (1.2%)</td>
<td>4 (1.6%)</td>
</tr>
<tr>
<td>8 (3.2 %)</td>
<td>Fungi</td>
<td>7 (2.8%)</td>
<td>1 (0.4%)</td>
</tr>
<tr>
<td>33 (13.2%)</td>
<td>Contamination</td>
<td>19 (7.6%)</td>
<td>16 (6.4 %)</td>
</tr>
<tr>
<td>35 (14%)</td>
<td>No growth</td>
<td>15 (6%)</td>
<td>18 (7.2%)</td>
</tr>
</tbody>
</table>
### Table 6. Comparison between microscopy and culture results

<table>
<thead>
<tr>
<th>Microscopy</th>
<th><em>M. tuberculosis</em></th>
<th>Fungi</th>
<th><em>Nocardia</em> sp.</th>
<th>MOOT</th>
<th>Contamination</th>
<th>No growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive AFB</td>
<td>147 00 07 14 16</td>
<td>00</td>
<td>00</td>
<td>17</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Negative AFB</td>
<td>03 08 00 00 21 17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5

DNA fingerprinting of IS6110 of *M. tuberculosis* isolates, digested by *PVUII*. Lanes 1 and 8 contain standardized DNA producing fragments of known molecular weights (Hae III), lanes 5, 6, and 17 show few amount of DNA; patterns in lanes 4 and 7 match exactly (*M. tuberculosis* strains with *rpoB* mutant gene), patterns in lanes 11 and 15, and so patterns in lanes 8, 10, 12, 13 and
lane 16 of *M. tuberculosis* (sensitive strain) patterns in lanes 2 and 3 match with exception of an extra band in lane 2, lane 14 contain DNA of *M. tuberculosis* with mutant types of *rpsL* gene, lane 9 contain DNA of *M. tuberculosis* sensitive strain.

**Figure. 6**

1.8 % agarose gel electrophoresis of PCR amplified *rpoB* gene (primers TR8 and TR9) from *M. tuberculosis* isolates.

Lanes 1 and 19, *rpsL* negative control; lanes 2 and 16 positive controls; lanes 15 and 30 DNA marker (100 bp), lanes 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 and 29 clinical isolates of *M. tuberculosis*. 
Figure 7
Agarose gel electrophoresis of PCR amplified rpoB genes (157bp) from *M. tuberculosis* isolates.
Lanes 1, *rpoB* positive control; lane 3, negative control; lane 6, DNA marker (100 bp), lanes 2, 4, 5, 7, 8, 9, 10, 11 and 12 clinical isolates of *M. tuberculosis*.
Figure 8

6% polyacrymaide gel electrophoresis of PCR amplified rpoB from M. tuberculosis isolates. Lanes 1, *rpoB* negative control; lane 7, *rpoB* positive control; lane 11, DNA marker (100bp), lanes 2, 3 and 10 sensitive strains of *M. tuberculosis*; lanes 4, 6, 7 and 9 mutant rpoB genes showing mobility shift; lanes 5 and show a few mount of DNA.
Figure 9

6% polyacrylamide gel electrophoresis of PCR amplified rpoB from M. tuberculosis isolates. Lanes 1 *rpoB* negative control; lane 8 *rpoB* mutant gene control; DNA marker (100 pb) was used as molecular weight (not shown). Lanes 2, 3, 4, 5, 7, 9, 12 and 13 sensitive strains of *M. tuberculosis*; lanes 11 mutant *rpoB* genes showing mobility shift; lanes 6 and 10 show a few mount of DNA.
3.4 PCR –SSCP analysis of rpoB gene

PCR was done to the 150 isolates which were AFB and showed typical morphological characteristics of M. tuberculosis. The reaction was done using set of primers (TR8 and TR9) to amplify rpoB gene and were successfully amplified, (Fig. 7 and 8).

SSCP of amplified rpoB gene, showed that 4 strains exhibited mobility shift of rpoB (strains; M.tb-SD7, M.tb-SD44, M.tb-SD78 and M.tb-SD113). Out of 150 strains of M. tuberculosis. (Table 7, 8 and Fig. 9, 10).

3.5 PCR - RFLP analysis of rpsL gene

PCR were done to the 150 isolates which were AFB and shown typical morphological characterization of M. tuberculosis, using set of primers (STR31 and STR51) to amplify rpsL gene, and were successfully amplified. (Table7, 8 and Fig. 11).

By using Restriction Enzyme Mob II, two strains 1.3% (M.tb-SD78 and M.tb-SD205) out of 150, which were amplified, did not cut and that mean they contain rpsL mutant gene (Fig. 12 and 13). Only one strain (0.7%) showed mutations to both rpoB and rpsl genes (M.tb-SD78). (Table 7 and 8).
Figure 10
Show a gorse gel electrophoresis of PCR amplified *rpsL* (272 bp) gene with primers STR52 and STR34 from *M. tuberculosis* isolates.
Lanes 1 rpsl positive control (DNA ready made) and lane 7 negative control; lane 4 DNA marker (100 bp); lanes 2, 3, 4, 5, 6, and 8 clinical isolates of *M. tuberculosis*.

**Figure 11**

PCR – RLFP analysis of amplified rpsL gene digested with *Mbo II* restriction enzyme on 1 % agarose gel.

Lanes 1 and 9 DNA as molecular size marker (100 bp); lane 8 and 12, *rpsl* positive control; and lane 16, negative control; lane 14 and 15, *rpsL* mutant genes, lanes 2, 3, 4, 4, 6, 7, 10, 11, 12 and 13 *M. tuberculosis* isolates (no mutations).
Figure 12

Restriction enzyme digest of *rpsl* gene 12% page gel electrophoresis analysis of the gene with restriction enzyme *Mbo II*.

Lanes 1, *rpsl* positive control; lane 2, *rpsL* negative control; lanes 3 and 5 *rpsL* mutant types; lane 4, 6, 7, 8, 9, and 10 clinical isolates of *M. tuberculosis* (sensitive strains, no mutant genes).
Table 7. Drug resistance pattern and mutations to *rpoB* and *rpsL* genes among five *M. tuberculosis* isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Drug Pattern</th>
<th>Mutations genes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em> - SD7</td>
<td>rifampicin</td>
<td><em>rpoB</em> 531</td>
<td></td>
</tr>
<tr>
<td><em>M. tuberculosis</em> - SD44</td>
<td>rifampicin</td>
<td><em>rpoB</em> 531</td>
<td></td>
</tr>
<tr>
<td><em>M. tuberculosis</em> - SD78</td>
<td>rifampicin, streptomycin</td>
<td><em>rpoB</em> 531 &amp; <em>rpsL</em> 43</td>
<td></td>
</tr>
<tr>
<td><em>M. tuberculosis</em> - SD113</td>
<td>rifampicin</td>
<td><em>rpoB</em> 531</td>
<td></td>
</tr>
<tr>
<td><em>M. tuberculosis</em> - SD205</td>
<td>streptomycin</td>
<td><em>rpsL</em> 43</td>
<td></td>
</tr>
</tbody>
</table>
Table 8. Percent of drug resistant of mutations in *rpoB* and *rpsL* genes among 150 *M. tuberculosis* isolated from patient at Khartoum

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mutant gene</th>
<th>Number</th>
<th>percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td><em>rpoB</em> gene</td>
<td>4</td>
<td>2.7 %</td>
</tr>
<tr>
<td></td>
<td><em>rpsL</em> gene</td>
<td>2</td>
<td>1.3 %</td>
</tr>
<tr>
<td></td>
<td><em>rpoB</em> &amp; <em>rpsL</em>  gene</td>
<td>1</td>
<td>0.7 %</td>
</tr>
<tr>
<td>Streptomycin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed (Streptomycin &amp; Rifampicin)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


CHAPTER FIVE
DISCUSSION

There has been a resurgence of interest in rapid diagnosis of M. tuberculosis infection following the increasing number of tuberculosis cases and outbreak of multi-drug resistant tuberculosis strains (MDR) over the past few years (Nolte and Mitchock, 1995).

The aims of the current study were to detect the primary drug resistance due to mutations in Mycobacterium tuberculosis to streptomycin and rifampicin, using molecular techniques as rapid and sensitive methods and to estimate the prevalence of mutant genes that are responsible for drug resistance of Mycobacterium tuberculosis.

A total of 250 samples were studied; 201(80.5%) of the total were found to be acid fast bacilli (AFB), this high percentage can be explained by the fact that the all studied samples were already diagnosed as having AFB positive.

150 out of the 250 were Mycobacterium tuberculosis complex (147 (73.1%) out of the 201 AFB positive gave growth of M. tuberculosis complex and three (6.1%) out of 49 which were AFB negative), 17 (8.5%) of the growth culture were rapid grower MOTTs. These results was supported by the results done by Eldawi (2000) who found that 10% were acid fast bacilli other than tuberculosis, included rapid growing mycobacteria.

Seven (3.5 %) were Nocardia spp. which was fully described and named Nocardia africana as a new species, and this results agree with the results of Sharaf Eldeen (2001), reported that 3.2% were Nocardia africana.

14 (6.9 %) were contaminated cultures and 16 (7.9%) were not grown. eight (16.3%) out of 49 of the samples were fungi.

Although a presumptive diagnosis of pulmonary tuberculosis can be made on the basis of patients' histories and clinical and radiological findings, the definite bacteriological diagnosis of tuberculosis continues to depend on the microscopic examination of acid fast stained sputum smear according toWHO (1998). Direct microscopy with Ziehl Neelsen staining to identify acid fast bacilli is the most rapid method, but it lacks sufficient sensitivity and specificity as concluded in this study 150 (60%) out of 250 were M. tuberculosis, 17 (8.5%) out of 201 acid fast bacilli were MOTTs and 7(3.5%) were Nocardia sp., all can be judged AFB on microscope, by proceeding to more specific techniques such as culture, biochemical test and sequencing according to Miller et al., (1994)
The conventional bacteriological procedures used for the diagnosis of TB and detection of MDR in clinical laboratories either show poor sensitivity (microscopy) or time consuming (culture) which requires 4 - 8 weeks to isolate M. tuberculosis complex and the same time for sensitivity test (Horsburgh et al., 1986; Siddigi et al., 1985), this delayed diagnosis not only affects the treatment of the patient but may also lead to increasing of spread of MDR strains. For all above reasons the molecular techniques were used in this study, which decrease the required time to nearly half.

The microscopic examination revealed the presence of AFB repeating the examination three times; only 60% of them were tuberculosis patients where as2.8% were pulmonary nocardiosic and10% were MOTT chest infections, this disagree with (WHO, 1996; Andrews and Radhakarishna, 1959 and Mitchison, 1968), which reported that it requiring at least two positive smears to classify a patient as having-sputum smear positive tuberculosis.

In this current study 17 (8.5%) out of 201 were rapid growers MOTTs, inspite of it was smear positive, but they show culture negative of TB. This result disagreed with Debrunner et al. (1992) and Maiko et al. (1994) who found that the efficiency of smear examination in detecting cases of tuberculosis subsequently shown to be culture positive and the high specificity of a positive sputum smear predicting M. tuberculosis is due to environmental mycobacteria which were supposed to be very rare (Chan et al., 1971).

Seven strains (3.5%) were Nocardia sp. with similar symptoms to tuberculosis, this agreed with Wayne (1992) who noticed that there were some MOTTs and Nocardia spp. that can cause chest infections with similar symptoms to tuberculosis.

14 (6.9%) of the culture bottles showed contamination and eight (3.2%) were fungi. This may be due to the decontamination method used in this study (4% NaOH), while by using 2%NaOH N-acetyl-L-cystein which is described by Kubica et al. (1963) which is more effective but it is not recommended in drug resistance detections, so it was avoided in this study.

16 (7.9%) showed no growth and that may be explained by the harsh decontamination procedures used (4% NaOH), or may be attributed to a few number of bacilli, or patients have been treated from other disease with streptomycin and / or rifampicin.

Using molecular procedure such as polymerase chain reaction (PCR) IS6110, 150 out of 250 were confirmed to be M. tuberculosis complex. This results match with the culture results conducted in the current study. It is enforced by results of a previous study done by El Dawi (2000) which evaluated IS6110 for the diagnosis of tuberculosis in Sudan and concluded that PCR – amplified IS6110
increased the sensitivity to 88% compared to 65% microscopy.

DNA fingerprinting designated IS6110, first reported by Thierry et al., (1990) is usually used to identify M. tuberculosis complex. This ability to accurately identify related strains of M. tuberculosis complex can help to define the people and places involved in M. tuberculosis transmission. However, in this study fingerprinting technique were used to confirm the results on strains, which have been microscopically and by culture classified as M. tuberculosis.

M. tuberculosis DNA fingerprinting is based on small piece of repitive DNA, named IS6110, that may be present from 1 to 20 or more times in the M. tuberculosis genome. Jan et al., (1993) noted that such data might provide impenitent insights into the global transmission of M tuberculosis and identify strains with particular properties. The physical map of the IS6110 sequence indicates that various restriction enzyme cleaves within the 1,355- 6Pelment- Bam Ht, SstII, Pst I, BstEII, BssH II and PVU II have all been successfully used to generate restriction fragment length polymorphism (Zainuddin and Dale, 1988; Van et al., 1991; Cave et al., 1991 and Mazurek et al., 1991), but in this study PVUII was used as it has been used by the majority of laboratories and it cleaves IS6110 sequence only once (Van et al., 1991).

The use of conventional culturing methods in diagnosis and susceptibility testing of clinical isolates of M. tuberculosis is protracted, and can take up to 12 weeks before a prediction of a resistance pattern can be made. This has negative consequences for the treatment of infected individuals and can contributes towards the spread of MDR organisms according to Sinder et al., (1981); Siddgi et al. (1985) and Rastogi et al. (1989). Thus, in this study, instead of culture detection for susceptibility testing of M. tuberculosis, more useful and rapid assays on nucleic acid amplification procedures such as single strand confirmation polymorphism (SSCP) and RFLP were used.

Single strand confirmation polymorphism (SSCP) procedure was used in this study as previously described (Telenti et al. 1993). SSCP methods can detect mutations in 95% of rifampicin - resistant clinical isolates of M. tuberculosis while sequencing can detect all mutations, but, the latter is too expensive. The basic cost per sample is more than 10 US $ and hand - on time eight hours, turn around time 48 hours and specific equipment needed cost approximately 100 000 – 120 000 US $, in sequencing. Whereas in manual SSCP the basic cost per sample is 1.75 $ and hand - on time 5.5 hours, turn around time less than 24 hours and specific equipment needed cost approximately 3 000 US $ according to Telenti and Persing (1996). SSCP is less demanding technique than sequencing; however automated SSCP would be best suited for reference laboratories serving a population with a high
prevalence of MDR tuberculosis. In contrast, SSCP using silver staining could represent a low cost option for other settings (Delgalo and Telenti, 1990). Therefore SSCP was used in this study.

In this study four isolates (2.3%) were found to contain (rpoB) mutant genes that fall within the range 0-3.0% and medium (0.2%) of WHO/ IUATLD (David et al., 1997). This result might not be exactly the real situation because mutations may be located outside of the region of analysis. In rifampicin-resistant M. tuberculosis complex strains, resistance results from point mutations or from small deletions or insertions in a limited region of the gene encoding for the β-subunit of the RNA polymerase (rpoB). More than 30 different mutations have now been described in a region spanning 81 bp corresponding to a stretch of 27 amino acids (Kapur et al., 1995), hence could not be detected. If sequencing or INNO-LiPA were used, the percentage might be higher or accurate as concluded by Peggy et al. (1998).

In Sudan Sharef eldeen (2001) found that the acquired resistance to rifampicin was 8% and it is more than the result of this study.

A previous survey on drug resistance in the Western Cape indicated 10.8% and 4.2% for acquired resistance, and overall incidences of 6.8% and 2.4% (N=6 266) for INH and RMP respectively, when both initial and acquired resistance were taken into account.

On the other side the rate of primary drugs resistance involved in this study was more than that detected by Robert et al., (2000), in France during surveillance of M. tuberculosis resistance (0.2%) from rpoB mutation gene mono-response in untreated patients. While the acquired resistance was higher (2.2%) which is similar to initial resistance result and as Robert et al (2000) concluded, there has been no increase in resistance to any drug among previously untreated patients.

It has previously been shown that 90% of clinical isolates resistant to RMP have mutation in the rpoB gene Vareldzis et al. (1994). This allows the attractive opportunity of using this locus as a marker for rifampicin resistance. However, results from pervious studies were collectively obtained from both SSCP analysis and sequence data. Results from this study showed that by using a combination of two optimised SSCP procedures, it was possible to detect mutations in 95% of 20 rifampicin – resistant clinical isolates of M. tuberculosis. It is possible that the remaining 5% of the samples analysed, not showing SSCP mobility shifts with any of these two optimised procedures, might not be optimised for detection of some uncommon mutations. Other methods, such as dideoxy- fingerprinting (ddf) and denaturing gradient gel electrophoresis (DGGE) might assist in detection of these mutations. If present
for diagnostic purposes it is important to evaluate these procedures, since automated sequencing is not economical or practical for all institutions Williams et al (1994).

The results of Vareldzis et al. (1994) suggest that it is not only possible to use mutations in the rpoB gene to predict rifampicin resistance but since there is a 95% chance that the particular organisms are also INH – resistant. This locus (rpoB) may also be used in the prediction of MDR strains. It was also shown in this study that conventional methods (culture) may be unnecessary, and that mutational screening can be done after short culture periods in BACTEC medium, or even directly from sputum samples. This may have immediate implications for the decision on chemotherapy, it is currently not feasible to test all tuberculosis cases, and it is recommended that only high-risk cases be selected.

SSCP analysis allows the detection of single base changes in short DNA fragments due to mobility differences of single stranded DNA molecules. Since it was first used by Orita et al. (1989), in this study SSCP was used to detect primary resistant of M. tuberculosis to rifampicin due to mutation in rpoB gene (β - Subunit of the RNA polymerase).

Other species of the genus Mycobacterium as M. leprae (Honore, et al., 1993. Guerrero, et al., 1994.) , M. avium (Williams, et al., 1994) and M. africanum (Williams, et al. 1994 and Vareldzis et al. 1994),were found to have the same mutations in the same gene but in different locations.

PCR was used in this study to amplify the ribosomal protein S12 (rpsL gene) from M. tuberculosis around codon 43 as most of the mutations associated with streptomycin resistance involve this gene according to Finken, et al. (1993) who used PCR to amplify encoding 16S rRNA and the ribosomal protein S12 from M. tuberculosis and concluded that in total of 38 STR –resistant 29 had mutations; in rrs (n=9=31%) or rpsL (n=20=69%). The 20 strains with rpsL gene mutations had variant codons for amino acids 43 (n=14=70%) and 88 (n=6=30%). All mutations observed in the rpsL gene resulted in either Lys→Arg or Lys→Thr substitutions.

In the current study streptomycin resistance found to be 1.3% which lies within the WHO/ IUATLD range 0.1% – 23.5 %, but less than the medium (3.5%) (David et al., 1997). This can be explained by the fact that the mutant gene (rrs gene) was not detected in this study. Moreover, the ribosomal protein S12 rpsL related mutations were only detected around codon 43 and those around codon 88 were not included in this study.

Other studies detected streptomycin resistance; in Ethiopia (1987) (Lemma et al. 1989); Kenya (Githui et al. 1993) and Tanzania (WHO, 1995) who revealed approximately similar results to this current study which were , 1.9%, 0.8 - 1.8 % and 0.3 - 1.0 % respectively.
Other studies done in Algeria (Chaulet, 1993) and Kenya (Githui et al., 1992) showed less prevalence: 0.9 and 0.8 respectively.

In contrast to other studies done in Sudan Sharaf Eldeen (2001); Libya (Elghoul et al. 1989); South Africa – Western Cape (Weyer and Kleeberg, 1992); Mauritania (WHO/IUALTD, 1998) and Kalima-Zaire (1983-1986) by Caluwe (1989), who recorded higher ranges of resistance prevalence 30%, 7.8-6.8%, 12.1%, 17.5% and 23.5% respectively.
CHAPTER SIX

CONCLUSIONS AND RECOMMENDATION

CONCLUSIONS

⇒ The present study concluded that, there were number of primary mutations associated with resistance to streptomycin and rifampicin.

⇒ Using molecular methods, 3.9% of the studied strains were found to be resistant to anti-tuberculosis drugs was 2.6% of them were rpoB mutant gene associated with rifampicin resistance and 1.3% rpsl mutant gene associated with streptomycin resistance.

⇒ The above mentioned may not reflect the actual incidence of resistance at study aria, the reasons behind that could be;

  o The numbers of studied samples were not quite enough analytically to represent Khartoum State.
  o The study concentrated on mutations most frequently described, ignoring other mutations such as rpsL around codon 88 and rrs gene.

⇒ It is unwise to depend only on microscopic examination for the diagnosis of tuberculosis since using other methods revealed that 3.5% of the samples were found to be Nocardia africana and 8.5% were MOTT, which was previously identified microscopically as Mycobacterium tuberculosis.

⇒ The PCR is more precise and less time consuming regarding the identification of Mycobacterium tuberculosis than other conventional methods.
RECOMMENDATIONS

⇒ The present study recommended for future works that, More studies are need for detecting primary drug – resistant, due to mutation to all frontline anti tuberculosis drugs in a large number of clinical isolates from more states of the Sudan.

⇒ There is a need to rapidly and reliably diagnose of tuberculosis and to distinguish it from MOTTs and Nocardia spp. Which are also causes of pulmonary infection.

⇒ There is a need for rapid methods to detect resistant mutation not only to treat the patients early but also to reduce the spread of outbreak of multi – drug resistance tuberculosis.

⇒ There is a need to correlate between primary and secondary drug resistance in order to know the transmission of commonly genes of drug resistance.

⇒ The study recommended that, DNA fingerprinting need to be used to detect and distinguish between the species of Mycobacterium tuberculosis and other species, and to determine the transmission of cluster within Sudan and other neighbour countries.
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Appendix 1

Equipment:

Analytical balance, automatic calibration                          Fisher scientific
Block thermostat (Analog Hot Block base)                        Boekel industries, inc
Centrifuge                                                      Hettich universal 11
High power pack                                                 Pharmaicia
Horizantal Gel electrophoresis apparatus                       Biometra
Incubator (Iso-temp)                                            Fisher scientific
Low power pack P25                                             Biometra
Microcentrifuge                                                Roth, Chemikalien laborbedarf
Micropepitites                                                 Roth, Chemikalien laborbedarf
Microscope Olympus                                             Olympus co.
pH meter                                                       Fisher brand
Polaroid gel camera                                             Sigma
Safety cabinet class II for culture                            Heraeus instruments
Safety cabinet for PCR mixture                                 M J Research
Sequencing apparatus model 377                                 Perkin Elmer
Stirrer, magnetic, with hot plate Alminum                       Fisher scientific UK
Thermal cycler                                                  Biometra
Transilluminator                                               Biometra
Ultra violet protective eyes glass                             Nycomed Amersham
Ultra violet protective face shields                           Nycomed Amersham
Vortex mixer                                                   Fisher scientific
Appendix 2

Materials

Aerosol barrier tips                        Sigma
Glass microslides with cut edges             Superior - German
Clinical Autoclave            Labsco
Disposable sterilized Eppendorf tubes                              Sigma
                          (1.5-0.5 ml)
Polaroid film                        Labsco
Plastic disposable Conical tubes 15-50ml                   Falcon
Plastic disposable pipettes           Roth, Chemikalien laborbedarf
Sputum container            Vderman International

Appendix 3

Chemicals and Reagents

Acetic acid              Sigma
Agarose       Mresco Agarose, Biotechnology
Ammonium persulphate             Sigma
Bis-Acrylamide .              Sigma
Boric acid               Sigma
Bovine serum albumin fraction V            Promega
Bromo-phenol blue
Carbol Fuchsin     BDH laboratory
Deoxynucleotides (100mM )        Boehringer Mannheim
EDTA                Sigma
Acrylamide              Sigma
Ethanol               Sigma
Ethidium bromide              Sigma
Glyserrol                          Sigma
L - Asparagine              Sigma
Magnesium chloride              Sigma
Magnesium Chloride (100 mM )   Boehringer Mannheim
Magnesium sulphate                          Sigma
Malachite green     Aldrich chemical company, INC.
Formaldehyde     Sigma
Formamide     Sigma
Methylene blue     Sigma
Oligonucleotides     BDH laboratory
10X PCR Reaction buffer     Boehringer Mannheim
Phenol     BDH laboratory
Polyvinyl pyrrolidone-360     Sigma
Potassium chloride     Sigma
Primers     (South Africa)
Silver nitrate     Sigma
Sodium Borohydride     Sigma
Sodium chloride     Sigma
Sodium citrate-N-acetyl-L-cysteine     Sigma
Sodium deodcyle sulphate     Sigma
Sodium hydroxide     Sigma
Sodium hydrogen phosphate     Sigma
Sodium hydroxide     Sigma
Sulfuric acid     BDH laboratory
Taq polymerase     Boehringer Mannheim
Tricine     Sigma
Tris     Sigma
Triton X     Sigma
Xylene cyanol     Sigma

Ziehl Neelsen stain:-

Solution A (Saturated alcoholic solution of fuchsin )

Basic fuchsin 3 g
Ethanol 96 % 100 ml

Solution B (Phenol solution, 50g/L (5%)

Phenol crystals 10 g
Water, distilled if possible 300 ml
Sulfuric acid, concentrated 100 ml

**Formula for counter staining Solution**

Methylene blue 0.3 g
Water (distilled) 100 ml

**Löwenstein-Jensen medium:-**

**Buffer salt solution**

- Potassium dihydrogen orthophosphate 28 g
- Disodium hydrogen orthophosphate 16 g
- Magnesium Sulphate 1.6 g
- Citric acid 12 g
- L-Asparagine 40 g
- Glycerol 80 ml
- Distilled water 4 litres

**Complete medium**

- Buffer salt solution 1200 ml
- Whole egg 2000 ml
- 1% Malachite green 80 ml

**Appendix 4**

**Molecular reagents**

**10X Reaction buffer**

500mM KCl
100mM Tris
1% Triton

Deoxynucleotides
100mM dATP, dCTP, dGTP, dTTP
Stock: 10µl of each of the above nucleotides up to 400 µl water
(concentration = 2.5mM)

Magnesium chloride
100 mM
(Stock = 25mM)

Taq polymerase
5U/µl

Sets of Primers
TR 8&TR 9
STR 52& STR 34
(Stock : 25mM solution)

Gel electrophoresis
10XTBE
108g Tris
55g Boric Acid
7.4g EDTA
1000mL water: pH 8.30

1.8% Agarose gel
1.8 g Agrose
100 ml 1X TBE buffer

Loading Buffer
0.25% Xylene cyanol
30% Glycerol
Ethidiumbromide
10mg/ml

**Single strand conformation polymorphism (SSCP):**

**Ammonium persulphate 3%**
0.3g Ammonium persulphate
10mL water

**Stop solution**
95% Formamide
20mM EDTA
0.005% Bromo- Phenol blue
0.005% Xylene cyanol.

**6% page gel**
Distilled water 135 ml
30% Acrylamide 40 ml
6XTBE 20 ml
3% Ammonium persulphate 4.2 ml
Glycerol 10 ml
TEMED 100µl

**Silver staining procedure:**

**Sodium hydroxide (stock)**
45g sodium hydroxide
300mL water
Sodium Borohydrate (stock)
0.3g sodium Borohydrate
300mL water

Solution 1
100 ml Ethanol (10%)
5mL Acetic acid (0.5%)
895ml water

Solution 2
1g silver nitrate
1000 ml water

Solution 3
60 ml sodium hydroxide (stock)
60 ml sodium Borohydrate (stock)
2.4 ml Formaldehyde
Water up to 600 ml

Solution 4
7.5g sodium carbonate
1000 mL water
Solution 1 and 2 stable for ± 1 week
Solution 3 fresh as needed.

Restriction Fragment Length Polymorphism

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
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<tbody>
<tr>
<td>PCR Product</td>
<td>10 ul</td>
</tr>
<tr>
<td>Enzyme Mob II</td>
<td>1 ul</td>
</tr>
<tr>
<td>10X enzyme buffer</td>
<td>2 ul</td>
</tr>
<tr>
<td>water</td>
<td>7 ul</td>
</tr>
<tr>
<td>Total</td>
<td>20 ul</td>
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