

Sudan University of Science and Technology

College of Graduate Studies

**The Use of the Insertion Sequence IS6110 to Characterize
Mycobacterium tuberculosis Detected among HIV Patients in
West Darfur State**

**استخدام الجين IS6110 في التعرف على المتفطرة الدرنية عند مرضى الأيدز بولاية
غرب دارفور**

A thesis submitted in fulfillment for the requirement of Ph.D degree in Medical
Laboratory Science (Medical Microbiology)

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June 2015

الآية

(سَنُرِيهِمْ آيَاتِنَا فِي الْأَفَاقِ وَفِي أَنفُسِهِمْ حَتَّىٰ يَتَبَيَّنَ لَهُمْ أَنَّهُ الْحَقُّ ۗ أَوَلَمْ يَكْفِ بِرَبِّكَ أَنَّهُ عَلَىٰ كُلِّ شَيْءٍ شَهِيدٌ).

سورة فصلت الآية (53)

Dedication

To the soul of my respectful mother

To my grateful father

To my dear wife

To my amazing sons: Mohamed and Omer

To my beloved daughters: Amel and Nana

To all family and friends

Abdul Rahim

Acknowledgement

First, I would like to thank ALMIGHTY ALLAH for his help to conduct this study. I would like to express my gratitude to my supervisor Prof. Al Fadhil Al Obeid Omer for his patience and guidance throughout this study. My thanks go to Dr. Hanan Babikir Al Tahir for her direct technical contribution to this study. I would like also to send my sincere thanks to Dr. Mansour Mohammed Mansour, Prof. Yousif Fadhalla and Dr. Mugahid Mohammed Al Hassan who had helped me in the preparation of the thesis. My appreciation also goes to the Ministry of Health of West Darfur State, Tuberculosis (TB) and Human Immunodeficiency Virus (HIV) program co- coordinators, People Living With HIV/AIDS (PLWHA) Association of Al Geneina, Voluntary Counseling and Testing (VCT)Center, and Al Geneina TB Center for their support during the collection of data and samples, as well as all patients who participated in this study. My great admiration is sent to all physicians who provided the clinical data and medical information.

Abstract

Background: Today it is known that TB and HIV/AIDS are the two of the world's major pandemics. HIV infection is now prevalent, to varying degree, in all areas of the world where TB is prevalent. The pandemic of HIV/ AIDS opened a new chapter on the role of *mycobacteria* in causing human disease in both the developed and less – developed world.

Objective: The aim of this study was to characterize and genotype *Mycobacterium* TB isolated from HIV/AIDS patients. The study was conducted in West Darfur State during August 2012 through June 2015.

Materials and Methods: The study samples (sputum) were collected from two groups: people living with HIV/AIDS (PLWHA) (N=158) and TB suspected patients that were negative for HIV (N=1160). The study samples have been stained by ZN stain and screened for the presence of AFB using the microscope. All ZN stain positive samples (N=206) with other negative sputum samples (n=15) taken from PLWHA were subjected to DNA extraction by the modified Guanidine Chloride Method. The samples then processed by PCR technique which confirmed the results of the microscopy. Selected PCR amplified DNA samples were sent to Macrogen Company in Korea for DNA sequencing.

Results: Six of the (PLWHA)(3.8%) were found to be positive for AFB, of which (66.7%) were males and(33.3%) were females , the most affected age group was 31-40 years (50%) , followed by 41-50 years (33.3%) which indicate negative impacts from the economical and social point of view. Of the total sputum samples taken from TB suspected patients and negative for HIV (200) samples were found positive for AFB (17.2%). the results of DNA sequencing achieved from Macrogen

Company in Korea revealed high similarity to the reference laboratory strain of *Mycobacterium tuberculosis* (H37Rv).

Conclusion: The study concluded that the prevalence of TB among (PLWHA) in the study area is low. Further comprehensive studies applying advanced molecular techniques were highly recommended nationally and in the study area as well.

الخلاصة

الخلفية: يعتبر مرض السل و الايدز اليوم من المشاكل الصحية العالمية، العدوى بفيروس العوز المناعي البشري (الايدز) منتشر الآن بدرجات متفاوتة في كل الاماكن التي ينتشر فيها مرض السل . مشكلة انتشار الاصابة بفيروس مرض الايدز عالمياً فتح مجالاً جديداً للمعرفة حول دور بكتيريا السل في التسبب في أمراض الانسان في الدول النامية و الاقل نمواً.

الاهداف: هدفت هذه الدراسة الي التمييز والتوصيف الجيني للبكتيريا المسببة لمرض السل في المرضى المتعايشون مع فيروس ومرض الأيدز. جمعت عينات الدراسة (التفاف) من مجموعتين من المرضى : الأولي المرضى المتعايشون مع فيروس ومرض الأيدز وعددهم (158) والثانية المرضى الذين يشكون من اعراض مرض السل والسليبين لفيروس مرض الايدز وعددهم (1160) مريضاً .

المواد والطرق: صبغت العينات بصبغة (زيل - نيلسون) وفحصت بواسطة جهاز الميكروسوب . خضعت كل العينات الموجبة لصبغة (زيل - نيلسون) وعددهم (206) بالاضافة لعدد (15) عينة اخرى سالبة لصبغة (زيل - نيلسون) جمعت من المرضى المتعايشون مع فيروس ومرض الايدز لفصل المادة الوراثية (الحمض النووي منقوص الأكسجين) لعينات الدراسة باستخدام طريقة كلوريد القوانيين المعدلة ، وبعدها تم تحليل العينات بتقنية تفاعل البلمرة المتسلسل والتي أكدت نتائج الميكروسكوب ومن ثم تم إرسال عينات مختارة من حصيلة تفاعل البلمرة المتسلسل الي دولة كوريا لإجراء تحليل التسلسل الوراثي .

النتائج: كانت عدد العينات الايجابية لميكروب السل في المرضى المتعايشون مع فيروس ومرض الايدز ستة عينات (3.8%) بينهم 4 من الذكور بنسبة (66.7%) واثنان من الاناث بنسبة (33.3%) . أكثر الفئات العمرية عرضة للإصابة ، الفئة العمرية بين 31-40 سنة بنسبة (50%) و تلتها الفئة العمرية بين 41-50 سنة بنسبة (33.3%) مدلة على الأثر السلبي للمرض من الناحيتين الاقتصادية والاجتماعية . وكانت عدد العينات الايجابية لميكروب السل في المرضى الذين يشكون من اعراض مرض السل والسليبين لفيروس مرض الايدز (200) عينة بنسبة (17.2%) . نتائج تحليل التسلسل الوراثي التي جاءت من شركة ماكروجين في دولة كوريا اوضحت تطابقاً تاماً لبكتيريا السل النموذجية (H37Rv) .

الاستنتاجات: خلصت الدراسة الي أن نسبة الإصابة بميكروب مرض السل وسط المرضى المتعايشون مع فيروس ومرض الايدز قليلة في منطقة الدراسة وأوصت الدراسة بإجراء بحوث أخرى شاملة بإستخدام تقنيات التحليل الوراثي والجيني المتقدمة علي المستوى القومي وفي منطقة الدراسة على حد سواء.

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List of Abbreviations

AFB	Acid-Fast Bacilli
AIDS	Acquired Immunodeficiency Syndrome
ART	Anti-Retroviral Therapy
BCG	Bacille-Calmette-Guérin
Bp	Base pair
CD4	Colony of Differentiation 4
CFPs	Culture Filtrate Proteins
CPT	Co-trimoxazole Preventive Therapy
DNA	Deoxyribo-Nucleic Acid
DNTPs	Deoxyribo-Nucleoside Tri-Phosphates
DOTS	Directly Observed Treatment Strategy
DTH	Delayed-Type Hypersensitivity
EDTA	Ethylene Diamine Tetra-Acetic Acid
FAS1	Fatty Acid Synthase
HAART	Highly Active Antiretroviral Therapy
HBC	High-Burden Country
HIV	Human Immunodeficiency Virus
INAH	Iso-Nicotinic Acid Hydrazid
IPT	Isoniazid Preventive Therapy
IUATLD	International Union for Tuberculosis and Lung Diseases
JUNPAIDS	Joint United Nations Programme on HIV/AIDS
LIC	Low-Income Countries

L-J medium	Lowenstein-Jensen Medium
LTRs	Long-Terminal Repeats
MDGs	Millennium Developmental Goals
MDR-TB	Multi-Drug-Resistant Tuberculosis
MDR-TB	Multi-Drug-Resistant Tuberculosis
MTB/RIF	Mycobacterium Tuberculosis /Rifampicin
MW	Molecular Weight
NGO	Non-Governmental Organization
PCR	Polymerase Chain Reaction
PGRS	Polymorphic GC-Repetitive Sequence
PLWHA	People Living With HIV/AIDS
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribo-Nucleic Acid
RT-PCR	Real-Time PCR
SNAP	Sudan National AIDS Control Program
STS database	Society of Thoracic Surgeons database
TB	Tuberculosis
TB-IC	Tuberculosis Infection Control
U.V. light	Ultra-Violet Light
USAID	United States Agency for International Development
VCT	Voluntary Counseling and Testing
VL	Viral load
WHO	World Health Organization
XDR-TB	Extensively Drug-Resistant Tuberculosis
ZN	Ziehl-Neelsen

CHAPTER ONE

Introduction and Literature Review

Introduction

1. 1. Introduction

The etiologic agent of AIDS has infected an estimated 30 to 40 million people worldwide. Of the two major forms of HIV, infection with type 1 (HIV-1) is prevalent throughout the world and is characterized by a slow, progressive deterioration of the immune system that is almost uniformly fatal. By contrast, infection with type 2 (HIV-2), which is found primarily in West Africa, generally has a more benign clinical course (Richman *et al.*, 2002).

Human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) epidemic is now a global crisis, and constitutes one of the most formidable challenges to development and social progress. In the most affected countries, the epidemic is eroding decades of development gains, undermining economies, threatening security and destabilizing societies. In addition, HIV/AIDS is affecting fundamental rights, particularly with respect to discrimination and stigmatization aimed at people living with and affected by HIV/AIDS. The epidemic and its impact strikes hard at vulnerable groups including women and children, thereby increasing existing gender inequalities and exacerbating the problem of child labor (MHESR, 2006).

Despite the fact that TB is a disease of antiquity and it is probably one of the illnesses most dealt with in the literature, there has been surprisingly little sound knowledge of the disease through the course of history, which has not helped contemporary efforts to combat the illness. From the time of Hippocrates (460-377 BC) up until the nineteenth century, the infectious nature of the disease was not even acknowledged; rather, TB was considered a hereditary disorder. However, air-a

common vehicle for the transmission of live germs-was included among the possible origin of the disease (Pratt *et al.*, 2005).

For this reason, the dietary regimen proposed by Hippocrates and Galen (130-200 AD) remained the basis of treatment up until the Renaissance. This practice changed very little in the seventeenth century, the sole difference being the recommendation of physical exercise and the use (as with other diseases) of new medicinal substances introduced in Europe at the time, such as quinine, coffee, tea, cocoa, and even tobacco. Such lack of understanding partly explains why humankind has been unable to defend itself against this terrible illness for the most of history-the only option being to fall ill and ultimately die. Only towards the latter half of the nineteenth century did the infectious nature of TB become apparent, as a result of the studies by Villemin (1865) and, particularly, Robert Koch (1843-1910).

Koch was the first to suggest the possibility of controlling this endemic disease, with the presentation of the results of his research (in 1882) that showed that TB was a contagious disease. He not only isolated the bacterium, which was latter named after him (Koch's bacillus), from the sputum of infected patients, but also proposed that the principal measure for controlling the disease in the community would be to isolate affected patients. This suggestion paved the way for the "sanatorium" era of TB, during which prolonged confinement of patients in sanatoriums was believed to be the only effective way to cure TB and control its transmission (Luna, 2004).

The name of the genus, *Mycobacterium* (Fungus bacterium), is allusion to mould – like pellicles formed when members of this genus are grown in liquid media. This hydrophobic property is due to their possession of thick, complex, lipid – rich, waxy cell walls. A further important characteristic, also due to waxy cell walls, is acid – fastness, or resistance to decolorization by a dilute mineral (or alcohol) after staining

with hot carbol fuchsin or other aryl methane dyes. There are over 80 named species of mycobacteria, which are divisible into two major groups, the slow and rapid growers, although the rate of the latter is slow relative to that of most other bacteria. The leprosy bacillus *Mycobacterium leprae*, which has never convincingly been grown in vitro, and members of the *Mycobacterium tuberculosis* complex are obligate pathogens. The other species are environmental saprophytes, some of which can cause opportunistic diseases (Greenwood *et al.*, 2002).

1.1.1. Rationale

HIV/AIDS and tuberculosis became a major global health problem (Pawan Sharma, 2004). The geographic location of West Darfur State which has international open borders with Republic of Chad and the Republic of Central Africa, where people from most West African countries with high prevalence of HIV/AIDS infections, travel freely across these borders. In addition, the current conflicts in Darfur create suitable environment for HIV/AIDS transmission (Spiegel ,2004) .

Molecular diagnosis (PCR) will lead to good results compares to the previous conventional procedures. The proper diagnosis of disease will help the health professions to be sure that they are treating the patients properly. This study will follow the feasible and the most adequate diagnostic procedures for HIV and TB.

Furthermore, PCR helps in determining the real prevalence of tuberculosis among people living with HIV/AIDS (PLWHA) and the determination of *Mycobacterium tuberculosis* to the genus level. These findings will add to our understanding of the co-pathogenesis of these two infections and may have therapeutic implications.

1.1.2.Objectives

1.1.2.1 General objective:

To use the insertion sequence (IS6110) to characterize *Mycobacterium tuberculosis* detected among HIV patients in West Darfur State

1.1.2.2 Specific objectives:

- 1- To perform ZN microscopy and molecular techniques to detect TB infection in West Darfur State.
- 2- To study the TB prevalence rate among TB and AIDS patients using molecular techniques.
- 3- To determine the factors leading to HIV/TB co-infection in West Darfur State population.

1.2. Literature Review

1.2.1. Human Immunodeficiency Virus (HIV):

1.2.1.1. Morphology and Structure:

By electron microscopy, the HIV-I virion measures approximately 100 to 150 nm in diameter. Mature viral particles are characterized by an electron-dense cylindrical core surrounded by a lipid envelope that is acquired as the virion buds from an infected cell. The virion core houses two copies of single-stranded viral genomic RNA encoding a series of structural, enzymatic, and regulatory proteins that are required for efficient completion of the viral life cycle. The HIV genome is approximately 10 kb long and shares the same general organization with all retrovirus. Flanked by two long-terminal repeats, (LTRs), the 5'-3' sequence consist of the gag gene, which encodes the virion structural components, the pol gene, which encodes several viral enzymes, and the *env* gene, which encodes the envelope glycol proteins. Unlike other non-primate retrovirus, the HIV-1 genome also contains at least six additional genes:

tat, *rev*, *nef*, *vpr*, *vpu* (or *vpx* in HIV-2), and *nif*. Proteins encoded by these genes function both early and late in the replication cycle to regulate transcription and translation of viral proteins and to enhance assembly and release of particles from infected cells (Richman, 2002).

1.2.1.2. Pathogenesis:

The major determinant in the pathogenesis and disease caused by HIV is the virus tropism for CD4-expressing T cells and macrophages. HIV-induced immunosuppression (AIDS) results from a reduction in the number of CD4 T cells, which decimates the helper and delayed-type hypersensitivity (DTH) functions of the immune response.

During vaginal or anal sexual intercourse, HIV-I infects Langerhans dendritic cells the epithelium, and these can then travel to lymph nodes. Anal sex may be of greater risk than other routes of infection. Special T cells bearing a greater number of co – receptors for the virus are separated by only a single layer of cells from the colon. On injection of the virus into blood, the virus is likely to infect dendritic and other monocyto-macrophage lineage cells (Murray, 2002).

1.2.1.3. Diagnosis:

HIV infections are routinely detected by serology (antibodies or viral antigen). The circulating viral count or viral load (VL) is determined by means of quantitative RT – PCR. The AIDS diagnosis is a clinical procedure that presupposes positive confirmation of HIV infection (Kayser *et al.*, 2001).

The diagnosis of HIV infection is usually made on the basis of the detection of HIV antibodies and/or antigen. Serological tests for detecting antibodies to HIV are generally classified as screening tests (sometimes referred to as initial tests) or confirmatory tests (sometimes referred to as supplemental tests). Those tests which simultaneously detect HIV antibody and antigen are usually referred to as enhanced screening tests. Initial tests provide the presumptive identification of antibody-

positive, or antibody/antigen positive, specimens, and supplemental tests are used to confirm whether specimens found reactive with a particular screening test contain antibodies specific to HIV and/or the presence of HIV antigen (WHO, 2004).

1.2.1.4. Opportunistic Infections in AIDS:

Multiple recurrent bouts of infections with fungi, bacteria, and viruses occur as the CD4+ cell count declines. For example, the nervous system can be the site of opportunistic infection with *Toxoplasma*, *Cryptococcus*, *JC* virus, and *Mycobacteria*. The lungs are also primarily affected by opportunistic infections, *Pneumocystis carinii* pneumonia being one of the most common. Mycobacterial infections are also a common problem in the lung; for example currently thirty percent of AIDS patients die from tuberculosis (Strohl *et al.*, 2001).

1.2.1.5. HIV/AIDS Prevalence in Sudan:

Sudan National AIDS Control Program (SNAP) National Survey Report of 2002 showed that the prevalence of HIV infection is estimated to be 1.6% of the population, which translates to 540,000 people living with HIV/AIDS in Sudan. Recently, the last SNAP survey indicates that HIV/AIDS in Sudan is low/concentrated rather than generalized epidemic (SNAP, 2002).

1.2.1.6. TB/HIV Dual Infection in Sudan:

Sudan has the highest pulmonary TB incidence among the Eastern Mediterranean countries, as reported by the World Health Organization. The current TB control programme in Sudan is based on passive case detection and treatment (El-Eragi *et al.*, 2007).

Tuberculosis (TB) continues to be a public health challenge in Sudan and it is estimated that the annual risk of infection is 1.8; 242 cases of TB per 100,000 occur in Sudan annually (UNAIDS). Active TB disease is the commonest opportunistic infection amongst HIV-infected individuals; in OMACU (TB/HIV centre in Khartoum), the prevalence of TB amongst HIV patients is 15% in 2008. (SNAP, 2002).

1.2.2. *Mycobacterium tuberculosis*:

1.2.2.1. Morphology and Identification:

In tissue, tubercle bacilli are thin straight rods measuring about 0.4/3 mm. On artificial media, coccoid and filamentous forms are seen with variable morphology from one species to another. *Mycobacterium* cannot be classified as either Gram – positive or Gram negative. Once stained by basic dyes they cannot be decolorized by alcohol, regardless of treatment with iodine. True tubercle bacilli are characterized by "acid fastness" i.e. 95% ethyl alcohol containing 3% hydrochloric acid "acid – alcohol" quickly decolorizes all bacteria expect the Mycobacteria. (Brooks *et al.*, 2002).

Members of the *M. tuberculosis* complex (tubercle bacilli) are non-motile, non-sporing, non-capsulate, straight or slightly curved rods. In sputum or other clinical specimens they may occur singly or in small clumps, and in liquid cultures they often grow as twisted rope-like colonies termed serpentine rods (Wood *et al.*, 2002).

1.2.2.2. Cell Wall:

These lipid-rich cell walls offer a high degree of protection to the cells and account for many of the properties that set Mycobacteria apart. For example:

- Resistance to drying and osmotic lysis.
- Resistance to many antibiotics and disinfectants.
- Resistance to acids and alkalis.
- Impermeability to stains.
- Survival within macrophages. The most notable lipids in the Mycobacteria cell wall are mycolic acids (Michael , 2010).

1.2.2.3. The Genome:

The genetic element (genome) of a *Mycobacterium* is, in common with that of all bacteria, a single circular chromosome composed of a "double helix" of DNA. Some mycobacteria have small additional closed rings of DNA called plasmids or episomes. In some bacterial genera, plasmids carrying important genes determining, for example, resistance to antibiotics have been identified. In the case the mycobacteria, however, no important genetic function has convincingly been ascribed to them. Each mycobacterial genome contains many genes that are found in all bacteria. Some of the genes are in fact structurally closely related to some found in higher animals, including human beings. Other genes are more specific to groups within the genes. Thus, the slowly growing and rapidly growing *mycobacteria* have genetic differences that are reflected in differences in proteins in their cytoplasm. Finally some genes are unique to each mycobacterial species and to variants within those species (Pratt *et al.*, 2005).

The *Mycobacterium tuberculosis* genome is one of the largest yet found (4.40 Mb), exceeded only by *E. coli* (4.60 Mb) and *Pseudomonas aeruginosa* (6.26 Mb) and contains around 4000 genes (Prescott *et al.*, 2005).

1.2.2.4. Viability:

The thermal death point is 60° C for 15-20 minutes. Many bacilli resist dryness for several weeks or months if protected from day light. It can resist 5% phenol for several hours when the bacillus is in the sputum. It is highly sensitive to sunlight and U.V. light, and ordinary daylight, even through glass, and has a lethal effect. It is sensitive to ordinary disinfectants. When outside the sputum it is sensitive to streptomycin, vancomycin and cycloserine among the antibiotic, and to Para-amino-salicylic acid (PAS), iso-nicotinic acid hydrazide (INAH), thiosemi-carbozone, ethio-namide and pyrazinamide among chemo-therapeutic agents (Omer, 1990).

The entire genome of *M. tuberculosis* H37 Rv, a widely used reference strain, was completely sequenced in 1998. This analysis showed that the genome of this strain contained genome 4, 411, 529 base pairs and a round 4,000 genes. This genome is larger than that of many bacteria, though not as large as of *E. coli*. It has a high guanine+cystosine content (65.6%) and this is uniform throughout the genome suggesting that it has evolved with little or no incorporation of DNA from other bacteria genera. In contrast to other bacteria, many of the genes of *M. tuberculosis* are involved in synthesis and metabolism of lipids. Indeed every known lipid biosynthetic system, including those in plants and animals, as well as some unique ones, are found in mycobacteria and *M. tuberculosis* contains around 5250 enzymes involved in lipid metabolism, compared to 50 in *E.coli*. Mycobacteria are unique among the bacteria in possessing the enzyme fatty acid synthase 1 (FAS1), which is also found in many eukaryotic cells, as well as fatty acid synthase II (FAS II) which is found in most other prokaryotes and plants. Much of this metabolic capability enables the mycobacteria to synthesize this very complex lipid – rich cell walls. Another unusual feature of the mycobacterial genome is the large number of genes, over 4% of the total; containing polymorphic GC-repetitive sequence (PGRS) and coding for two unrelated families, PE and PPE, of acidic, glycine- rich proteins. The function of these proteins is unknown but, as many of them found in the cell membrane and cell wall, they may mediate antigenic variation and there by effect virulence and pathogenicity (Schaaf and Zumla, 2009).

1.2.2.5. Reaction to Physical and Chemical Agents:

M. tuberculosis is relatively resistant to acids and alkalis. NaOH is used to concentrate clinical specimens; it destroys unwanted bacteria, human cells, and mucus but not the organism. *M. tuberculosis* is resistant to dehydration and so survives is dried expectorated septum; this property may be important in its transmission by aerosol (Levinson, 2008).

1.2.2.6. Antigenic Structure of *Mycobacteria*:-

Mycobacterial antigens are divisible into three main groups, actively secreted, cell wall-bound, and cytoplasmic. The first two are those initially encountered by the immune system and are thus likely to play a role in the induction of protective defense mechanism and in pathogenicity. The nomenclature and classification of mycobacterial antigens is confusing. Some, especially the various polysaccharide antigens, are known by their chemical names, such as lipoarabinomannan, phenolic glycolipid and trehalose dimycolate. Protein antigens are defined by their size in kilo Daltons, their N-terminal amino acid sequencing, and their mass spectroscopic patterns. Numerous soluble and cell – mycobacterial antigens have been described but only a few have been well characterized functionally. Some have been utilized for the development of various diagnostic tests based on antibody or cell-mediated host immune responses. Around 200 antigenic culture filtrate proteins (CFPs) are demonstrable in double dimensional immuno-electrophoresis in gel. Double immune diffusion in gel reveals around 15 soluble antigens in each species which form the basis of taxonomic system particularly developed by Stanford and Grange in early 1970s. In this system, soluble antigens are divisible into those shared by all mycobacteria and variably by related genera (group I), those restricted to slowly growing species (group II), those found in rapidly growing species and shared with nocardiae (group III) and those unique to each species (group IV) (Schaaf and Zumla, 2009).

1.2.2.7. Sources and Spread of Infection:

The sources of infection are two:-

1- From drinking infected milk and direct contact with infected cattle. Nowadays this source is rare.

2- From human cases, this is the commonest; usually the human type of mycobacteria originates from open cases. The spread of infection starts from the open human case when the patient expectorates large numbers of bacilli in sputum, coughing, speaking and sneezing. The bacilli may settle on the dust and infect people indirectly in dried dust particles. Sputum dried in handkerchiefs, clothing, bedclothes, furniture or floor can be broken when dry into fine dust and in the course of the body movements and disturbance of these articles, they are inhaled by the victim (Omer, 1990).

1.2.3. Tuberculosis:

Approximately one-third of the world's population is infected with *Mycobacterium tuberculosis*, making this microbe one of the most successful human pathogens. This is amongst others, attributable to its ability to survive in the host for prolonged periods of time without inducing any symptoms and its capability to switch between this asymptomatic non-infectious phase and a clinically apparent infectious phase. Its close association with poverty and human immunodeficiency makes it into one of the most important public health problems in under developed parts of the world (Schaaf and Zumla, 2009).

After the discovery of streptomycin in 1943, tuberculosis in the developed world declined rapidly. But since the mid-1980s, it has reemerged as a serious threat (Cowan and Talaro, 2006).

1.2.3.1. Primary Pulmonary TB:

Primary TB refers to the infection of a previously uninfected (tuberculosis – negative) individual. A few patients develop a self-limiting febrile illness but clinical disease only occurs if there is hyper sensitivity reaction or progressive infection. Progressive primary disease may appear during the course of initial illness or after a latent period of weeks or months (Boon *et al.*, 2006).

1.2.3.2. Miliary TB:

Blood-borne dissemination gives rise to miliary TB which may present acutely but more frequently is characterized by 2-3 weeks of fever, night sweats, anorexia, weight loss and a dry cough. Hepatosplenomegaly may be present and the presence of a headache may indicate co – existent tuberculosis meningitis. The classical appearances on chest X –ray are those of fine 1 – 2 mm lesions (millet seed) distributed throughout the lung fields, although occasionally the appearances are coarser (Boon *et al.*, 2006).

1.2.3.3. Post-Primary Pulmonary TB:

Pulmonary TB is the most frequent form of Post – Primary disease. The onset is typically insidious and develops slowly over several weeks. Systemic symptoms include fever, night sweats, malaise, loss of appetite and weight (Boon *et al.*, 2006).

1.2.3.4. Laboratory Diagnosis:

When Robert Koch published his seminal paper in 1882 on the identification of the aetiological agent of tuberculosis, he presented methods for both staining and culturing. His staining method used a primary stain and a mordant (to facilitate diffusion of the stain into the cell through the lipid-rich cell wall), a decolorizing agent, and a counterstain. These were required because the lipidity makes the cell wall hydrophobic and difficult for the stain to penetrate. Koch used methylene blue as the primary stain, and an alkaline potassium hydrate solution as the mordant. He skipped the decolorizing agent and used vesuvius as both a declarant and a counterstain. Ehrlich proposed (among other alternatives) the use of fuchsin as the primary stain, and used an alkaline aniline solution as the mordant. He introduced nitric acid as a declarant and proposed a blue counterstain if the primary stain was red (as is the case with fuchsin). Ziehl challenged the use of the mordant and proposed phenol. Neelsen combined the best of all, proposing to use Ehrlich's approach to the primary stain, but using Nielsen's proposal of the mordant (phenol) and changing the declarant from

nitric acid to sulphuric acid, thus establishing the basis of the current Ziehl-Neelsen staining technique, which was born as early as 1882 (Rieder *et al.*, 2007).

1.2.3.5. Collection of Specimens for Smear Examination:

The collection of sputum specimens for smear examination should be as efficient and as convenient as possible for both patients and laboratory workers. Numerous studies indicate that the incremental yield in detection of cases decreases with each succeeding specimen collected. Most countries have adopted the policy of collecting three specimens as an optimal means of identifying infectious cases of tuberculosis, following the recommendations of the WHO and The Union. Of patients ultimately positive on sputum smear examination, it has been reported that approximately 80% are positive on the first, another 15% on the second, and an additional 5% on the third specimen. However, several recent studies have suggested that the yield of the third specimen might be only in the range of 1% to 3%, thus constituting a high workload to identify the remaining additional cases (Rieder *et al.*, 2007).

1.2.3.6. Culture:

Mycobacteria that cause respiratory disease, particularly in patients with evidence of cavitations, are abundant in the respiratory secretions (e.g. 10^8 bacilli per ml or more). Recovery from the organisms is virtually assured in patients from whom early morning respiratory specimens are collected for 3 consecutive days. It is more difficult, however, to isolate *M. tuberculosis* and other mycobacteria from other sites in the patients with disseminated disease. The in vitro growth of mycobacteria is complicated by the fact most isolates grow slowly and can be obscured by the rapid – growing bacteria that normally colonize people. Thus, specimens such as sputum are initially treated with a decontaminating reagent (e.g., 2% sodium hydroxide) to remove organisms that could confound results. Mycobacteria can tolerate brief alkali treatment, which kills the rapid-growing bacteria and permits the selective isolation of

mycobacteria. Extended decontamination of the specimen kills mycobacteria, so the procedure is not performed when normally sterile specimens are being tested or when few mycobacteria are expected. In former times, when specimens were inoculated on to egg based (e.g., Lowenstein-Jensen) and agar based (e.g., Middle brook) media, it generally took a long time for *M. tuberculosis*, *M. avium* complex, and other important slow growing *mycobacteria* to be detected. However, this time has been shortened through the use of specially formulated broth cultures that support the rapid growth of most mycobacteria. Thus, the average time to grow *mycobacteria* has been decreased from 3 to 4 weeks to 10 to 14 days (Murray *et al.*, 2002).

1.2.4. PCR:

Polymerase Chain Reaction (PCR), discovered in 1985 by Kary Mullis at Cetus Corporation, proved evidence to the genome project in several ways. First, it provided a simple means to obtain DNA markers and to genome chromosome fragments, without the need of exchanging cloned. Second, it provided a new chemistry that allowed DNA sequencing to be automated. PCR provided a simple means of isolating pure quantities of specific DNA sequences. PCR uses enzymatic amplification to increase the copy number of any DNA fragment up to 6000 bp in length. Using data available from an STS database, a researcher anywhere in the world can use PCR to assay a DNA fragment for a particular STS in several hours. PCR is based on the phenomenon of primer extension by DNA polymerase, discovered in 1950s. Synthesis of each DNA strand is primed by a short double – stranded stretch. Working from this primer, the polymerase adds nucleotides complementary to the template DNA, extending the double - stranded region. First a primer of DNA oligonucleotide primers approximately 20 nucleotides in length are synthesized that bracket the target region to be amplified. The primers are designed to anneal to complementary DNA sequences at the 5 – end of each strand of the DNA molecule. The two primers are mixed in excess with a DNA sample containing the

target sequence, along with a dDNA polymerase. The cofactor magnesium (Mg^{++}) and the four Deoxyribonucleoside tri-phosphates (dNTPs) are also provided. The reaction mixture is then taken through multiple synthesis cycles consisting of the following:

1- **Denaturing:** Heating to near boiling $94^{\circ} C$ denatures the target DNA and creates a set of single-stranded templates. Heating increases the kinetic energy of the DNA energy needed to maintain hydrogen bonds between base pairs, and the double – stranded DNA separates into single strands.

2- **Annealing:** Cooling to approximately $65^{\circ} C$ encourages oligonucleotide primers to anneal to their complementary sequences on the single – stranded templates. The optimum annealing temperature varies according to the proportion of AT to GC pairs in the primer sequence. Because the primers are added in excess and are short, they will anneal to their long target sequences before the two original strands can come back together.

3- **Extension:** Heating to $72^{\circ} C$ provides the optimum temperature for the DNA polymerase to extend from the oligonucleotide primer. The polymerase synthesizes a second strand complementary to the original template. During each synthesis cycle of approximately 2 minutes, the number of copies of the target DNA molecule is doubled. The high temperature used in PCR required using a heat – stable DNA polymerase. In the late 1980s, the first heat – stable DNA had been isolated from a cultured sample of the bacterium *Thermus aquaticus*, originally obtained in the 1960s from the mushroom pool in Yellowstone National Park. This bacterium is adapted for life in the $80^{\circ} C$ waters of the thermal pool, and its DNA polymerase (called Taq polymerase) maintains activity throughout repeated exposure to near-boiling temperatures, making it ideal for PCR (Micklos *et al.*, 2006).

The PCR technique has already proven exceptionally valuable in many areas of molecular biology, medicine, and biotechnology. It can be used to amplify very small

quantities of specific DNA and provide sufficient material for accurately sequencing the fragment or cloning it by standard techniques. PCR based diagnostic tests for AIDS, Lyme disease, *Chlamydia*, Tuberculosis, hepatitis, the human papilloma virus, and other infectious agents and diseases are being developed. The tests are rapid, sensitive and specific (Prescott *et al.*, 2005).

1.2.5. Gel Electrophoresis:

Gel electrophoresis separates charged macromolecules by their rate of migration in analectic field. The migration of charged molecules through the agarose or poly acrylamide gel under the influence of an electric field is called gel electrophoresis. An electric field is applied and the negatively charged DNA molecules penetrate and move through the complex network of molecules that comprise the gel and towards the positive electrode or anode (Tropp, 2008).

1.2.6. Improving Laboratory Diagnosis of TB:

Consequently, the need for rapid identification of mycobacteria is vital for effective case management and control. Recently, molecular techniques have begun to replace the time-consuming phenotypic assays for characterization of mycobacteria. Techniques such as polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), duplex PCR and DNA sequence analysis were used for detection and characterization of mycobacteria from clinical specimens with reported higher sensitivities and specificities than those of conventional techniques and the cost of reagents used in molecular diagnosis is decreasing due to the increased availability in the international market (El-Eragi *et al.*, 2007).

Amplification and detection of *M. tuberculosis* DNA is one of the fastest and most sensitive ways to detect TB and it allows the detection of genetic mutations associated with drug resistance. The Xpert MTB/RIF assay is a fully automated system that allows a relatively untrained operator to perform sample processing, DNA amplification and detection of *M. tuberculosis* and screening for rifampicin resistance

in less than 2 hours and only minutes of hands-on time. Xpert MTB/RIF can be done outside of conventional laboratory settings. WHO strongly recommends that countries use the Xpert MTB/RIF as the initial diagnostic test in individuals suspected of having MDR-TB or HIV-associated TB and (when appropriate) as a follow-on test to microscopy in settings where MDR-TB and/or HIV is of lesser concern, especially in smear-negative specimens. Guidance exists to support implementation of the Xpert MTB/RIF assay. Adoption and scale-up of Xpert MTB/RIF has significant resource implications: the GeneXpert device that automates the procedure is a computer-driven, sophisticated piece of equipment that requires security, uninterrupted and stable electrical power and annual calibration. Xpert MTB/RIF is suitable for all levels of laboratories but capacity of one device is limited to 20 specimens per day. Higher -volume settings may require more than one device. Test costs are much higher than microscopy; however, once implemented the running costs of Xpert MTB/RIF are much lower than liquid culture or line probe assays. Xpert MTB/RIF does not eliminate the need for conventional microscopy culture and DST, which are required to monitor treatment progress and to detect resistance to drugs other than rifampicin. Recommended coverage is dependent on local prevalence of TB, MDR-TB and HIV (WHO, 2011).

1.2.7. Tuberculin Skin Test:

A positive test does not necessarily indicate active disease. In this test, a purified protein derivative of tuberculosis bacterium, derived by precipitation from both cultures, is injected cutaneously. If the injected person has been infected with TB in the past, sensitized T cells react with these proteins, and delayed hypersensitivity reaction occurs in about 48hours. This reaction appears as an indurations (hardening) and reddening of the area around the injection site. A positive tuberculin skin test (TST) in the very young is probable indication of active case of TB. In older

individuals, it might indicate only hypersensitivity resulting from a previous infection or vaccination, not current active case (Gerard *et al.*, 2010).

1.2.8. Vaccination with BCG:

The Bacille Calmette-Guérin (BCG) vaccine is a live vaccine derived from a strain of *Mycobacterium bovis* (similar to *M. tuberculosis*) first administered to humans in 1921. Since that time, many different strains have been derived and are used today throughout the world to prevent TB disease. BCG vaccination reduces the risk for progression from latent TB infection to TB disease, especially disseminated or central nervous system disease in children. BCG vaccination may cause a positive reaction to a tuberculin skin test (CDC, 1999).

1.2.9. Prevention and Control:

The steady decline in incidence of tuberculosis since the beginning of the Twentieth century, and before specific preventive measures were available, underlines the importance of improvements in social conditions in the prevention of this and many other infectious diseases. However there has been an increase in the number of cases associated with AIDS, in some countries in the resource-poor world, HIV infection and AIDS are threatening to overwhelm tuberculosis control programmes (Goering *et al.*, 2013).

1.2.10. Treatment of Tuberculosis:

The treatment of tuberculosis (TB) is based on two bacteriological considerations: the combination of drugs to avoid the selection of drug resistance, and the need for prolonged treatment to ensure that all bacteria in their different phases of metabolic growth are effectively destroyed. Clinical and microbiological research between 1950 and 1970 led to the conclusion that the best treatment for a patient with sensitive TB is 2HRZ/4HR. However, considering that in many parts of the world pharmacological

treatments have been used indiscriminately, resistance to isoniazid (H) is usually more than 4%. Thus, to prevent treatment failure and further drug resistance in this setting, ethambutol (E) should always be used during the first 2 months of therapy. It has also been demonstrated that if strict supervision of observed therapy is not established for the full duration of treatment, there is a high risk of developing resistance to rifampicin (R). For this reason, in areas where supervision of drug administration cannot be guaranteed in the second phase, ethambutol should be prescribed instead of rifampicin during the second phase, and treatment should be extended to 8 months. These inconveniences with rifampicin are clearly obviated if several drugs are used in combination (especially H+R and H+R+Z), at fixed doses, and incorporated in the same tablet—an approach that should always be recommended. Thus, the treatment scheme advised for all initial patients should be 2HRZE/4HR or 2HRZE/6HE. Although all first-line drugs (isoniazid, rifampicin, pyrazinamide, ethambutol, and streptomycin) are well tolerated, personnel treating and caring for patients should be aware of the few associated side effects. It is important to know how to proceed in the event of side effects, to deal with food-drug interactions, and to manage patients presenting with special conditions, such as renal failure, severe liver impairment, or pregnancy. Lastly, it must be emphasized that extrapulmonary TB should be treated in the same way as pulmonary TB, and that it is essential to know when surgery is indicated, as well as when the use of corticoid therapy is warranted. Hospitalization of patients with TB disease should be based on the severity of the disease, and occasionally to ensure adherence to therapy; hospitalization should never be based only on the patient having TB (Frieden, 2004).

1.2.11. TB / HIV Co-Infection:

Tuberculosis is particularly insidious problem to those who have AIDS .In these patients, the T lymphocytes, immune system cells that normally mount a response to *M. tuberculosis*, are being destroyed, and the patient cannot respond to the bacterial infection. Unlike most other tuberculosis patients, those with HIV usually develop tuberculosis in the lymph nodes, bones, liver, and numerous other organs (Pommerville, 2004).

In the words of the WHO, HIV infection is "fueling the tuberculosis epidemic" as it is now the most important predisposing factor for the development of active tuberculosis. As described above, a non – immuno-compromised person who has overcome primary tuberculosis infection has about a 5 percent chance of developing post – primary tuberculosis later in life. In a person infected with both *M. tuberculosis* and HIV, the risk of developing active tuberculosis rises to around 8 percent each year – a greatly increased risk. If an HIV – infected person becomes primarily infected or co – infected by the tubercle bacillus, the risk of progressing to active tuberculosis is very high, especially if he or she has had AIDS defining condition. Furthermore, the disease process which usually takes years or even decades to develop, is telescoped down to just a few months. Little wonder, therefore, that co – infection with *M. tuberculosis* and HIV has been termed "The cursed duet" and that in addition to using the term "apocalyptic" the WHO has stated that " HIV and TB co – infection represents an unprecedented global public health crisis (Pratt *et al.*, 2005) Today it is known that TB and HIV/AIDS are the two of the world's major pandemics. HIV infection is now prevalent, to varying degree, in all areas of the world where TB is prevalent. The pandemic of HIV/ AIDS opened a new chapter on the role of mycobacteria in causing human disease in both the developed and less – developed world. Worldwide, TB remains a problem of enormous proportions, with the

interaction of TB and HIV causing huge and increasing burden of illness and death in much the developing world. The number of people co – infected with TB and HIV already stands of over 10 million and is expected to increase dramatically over the next 10 years. Evidences suggest that one third are 30 to 50 times more likely to develop active TB, making TB the biggest AIDS – related killer in the world today. To make matters worse, global rates of MDR – TB are also on the rise. MDR – TB is very complicated and difficult to treat, very expensive to treat and often fatal (Sharma, 2004).

1.2.11.1. Impact of HIV on TB:

Infection with HIV -1 has emerged as the single strongest risk factor for development of active TB. This may be the result of number of different processes and underlying immunological mechanisms. HIV infected individuals may be more frequently exposed to *Mycobacterium tuberculosis* infection and outbreaks of TB have been described among patients attending healthcare facilities in both high – income and resource – limited settings. It is not known, however, whether HIV-infected individuals have greater risk of acquiring *M. tuberculosis* infection than HIV-uninfected individuals following a similar exposure to the organism. The fact that certain species of innate immune function are impaired by HIV infection might potentially increase host susceptibility to *M. tuberculosis* infection following exposure. Data from outbreaks among HIV – infected. This study was limited though. As these data were derived using the tuberculin skin test "TST" which has lowered sensitivity to detect *M. tuberculosis* infection in the context of HIV co infection.

The high incidence of active TB reported in outbreaks among "HIV" – infected people may instead reflect more rapid progression of *M. tuberculosis* infection. The risk of progressive primary diseases of is indeed high following infection with *M. tuberculosis*. Attack rates of 30 – 40 % have been reported among HIV – infected patients exposed to *M. tuberculosis* in healthcare settings, with many patients develop

disease within 1-2 months of initial exposure. This suggests that the host granulomatous response to early *M. tuberculosis* infection is impaired by HIV infection. A great majority of HIV – uninfected individuals with latent *M. tuberculosis* infection do not develop TB disease as their cellular immune function is adequate to maintain the mycobacterial infection in a clinically latent *M. tuberculosis* infection is estimated to be 2 – 23 %. In contrast, HIV – infected patient, with latent *M. tuberculosis* infection have a high risk of reactivation with approximately 10 % of such individuals developing active TB disease each year. This observation strongly suggest that the ability to maintain *M. tuberculosis* latency with in granulomas long term is abrogated by HIV – 1 co – infection. As mentioned one – third of the world population is infected with *M. tuberculosis*, with such higher rates in poor communities in resource – limited settings that have high HIV prevalence. The broad geographical overlap of these infections is a critical factor explaining the huge impact of HIV on global TB control (Schaaf and Zumla, 2009).

The HIV -1 epidemic has not only had a great impact on the epidemiology of TB, but the clinical, radiological, and histo-pathological features of the disease are often modified in co – infections patients, in contrast to many opportunistic infections. TB occurs across a wide spectrum of immunodeficiency. This reflects the greater virulence of *M. tuberculosis* compared with other opportunistic pathogens such as *mycobacterium avium* complex, *Pneumocystic jiroveci*, and *Cryptococcus neoformans*, which typically only cause disease among patients with advanced immunodeficiency. The features of TB in HIV – 1 infected individual with well - presented CD4 T cell counts are indistinguishable from those of individuals with TB but no HIV -1 co infection. However, progressive immunodeficiency is associated with an increasing frequency of military and disseminated forms of disease, reflecting failure of containment of mycobacterial infection. Impaired tissue inflammatory response to infection affects the radiographic appearances of pulmonary TB, with

reduced consolidation, fibrosis and cavitations, and also results in sputum smear Microscopy for acid fast bacilli (AFB) being less frequently positive. Mortality rates among patients with TB and HIV-1 co-infection are high and may be due to either TB itself or another HIV – associated illness.

HIV co – infection is also associated with high rates of coetaneous energy to TST indicating failure of delayed type hypersensitivity responses do not gradually diminish with progressive immunodeficiency as one might expect but instead appear to be an "all-or-nothing" phenomenon. Although the proportion of HIV – Infected patients who have coetaneous energy to TST increases with progressive immunodeficiency positive TST responses of HIV – Infected patients are nevertheless similar in magnitude to the positive responses of HIV – Infected patients (Schaaf and Zumla, 2009).

1.2.11.2. Impact of Tuberculosis on HIV:

Data from various studies support the hypothesis that active TB causes an accelerated rate of immunological decline among HIV – infected patients. The first such study conducted in the USA by Whalen *et al* was retrospective analysis. Survival among 106 HIV – infected patients with TB cases was compared with survival among HIV – infected control patients who remained TB free and who were matched for baseline degree of immunodeficiency and patients characteristics. The adjusted odds for mortality among cases were significantly increased (approximately two fold). Subsequent prospective studies in France, Uganda, and South Africa have produced finding consistent with this. In the two studies from sub – Saharan Africa significantly increased mortality risk was observed only among TB patients with CD4. Cells counts ≥ 200 cells / Ml, suggesting that any long- term impact of TB on HIV. Pathogenesis occurs among those with greater immunological reserve. A substantial body of laboratory data is consistent with the hypothesis that TB has a co-factor effect on the pathogenesis of HIV- I infection (Schaaf and Zumla, 2009).

Activation associated with *M. tuberculosis* increases HIV - I replication in vitro. *M. tuberculosis* and certain mycobacterial cell wall components not only induce HIV – I replication within cells of the monocytic lineage and CD4 T cells but also enhance viral infectivity for this cell type and increase transmission of HIV – I from antigen – presenting cells to lymphocytes (Schaaf and Zumla, 2009).

1.2.11.3. TB/HIV Co-Infection in Sub-Saharan Africa:

Globally, approximately 9% of all new TB cases in adults are attributable to HIV infection. The proportion is much greater in sub – Saharan Africa, but is about 4% in U.K. The close links between HIV and TB, and the potential for both diseases to overwhelm health – care funding in resource. Poor nations have been recognized with the promotion of programmes that link detection and treatment of TB with detection and treatment of HIV (Boon *et al.*, 2006). The tuberculosis epidemic in a large number of sub-Saharan African countries has been seriously affected by the HIV epidemic. It has been estimated that approximately one third, ranging from 0 to 72 per cent, of tuberculosis cases between 1985 and 1993 would not have occurred in sub-Saharan Africa if pre-1985 trends had continued. In Burundi, tuberculosis notifications more than doubled from 1984 to 1991. In a hospital in Brazzaville, Republic of Congo, the prevalence of HIV infection among tuberculosis patients increased from 3 to 24 per cent just in the 4-year period between 1988 and 1992. In Côte d'Ivoire, an autopsy study in patients who died with HIV infection revealed that tuberculosis was the cause of death in 32 per cent. In Malawi, the number of notified tuberculosis cases almost quadrupled from 1985 to 1995. Large increases in tuberculosis notifications attributed at least partially to HIV have also been reported from Kenya, Zambia, Zimbabwe, and other countries. In Tanzania, tuberculosis case notifications increased virtually exponentially from 1984 to 1995. In addition to the increase in the case rates, it is of particular concern that the age distribution of sputum smear-positive cases also changed. From 1984 to 1995, the peak rate of sputum smear-positive cases shifted

very clearly to a younger age group. This is an epidemiologically ominous sign, as improvements in the epidemiological situation must invariably be associated with an increase in the median age of tuberculosis patients, while conversely a lowering of the median age would indicate a higher perpetuation of transmission to younger population segments. The large impact of HIV on the tuberculosis situation in Tanzania (indicative of the situation in many other sub-Saharan countries) has been shown in a country-wide, representative HIV seroprevalence study among tuberculosis patients. The results indicate that between 30 and 45 per cent of patients with tuberculosis had HIV infection. The risk of HIV infection is unevenly distributed in the country. This investigation showed that the likelihood of a tuberculosis patient being infected with HIV was greatest in the 25- to 34-year-old age group. Female tuberculosis patients were more likely to be infected with HIV. Extrapulmonary tuberculosis cases were much more likely to have HIV infection. BCG vaccination appeared to provide relative protection against tuberculosis among adults as long as they were not HIV-infected. A second round of the survey has shown a rapid progression of HIV infection even over such a short span as 3 years. The rapid increase is particularly conspicuous in rural areas (Rieder, 1999).

1.2.11.4. Tuberculosis in HIV-Infected Children:

The natural history of tuberculosis in an HIV -infected child depends on the stage of the HIV infection. If the HIV infection is not yet clinically evident and the child still has good immunity, we can expect the same signs of tuberculosis as in an un-infected child. In an HIV-infected child, however, tuberculosis is more likely to spread to other parts of the body, resulting in tuberculous meningitis, miliary tuberculosis and general enlargement of lymph nodes. The overlap in symptoms and signs between tuberculosis and HIV infection means that the diagnosis of tuberculosis in a HIV-infected child can be difficult. For this reason, always consider the

possibility of tuberculosis in an HIV -infected child. Children who are HIV infected are at great risk of developing tuberculosis. Thus, all children diagnosed with HIV infection must be screened for tuberculosis. If they are found to be infected on the basis of a positive tuberculin test but do not have active disease, this latent infection should be treated with isoniazid (5 mg/kg/ day) for 9 months. The decision to treat a child should be considered carefully; once started, the full course of isoniazid must be completed (Frieden, 2004).

1.2.12. Diagnosis of Childhood Tuberculosis:

As in adults, pulmonary tuberculosis is the most common manifestation of tuberculosis in HIV-positive children. The diagnosis of pulmonary tuberculosis in children under 4 years old has always been difficult, and HIV infection further compounds this diagnostic challenge. There is a high incidence of cutaneous anergy in HIV-positive children with tuberculosis, and most cases are diagnosed according to nonspecific clinical and radiographic criteria. Because it is often difficult to distinguish HIV-related pulmonary disease from pulmonary tuberculosis, childhood pulmonary tuberculosis is probably over-diagnosed in many areas (Harries *et al.*, 2004).

1.2.13. Infections with Environmental Mycobacteria:

These are sometimes called 'atypical', 'opportunistic' or 'mycobacteria other than tubercle bacilli': There are a number of different sorts of these bacteria. They are common in our environment, in the water and soil for example, and some cause tuberculosis-like disease in various animals. They occasionally infect people and cause chest disease that resembles tuberculosis. Diagnosis can only be made by culture. Rarely, particularly in patients with AIDS, the bacteria spread through the bloodstream. They may then cause multiple bone abscesses etc. In countries with a high prevalence of tuberculosis these infections are less frequent, while tuberculosis is

common. They will therefore be less important in your practice. In any case, you need culture facilities to diagnose infection with these mycobacteria. Most people easily control the infection and do not become ill. But patients with AIDS have severely damaged immune defenses and are particularly prone to these infections (Rieder *et al*, 2009).

1.2.14. Treatment of Tuberculosis in HIV-Positive Patients:

Modern short-course treatment of tuberculosis is just as effective in HIV-positive patients as in HIV-negative patients. The sputum becomes negative just as quickly but relapse rates are somewhat higher. Weight gain may be somewhat less than in HIV-negative patients. But with former long-term 'standard' treatment, not including rifampicin, treatment was less successful and relapse much more frequent. Some of the relapse may have been due to re-infection because of the patient's lowered defenses due to the HIV (Rieder *et al*, 2009).

1.2.15. Collaborative TB/HIV Activities:

Collaborative TB/HIV activities are essential to ensure that HIV-positive TB patients are identified and treated appropriately, and to prevent TB in HIV-positive people. These activities include establishing mechanisms for collaboration between TB and HIV programmes (coordinating bodies, joint TB/HIV planning, monitoring and evaluation, HIV surveillance); infection control in health-care and congregate settings; HIV testing of TB patients and, for those TB patients infected with HIV, cotrimoxazole preventive therapy (CPT) and antiretroviral therapy (ART); and intensified TB case-finding among people living with HIV followed by isoniazid preventive therapy (IPT) for those without active TB (WHO, 2009).

1.2S.16. Genotyping Background:

Tuberculosis (TB) genotyping is a laboratory-based analysis of the genetic material of the bacteria that causes TB disease, *Mycobacterium tuberculosis*

complex. The total genetic content is referred to as the genome. Specific sections of the genome contain distinct genetic patterns that help distinguish different strains of *M. tuberculosis*. TB genotyping results, when combined with epidemiologic data, help identify persons with TB disease involved in the same chain of recent transmission. This information adds value to conventional TB control activities.

CHAPTER TWO

Materials and Methods

2. Materials and Methods

2.1. Study Approach:

This was a qualitative, prospective study.

2.2. Study Design:

The study design was a hospital-based, analytical study.

2.3. Study Type:

The study type was a descriptive, cross-sectional study.

2.4. Study Setting:

It was a hospital study setting. Specimens were collected from participants suspected of tuberculosis and HIV infections.

2.5. Study Duration:

This study was conducted during August 2012 to June 2015, including the pilot study, literature review, specimens' collection, laboratory investigations, data analysis, and thesis writing.

2.6. Study Area:

This study was carried out in the West Darfur State which is located in the Sudan's far western borders. It is delimited by North Darfur State in the north, Middle Darfur

State in the east and southeast. It has international borders with Republic of Chad and Republic of Central Africa in the west and southwest. The area is 1.531.682 kilometers square. Population: 750.000 persons and livestock resources are 5.083.599 heads. The most important towns are Morni, Kulbus, and Algineina which is the capital of the State.

2.7. Study Population:

Population studied was divided into two groups: patients with HIV and AIDS and tuberculous patients negative for HIV and AIDS; covering males and females at different age groups.

2.8. Inclusion Criteria:

- 1- All HIV positive patients in West Darfur State.
- 2- TB positive-HIV negative patients in West Darfur State.

2.9. Exclusion Criteria:

- 1-HIV-negative individuals attending the VCT Center.
- 2- TB-negative individuals attending Algineina TB Center at Algineina Hospital.

2.10. Study Variables:

- Demographic data: age, occupation, residence, education.
- Molecular sensitivity pattern.
- Molecular genotypes and sequencing

2.11. Sampling:

- Non- probability purposive sampling.

- Sample strategy: convenience where participants were chosen on the basis of accessibility.
- Sample frame: participants with patients with HIV and AIDS and tuberculous patients negative for HIV and AIDS in West Darfur State.
- Sample size: A total of 1318 sputum samples had been collected from the two population groups.

2.12. Validity and Pre-Testing:

- All reagents and primers were pre-tested using control strains; and equipment was calibrated.

2.13. Ethical Considerations:

- Maintaining confidentiality of information obtained from participants investigated.
- Consent of the participants was obtained before being enrolled in the study.
- Laboratory results of specimens collected were handed to all participants included in the study or dispatched to physicians treating those participants for prescription.
- Permission to collect the specimens was obtained from the Federal Ministry of Health (Khartoum) and Algeneina TB Center at Algeneina Hospital (Appendix 2 and 3).
- Complete information regarding risk factors, if any, was handed to all participants under the study and no concealment whatsoever.

2.14 Sample Collection and Processing:

Demographic and clinical data were collected from all participants using a structured questionnaire with a written informed consent (Appendix 1).

2.14.1 Patients with HIV and AIDS:

Samples collected from this group were 158. Patients of this group were attending VCT Center (attached to Algeneina Hospital)(n=87) and PLWHA Center at Algeneina Hospital (n=71) . Specimens were microscopically examined for AFB. TB positive sputum samples were stored at -20°C until transported to Khartoum for further molecular techniques.

2.14.2 TB Patients:

Samples collected from this group were 1160. Patients of this group were pulmonary suspected TB patients attending Algeneina TB Center. Specimens were microscopically examined for AFB. TB positive sputum samples were stored at -20°C until transported to Khartoum for further molecular techniques.

Two sputum samples were collected from the suspected cases of pulmonary TB. into a well-labeled wide-mouth containers covered with lid. Diagnosis of TB among TB suspects was as per WHO guidelines for TB microscopical diagnosis. Two consecutive sputum samples (spot-morning) were collected and examined for the presence of Acid Fast Bacilli (AFB) using the standard Ziehl-Neelsen method. The specimens were transported to the laboratory for immediate processing while any sample that was poorly collected (sputum containing saliva) was discarded.

2. 15. Ziehl-Neelson (ZN) Staining Technique:

The sputum containers were taken corresponding to the number on the slides. The containers were carefully opened to avoid aerosol production. A wood-stick applicator was broken and yellow, purulent particle of sputum with jagged end of the broken wood was selected. The sputum was evenly spread over the central area of the slide using a continuous rotational movement. Finally the stick applicators were discarded by placing them in a waste receptacle containing 5% aqueous phenol solution and incinerated. Dried smears were fixed by holding them with forceps and passing them smear side up over the flame 5 times for about 4 seconds avoiding overheat. The Fixed slides were placed on the staining rack in serial order, smeared side up. Slides were separated by a 1 cm gap, so that the slides should never touch one another. The slides were covered individually with filtered 0.3% ZN carbol fuchsin working solution. The slides were heated from underneath with a flame until vapour starts to rise and left for 5 min. Staining solution should never be allowed to boil. The slides were gently rinsed with tap water to remove excess carbol fuchsin. Then slides were covered with 25% sulfuric acid solution. and allowed to stand for 2 minutes, after which the red colour has almost completely disappeared. The sulfuric acid was then gently washed away with water. The slides were individually covered with 0.3% methylene blue counter-staining solution and allowed to stand for 1 minute. Water was drained off the slides, and allowed to air dry.

Microscopic examination of smears was carried out using a binocular microscope with two objectives (a regular 40 x magnification objective and an oil immersion 100 x magnification objective, and eyepiece of moderate magnification 8x or 10x) is used. A drop of immersion oil was placed on a dry stained slide to increase the resolving power of the objective. Acid-fast bacilli appeared bright red or pink against the blue

counterstained background. They vary greatly in shape, from short, coccoid to elongated filaments. They were uniformly or unevenly stained, and also even appear granular. They occurred singly or in variable sized clumps, and typically appear as long, slender curved rods. The microscopic examination was performed systematically and standardized. It started at the left end of the smear. The reading begins at the periphery of the field and ends at the center.

2.16. DNA Extraction:

Sputum samples from all ZN stain positive TB patients (N= 200 patients) and from PLWHA (N= 6) were subjected to DNA extraction by the Guanidine chloride method.

2.16.1. Guanidine Chloride Method: P

This method was introduced by Piotr and Nicoletta in (1987), Guanidine is added to the organic phase to aid in the denaturation of proteins (Chomczynski and Sacchi,1987).

- 1- Sputum samples were taken in a tube and equal volume of sodium hydroxide (4%) was added for decontamination, then transferred to a Falcon tube and mixed well and centrifuged for 15min-at 4000rpm (appendix.8).
- 2- The supernatant was discarded and the quantities of the following reagents were added: 2000 µl of WBCs lysis buffer which contains ;(10 mM Tris-HCl+0.1 mM EDTA + 100 mM NaCl + 20% SDS) (appendix 8,9) , 10 µl of proteinase K (Stock solution 100mg/ml+ Working solution 10mg/ml), 300 µl of ammonium acetate (MW=77.08+57.81g in 100 ddH₂O) and 1000 µl of guanidine(MW=95.53+57.318g in 100ml ddH₂O).
- 3- After incubation at 65 °C for 2 hours, 2000 µl of chloroform was added, mixed and centrifuged at 3000rpm for 10 minutes.

- 4- The upper layer was carefully transferred to another clean falcon tube, and 10 ml of 95% cold ethanol was added and incubated overnight at -20°C.
- 5- Then the tubes were centrifuged at 3000rpm for 10 minutes and the supernatant was discarded off, and the tubes were then dried in a vertical position.
- 6- Four ml of 70% ethanol was added to the tubes, mixed well and centrifuged at 3000rpm for 10 minutes and the supernatant was discarded.
- 7- The pellet was allowed to dry for 15minutes and 50 µl of distilled water was added.
- 8- The concentration of the DNA and its purity were measured using the Nanodrop machine (code No.3516, IMPLEN, Germany). The samples with DNA concentration above 50and purity above 1.7 were used for next steps.
- 9- The extracted DNA was stored at -20°C till used.

2.17. PCR Technique:

The extracted DNA was processed by PCR technique as follows:

2.17.1 Primers Design:

Oligonucleotides derived from the IS6110 sequence were used as primers (Eurofins Genomics) in polymerase chain reaction technique for the detection and identification of *M. tuberculosis* bacilli in this study with amplification of DNA which performed with primers:-

IS6110-Forward 5'-CCTGCGAGCGTAGGCGTCGG-3'

IS6110-Revers- 5'CTCGTCCAGCGCCGCTTCGG-3' (Appendix.4).

To amplify 123 bp fragment of insertion element IS6110 of *M. tuberculosis* complex the following was prepared:

- 1- A template DNA and primers was added into Maxime PCR Pre-Mixtubes (i-Taq) (iNtRONBIOTECHNOLOGY-Korea) which contains (i-TaqTMDNA Polymerase, dNTP mixture and reaction buffer) (Appendix.5).
- 2- A volume of 16 μ l Distilled water then added into the tubes to a total volume of 20 μ l. As illustrated in (Table 1).

Table 1. PCR Materials and Preparation

PCR reaction mixture	Volume
Template DNA	2 μ l
Primer (F : 10pmol/ μ l)	1 μ l
Primer (R : 10pmol/ μ l)	1 μ l
Distilled Water	16 μ l
Total reaction volume	20 μ l

- 3- Then the PCR procedure was performed in line with the following cycling parameters (Table 2).

Table 2. PCR Technique Procedure

PCR cycle		Temp.	PCR product size (100-500 bp)
Initial Denaturation		94°C	5min.
30 cycles	Denaturation	94°C	30 sec.
	Annealing	50-65°C	30 sec.
	Extension	65-72°C	30 sec.
Final Extension		72°C	7 min.

- 4- The samples were then loaded on 2.0% agarose gel without adding a Loading-dye buffer and electrophoresis was performed.
- 5- The electrophoresis product was visualized by UV light.

2.17.2. Electrophoresis Technique for DNA Detection:

DNA was detected by electrophoresis on gels and stained with ethidium bromide, which has an intense fluorescence excited by ultra-violet radiation when it complexes with nucleic acids.

2.17.2.1 Gel Preparation (2.0% Agarose Gel):

The gel was prepared by mixing 2.0 gm agarose, 100 ml 1X TBE buffer and 4 µl of ethidium bromide (10 mg/ml).

2.17.2.2 Loading of the Samples:

3 µl of the extracted DNA mixed with 3 µl of loading buffer. 4 µl DNA was loaded on the gel. A molecular weight DNA marker (Ladder) is run on every gel. The gel was run in 1X TBE running buffer and electrophoresis was carried out at 100 volts for 20 min. then the gel is viewed under UV light and photographed.

2.18. DNA Sequencing:

Amplified DNA extracted from some selected study samples from (PLWHA) and TB patients negative for HIV have been sent to Korea (Macrogen Company) for DNA sequencing.

2.19. Data Analysis:

The software used for the analysis of data was the Statistical Package for Social Sciences (SPSS) program (version 14). For categorical variables, proportions were compared by the Chi-square test as appropriate. The means and medians of the continuous variables were compared by Student's *t* test program depending on the sample distribution. Frequencies, percentages, tables and graphs were used for presentation of the data.

2.20. Plan of Dissemination of Results:

The study findings may be shared with others by the following means:

- Presentations in conferences, symposia, workshops, and scientific meetings.
- Publishing results in scientific journals, memoranda, textbooks, and websites.
- Lectures, seminars, and tutorials addressed to undergraduate and postgraduate students as well as university graduates.

CHAPTER THREE

Results

Results

3. Results

Of the total 158 PLWHA patients tested for TB in this study 6/158 (3.8%) were found smear positive by microscopic ZN examination (Fig.1).

As shown in Fig. 2, the prevalence rate of TB among HIV-infected patients in this study was significantly higher in males (66.7%) compared with females (33.3%).

Table 3 shows the TB prevalence among PLWHA patients. It was observed to be higher in the age group 31-40 years (50%) and 41-50 years (33.3%) compared to younger age groups of 21-30 years (16.7%).

Of the total 1160 sputum samples collected from TB patients, 200 samples were smear positive by ZN microscopy. From these males were more infected by TB than females (Fig. 3).

Fig. 4 demonstrates the TB prevalence among TB patients. The most affected age group was 31-45 years followed by the age group 46-60 years.

It was also noticed that most of the patients studied were residents of rural areas of Algeneina city (Fig. 5).

Of the 200 control smear-positive sputum samples collected from the TB patients who were negative for HIV infection, (85 %, 170/200) were found PCR positive (Table 4).

Also all the smear positive sputum samples collected from PLWHA patients were found positive by PCR technique (6/6, 100%).

The modified guanidine chloride method used for DNA extraction from the sputum samples had given good results by the PCR technique, indicating

that it has no noticeable PCR inhibitors and it is suitable for *M. tuberculosis* DNA extraction.

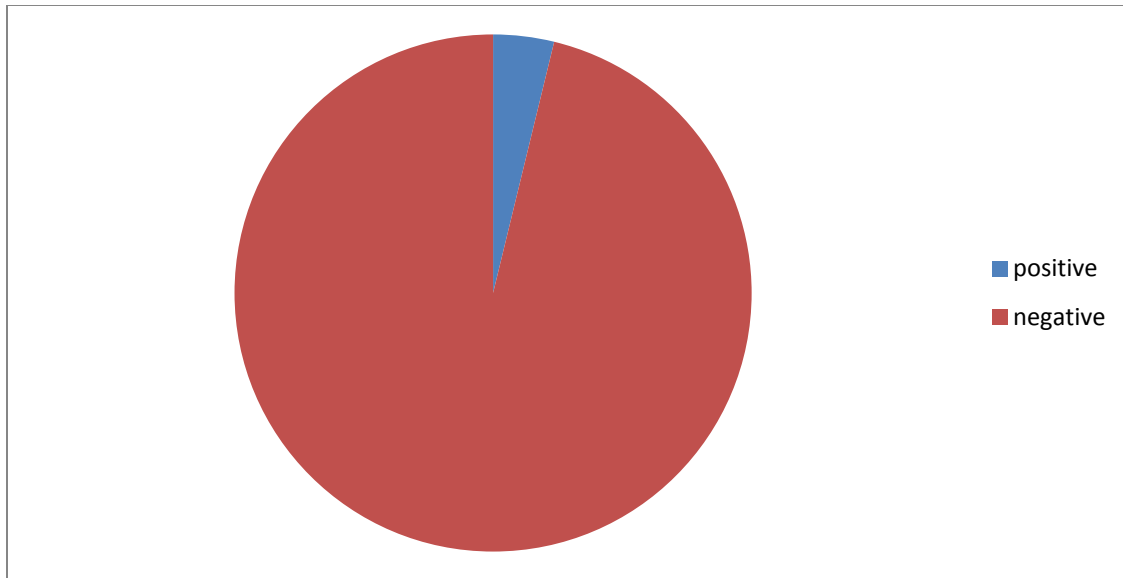
PCR-IS6110 primer used for detecting *M. tuberculosis* in this study showed clear and obvious bands on gel electrophoresis revealing a good PCR product (Fig. 6).

Most of the TB positive PLWHA patients were from the VCT Center (5/6, 83 %); and only one patient (1/6, 17%) was from the Center of PLWHA Association (Fig. 7) .

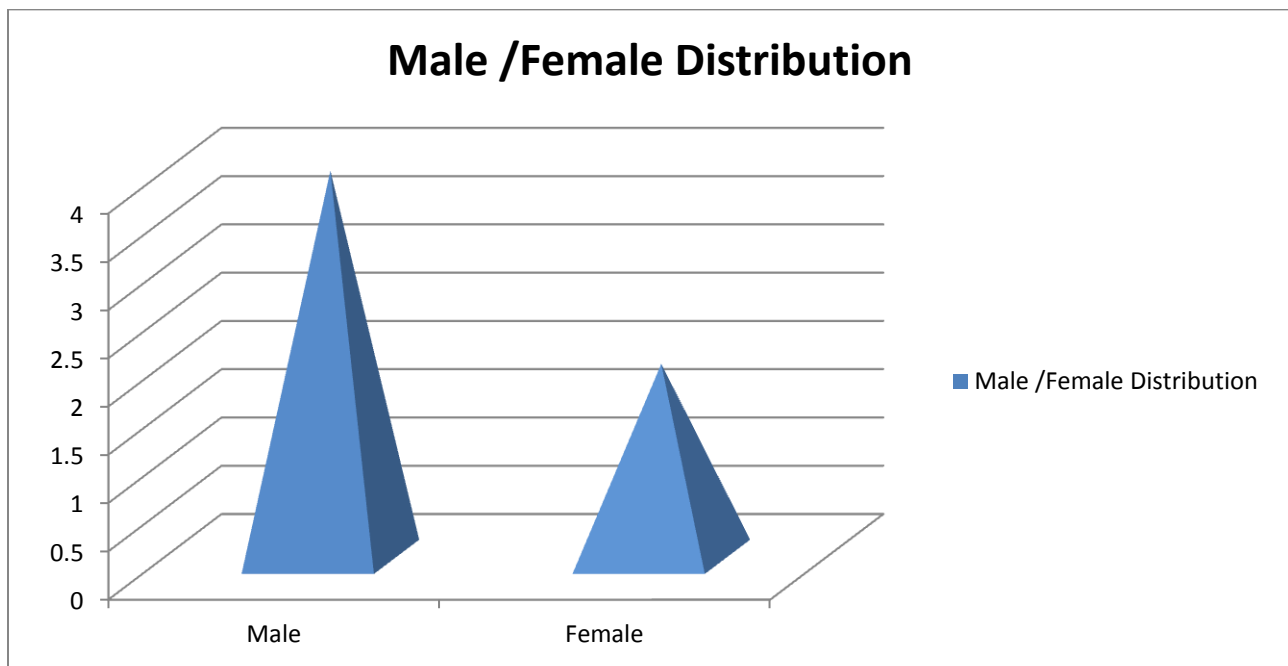
BLAST technique was used to identify the sequence polymorphisms of selected genes related to *Mycobacterium tuberculosis* strain H37Rv. The DNA sequencing results showed that the DNA sequence of these selected samples had a pattern matching those genes in the database and close to or identical to the *Mycobacterium tuberculosis* H37Rv genome by 100% for sample 1, (Fig. 8), by 99% for samples 2, 3, 4 respectively, and by 96% for sample 5 (Fig. 9).

A high similarity of sequences retrieved from NCBI database with the deletion of C nucleotide in position 5 in samples 2, 3 and 4 with the deletion of four nucleotides in sample (5) was observed. The alignment sequence of the four nitrogenous bases was drawn by base spacing computerized programmes showing the steady distribution of these bases along a piece of *Mycobacterium tuberculosis* H37Rv genome colored with distinct green, blue, black and red colors (Fig. 10, 11, 12, 13 and 14)and Appendix 12,13,14 .

The phylogenetic tree of the selected study strains revealed the evolutionary relation between the sequences. The phylogenetic analysis of revealed two distinct phylogroups which include some MTB mutant strains with the deletion of one or more nucleotides (Fig. 15).



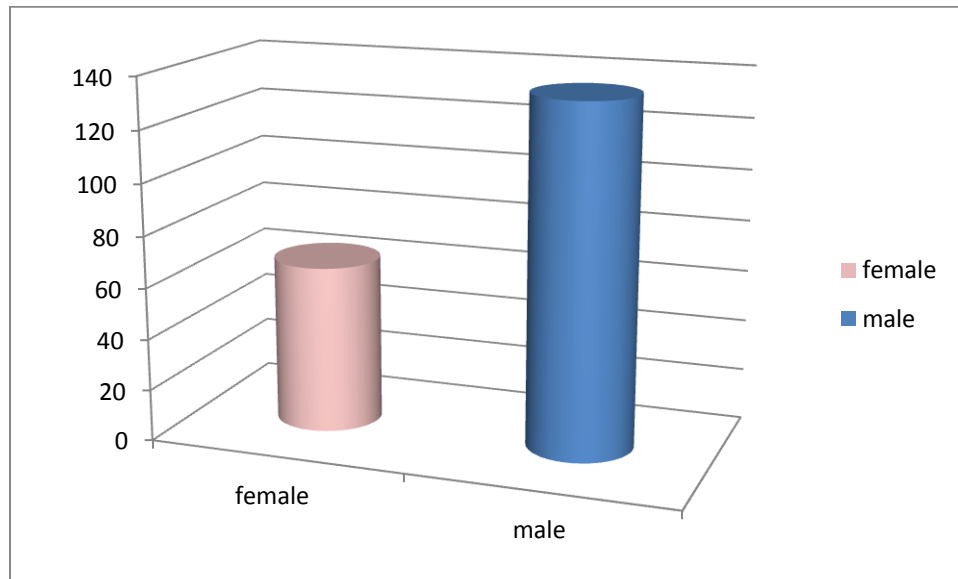
(Fig. 1) Positive Microscopic ZN Specimens among PLWHA Patients



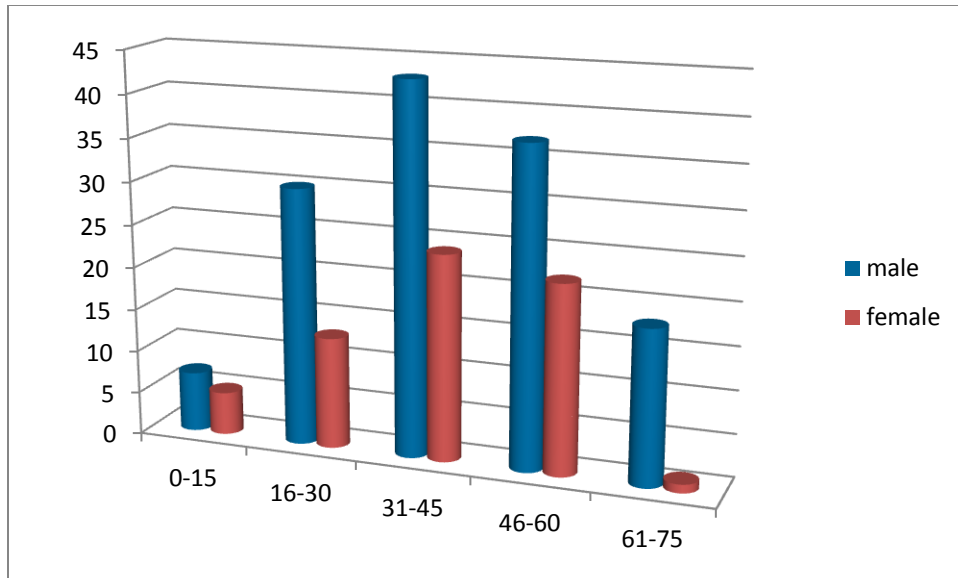
(Fig. 2) Distribution of TB Positive Specimens among PLWHA Patients According to Gender

**(Table 3) The TB Prevalence among PLWHA Patients
According to Age Incidence**

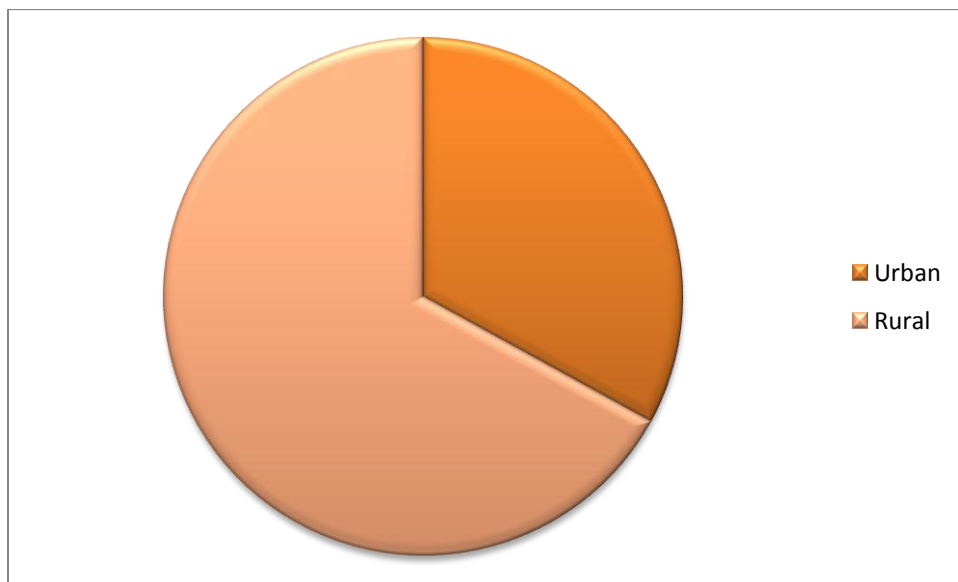
Age group	Number	Percentage
20-30	1	16.7
31- 40	3	50.0
41-50	2	33.3
51-60	0	0
Total	6	100



(Fig. 3) Distribution of TB Positive Patients According to Gender.



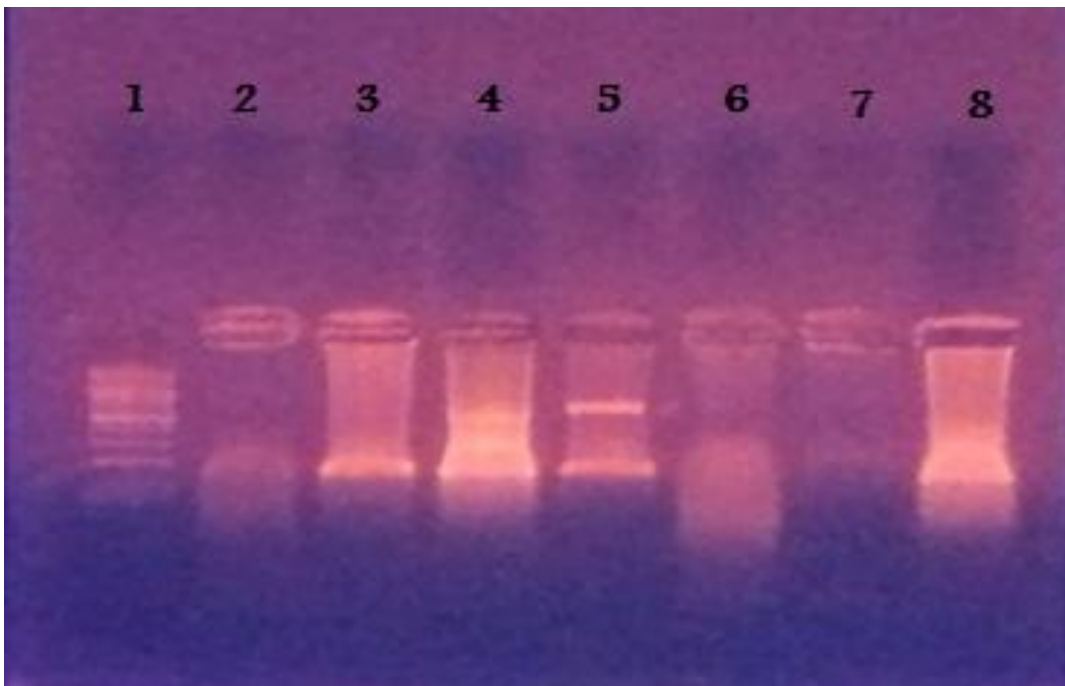
(Fig. 4) Distribution of Positive Specimens among TB Patients According to Age Incidence



(Fig. 5) Distribution of TB Positive Patients According to Residence.

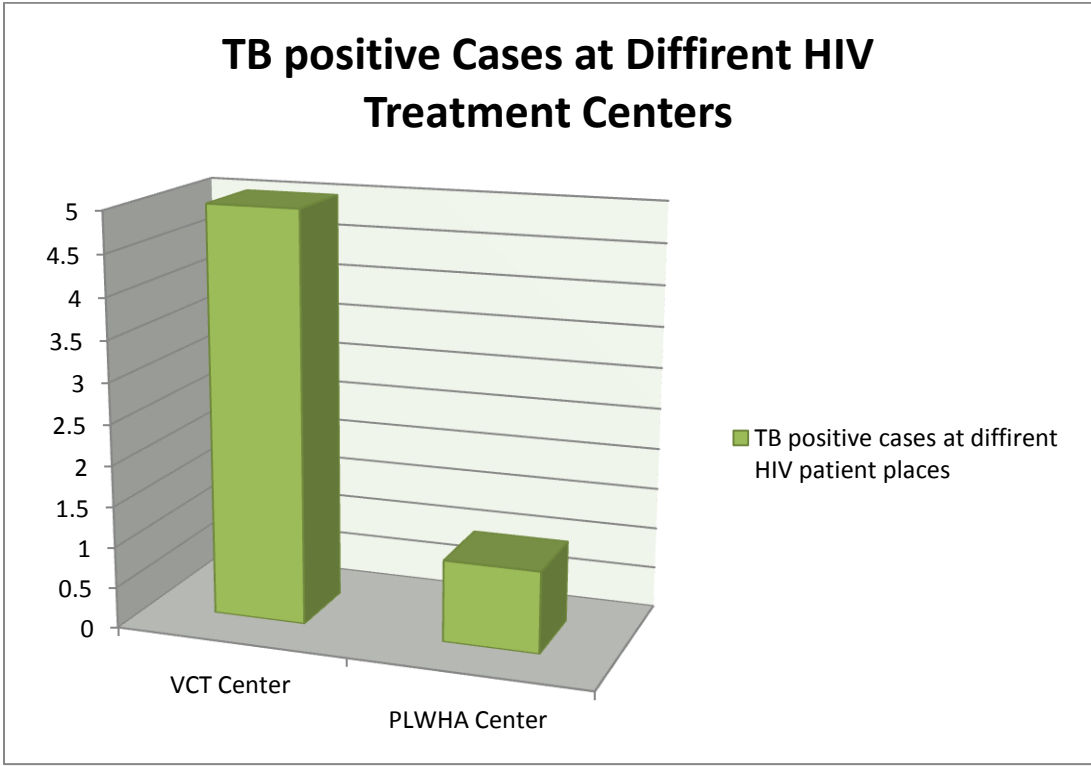
(Table 4) Detection of TB Positive Cases by PCR and ZN Microscopy.

Technique	Number of positive cases	Percentage
ZN microscopy	200	100
PCR	170	85



(Fig. 6) PCR bands on Gel Electrophoresis

(Sample 1 DNA marker (ladder), samples 2, 6 and 7 are negative, samples 3, 4, 5, and 8 are positive).



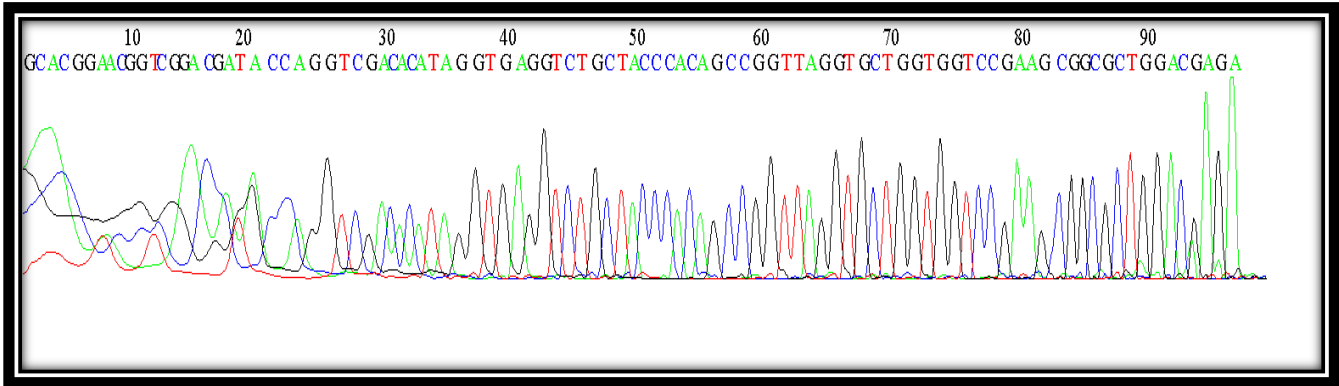
(Fig. 7) TB Positive Cases at Different HIV Treatment Centers.


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114. CCAGGTCGACACATAGGTGAGGTCTGCTACCCACAGCCGGTTAGGTGCTGGTGGTCCGAAGCGGCGCTGGACGAGA
|||||
Ref. CCAGGTCGACACATAGGTGAGGTCTGCTACCCACAGCCGGTTAGGTGCTGGTGGTCCGAAGCGGCGCTGGACGAGA
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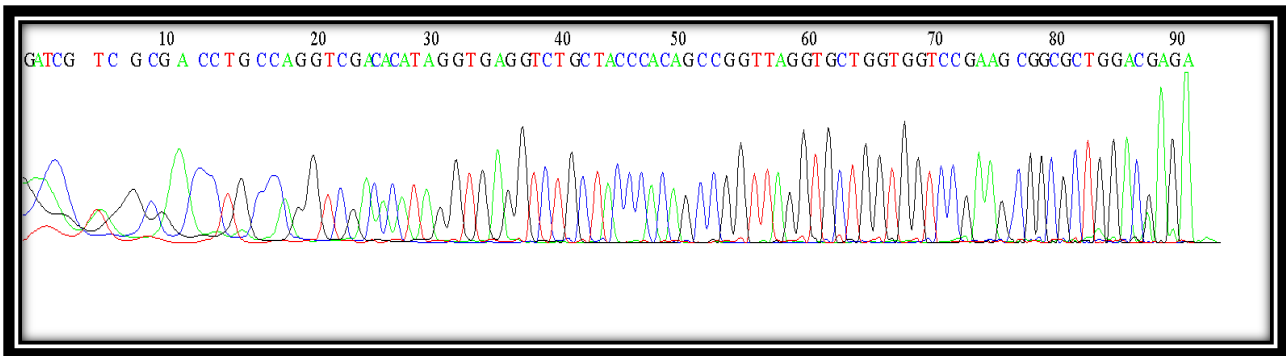
(Fig. 8) 100% Identity of Sample 1 (TB114) to *M. tuberculosis* H37Rv.

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47. CCTG-CCAGGTCGACACATAGGTGAGGTCTGCTACCCACAGCCGGTTAGGTGCTGGTGGTCCGAAGCGGCGCTGGACGAGA
|||
Ref. CCTGCCAGGTCGACACATAGGTGAGGTCTGCTACCCACAGCCGGTTAGGTGCTGGTGGTCCGAAGCGGCGCTGGACGAGA
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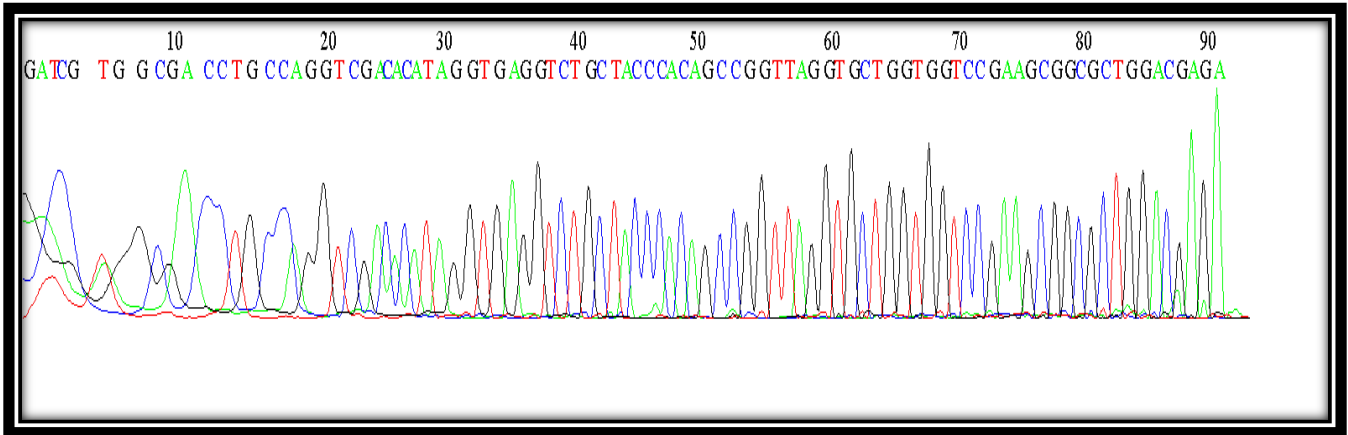
**(Fig. 9) 99% Identity of Sample 2 (TB 47) to
Mycobacterium tuberculosis H37Rv.**



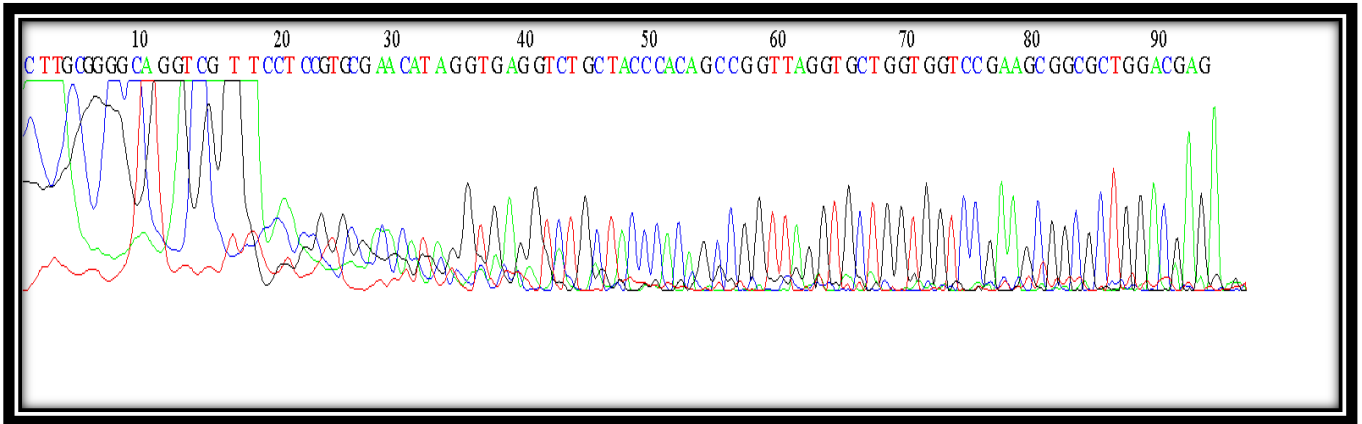
(Fig. 10) A Chromatogram Showing the Four Nitrogenous Bases Distribution in Different Colors for Sample1. (TB114)



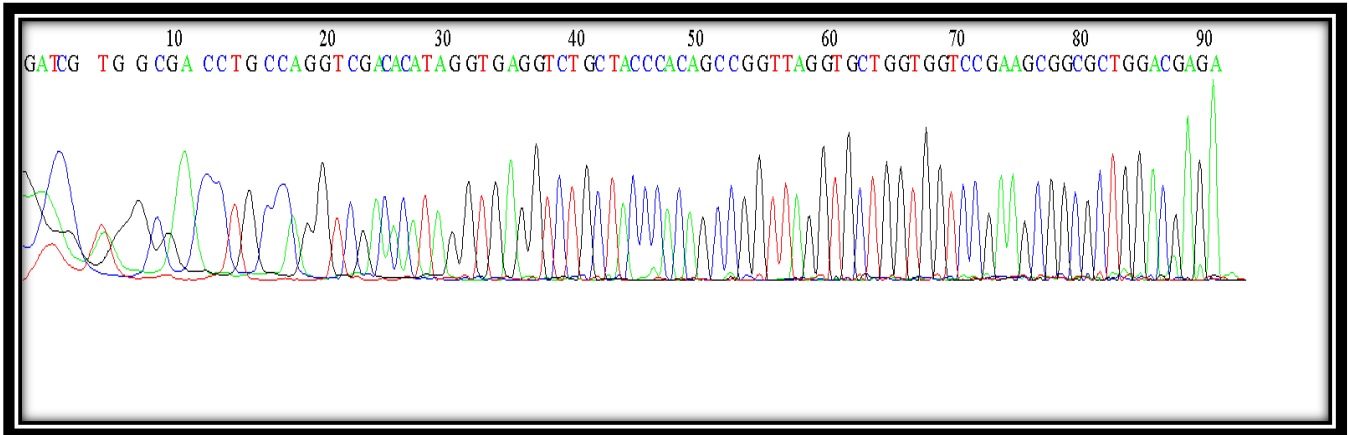
(Fig. 11) A Chromatogram Showing the Four Nitrogenous Bases Distribution in Different Colors for Sample 2.



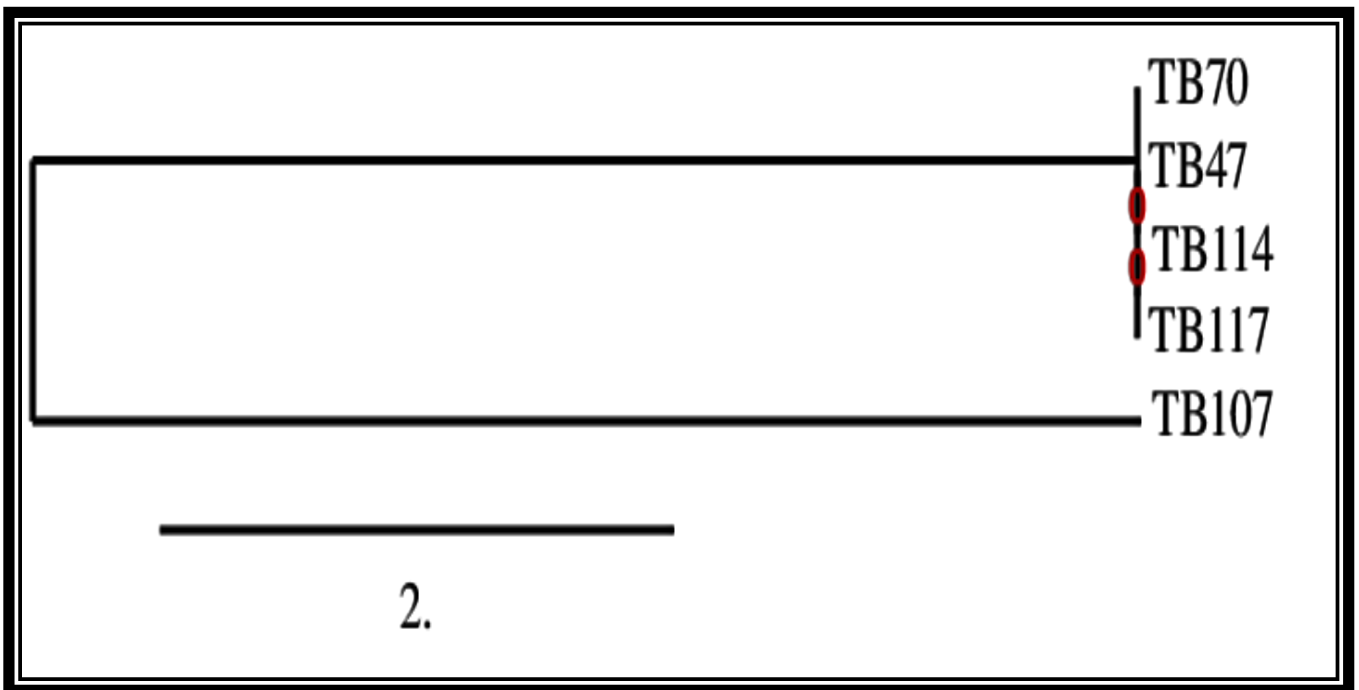
(Fig. 12) A Chromatogram Showing the Four Nitrogenous Bases Distribution in Different Colors for Sample 3. (TB70).



(Fig. 13) A Chromatogram Showing the Four Nitrogenous Bases Distribution in Different Colors for Sample 4. (TB107)



(Fig. 14) A Chromatogram Showing the Four Nitrogenous Bases Distribution in Different Colors for Sample 5. (TB117)



(Fig. 15) The Phylogenetic Tree of the Sequenced *Mycobacterium tuberculosis* Strains with Code Numbers.

CHAPTER FOUR

Discussion , Conclusion and Recomendations

4.1. Discussion

A total 158 PLWHA patients were investigated for TB infection in this study. The prevalence rate found was 6/158 (3.8%). The result was similar to other studies conducted in Accra (Ghana) where 3.6% prevalence of TB among HIV sero-positive patients was reported (Essiam, 2013).

The prevalence rate of TB in this study was less than the 7.2 % rate of a study carried out in Uganda to evaluate the prevalence, incidence and mortality associated with tuberculosis in HIV-infected patients in Rural Uganda (Moore *et al.*, 2007).

However, the prevalence rate observed in this study was to a significant extent lower as compared to another study conducted in Gujarat (India) where 49.2% prevalence rate of TB was reported among HIV-positive subjects (Ghiya, *et al.*, 2009). This result reflects a very high TB prevalence rate among PLWHA in Gujarat region in India.

Our Study results also disagreed with other findings reported by Cain and his colleagues from Rural Cambodia where the TB prevalence rate was 38.0% among HIV infected patients (Cain *et al.*, 2007) and with the result (33.0%) reported by Mihir and his co-workers among HIV-sero-positives attending a counseling center in Kolkata, India (Mihir *et al.*, 2011).

The variations in results of this study results and the above-mentioned findings is multifactorial. They may be due to the different methodologies carried out in these studies; or due to the diagnostic techniques adopted; or due to the different populations studied. Most of these studies covered all forms of tuberculosis, while our present study focused on pulmonary tuberculosis only. Also the variation could be due to the difference in the general HIV prevalence

rates reported in these countries. Furthermore, the differences between the prevalence rate of this study and the other studies mentioned above may be due to differences in the selection of the inclusion and exclusion criteria. In turn, the diagnosis of TB in PLWHA patients can be problematic, depending on the degree of the immuno- suppression state at the time of diagnosis. In HIV-infected patients with good immune defenses (CD4+lymphocyte counts >300 cells/mm³), the microbiological tests afford similar performance and maximum care is necessary to ensure collection of good multiple samples.

The current prevalence rates of TB among PLWHA patients in West Darfur State (Sudan) were still low when compared with prevalence rates (8.2%) reported in Northern Tanzania (Ngowi *et al.*, 2008). This may be probably attributed to the type, time, and area of studies.

Our findings are closer to the 3.4% result reported by Sudre and his colleagues in Switzerland (Sudre *et al.*, 2002). On the other hand, our result is quite similar to prevalence rate 3.4% reported in rural areas of Maharashtra (India) among HIV-infected patients (Tamboli *et al.*, 2010).

The decrease in the prevalence rate of TB in HIV-infected patients may be due to the introduction of highly active antiretroviral therapy (HAART) which was found to have remarkable changes towards improving the management of HIV patients. We believe that, the rate of pulmonary tuberculosis could be higher if new diagnostics, particularly Xpert MTB/RIF was performed routinely for detection of *Mycobacterium tuberculosis*.

In a study conducted in Islamic Republic of Iran, the rate of pulmonary tuberculosis in HIV-infected patients was found to be 11.7 % (Mansoori *et al.*, 2002). In another study conducted in Nigeria (2013) to study the factors affecting treatment outcomes of tuberculosis in a tertiary-health center, the rate of pulmonary tuberculosis among HIV-infected patients was 20.0% (Babatunde

et al., 2013). This result was obviously higher compared to our 3.8% prevalence rate.

WHO guidelines call for culture of sputum-smear-negative HIV-infected patients especially those with a clinical suspicion of TB. However culture is more expensive to perform and require upgraded infrastructure and skilled laboratory staff (Parsons *et al.*, 2011). In our study area it was difficult to apply due to the limited resources. The clinical care and close follow-up provided for HIV infected patients in the VCT centers and clinics in our study area might have further reduced transmission and might explain the lower prevalence of TB among HIV-infected patients compared to HIV-negative patients. Concerning the high TB infectivity among the rural residences compared to the urban may be due to the poverty, malnutrition, and the lack the health care facilities more prevalent in the rural areas.

The DNA sequence results obtained in this study for detection of *M. tuberculosis* using insertion sequence IS6110 had been employed successfully by Osman and his co-workers (Osman *et al.*, 2014). The IS6110 gene was used as a target by the majority of the investigators performing PCR-based diagnosis of TB. The principal reason for using IS6110 gene is because it is considered to be a good target for application as an insertion sequence in most strains of *Mycobacterium tuberculosis* liable to confer high sensitivity (Gasmelseed *et al.*, 2014).

Concerning the 85% positivity of our study control samples collected from TB-negative HIV patients was closer to that reported by El Dawi and his colleagues in their study that aimed to assess and evaluate a PCR-amplified IS6110 insertion element in the rapid diagnosis of pulmonary tuberculosis in comparison to microscopic methods in Sudan. They reported a 88.5% sensitivity and 98.6% specificity rates for IS6110-based PCR for identification of *M.*

tuberculosis isolates in Khartoum (El Dawi *et al.*, 2004). Also ZN microscopy detects most acid fast bacilli in the sputum samples examined. These points indicate that the techniques used in this study are reliable and can be used for diagnosis and characterization of *Mycobacterium* strains.

In this study the TB infectivity rate was found higher (66.7%) among males as compared with females (33.3%). This rate is closer (66.2% among males and 33.8% in females) to the results reported by Abdallah and his co-workers in their study conducted to investigate the sero-prevalence of HIV among TB patients at Kasala Teaching Hospital (Abdallah *et al.*, 2012).

The globally low frequency of women TB infection may be due to the gender factor as a biological determinant; or as a socio-cultural determinant factor influencing access to TB examination. Some workers attribute that to social barriers and others ascribe it to the natural epidemiology of the disease (Ottmani and Uplekar, 2008). In this study area it is socially unwise for women to cough or to spit up sputum deeply on collection of sputum samples. Hence the probability of finding positive AFB in the sputum given by these women will be lower than by men.

In this study the TB prevalence among PLWHA patients was found to be higher (50%) in the age group 31-40 years and (33.3%) in the age range 41-50 years as compared to younger age groups of 21-30 years (16.7%). This age distribution is similar to the global and national frequency where the majority of TB cases are in their productive ages (SNTCP, 2011).

The official prevalence rate of TB infection in Sudan is about 209 cases per 100,000 of the population in 2009 (SNTCP, 2011). This high incidence ranks Sudan among the high prevalence countries for TB in the Eastern Mediterranean Region and accounts for 14.6% of the total TB burden (Khalid *et al.*, 2015). However, the prevalence rates of TB/HIV co-infection in West Darfur State in

the years 2013 and 2014 was 3.0 % and 4.5% respectively (W.D.S.TB Records, 2015). This frequency rate is very closer to the results of the present study. Thus this study would probably give a representative sample of tuberculosis prevalence among HIV co-infection in West Darfur State.

The 100% and 99% similarity of the genetic sequence detected in this study as regard *M. tuberculosis* H37Rv strain strongly indicate that all sample processing procedures (i.e. ZN microscopy and molecular techniques) were performed properly. Pair-wise alignment of the sequences showed that the studied TB strains were closely related to each other. In general, the 96.3–100% similarity reported in this study was also reported in the study carried by El-Eragi and his colleagues in Sudan (El-Eragi *et al.*, 2007).

The 99% and 96% similarity *M. tuberculosis* genetic sequence reported in this study among PLWHA patients were closely similar to that found among negative HIV-TB patients. The deletion of one and four nucleotides observed in the present study as compared to the reference strain nucleotide sequence is a common phenomenon. However, it has been observed that many of the differences between strains within the *M. tuberculosis* complex involve just single nucleotide, the so-called single nucleotide polymorphisms (SNP). These differences between the *M. tuberculosis* strains of this study were small on considering that there are over 4 million base pair (bp) in the genome of members of *M. tuberculosis* complex (Schaaf and Zumla, 2009).

A mutation in one nucleotide (C) was reported in some studies carried out in India (Thomas *et al.*, 2011). The genotyping results of the present study agreed with recently published work reported by Brudey and his colleagues. These authors commented that the genetic diversity of *M. tuberculosis* genomes and hence their population structure are strongly linked to geography at a fine geographical scale; thus reinforcing the importance of the localized effort to

control tuberculosis and to consider the global tuberculosis pandemic as the sum of very different and genetically separate individual outbreaks (Brudey *et al.*, 2006).

The mechanisms of the virulence of *M. tuberculosis* may vary according to the genotyping lineages of the strains which may have diversified as adaptations to human population of differing genetic constitution, as this study was aimed to characterize the predominant genotypes responsible for TB in the study area and to generate a preliminary baseline data for further epidemiological and infection control studies.

The primary branches of this study strains sequence-based phylogenetic tree were also consistent with earlier studies that classified *M. tuberculosis* into “ancient” and “modern” forms based on the presence or absence of a genomic deletion known as TbD1 (Hershberg *et al.*, 2008). This reported pattern of phylogenetic diversity may be due to the results of both a deep ecological differentiation and a more recent demographic and epidemic history. This observation may suggest the introduction of new genotypes due to casual contact and/or increased international travel. Also, these findings suggest that the evolutionary characteristics of tuberculosis bacteria could synergize with the effects of increasing globalization and human travel to enhance the global spread of drug-resistant tuberculosis.

It is of great importance to say that, should the TB/HIV co-infection rate increases, it may be anticipated that providing comprehensive HIV/AIDS care and support to HIV-positive TB patients such as antiretroviral therapy (ART) and monitoring and management co-infection cases, will be made more difficult. On top of this, health care services in remote areas of the study area suffer from a lack of infrastructure, aging equipment and a shortage of adequately trained healthcare workers, resulting in additional overburdening of

already strained health care institutions. Because the data of *Mycobacterium tuberculosis* sequencing in Sudan is very limited, a comprehensive national and state level epidemiological surveys are needed applying the combination of genomics and bioinformatics which has the potential to generate the information and knowledge that will enable the conception and development of new therapies; and interventions needed to treat this airborne disease and to elucidate the unusual biology of its etiological agent.

4.2. Conclusions

The study was concluded in the following important points:

Conventional and molecular techniques performed in this study showed that the people living with HIV/AIDS in West Darfur State are also co-infected with tuberculosis.

Molecular techniques are rapid and sensitive diagnostic tools for diagnosis of tuberculosis infection; and the distribution of the major phylogenetic families.

4.3. Recommendations

The following are the essential recommendations for the stakeholders to control the TB and HIV/AIDS.

- 1- Prompt diagnosis and appropriate treatment of TB are highly recommended to hinder HIV disease progression.
- 2- Concerted efforts are needed to adopt rapid and accurate diagnostic tools for diagnosis of active and latent TB infection.
- 3- Effective and feasible strategies should be established to optimize management of TB and HIV in the co-infected patient.
- 4- More research studies should be conducted to identify MTB strains in order to help prevent MDR cases among HIV patients.

- 5- Strengthening the links between TB services and HIV testing and HIV care services
- 6- Efforts are needed to develop new technologies to improve diagnosis and therapy, particularly Xpert MTB/RIF methodology.

REFERENCES

References

Abdallah, T.M. ,Siddig, M.F. and Ali, A.A. (2012). Seroprevalence of HIV Infection among Tuberculosis Patients in Kassala, Eastern Sudan. *Journal of AIDS and HIV Research* , 3(8) :161-163.

Babatunde, O.A., Elegbede, O.E. , Ayodele, A. Joseph O. , Ayodeji O.I. Demilade O. I., and Akinyandenu, J. (2013). Factors Affecting Treatment Outcomes of Tuberculosis in a Tertiary Health Center in Southwestern Nigeria. *International Review of Social Sciences and Humanities*. 4 (2) : 209-218.

Bhattacharya, M.K. ,Naik, T.N. ,Ghosh, M. ,Jana, S. and Dutta, P. (2011). Pulmonary Tuberculosis among HIV Seropositives Attending a Counseling Center in Kolkata, *Indian Journal of Public Health*, 55(4):145-153.

Boon, N.A. and College, N.R. (2006) . David Son's Principles and Practice of Medicine.Churchill Livingston , Elevier, London 280-291.

Brooks, G.F. ,Carroll,K.C. ,Butel, J.S., Morse, S.A. and Mietzner,T.A. (2004). Jawetz, Melink and Adleberg's Medical Microbiology.25th edition , Mc Graw Hill Press,New York.

Brudey, K. ,Driscoll, R. ,Rigouts , L. and Prodinger, M. (2006). Mycobacterium tuberculosis complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. *BMC Microbiology* 6(23) : 21-24.

Cain, K.P. ,Kanara, N. ,Laserson, K.F. ,Vannarith, C. ,Sameourn, K. ,Samnang, K. ,Qualls, M.L. ,Wells, C.D. and Varma, J.K. (2007). The Epidemiology of HIV-Associated Tuberculosis in Rural Cambodia., *Int J Tuberc Lung Dis.*, 11(9):1008-13.

Centers for Disease Control (CDC) (1999). Tuberculosis Infection Control in the era of Expanding HIV Care and Treatment , Addendum to WHO Guidelines for the Prevention of Tuberculosis in Health , Care Facilities in Resource-Limited Settings, 1999 and Prevention , p7.

Chomczynski, P. and Sacchi, N. (1987). "Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction". *Anal. Biochem.*8(11): 156–159.

Cowan, M.K. and Talaro, K.P. (2006). Microbiology a Systems Approach, Mc Graw Hill, San Francisco, p 276 -278.

El-Dawi, T.G. ,Saeed, N.S. ,Hamid M.E. (2004). Evaluation of a PCR-Amplified IS6110 Insertion Element in The Rapid Diagnosis of Pulmonary Tuberculosis in Comparison to Microscopic Methods in Sudan. *Saudi Med. J.* 25(11):1644-7.

El-Eragi, M. A. ,Hamid M.E. ,Saeed, N.S. ,Ahmed, H.A. ,Lee, K. ,Kook Y.H. and Mukhtar, M. M. (2007). Comparative assays of the rpoB gene for identification of *Mycobacterium tuberculosis* isolated from patients in Sudan. *INT J TUBERC LUNG DIS.*, 11(6):671–675.

Essiam, J.O., (2013). Clinical Outcomes and Immunohaematological Markers of People Living with HIV and AIDS with or without *Mycobacterium tuberculosis* in Selected Anti-Retroviral Clinics in the Greater Accra Region of Ghana. A thesis submitted to Kwame Nkrumah University of Science and Technology , Dep. Of Theoretical and Applied Biology to award a master degree in Science . p75-79.

Frieden, T. (2004).Tomans Tuberculosis, Cases Detection ,Treatment and Monitoring- questions and answers. Second Edition , WHO, Geneva , p 82.

Gasmelseed, N. ,Aljak M.A. , Elmadani A.E. ,Elgaili M.E. and Saeed O.K. (2014) . Assessment of Different Samples for Molecular Diagnosis of Extra-Pulmonary Tuberculosis. *International Journal for Biotechnology and Molecular*

Gerard, J.T. ,Berdell R.F. and Christine L.C. (2010). *Microbiology, An Introduction*, Tenth Edition , Benjamin Cummings , San Francisco, , P 683 – 684.

Ghiya, R. ,Naik, E. ,Casanas, B. ,Izurieta, R. and Marfatia, Y. (2009). Clinico-Epidemiological Profile of HIV/TB Co-Infected Patients in Vadodara, Gujarat. *Indian J Sex Transm. Dis.* 30 (1):10-15.

Goering, R.V. ,Zuckerman, H.M. ,Roitt, I.M. and Chiodini, P.L. (2013). *Mims Medical Microbiology*. Fifth Edition, Elsevier, London. p 211-213.

Greenwood, D. ,Slack, R.C., and Peutherer, J. F. (2002). *Medical Microbiology, A Quid to Microbial Infections, Pathogenesis, Immunity, Laboratory Diagnosis and Control*. Sixteenth Edition, Churchill Livingstone, Edinburgh.200-2013.

Harries, A. ,Maher, D. and Graham, S. (2004). *TB/HIV: A Clinical Manual* . 2nd Ed. ,World Health Organization, Geneva, 61-71.

Hershberg, R. , Lipatov, M. ,Small, P.M. ,Sheffer, H. ,Niemann, S.(2008). High Functional Diversity in *Mycobacterium Tuberculosis* Driven by Genetic Drift

and Human Demography . *PLOS BIOLOGY*, 10(13):71- 85.

Kayser, F.H. ,Bienz, K.A. ,Eckert, J. ,Zinkernagel, R.M. (2001). *Medical Microbiology* ,Thieme, New York, p 448 .

Khalid, F.A. ,Hamid, Z.A. and Mukhtar, M.M. (2015). Tuberculosis Drug Resistance Isolates from Pulmonary Tuberculosis Patients, Kassala State. *International Journal of Mycobacteriology* , 7(43): 44 –47.

Levinson, W. (2008). *Review of Medical Microbiology and Immunology*. Tenth Edition, Mc Graw Hill, New York. pp: 161.

Luna, J.C. (2004). *A Tuberculosis Guide for Specialist Physicians*. International Union against Tuberculosis and Lung Disease, Paris , France, p16-18.

Mansoori, D. ,Alaei, K. ,Alaei, A. (2002). Prevalence of Clinical Tuberculosis in HIV Infected Patients from Kermanshah Province, IRAN . *National Research Institute of Tuberculosis and Lung Disease, Iran*. 1(2): 27-33.

Michael, F. (2010). Medical Microbiology. Institute of Biomedical Science , Oxford University Press, p 214-216.

Micklos, D.A. ,Freyer, G.A. and Crotty, D.A. (2003). DNA Science - a first Course. Second Edition ,Cold Spring Harbor Laboratory Press . p192 – 195.

Ministry of Higher Education and Scientific Research (MHESR) Sudan (2006). The Strategic Plan for HIV –AIDS Control (2005-2015).p 5-8.

Moore, D. ,Liechty, C. ,Ekwaru, P. ,Were, W. ,Mwima, G. ,Solberg, P. ,Rutherford, G. and Mermin, J. (2007). Prevalence, Incidence and Mortality Associated with Tuberculosis in HIV-Infected Patients Initiating Antiretroviral Therapy in Rural Uganda. *AIDS*.14 (21):26-33.

Murray, P.R. ,Rosenthal, K.S. ,Kobayashi, G.S. and Pfaller, M.A. (2002). Medical Microbiology . 4th Edition, Mosly, London, p 580.

Ngowi, B. J. ,Mfinanga, S. G. ,Bruun, J. N. and Morkve, O. (2008). Pulmonary Tuberculosis among People Living with HIV/AIDS Attending Care and Treatment in Rural Northern Tanzania. *biomedcentral.com*,8(147):338-341.

Omer, O .E. (1990) . Review of Medical Bacteriology. Al morwah Printing Press ,Mukka,Saudi Arabia .

Osman, A.L. ,Saeed, N. S. ,Elmekki, M. A. ,Elhassan, M. M. (2014). Improved Detection of *Mycobacterium Tuberculosis* in Sudanese Children by Use of Insertion Sequence IS6110 . *Egyptian Academic Journal of Biological Sciences . Physiology & Molecular Biology.* 6 (2): 65– 70.

Ottmani, S. E. and Uplekar, M.W.(2008). Gender and TB: Pointers from Routine Records and Reports. *The International Journal of Tuberculosis and Lung Disease.* 12(7) : 825 -826 .

Parsons, L.M. ,Somoskövi, A. , Lee, C.G. ,Paramasivan, C.N. ,Abimiku, A. , Spector, S. ,Roscigno, G. and Nkengasong, J. (2011). Laboratory Diagnosis of Tuberculosis in Resource-Poor Countries: Challenges and Opportunities, *Clin. Microbiol. Rev.* 24(2):314.

Pommerville, J.C. (2004). Alcamos Fundamentals of Microbiology ,7th Edition , Janes and Bartlett Publishers ,Boston, p122-128.

Pratt, O. J. ,Grange, J.M. and Williams, V.G. (2005). Tuberculosis A foundation for Nursing and Health Care Practice. Honder Arnold Press. London. p17-22.

Prescott, L.M. ,Harley, J.P. and Klein, D.A. (2005). Microbiology. Sixth Edition, MC Graw Hill, Boston p54-56.

Richman, D.D. ,Whitley, R.J. ,Hayden, F.G.(2002). Clinical Virology .second edition ,ASM Press,Washington, 685-692.

Rieder, H. ,Yuan, C.C. ,Gie, R.P. and Enarson, D.A. (2009) .Crofton's Clinical Tuberculosis. Third Edition, International Union against Tuberculosis and Lung Disease , Paris, France p71.

Rieder, H.L. (1999). Epidemiologic Basis of Tuberculosis Control. First edition, International Union Against Tuberculosis and Lung Disease , Paris, France, p112.

Rieder, H.L. ,Deun, R.V. ,Kam, K.M. ,Kim, S.J. ,Trébucq M. C. and Urbanczik R. (2007). Priorities for Tuberculosis Bacteriology Services in Low-Income Countries. International Union Against Tuberculosis and Lung Disease, Second edition, Paris, France, p11- 22.

Schaaf, H.S. and Zumla, I. A. (2009). Tuberculosis A Comprehensive Clinical Reference. Elsevier. p 66-80.

Sharaf Eldin, G.S. ,Fadl-Elmula, I. ,Ali, M.S. ,Ali, A.B., Salih A.G., Mallard, K. ,Bottomley, C. and Mc Nerney R. (2011). Tuberculosis in Sudan: a Study of *Mycobacterium Tuberculosis* Strain Genotype and Susceptibility to Anti-Tuberculosis Drugs, *BMC Infectious Diseases* , 11:219.

Sharma, P. (2004). Tuberculosis, Current Research Trends. Allied publishers pvt. limitid, New Delhi. India. p 55-68.

Spiegel, B.P. (2004). HIV/AIDS among conflict affected and displaced populations :dispelling and taking action. Oxford press ,UK. p322-328.

Strohl, W.A. ,Rouse H. and Fisher, B.A. (2001). Lippin Cott's Illustrated Reviews Microbiology. Williams and Wilkins press ,Sydney. p 368.

Sudan National AIDS Program (SNAP). (2002).TB/HIV Background and Situation ,Sudan Federal Ministry of Health, Care and Treatment Unit, TB/HIV Section.

Sudan National TB Program(2011). Republic of Sudan, Federal Ministry, General Directorate of Primary Health Care of Health, Sudan National Tuberculosis Control Program.

Sudre, P. ,Hirschel, B. ,Toscani, L. ,Ledergerber, B. and Rieder, H.L. (2002). Risk Factors for Tuberculosis among HIV-Infected Patients in Switzerland. *European Respiratory Journal*, 1(2): 27-33.

Tamboli, S.S. ,Deshpande, S.G ,Pichare, A.P. ,Lamture, A.R. ,Kendre, P.B. ,Dharne, S.M. (2010). Prevalence of Tuberculosis in The Rural Area of Maharashtra among HIV Infected Persons. Dept. of Microbiology, MIMSR Medical College, Latur, Maharashtra, India. p32-35.

Taylor, D.J. ,Green, N.P. ,Stout, G.W. ,Soper R. (2006). Biological Science. Third edition ,Cambridge University Press, p63-67.

Thomas, S.K. ,Iravatham, C.C. ,Moni B.H. , Kumar, A. ,Bandaru V. , Majid M., Priyadarshini, Y. ,Rani P.S. ,Valluri V. ,Hasnain, S.E. ,Ahmed N. (2011). Modern and Ancestral Genotypes of Mycobacterium Tuberculosis from Andhra Pradesh. *India, Plos One*, DOI. 10 (13):69-71.

Tropp, E.B. (2008). Molecular Biology :Genes to Proteins , 3rd Edition , Jane's and Bartlett Publishers ,Boston.

W.D.S.TB Records (2014). TB Center Registration Records 2012-2013 .
Tuberculosis Control Program , Algeneina ,West Darfur State .

WHO (2009). A guide to Monitoring and Evaluation or Collaborative TB/HIV Activities. Stop TB Department and Department of HIV/AIDS, World Health Organization, United States President's Emergency Plan for Aids Relief, The Joint United Nations Program on HIV/AIDS p 14-17.

World Health Organization (WHO) (2011).Tuberculosis Prevention, Care and Control, A Practical Directory of New Advances, Stop TB Partnership, p 12-13.

APPENDIX

Appendix -1

Sudan University of Science and Technology
College of Graduate Studies and Scientific Research

Study Questionnaire

Patients Name :.....

Lab No.....

Sex: Male

Female

Age:

Occupation:.....

Education:.....

Social Status:.....

Residence:

AL Geneina Locality

Out-side AL Geneina locality

HIV Test - positive

Negative

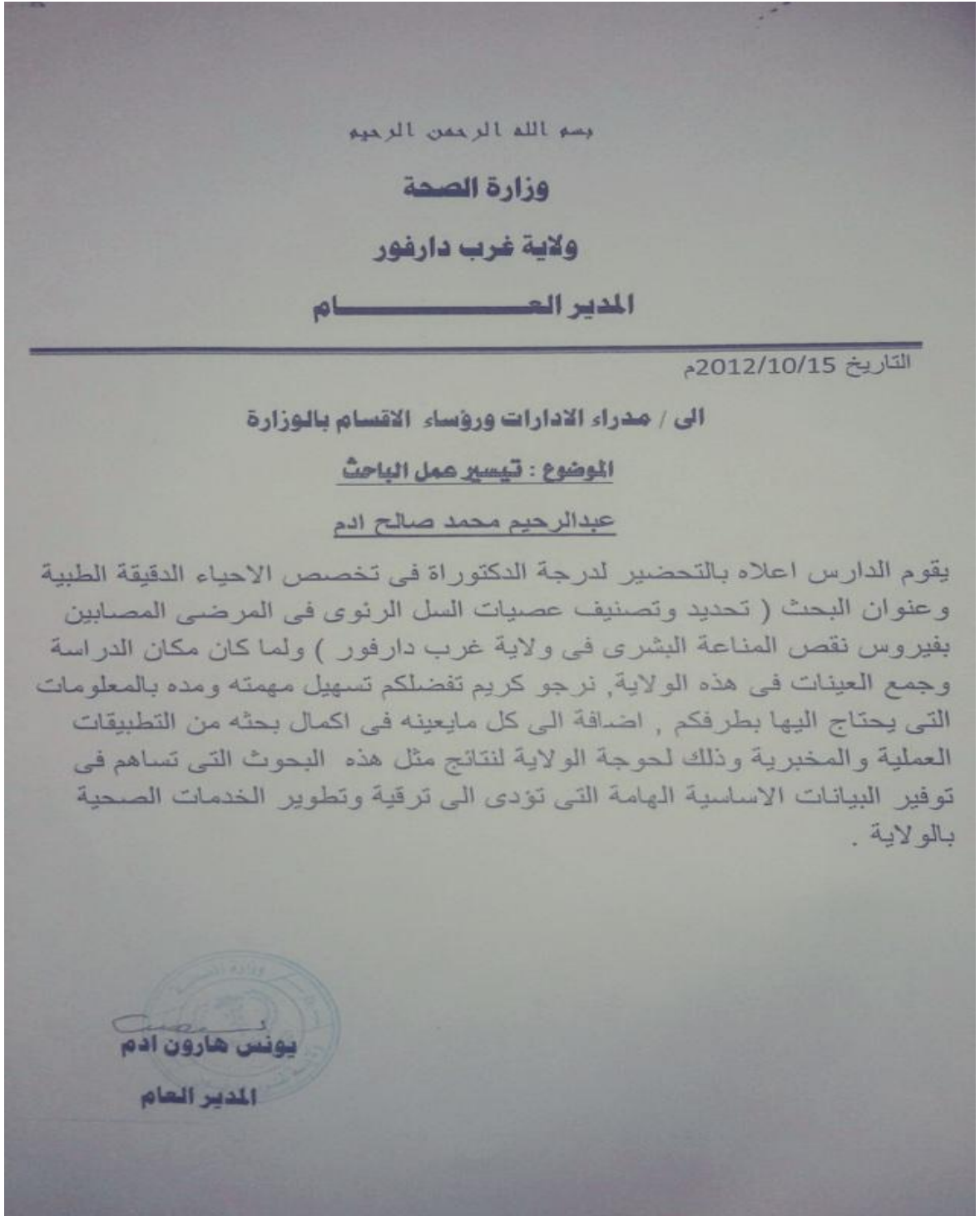
History and Clinical Features:-

No.	Question	Answer		
		Yes	No	Don't know
1	Have you ever had a BCG injection?			
2	Do you have a cough that has lasted longer than three weeks?			
3	Have you had fever, chills or night sweats?			
4	Have you ever been told you have TB?			
5	Have you ever had an abnormal chest X- Ray?			
6	Have you ever taken a TB Therapy?			

Microscopy (ZN) Result:-..... PCR Result -

أنا أقر بانني قد تبرعت لأن أكون من ضمن أفراد عينة هذه الدراسة
وقبلت بأن تجمع مني عينات التفاف وذلك خدمة للبحث العلمي .

Appendx-2



Appendix-3

MINISTRY OF HEALTH

WEST DARFUR STATE

DG . OFFICE

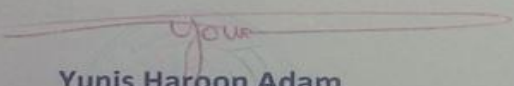
TO WHOM IT MAY CONCERN

This is to certify that :**ABDUL RAHIM MOHAMED SALIH ADAM**

Is doing a research in :**Characterization and genotyping of mycobacterium Tuberculosis in HIV infected patients in west Dar Fur State**

As a requirement of a PHD degree & this research is going to be done in WD state so please kindly provide as much as possible facilitation that can help him to complete this research as it help in building a clear & important data in west Darfur .

Our best regards


Yunis Haroon Adam

DG/S.M.O.H

Appendix-4

eurofins		Oligonucleotide Synthesis Report													
Genomics															
No.	Oligo Name	Sequence (5' -> 3')	Yield [OD]	Yield [µg]	Yield [nmol]	Concentration [pmol/µl]	Vol. for 100pmol/µl	Tm [°C]	MW [g/mol]	GC-Content	Synthesis Scale	Purification	Modification	Barcode IDO	QC Rept
10	GOTAQ 2	CCGTTGTTGCCTAAAC TCC (21)	6.8	207	32.8	-	328	57.9	6323	47.6 %	-	-	-	018163318	MALD
11	LITSRn	CTGGATCATTCCGATG (18)	6.7	196	35.8	-	358	51.4	5480	44.4 %	-	-	-	018163310	MALD
12	LE-65	TGATCCACTIATCGCAC TT (20)	6.3	181	30.0	-	300	53.2	6026	40 %	-	-	-	018163311	MALD
13	IS6110 F	CCTCCGAGCGTAGGCGT CCG (20)	5.7	169	27.3	-	273	67.5	6175	75 %	-	-	-	018163312	MALD
14	IS6110 F-2	CCTCCGAGCGTAGGCGT CCG (20)	6.3	186	30.2	-	302	67.5	6175	75 %	-	-	-	018163319	MALD
15	IS6110 R	CTCGTCCAGCGCCGCT CCG (20)	6.1	194	31.1	-	311	67.5	6045	75 %	-	-	-	018163313	MALD
16	IS6110 R	CTCGTCCAGCGCCGCT CCG (20)	5.9	188	31.1	-	311	67.5	6045	75 %	-	-	-	018163320	MALD

Eurofins Genomics is certified according to ISO 9001:2008

Customer Support: support.eu@eurofins.com | +49 8092 8208-77

Appendix-5

Maxime PCR PreMix Series

Research Use Only

ISO 9001/14001 Certified Company

Maxime PCR PreMix Kit (i-Taq)

for 20µl rxn / 50µl rxn

Cat. No. 25025(for 20µl rxn, 96 tubes) Cat. No. 25026(for 20µl rxn, 480 tubes)
 Cat. No. 25035(for 50µl rxn, 96 tubes)

DESCRIPTION

iNTRON's Maxime PCR PreMix Kit has not only various kinds of PreMix Kit according to experience purpose, but also a 2X Master mix solution. Maxime PCR PreMix Kit (i-Taq) is the product what is mixed every component: i-Taq™ DNA Polymerase, dNTP mixture, reaction buffer, and so on in one tube for 1 rxn PCR. This is the product that can get the best result with the most convenience system. The first reason is that it has every components for PCR, so we can do PCR just add a template DNA, primer set, and D.W.. The second reason is that it has Gel loading buffer to do electrophoresis, so we can do gel loading without any treatment. In addition, each batches are checked by a thorough Q.C., so its reappearance is high. It is suitable for various sample's experience by fast and simple using method.

STORAGE

Store at -20°C; under this condition, it is stable for at least a year.

CHARACTERISTICS

- High efficiency of the amplification
- Ready to use: only template and primers are needed
- Stable for over 1 year at -20 °C
- Time-saving and cost-effective

CONTENTS

• Maxime PCR PreMix (i-Taq, for 20µl rxn)	96 (480) tubes
• Maxime PCR PreMix (i-Taq, for 50µl rxn)	96 tubes

Component in	20 µl reaction	50 µl reaction
i-Taq™ DNA Polymerase(5U/µl)	2.5U	5U
dNTPs	2.5mM each	2.5mM each
Reaction Buffer(10x)	1x	1x
Gel Loading buffer	1x	1x

PROTOCOL

- Add template DNA and primers into Maxime PCR PreMix tubes (i-Taq).

Note 1 : Recommended volume of template and primer : 3µl-9µl
 Appropriate amounts of DNA template samples

 - cDNA : 0.5-10% of first RT reaction volume
 - Plasmid DNA : 10pg-100ng
 - Genomic DNA : 0.1-1ug for single copy

Note 2 : Appropriate amounts of primers

 - Primer : 5-20pmol/µl each (sense and anti-sense)
- Add distilled water into the tubes to a total volume of 20µl or 50µl . Do not calculate the dried components

Example	Total 20µl or 50µl reaction volume	
PCR reaction mixture	Add	Add
Template DNA	1 ~ 2µl	2 ~ 4µl
Primer (F : 10pmol/µl)	1µl	2 ~ 2.5µl
Primer (R : 10pmol/µl)	1µl	2 ~ 2.5µl
Distilled Water	16 ~ 17µl	44 ~ 41µl
Total reaction volume	20 µl	50 µl

Note : This example serves as a guideline for PCR amplification. Optimal reaction conditions such as amount of template DNA and amount of primer, may vary and must be individually determined.

- Dissolve the blue pellet by pipetting.

Note : If the mixture lets stand at RT for 1-2min after adding water, the pellet is easily dissolved.
- (Option) Add mineral oil.

Note : This step is unnecessary when using a thermal cycler that employs a top heating method(general methods).
- Perform PCR of samples.
- Load samples on agarose gel without adding a loading-dye buffer and perform electrophoresis.

SUGGESTED CYCLING PARAMETERS

PCR cycle	Temp.	PCR product size		
		100-500bp	500-1000bp	1Kb-5Kb
Initial denaturation	94 °C	2min	2min	2min
30-40 Cycles	Denaturation	94 °C	20sec	20sec
	Annealing	50-65 °C	10sec	10sec
	Extension	65-72 °C	20-30sec	40-50sec
Final extension	72 °C	Optional. Normally, 2-5min		

EXPERIMENTAL INFORMATION

• Comparison with different company kit

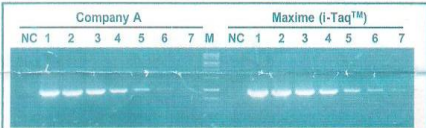


Fig.1. Comparison of Maxime PCR PreMix (i-Taq) and Company A's PreMix system by amplifying 1 Kb DNA fragment.
 After diluting the ADNA as indicates, the PCR reaction was performed with Maxime PCR PreMix (i-Taq) and company's A product.
 Lane M, Sizer-1000 DNA Marker; lane 1, undiluted ADNA; lane 2, 200 ng ADNA; lane 3, 40 ng ADNA; lane 4, 8 ng ADNA; lane 5, 1.6 ng ADNA; ; lane 6, 320 pg ADNA; lane 7, 64 pg ADNA; lane NC, Negative control

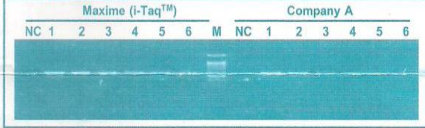


Fig.2. Comparison of Maxime PCR PreMix (i-Taq) and Company A's PreMix system by amplifying 570 bp DNA fragment (GAPDH).
 Total RNA was purified from SNU-1 using easy-BLUE™ Total RNA Extraction Kit (Cat. No. 17061). And then, the first strand of cDNA was synthesized using Power cDNA Synthesis Kit (Cat. No. 25011). After diluting the cDNA mixture as indicates, the RT-PCR reaction was performed.
 lane M, Sizer-100 DNA Marker; lane 1, undiluted cDNA; lane 2, 1/2 diluted cDNA; lane 3, 1/4 diluted cDNA; lane 4, 1/8 diluted cDNA; lane 5, 1/16 diluted cDNA; lane 6, 1/32 diluted cDNA; lane NC, Negative control

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