TUBERCULOSIS AND HUMAN IMMUNODEFICIENCY VIRUS CO-INFECTION AND CLINICAL SIGNIFICANCE OF NON-TUBERCULOUS MYCOBACTERIA IN WESTERN KENYA

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2012

Tuberculosis and human immunodeficiency virus co-infection and clinical significance of non-tuberculous mycobacteria in western Kenya

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A thesis submitted in partial fulfillment for the degree of Doctor of Philosophy in Medical Microbiology in the Jomo Kenyatta University of Agriculture and Technology

2012

DEDICATION

This thesis is dedicated to my late father Mzee Dismas Nyamogoba, my mother Mama Elizabeth Bange, my wife Jane Ayuma, my daughter Laureen Nyakerario and my son Amos Oyugi.

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ABBREVIATIONS AND ACRONYMS

ACETB   Advisory Council for the Elimination of Tuberculosis

ACP   Acyl carrier protein

AFBs   Acid-fast bacilli

AIDS   Acquired immunodeficiency syndrome

AP   Alkaline phosphatase

ART   Antiretroviral therapy

ATS   American Thoracic Society

ATS-CDC   American Thoracic Society-Centres for Disease Control and Prevention

ARVs   Antiretrovirals

BCG   Bacille-Calmette Guerin

BCG vaccine Bacille-Calmette Guerin vaccine: an attenuated strain of *Mycobacterium bovis* used to immunize against tuberculosis

BD   Becton Dickinson

CDC   Centres for Disease Control and Prevention

DEN   Denaturation solution

DLTLD   Division of Leprosy, Tuberculosis and Lung Disease

DNA   Deoxyribonucleic acid

dATP   Deoxyadenosine triphosphate

dCTP   Deoxycytidine triphosphate

dGTP   Deoxyguanosine triphosphate

dTTP   Deoxythymidine triphosphate

DOTS	Directly observed therapy short course
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EMB	Ethambutol
env A	Gene marker for identifying human immunodeficiency -1 clade A
env C	Gene marker for identifying human immunodeficiency -1 clade C
env D	Gene marker for identifying human immunodeficiency -1 clade D
HaeII	Restriction endonuclease enzyme produced by *Haemophilus aegyptius*
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HIV/AIDS	Human immunodeficiency virus/acquired immunodeficiency syndrome
HIV-O	HIV-1 outlier strains
HLA	Human leukocyte antigen
INH	Isoniazid
inhA	Gene encoding for NADH enoyl ACP reductase
IS6110	Insertion sequence 6110
IUATLD	International Union Against Tuberculosis and Lung Disease
katG	Gene encoding for the catalase peroxidase
LJ	Lowenstein-Jensen medium
MDR-TB	Multi-drug resistant tuberculosis
MgCl$_2$	Magnesium chloride

MGIT	*Mycobacterium* growth indicator tube
MIRU-VNTR	Mycobacterial interspersed repetitive units – variable numbers of tandem repeats
mM	Millimolar
MRL	Mycobacteria Reference Laboratory
MTRH	Moi Teaching and Referral Hospital
MUSOM	Moi University School of Medicine
MAC	*Mycobacterium avium* complex
MAI	*Mycobacterium avium- intracellulare* complex
N	New case of tuberculosis
NADH	Reduced nicotinamide adenine dinucleotide
NALC	N-acetyl-L-cysteine
NaOH	Sodium hydroxide
NCCLS	National Committee for Clinical Laboratory Standards
NIAID	National Institute of Allergy and Infectious Diseases
NICHD	National Institute of Child Health and Human Development
NIH	National Institute of Health
NIMH	National Institute of Mental Health
NLTP	National Leprosy and Tuberculosis Control Programme
NTM	Non-tuberculous mycobacteria
PANTA	Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim, Azlocillin antibiotic mixture

PAS	Para-aminosalycylic acid
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PNM	Primer /Nucleotide Mix
PvuII	Restriction endonuclease enzyme produced by *Proteus vulgaris*
PZA	Pyrazinamide
R	Resistant to anti-tuberculosis drug
RP	Relapse case of tuberculosis
RFLP	Restriction fragment length polymorphism
RIF	Rifampicin (rifampin)
rpoB	Gene encoding for the β-subunit of the RNA polymerase
S	Susceptible to anti-tuberculosis drug
SCC	Short course chemotherapy
SUB-C	Substrate Concentrate
TB	Tuberculosis
TF	Treatment failure
TI	Transfer in
TR	Treatment resumed
tRNA	Transfer ribonucleic acid
UNAIDS	United Nations and Acquired Immunodeficiency Syndrome
USAID	United States Agency for International Development
WHO	World Health Organization

XDR-TB   Extensively drug resistant tuberculosis

ZN stain   Ziehl Neelsen stain

DEFINITIONS OF TERMINOLOGIES

A new (N) TB case: A patient who has never been treated for TB.

A relapse (R) TB case: A smear-positive TB patient who has previously been treated and declared cured.

Bacteriophage: A virus that infects bacteria and causes the lysis of the bacterial cells.

Clade: Related organisms descended from a common ancestor (For example, isolate M of HIV-1 consists of at least ten clades).

Clinical significance of

NTM: Importance of NTM as human pathogens.

Lysogenic bacteria: Bacteria that carry prophages.

Lysogeny: The state of a bacterium that is carrying a prophage that is integrated into its chromosomal DNA.

Mycobacterioses: Diseases caused by atypical or non-tuberculous mycobacteria (NTM).

Out of control (OO)

TB case: A patient who has not attended two consecutive clinics and all efforts to motivate him to attend again have failed.

Retreatment: TB client who was treated for four (4) weeks or more in the past and who is now smear or culture positive or who has clinically been diagnosed with TB (failure, relapse,

return after default).

Recurrent TB case: TB who had been previously treated for TB and declared cured and re-notified at least 12 months from the date of the initial notification.

P-value: Level of marginal significance within a statistical hypothesis test, representing the probability of the occurrence of a given event.

Prophage: The viral DNA of a temperate phage which becomes incorporated into the host DNA.

Strain: All descendants of a pure culture; usually a succession of cultures derived from an initial colony.

Treatment failure (TF)

TB case: A patient with a positive smear at the end of 5 or 8 months despite being on anti-TB treatment.

Treatment resumed

(TR) TB case: A patient who interrupted his treatment and was declared out of control, but is now resuming treatment.

Transferred in (TI)

TB case: A patient who was initially registered in another district and has reported to continue treatment in another district.

Tuberculosis – Human

immunodeficiency virus

co-infection: Presence of active TB and HIV in a patient.

ABSTRACT

Tuberculosis and human immunodeficiency virus co-infections have a global prevalence with high morbidity and mortality rates. Non-tuberculous mycobacteria have emerged as opportunistic pathogens among human immunodeficiency virus / acquired immunodeficiency syndrome patients. Ziehl Neelsen smear microscopy initially used in the diagnosis of tuberculosis fails to differentiate between tuberculous and non-tuberculous mycobacteria. This cross-sectional study was carried out between 2007 and 2009 at one provincial, one Level 5 and eight district hospitals in western Kenya to determine the performance of Ziehl Neelsen smear microscopy and culture in the diagnosis of tuberculosis among human immunodeficiency virus / acquired immunodeficiency syndrome patients. It sought to determine the prevalence of tuberculosis and human immunodeficiency virus infection, and tuberculosis - human immunodeficiency virus co-infection rate in western Kenya. It also sought to determine the clinical significance of non-tuberculous mycobacteria in western Kenya, and the correlation between human immunodeficiency virus co-infection and resistance of *Mycobacterium tuberculosis* to first-line anti-tuberculosis drugs isoniazid, rifampicin, streptomycin and ethambutol. Sputa from 872 tuberculosis suspects underwent microscopy and culture on solid and liquid media. Isolates were identified as *Mycobacterium tuberculosis* or species of non-tuberculous mycobacteria using Hain's GenoType® Mycobacterium CM/AS Molecular Genetic Assay. Drug susceptibility testing was done using the BACTEC MGIT 960 incubator. Hain's GenoType MTBDR*plus* Molecular Genetic Assay was also used to determine resistance to

isoniazid and rifampicin. Blood samples from 695 out of the 872 tuberculosis suspects enrolled into the study were screened for human immunodeficiency virus infection using Uni-Gold[TM] rapid test and positives confirmed with enzyme linked immunosorbent assay. A questionnaire was used to collect demographic and medical history data of the tuberculosis suspects. The Ziehl Neelsen smear positivity rate was 42.7% among the human immunodeficiency virus infected cases and 57.3% among the non-infected cases. Culture positivity rate among the human immunodeficiency virus infected cases was 46.4% and 53.6% among the non-infected cases. Tuberculosis prevalence was 39.7% with disease significantly in males than females ($P < 0.05$). Tuberculosis recurrence rate was 44.8% with no significant difference between the genders ($P > 0.05$). Human immunodeficiency virus prevalence was 39.1% with infection significantly higher among females than males ($P < 0.05$). Tuberculosis - human immunodeficiency virus co-infection rate was 41.8% with no significant difference in co-infection between the genders ($P > 0.05$). Non-tuberculous mycobacterial disease prevalence was 1.72%. A total of 8 out of 12 *Mycobacterium tuberculosis* mono-drug resistant isolates were from human immunodeficiency virus infected cases. Ziehl Neelsen smear microscopy was inaccurate in the diagnosis of tuberculosis among human immunodeficiency virus infected patients compared to culture. Tuberculosis and human immunodeficiency virus / acquired immunodeficiency syndrome prevalence were high in western Kenya. Tuberculosis recurrence rate was high in western Kenya. Tuberculosis - human immunodeficiency virus co-infection rate was high in western Kenya. Non-tuberculous mycobacteria played a significant role in

causing tuberculosis-like disease which was misdiagnosed as tuberculosis. Anti-tuberculosis drug resistance was more among *Mycobacterium tuberculosis* isolates from human immunodeficiency virus infected patients suggesting a positive correlation. A more accurate diagnostic technique to augment Ziehl Neelsen smear microscopy is needed to improve tuberculosis diagnosis among human immunodeficiency virus infected patients. There is need to explore new approaches to childhood tuberculosis diagnosis in order to increase case detection rate. The high prevalence of tuberculosis and human immunodeficiency virus infection in western Kenya underscores the need for more efforts and resources to increase knowledge and access healthcare. The high tuberculosis recurrence rate observed in this study calls for studies to determine the proportions of the disease attributable to endogenous re-activation and exogenous re-infection. There is need to strengthen tuberculosis and human immunodeficiency virus / acquired immunodeficiency syndrome collaborative activities to reduce morbidity and mortality among co-infected patients. A more accurate diagnostic technique, a robust scoring system and algorithms for non-tuberculous mycobacterial disease need to be developed in order to enhance the diagnosis of the disease in Kenya. Large case-control studies are imperative to identify risk factors and determine the contribution of non-tuberculous mycobacteria to tuberculosis-like disease among human immunodeficiency virus / acquired immunodeficiency syndrome patients. Since no multi-drug resistant tuberculosis was observed in this study, continued use and surveillance of resistance trends to first-line anti-tuberculosis drugs would be prudent.

CHAPTER ONE

1.0   INTRODUCTION

1.1   Background information

With the advent of the human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) in the early 1980s, a variety of infections have emerged including opportunistic ones initially unknown. Other infections that were on the decrease are blossoming anew, with mycobacterial infections being in the lead. Tuberculosis (TB), caused by *Mycobacterium tuberculosis* complex is singled out here for its epidemiology, has been greatly altered by the HIV/AIDS epidemic (Mallory *et al*., 2000). Tuberculosis – human immunodeficiency virus co-infections have a global prevalence with devastating morbidity and massive mortality (UNAIDS, 1997; UNAIDS, 2009).

Tuberculosis (TB) is the most common opportunistic infection in HIV/AIDS patients, and is the common cause of hospitalization and death among HIV/AIDS patients (Nullis-Kapp, 2005). The HIV/AIDS and associated TB are robbing many countries of resources and capacities on which human security and development depend, dozens of resource poor countries already being in the grip of serious epidemics (UNAIDS, 2003, UNAIDS, 2009).

The HIV infection is currently the greatest risk factor for the progression of latent infection to active TB and for rapid progression of new TB infection. It is also a

potential risk for TB recurrence (Mallory *et al*., 2000). On the other hand, TB is the leading cause of morbidity and mortality in HIV/AIDS cases (UNAIDS/WHO, 2003). The current upsurge of HIV-associated TB epidemics and the resurgence of multi-drug resistant TB (MDR-TB) are adversely affecting patient care and public health globally (Poggio *et al*., 1997).

The TB-HIV/AIDS problem is compounded by the emergence of non-tuberculous mycobacteria (NTM) as opportunistic infections in the HIV/AIDS patients, and their treatment is not directly analogous to that of TB (Johnston and Ellner, 1999; Wolinsky, 1992). The situation is aggravated by emergence of multi-drug resistant TB (MDR-TB), reportedly affecting more TB-HIV/AIDS than non-HIV TB patients. It is also notable that TB and HIV interaction is complex, different strains of both *Mycobacterium* and HIV-1 are involved in TB-HIV co-infection. Both HIV provirus and *Mycobacterium* deoxyribonucleic acid (DNA) segment(s) have been detected in T-lymphocyte DNA (Lei, 1997; Lei and Xiong, 1997). However, unknown is the occurrence of HIV-*Mycobacterium* genomic interaction and the outcome of such interaction.

The *M. tuberculosis* complex, also known as tubercle bacilli are opportunistic conventional pathogens causing severe and disseminated disease among HIV/AIDS patients. Another interesting aspect of the tubercle bacilli is the isolation of L-forms from patients with destructive lung TB. These isolates have been seen to revert into

typical bacilli after 1-3 passages *in vitro* (Chernushenko *et al*., 1997). Tubercle bacilli

with such a deficiency in the cell wall are less refractory and could easily undergo

genetic phenomena such as transformation, transduction or lysogeny (Kourilsky and

Knapp, 1974).

1.2   Statement of the problem

Kenya with more than 132,000 new cases every year currently ranks 13th on the list of

22 high-burden TB countries in the world and has the fifth highest burden in Africa

(WHO, 2007; WHO, 2009). The HIV/AIDS pandemic has greatly contributed to the

increase of TB cases (WHO, 2009) and TB is a major killer of HIV/AIDS patients. The

problem is compounded by the emergence of NTM as opportunistic infections causing

TB-like syndromes among HIV/AIDS patients, which are often misdiagnosed as TB

(Wolinsky, 1992), and the continued stigma associated with the disease. Tuberculosis

treatment in many health facilities in Kenya is based on clinical diagnosis and Ziehl

Neelsen (ZN) smear microscopy for acid-fast bacilli (AFBs), which do not differentiate

TB from NTM disease (Buijtels, 2007). Some of the NTM disease cases may be

misdiagnosed as TB and their prevalence in high HIV/AIDS prevalent settings in Kenya

may be underestimated (Buijtels *et al*., 2009).

The resurgence of multi-drug resistant TB (MDR-TB) and extensively drug resistant TB

(XDR-TB) is worsening the situation. The XDR-TB and MDR-TB have been reported

to affect HIV-infected patients more compared to non-HIV TB patients (Munsiff *et al*.,

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2002). However, this observation has not been adequately explained.

1.3   Justification of the study

In the light of the worsening global TB situation due to the HIV/AIDS pandemic, periodic evaluation of TB and TB-HIV/AIDS morbidities in communities is important for effective control of the epidemics. Following the emergence of NTM as opportunistic pathogens among HIV/AIDS patients, and taking into account that TB treatment is invariably based on ZN smear microscopy which does not differentiate between TB and non-tuberculous mycobacterial disease, a study to determine the clinical significance of NTM in a given community will facilitate the management of both TB and NTM disease by clinicians. Since the increasing XDR-TB and MDR-TB are observed to more among HIV co-infected TB patients compared to non-HIV TB patients (NIH, 2007; WHO, 2009), a study to determine the association between HIV co-infection and anti-TB drug resistance could shed more light to this observation (NIH, 2007; WHO, 2009). The data generated would also provide baseline information for guided anti-mycobacterial chemotherapy in Kenya.

1.4.0   Null hypothesis

Human immunodeficiency virus infection is not associated with low ZN smear and culture sensitivity, high TB prevalence, clinical significance of NTM and anti-TB drug resistance.

1.5   Objectives

1.5.1   General objective

To establish the magnitude of TB and HIV co-infection in western Kenya and demonstrate the laboratory misdiagnosis of non-tuberculous mycobacterial disease as TB.

1.5.2   Specific objectives

1.   To determine the performance of ZN smear microscopy and culture in TB diagnosis among HIV – infected patients.

2.   To determine the prevalence of TB and HIV, and TB-HIV co-infection rate in western Kenya.

3.   To determine the clinical significance of NTM in western Kenya.

4.   To determine the correlation between HIV co-infection and anti-TB drug resistance.

1.6   Study assumptions and limitations

1. The ZN smear positive but culture negative cases were regarded and treated as TB cases. This could affect TB-HIV co-infection rate and NTM disease prevalence results.

2. The Hain's GenoType® Mycobacterium CM /AS Molecular Genetic Assay kits were able to identify only 29 species of *Mycobacterium*.

3. Some TB suspects / cases declined HIV test, which could affect TB-HIV co-infection rate result.

4. The TB-HIV co-infection was determined only once and CD4 cell counts were not done. Ideally CD4 cell counts should have been established for a defined period of time to determine the levels of immunity of the patients, which could have influenced ZN smear and culture positivity rates. However, this would have required a longitudinal study design which is expensive and outside the scope of the current study.

1.7   Ethical issues

The proposal for this study was approved by KEMRI's Scientific Steering Committee (SSC), and Ethical Review Committee (ERC) [SSC No. 837]. It was also approved by Moi University School of Medicine / Moi Teaching and Referral Hospital Institutional Research and Ethics Committee (IREC) [FAN No.00092]. Clearance was obtained from respective health authorities. Informed consent / assent were obtained from TB suspects or their guardians before they were enrolled into the study. The purpose of the study was explained to the suspects in English, Kiswahili or a local language (Turkana, Kalenjin, Luyhia, Dholuo, Ekegusii, Maasai) before consent was sought. Code numbers were used to identify TB suspects in order to maintain confidentiality (Appendix 1).

CHAPTER TWO

2.0   LITERATURE REVIEW

2.1   General introduction

Tuberculosis (TB) is an ancient disease having emerged some 15,000 to 35,000 years ago (Kapur *et al*., 1994). It has been called the "captain of all men of death" due its global prevalence with devastating morbidity and massive mortality for many centuries (Anathanarayan, 1994). The disease has re-emerged as a global public health problem, accompanied with the resurgence and transmission of multi-drug resistant (MDR) TB (Poggio *et al*., 1997). Tuberculosis kills more than 2 million people worldwide every year, more than 250,000 of these deaths being children. However, TB predominantly affects young adults in their most productive years of life and has substantial negative impact on economic development (Johnson and Ellner, 1999). The current upsurge of TB epidemics is HIV-associated and the WHO (2004) estimates that one billion people will contract TB, and 35 million will die of the disease by the year 2020.

The HIV/AIDS and associated TB are robbing many countries of resources and capacities on which human security and development depend, dozens of resource poor countries already being in the grip of serious epidemics (UNAIDS, 2003). The HIV infection is currently the greatest risk factor for the progression of latent infection to active TB and rapid progression of new infection. It is also a potential risk for TB recurrence. On the other hand TB is the leading cause of morbidity and mortality in HIV/AIDS individuals (UNAIDS/WHO, 2004).

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2.2   The biology of *Mycobacterium tuberculosis* complex

The genus *Mycobacterium* can be divided into *Mycobacterium tuberculosis* complex, *M. leprae*, and non-tuberculous mycobacteria (NTM). They comprises of non-motile rod-shaped bacteria that form cordlike groups. These bacteria produce a distinctive group of waxy substances called mycolic acids, which make standard staining procedures difficult. They stain weakly with Gram stain. However, they uniquely withstand acid-alcohol decolourization following staining with hot carbol-fuchsin, a property known as acid fast (Cheles *et al*., 2004). *Mycobacterium tuberculosis* (tubercle bacillus), an important member of this genus was discovered by Robert Koch in 1882 (Grange, 1996).

Mycobacterium tuberculosis complex is a group of closely related organisms, *M. tuberculosis*, *M. africanum*, *M. bovis*, the *M. bovis BCG* vaccine strain, *M. microti* and *M. canettii*. *Mycobacterium bovis* shares greater than 75% DNA homology with *M. tuberculosis* (Wayne *et al*., 1992). The complete genome sequence of the best-characterized strain of *M. tuberculosis*, H37Rv, has been determined and analyzed in order to improve our understanding of the biology of this slow-growing pathogen and to help the conception of new prophylactic and therapeutic interventions. The genome comprises 4,411,529 base pairs, contains around 4,000 genes, and has a very high guanine + cytosine (G + C) content that is reflected in the biased amino-acid content of the proteins (Cole *et al*., 1998).

The characteristic features of *M. tuberculosis* complex include slow growth, dormancy, complex cell envelope, intracellular pathogenesis and genetic homogeneity (Wheeler *et al*., 1994). The generation time of *M. tuberculosis* in synthetic medium or infected animals is typically 20-24 hours. This contributes to the chronic nature of the disease, imposes lengthy treatment regimens and represents a formidable obstacle for researchers. The state of dormancy in which the bacillus remains quiescent within infected tissue may reflect metabolic shutdown resulting from the action of a cell-mediated immune response that can contain but not eradicate the infection. As immunity wanes, through ageing or immune suppression, the dormant bacteria reactivate, causing disease often many decades after the initial infection (Chan *et al*., 1994). The molecular basis of dormancy and reactivation remains obscure but is expected to be genetically programmed and to involve intracellular signalling pathways (Cole *et al*., 1998).

The cell envelope of *M. tuberculosis* is unique among prokaryotes. It contains an additional layer beyond the peptidoglycan that is exceptionally rich in unusual lipids, glycolipids and polysaccharides (Brennan *et al*., 1994; Kolattukudy *et al*., 1997) (Fig. 2.1). Novel biosynthetic pathways generate cell-wall components such as mycolic acids, mycocerosic acid, phenolthiocerol, lipoarabinomannan and arabinogalactan, and several of these may contribute to mycobacterial longevity, trigger inflammatory host reactions and act in pathogenesis. Little is known about the mechanisms involved in life within the macrophage, or the extent and nature of the virulence factors produced

by the bacillus and their contribution to disease (Cole *et al*., 1998).
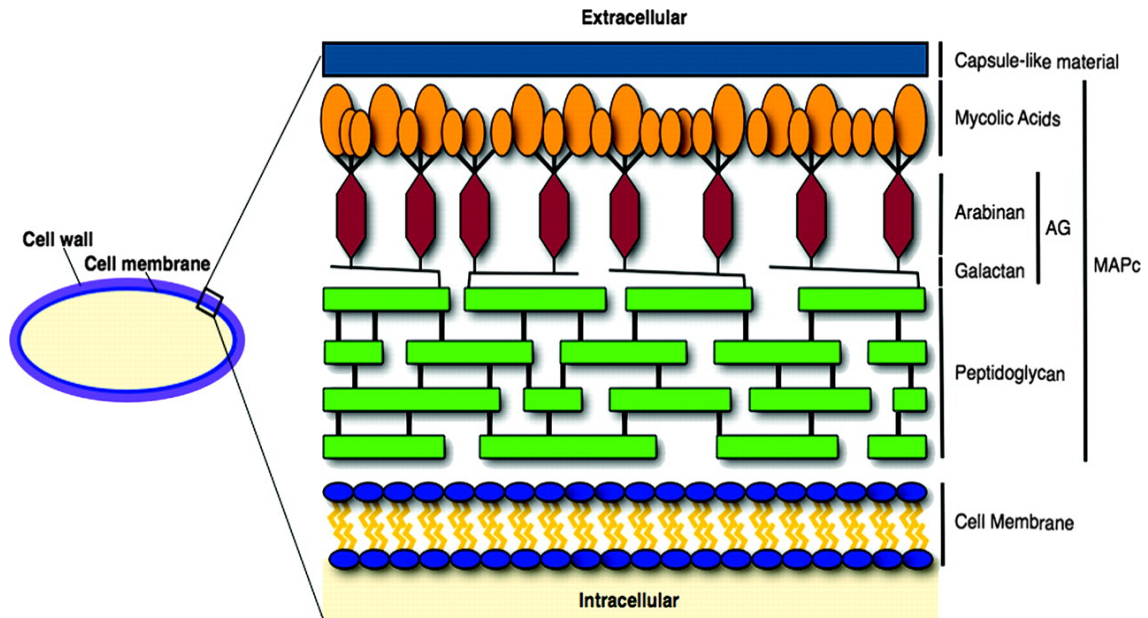
FIG. 2.1. Diagram of the basic components of the mycobacterial cell wall. MAPc, MA-AG-PG complex (Hett and Rubin, 2008).

Mycolic acids are unique high-molecular-weight (60-90 carbons) α-branched β-hydroxy fatty acids, which form a lipid shell around the cells of *Mycobacterium*. They also contain other substituted groups such as cyclopropane rings or methoxy groups (Gurr *et al*., 1991). The lipids represent over 60% of the dry weight of the cell envelope accounting for the strong hydrophobic nature of the organisms and probably for the high virulence (Glickman *et al*. 2000; Todar, 2002). It is also responsible for the relative impermeability to stains, acid fastness, and resistance to inactivation by acid and alkali

(Glickman *et al*., 2000; Gurr *et al*., 1991).

The genome of *M. tuberculosis* differs radically from other bacteria in that a very large portion of its coding capacity is devoted to the production of enzymes involved in lipogenesis and lipolysis, and to two new families of glycine-rich proteins with a repetitive structure that may represent a source of antigenic variation (Cole *et al*., 1998). Sixteen copies of the promiscuous insertion sequence IS*6110* and six copies of the more stable element IS*1081* reside within the genome of H37Rv, the prototype strain of *M. tuberculosis* (Philipp *et al*., 1996). A further 32 different insertion sequence elements have been identified, most of which have not been described previously. The 13E12 family of repetitive sequences has also been identified, which exhibit some of the characteristics of mobile genetic elements. These newly discovered insertion sequences belong mainly to the IS*3* and IS*256* families, although six of them define a new group. Most of the insertion sequences in *M. tuberculosis* H37Rv appear to have inserted in intergenic or non-coding regions, often near transfer RNA (tRNA) genes. Many are clustered, suggesting the existence of insertional hot-spots that prevent genes from being inactivated (Bardarov *et al*., 1997). The IS*6110* is widely used in restriction fragment length polymorphism (RFLP) typing of *M. tuberculosis* complex (van Embden *et al*., 1993).

At least two prophages have been detected in the genome sequence and their presence may explain why *M. tuberculosis* shows persistent low-level lysis in culture. Prophages

phiRv1 and phiRv2 are both ≃10 kb in length and are similarly organized. The site of insertion of phiRv1 is intriguing as it corresponds to part of a repetitive sequence of the 13E12 family that itself appears to have integrated into the biotin operon. Some strains of *M. tuberculosis* have been described as requiring biotin as a growth supplement, indicating either that phiRv1 has a polar effect on expression of the distal *bio* genes or that aberrant excision, leading to mutation, may occur. During the serial attenuation of *M. bovis* that led to the vaccine strain *M. bovis* BCG, the phiRv1 prophage was lost (Mahairas *et al*., 1996).

Despite intensive research efforts, there is little information about the molecular basis of mycobacterial virulence and pathogenicity (Collins, 1996). However, this situation should now change as the genome sequence will accelerate the study of pathogenesis because other bacterial factors that may contribute to virulence are becoming apparent. Before the completion of the genome sequence, only three virulence factors had been described (Collins, 1996). These are catalase-peroxidase that protects against reactive oxygen species produced by the phagocyte, the macrophage-colonizing factor gene *mce* (Arruda *et al*., 1993), and a sigma factor gene, *sigA* (aka *rpoV*), whose mutations can lead to attenuation (Collins, 1996).

2.3   Tuberculous and non-tuberculous mycobacterioses

The genus *Mycobacterium* causes more morbidity and mortality worldwide than all other bacterial infections combined. The most notorious is the *M. tuberculosis* complex

also known as tubercle bacillus, followed by the lepra bacillus, *M. leprae*, the aetiology of leprosy (Iseman, 1998). Despite the availability of an effective directly observed therapy short-course (DOTS), and the Bacille Calmette-Guérin (BCG) vaccine, the tubercle bacillus continues to claim more lives than any other single infectious agent (Snider *et al*., 1994). A recent World Health Organization report estimated that there were 8.8 million new cases of clinical TB diagnosed in the year 2005, with 1.6 million deaths directly attributable to this disease (WHO, 2007).

Mycobacterium tuberculosis complex are obligate opportunistic pathogens, causing both pulmonary and extra-pulmonary TB (Iseman, 1998). Their differentiation is necessary for the treatment of individual patients and for epidemiological purposes in areas where TB has reached epidemic proportions. It is also necessary where transmission of *M. bovis* between animals or animal products and humans is a problem. It also important to identify isolates of *M. bovis* BCG recovered from immunocompromised patients (Parsons *et al*., 2002).

Some TB-like syndromes (mycobacterioses) may be caused by non-tuberculous mycobacteria (NTM). Due to the spread of HIV, the role of NTM in mycobacterioses may be underestimated particularly in developing countries (Buijtels *et al*., 2009). The NTM are *Mycobacterium* species different from those belonging to *M. tuberculosis* complex (Dawson, 2000), most of them being environmental saprobes (Primm *et al*., 2004; Rautiala *et al*., 2004; Falkinham, 1996). However, some are opportunistic

pathogens, which may cause severe and fatal disease (Iseman, 1998). Skin test data suggest that a high proportion of people have been exposed to one or more NTM species. The predominant NTM species may vary from country to country and between different areas of a country (Wolinsky, 1981; Wolinsky, 1992; Falkinham, 1996).

Mycobacterium avium complex (MAC) also referred to as *Mycobacterium avium-intracellulare* (MAI) complex, is the most common cause of NTM disease (Buijtels *et al.*, 2009; Richter *et al.*, 1997). The MAC consists of 28 serovars of two distinct species, *M. avium*, and *M. intracellulare.* Three subspecies of *M. avium* have been proposed on the basis of phenotypic and genetic characteristics: *M. avium subsp. avium*, *M. avium subsp. paratuberculosis* and *M. avium subsp. silvaticum* (Thorel, *et al.*, 1990). The MAC is responsible for progressive and usually fatal disease if untreated, especially in immunocompromised patients (Iseman, 1998; Richter *et al.*, 2002). *Mycobacterium kansasii* is second to MAC in the causation of NTM lung disease (Iseman, 1998).

The American Thoracic Society (ATS) (2007) reports MAI complex, *M. kansasii*, *M. fortuitum* and *M. chelonae* as the most common NTM causing chronic respiratory disease, with *M. kansasii* causing chronic pulmonary disease similar to reactivation TB. The *M. kansasii* infection occurs worldwide but is most common in the USA and UK (Johnson and Ellner, 1999). In Kenya, *M. fortuitum*, *M. chelonae*, *M. szulgai*, *M. kansasii,* and *M. terrae* are among the NTM species that have been isolated from

patients who present with acute radiologically-confirmed pneumonia (Scott *et al.*, 2000).

Lately however, new NTM species have emerged as opportunistic pathogens in AIDS patients. The *M. genasense* was first isolated in 1990 from a Swiss patient, and is now being reported in other European countries, USA, and Australia. *Mycobacterium celatum*, which seems biochemically indistinguishable from *M. avium*, but shows mycolic acid patterns closely related to that of *M. xenopi*, is also being reported to cause infection (Garcia-Garrote *et al.*, 1997).

The other NTM species which have been associated with lung disease in HIV/AIDS patients include *M. malmoense*, *M. xenopi* (Pozniak, 1997), *M. abscessus*, *M. chelonae*, *M. fortuitum* (von Reyn, 1997), *M. asiaticum* (Bonard *et al.*, 1999), *M. haemophilum* (Sampaio *et al.*, 2002), *M. triviale*, *M. szulgai* and *M. smegmatis* (Saidi *et al.*, 1997). Death rates from NTM disease are high even with treatment (Pozniak, 1997). While previous studies in developed countries have reported the significant role the NTM play in the aetiology of non-tuberculous mycobacterioses in HIV/AIDS patients, their contribution to the aetiology of the same syndromes in Africa and other resource-poor settings has hardly been examined and documented (Buijtels *et al.*, 2009).

2.4   **Epidemiology of tuberculosis (tuberculous mycobacteriosis)**

Tuberculosis (TB) or tuberculous mycobacteriosis is an ancient disease re-emerging as a global public health crisis (Cantwell *et al*., 1994). While both preventable and curable, it remains one of the world's major causes of morbidity and mortality. In 1993, the WHO declared TB to be a global health emergency. One-third of the world's population (2 billion people), are infected with *M. tuberculosis* and are at risk of developing active disease. However, about 9.27 million of these proceed to develop active TB each year (WHO, 2009), up from 1.9 million cases in 1990s (Dye *et al*., 1999). Tuberculosis continues to predominantly affect young adults in their most productive years of life and the 15-44 year age-group continues to bear the highest burden (Johnson *et al*., 1999; WHO, 2009).

Vast majority (90%) of the TB morbidity and mortality occur in resource-poor settings, particularly those in Asia, Africa and South America (Sudre *et al*., 1992; WHO, 2009). This poses significant challenges to the livelihoods of individuals and developing economies as TB primarily affects people during their most productive years (WHO, 2005; WHO, 2008). India, China, Indonesia, Nigeria and South Africa rank first to fifth in terms of the total number of incident cases. Asia (the South-East Asia and Western Pacific regions) accounts for 55%, and the African Region for 31% of the global TB cases. Among the 15 countries with the highest estimated TB incidence rates, 13 are in Africa, a phenomenon linked to high rates of HIV co-infection. However, the disease has been increasing since mid 1980s in many developed countries including the USA,

by which time it was out of control across most of the poor countries, especially Central Africa and South Asia (WHO, 1994).

Tuberculosis is a problem of enormous dimension in Africa and Asia. The highest TB incidence rates (100/100,000 or higher) are observed in sub-Saharan Africa, India, China, and Southeast Asia. Intermediate rates of TB (26 - 100 cases/100,000) occur in Central and South America, Eastern Europe, and northern Africa. Low rates (less than 25 cases per 100,000 inhabitants) occur in the United States, Western Europe, Canada, Japan, and Australia (WHO, 2008).

In Kenya, about 50% of the population have latent TB infection (DLTLD, 2009). However, in the last decade the HIV/AIDS epidemic has led to the tripling of the number of new (adult) active TB cases. About 117, 000 active TB cases were reported in 2006 (DLTLD, 2007) down from about 123,000 cases in 2000 (WHO, 2002). Currently, Kenya ranks 13th on the list of 22 high-burden TB countries in the world and has the fifth highest burden in Africa. According to the WHO's Global TB Report (2009), Kenya had approximately 132,000 new TB cases and an incidence rate of 142 new sputum smear-positive cases per 100,000 population. This figure dropped to 110,065 in 2008 with an incidence of 1% in 2009 (DLTLD, 2009). However, the prevalence of TB caused by the bovine tubercle bacillus, *M. bovis*, in developing countries is largely unknown due to the complexities and prohibitive cost in differentiating between the mycobacterial species (Cosivi *et al*., 1998).

Many factors contribute to the increasing trends of TB. The emergence of HIV and large influxes of immigrants from poor countries with high prevalence of TB (Navin *et al*., 2002) and increasing homelessness are major factors in developed countries (De Cock *et al*., 1999). A high prevalence of HIV infection in a community leads to increased rates of TB reactivation as well as primary TB (De Cock *et al*., 1999; Farmer *et al*., 1997). The advent of HIV has also led to high rates of dissemination in those co-infected with *M. tuberculosis* (Iseman, 1993; Catwell *et al*., 1997). The association between poverty and TB is well established. Malnutrition, crowding and social disruption of various sorts including civil strive and natural disasters fan TB outbreaks (Farmer *et al*., 1998a; Spence *et al*., 1993).

In Kenya, the distribution of TB cases follows a pattern that is in line with poverty levels and HIV prevalence. In the year 2008, Nyanza province contributed to 20% of the TB load followed by Rift Valley province which contributed to 19%. The other provinces' contributions in decreasing order were: Nairobi (17%), Eastern (14%), Coast (10%), Central (10%), Western 8% and North Eastern (3%) (DLTLD, 2008).

2.5   The origin and biology of human immunodeficiency virus

The origin and evolutionary mechanisms of human immunodeficiency virus (HIV) remain controversial. The theories of the origin and evolutionary relationship between HIV-1, HIV2 and the simian immunodeficiency virus (SIV) are based on sero-epidemiological and viral genome molecular data. The existing data support the theory

of transmission of SIV to humans becoming HIV-2, followed by its rapid evolution to HIV-1 with an explosive escape from an isolated human population (Sharp *et al*., 1999; Wolfe *et al*., 2004).

The HIV belongs to the family *Retroviridae* with ribonucleic acid (RNA) as their genetic material. Within the retrovirus family, HIV belongs to a subgroup of lentiviruses, or "slow" viruses, which are known for having a long time period between initial infection and the beginning of clinical symptoms. The HIV is spherical in shape. The envelope (outer coat) of the virus is composed of two lipid layers of fatty molecules derived from the membrane of a human host cell when a progeny virus particle buds from the host cell. Embedded throughout the viral envelope are proteins from the host cell, as well as 72 copies (on average) of a complex HIV protein known as env. These env copies protrude or spike through the surface of the complete virus particle (virion). The env consists of a cap made of three glycoprotein 120 (gp120) molecules, and a stem consisting of three gyclycoprotein 41 (gp41) molecules that anchor the structure in the viral envelope. Much of the research to develop a vaccine to prevent HIV infection has focused on these envelope proteins (NIAID, 2009).
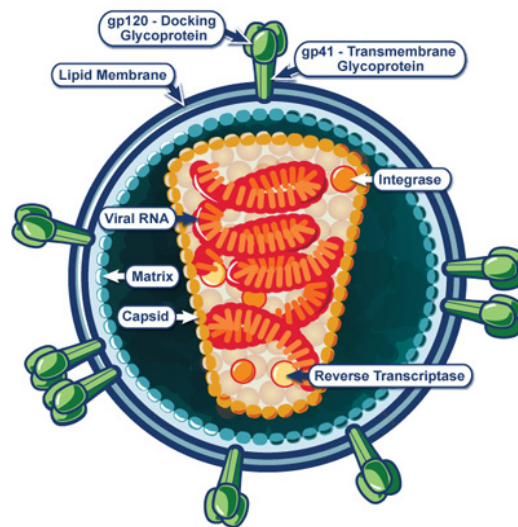
Fig. 2.3 Structure of HIV (NIAID, 2009).

Within the viral envelope is a bullet-shaped core or capsid, made up of 2,000 copies of the viral protein, p24. The capsid surrounds two single strands of HIV RNA, each of which has a complete copy of the virus's genes. The HIV has nine genes, compared to about 25,000 protein-coding genes in a human. The HIV has three structural genes (*gag, pol, and env*) that contain information needed to make structural proteins for new virus particles (virions). The *env* gene codes for gp160 protein that is broken down by a viral enzyme to form gp120 and gp41, the components of the env protein (Fig. 2.2). The HIV has six regulatory genes (*tat, rev, nef, vif, vpr, and vpu*) that contain information needed to produce proteins that control its ability to infect the host cell, produce new copies of the virus, or cause disease. The protein encoded by *nef*, for instance, apparently is necessary for the virus to replicate efficiently, and the *vpu-*encoded protein influences the release of new virus particles from infected cells (Avert,

2009; NIAID, 2009). Recent research findings indicate that vif (the protein encoded by the *vif* gene) interacts with an antiviral defense protein in host cells (APOBEC3G), causing inactivation of the antiviral effect and enhancing HIV replication. This interaction may serve as a new target for antiviral drugs (NIAID, 2009). The ends of each strand of HIV RNA contain an RNA long terminal repeat (LTR) sequence. Regions in the LTR act as switches to control production of new viruses and can be triggered by proteins from either HIV or the host cell (Avert, 2009; NIAID, 2009). The HIV's core also includes the p7 protein (nucleocapsid) (NIAID, 2009).
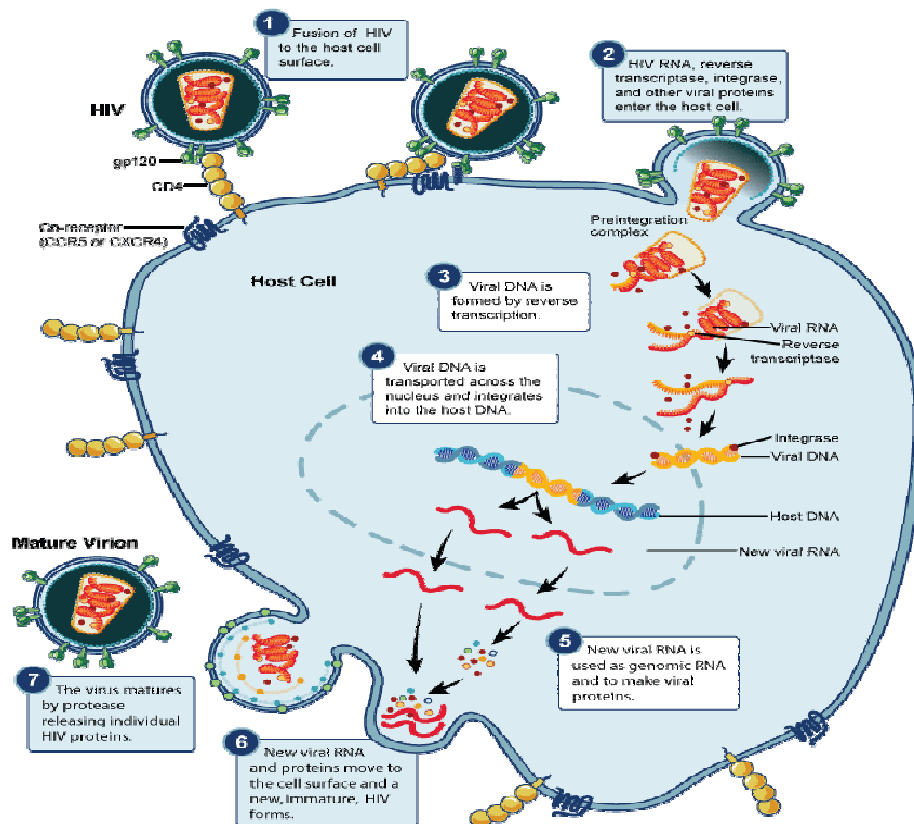
Fig.2.3 Replication cycle of HIV (NIAID, 2009).

Three enzymes carry out later steps in the virus's life cycle: reverse transcriptase (RT), integrase, and protease. The RT is involved in the conversion of single-stranded (+) viral RNA (+ssRNA) into double strand DNA (dsDNA). Integrase enzyme is responsible for integration of viral DNA into the host cell chromosome, a step that is essential for HIV replication. The enzyme protease plays a vital role in viral assembly step, by chopping up long strands of protein into smaller pieces, which are used to construct mature viral cores. Another HIV protein, the p17, or the HIV matrix protein, lies between the viral core and the viral envelope (NIAID, 2009). However, reverse transcription (RNA → DNA) in HIV lacks the proofreading capabilities of DNA replication or of normal DNA → RNA transcription. The resultant numerous errors in reverse transcription lead to mutations responsible for the frequent HIV genetic variability (Gao *et al*., 1999). Why mutations which are usually detrimental in most organisms appear to be beneficial for HIV is confounding. After infecting the host cell, HIV uses the RT enzyme to convert its RNA into deoxyribonucleic acid (DNA) and then proceeds to replicate itself using the host's biosynthetic machinery (NIAID, 2009).

2.6 Epidemiology of human immunodeficiency virus / acquired immunodeficiency syndrome and human immunodeficiency virus strain dynamics

The HIV spreads silently and unnoticed before it wreaks havoc and devastation. The HIV/AIDS kills people, tears apart families, destabilizes communities, slows economies, disrupts social services, and weakens democracies. It has a cross-generational impact, denying many children their parents' guard, guidance, protection

and love (UNAIDS, 2003). The global HIV morbidity currently stands at about 40 million people (WHO, 2009), more than 90% of them being in developing countries. Sub-Saharan Africa, the epicentre of the global pandemic has about 29 million of the HIV/AIDS cases (WHO, 2009).

Although men basically drive the pandemic, women and girls now make 60 % of those infected in Africa, with an average of 13 women infected for every 10 men. The ratios are even more lop-sided between the sexes for the 15-24 age-group, where 76 % of the infected are women. Recent population-based studies suggest that on average 36 women in this age group are living with HIV for every 10 infected young men (UNAIDS, 2004; UNAIDS/WHO, 2004).

Kenya has also been hard-hit by the HIV/AIDS pandemic, which has reduced live expectancy from 60 years in 1990 to 45.5 years in 2002. More than 2.5 million Kenyans live with HIV/AIDS, the adult prevalence currently standing at 7 % down from 13 % three years ago (WHO, 2009). About 63 % of the HIV/AIDS cases in Kenya are women (NASCOP, 2003). However, the prevalence is higher (about 20%) among girls and young women who are more vulnerable to infection (USAID, 2003). On overage 700 Kenyans die daily from HIV/AIDS and related conditions (National AIDS Control Council [NACC], 2000).

Molecular analysis of HIV-1 and HIV-2 subtypes has revealed the global epidemic to

be composed of multiple genetically distinct virus sub-epidemics (Kanki *et al.*, 1992; Kanki *et al*., 1994; Esteves *et al*., 2000). The HIV-2 prevalence is highest in the West African countries of Guinea-Bissau, Gambia, Senegal and the Cape Verde Islands (Kanki *et al*., 1992; Kanki *et al*., 1994). Genotyping of HIV-1 collected from around the world show that the prevalence of different clades in different countries are strikingly non-uniform. Clade B predominates in Western Europe and in North America; Clades A and D predominate in most of sub-Saharan Africa; Clade C predominates in Southern Africa, the horn of Africa and West Africa, and clade E/A predominates in Southeast Asia. The HIV-1 genotypes A through H have all been detected in the Equatorial African region bounded by Congo, Cameroun, Gabon and the Central African Republic. The "cosmopolitan clade B" predominates in other regions of the world including South America, Australia, Japan and China. The HIV-O (HIV-1 Outlier) strains have been detected only in Gabon, Cameroun, Equatorial Guinea and in some people who have visited these countries (Hu *et al*., 1996).

Studies on a patient from Cameroun have demonstrated co-infection with group M and a recombinant M/O virus. Recombination between strains with such distant linkage (65% overall homology) may contribute substantially to the emergence of new HIV-1 variants, with serious implications for serological and molecular diagnosis and treatment of HIV-1 infections (Peeters *et al*., 1999).

Genetic studies have also shown that HIV-1 and HIV-2 dual infection may not be a static condition. It is suspected that levels of HIV-2 may decrease with progression of disease or sequester in tissue reservoirs, or HIV-1 may effectively outgrow the HIV-2 in co-infected individuals (Sarr *et al*., 1998). In East Africa, studies show variations of HIV-1 subtypes by country, subtype A and AD recombinants being predominant in Kenya, subtype D and AD recombinants in Uganda and subtype C and AC recombinants in Tanzania (Dowling *et al*., 2002). In the coastal strip of Kenya, the HIV-1 subtype A1 is the most dominant subtype (86%) in circulation. The subtypes C, D and G constitute 5%, 8% and 1%, respectively (Khamadi *et al*., 2009). In the northern border with Ethiopia, subtypes A and C are dominant. The Ethiopian side is dominated mainly by HIV-1 subtype C, which incidentally is the dominant subtype in the border town of Moyale, an indication that cross-border movements play an important role in the circulation of the HIV-1 subtypes (Khamadi *et al*., 2005). In the Nairobi region, the HIV-1 subtypes A1/A1 (65%), A/C (9%), A1/D (7%), C/D (2%), D/D (2%), A1/A2 (2%), G/G (2%), A2/D (2%), C/C (2%), and CRF02_ AG (7%) are in circulation (Lihana *et al*., 2009). In western Kenya, the subtypes A (52.6%), D (9%), G (3.8%), C (1%), and unclassified unique recombinants were recently reported (Oyaro *et al*., 2009).

2.7   Epidemiology of tuberculosis and human immunodeficiency virus co-infection

The TB-HIV co-infections have a global prevalence with devastating morbidity and massive mortality (Raviglione *et al*., 1995; UNAIDS, 1997). Africa is the worst hit region in terms of the impact of TB-HIV where one-third of the estimated 40 million people living with HIV/AIDS are co-infected with TB (Nullis-Kapp, 2005). Among the 9.27 million new cases of TB that occurred in 2007, 1.37 million cases were HIV co-infected. Africa accounted for 79% of the TB-HIV cases, followed by South-East Asia with 11%. South Africa accounted for 31% of the TB-HIV cases in Africa. People infected with HIV are 20 or more times more likely to develop TB in a given year than HIV-negative people (WHO, 2009), fuelling an upsurge in the TB epidemic which kills an estimated 2 million people worldwide in a year (Nullis-Kapp, 2005). The resurgence (Rothe *et al*., 1996) and transmission (Menzies, 1997; Mendoza, 1997) of MDR-TB, and the emergence of non-tuberculous mycobacteria (NTM) as opportunistic infections in HIV/AIDS patients (Mendum *et al*., 2000) compound the problem. The MDR-TB has also been associated with explosive lethal outbreaks in the HIV/AIDS cases (Farmer *et al*., 1997).

Co-infection with HIV alters the microbiology, immunology and clinical outcomes of TB infection (Barnes *et al*., 1994; Wallis *et al*., 1996). Production of immune factors such as interferon-γ is reduced in HIV-1 infection which may contribute to the increased risk of TB in HIV-infected persons (Barnes *et al*., 1994). However, tumour

necrosis factor-α (TNF-α) is produced in excess in TB-HIV co-infection (Wallis *et al*., 1993). The TNF-α is expressed by macrophages on coming into contact with *M. tuberculosis* (Wallis *et al*., 1990; Wallis *et al*., 1993), and is essential for the inhibition of intracellular bacillary multiplication (Griffin *et al*., 1991; Kinter *et al*., 1990; Kindler *et al*., 1989). However, TNF- α promotes virus expression in HIV-infected human cells (Lederman *et al*., 1994), meaning that the inflammatory response triggered by *M. tuberculosis* undesirably accelerates HIV progression (Wallis and Ellner, 1994).

The HIV infection is currently the greatest risk factor for the progression of latent TB infection to active TB by a 100 fold (Gilks, 1999), rapid progression of new TB infection, and a potential risk for recurrence of TB (Daley *et al*., 1992; Villarino *et al*., 1992; Mallory *et al*., 2000). Development of active TB in HIV-infected persons appears to be associated with increased HIV replication (Lederman *et al*., 1994; Wallis *et al*., 1993) probably initiated by enhanced cytokine expression (Wallis *et al*., 1993) and is responsible for shortened survival in HIV- infected persons (Ackah *et al*., 1995; Whalen *et al*., 1995). Mortality is reported to increase up to 4-fold in HIV-infected TB patients (Nunn *et al*., 1992). The HIV infection may also alter the epidemiology of TB through transmission of the tubercle bacilli to the general population from patients who develop TB because of the HIV infection (Sutherland, 1990). In turn, TB is the leading cause of respiratory morbidity (Grant *et al*., 1998) and mortality in HIV-infected persons globally, and accounts for 44% of all AIDS-related deaths annually (WHO, 1995). More than 13 million individuals have TB-HIV/AIDS, 80% of them being in

sub-Saharan Africa, where up to 80% of hospitalised TB patients are HIV-infected (WHO, 2002). The HIV/AIDS has profound impact on the TB epidemic in Kenya also, where up to 60% of TB patients in the country are HIV-infected (DLTLD, 2007).

With the high prevalence of HIV/AIDS in the developing countries, there is the possibility that a substantial number of TB patients will be smear-negative. Similarly a number of the HIV/ADS cases will be smear-positive due to non-tuberculous mycobacteria and non-mycobacteria. Considering the relatively low sensitivity of ZN smear microscopy it is possible that a substantial number of HIV co-infected TB patients may already be missed to be initiated on treatment, a worrying situation since untreated TB from smear negatives contributes to ongoing transmission (Lienhardt *et al*., 2001; MacIntyre *et al*., 1995). The increased prevalence of extra-pulmonary forms of TB in HIV infected patients is a further challenge to the management of TB in resource-poor settings, where access to histopathology and advanced imaging tests are limited or unavailable (Mc Shane, 2005). As TB rates continue to increase in HIV endemic regions, there is merit for evaluation of the performance of diagnostic techniques as one of the TB control strategies. There also remains a need to develop more user friendly TB diagnostic techniques, which can be adapted for use in the high-burden and low-income countries (Chaudhary *et al*., 2010). There is need for large studies to identify selected clinical and laboratory parameters which could be used to identify individuals with TB who are likely to be co-infected with HIV (Lawson *et al*., 2008).

2.8   Sources and transmission of tuberculosis

Mycobacterium tuberculosis does not multiply outside the animal (human, bovine) body. Patients with active pulmonary TB (PTB) are the major reservoir of the spread of the tubercle bacilli, primarily by inhalation of aerosolised infectious droplet nuclei. Lungs are the main portals of entry of TB infection (Bermudez *et al.*, 1996). The droplet nuclei are generated during coughing, sneezing, talking and heavy breathing by persons with active PTB. Aerosols are fine sprays producing droplets that remain suspended in the air for some time (Pelczar *et al.*, 1986). Small aerosol droplets evaporate leaving droplet nuclei of the tubercle bacilli. Large aerosol droplets settle out rapidly from the air on various surfaces where they dry. Disturbance of this dried material by processes such as bed making or sweeping a floor in the patients' room can generate dust particles that add bacilli to the articulating air (Pelczar *et al.*, 1986; Deodhare *et al.*, 1994).

Direct transmission by heavily infected body secretions from fistulas and skin lesions occur infrequently (De Cock *et al.*, 1999). Infection through the pharyngeal mucosa has been reported where the bacilli are carried to the lymph nodes (Deodhare, 1994). Vertical transmission may occasionally occur when the bacilli are carried from a tuberculous mother to a foetus *in utero* across the placenta. In this case the lesion is situated in the liver of the foetus (Deodhare, 1994). The bacilli may also be directly inoculated on an abrasion or minor cut in the skin in which case non-progressive TB will result (Deodhare, 1994).

Infection can also occur through ingestion of the tubercle bacilli in raw milk and milk products from infected animals mainly cattle (De Cock *et al.*, 1999). This usually involves *M. bovis*, commonly affecting children and young people (Cosivi *et al.*, 1998). However, *M. bovis* is widely distributed and its zoonotic importance is a serious public health problem. This is particularly in areas badly affected by the HIV pandemic (Raviglone *et al.*, 1995) and where effective controls through pasteurisation and the slaughter of infected animals are not applied. Transmission occurs between animals, from animals to humans and vice versa, and rarely between humans (Cosivi *et al.*, 1998).

Crowded housing leads to increased transmission of TB due to prolonged exposure and decreased air exchange which would dilute and remove infectious nuclei. Most transmission occurs in household contacts of an infectious smear-positive case (Johnson *et al.*, 1999). Transmission of TB in institutions that concentrate persons with HIV infection such as prisons, hospital, schools and army barracks has been one of the serious public health problems in industrialized countries in the HIV/AIDS era (Perri *et al.*, 1989; Frieden *et al.*, 1995; Frieden *et al.*, 1996). Studies in Africa also indicate frequent nosocomial transmission (Harries *et al.*, 1997), healthcare workers are highly at risk, and TB is prevalent in prisons (Nyangulu *et al.*, 1997).

2.9   Virulence factors and pathogenesis of tuberculosis

Mycobacterium tuberculosis is not known to produce any toxin. Virulence is associated with the cell wall lipid components. The mycolic acid around the cells is a major determinant of virulence for the bacterium (Todar, 2002; Gurr *et al*., 1991). It is also responsible for their resistance to the bactericidal action of antibodies and complement proteins (Glickman *et al*., 2000; Gurr *et al*., 1991). Resistance to the action of antibodies and complement proteins is probably by preventing attack of the mycobacteria by cationic proteins, lysozyme and oxygen radicals in the phagocytic granule. They (cell wall lipid components) also protect extracellular mycobacteria from complement deposition in serum (Glickman *et al*., 2000).

The cord factor (tetrahalose dimycolate), consisting of two glycolipids is the major virulence factor that is toxic to macrophages. The ability of the mycobacteria to survive intracellularly in macrophages is due to the cord factor and probably due to the sulfatides. Sulfatides are sulfur-containing lipids, which promote intracellular survival of the bacilli by evading the bactericidal activity of macrophages through inhibition of the fusion of lytic enzyme-containing lysosome and bacilli-containing phagosome. The catalase-peroxidase enzyme has been reported to protect the mycobacteria against reactive oxygen species such as hydrogen peroxide produced by the phagocyte (Arruda *et al*., 1993). Other factors thought to play a role in the virulence of *M. tuberculosis* include the ability to acquire iron from the host and the presence of oxygen. The high oxygen tension in lung tissue may account for the predilection of the organism to this

site (Silva *et al*., 1985).

In the pathogenesis of TB infection, once the TB bacilli lodge in the lung tissue of an immunocompetent host, a cell-mediated immunity (CMI) is induced. T-lymphocytes migrate towards the bacilli, and upon contact liberate lymphokines, which attract macrophages to the area and cause them to accumulate. Eventually, a small tubercle forms consisting of the bacilli, several concentric layers of macrophages and an outer mantle of lymphocytes. This halts further advance of the infection and the tubercle becomes dormant (Wallis *et al*., 1994). However, small numbers of viable bacilli may survive in these foci for decades in a dormant state. Breakdown of these foci in the setting of waning immunity due to factors such as HIV/AIDS and immunosuppressive medications can result in reactivation of infection (Jones *et al*., 1993).

2.10   Tuberculosis disease phenotypes

It is generally accepted that 90% of infected immunocompetent subjects will never develop TB. One-in-two people of the remaining 10% will progress to disease during the first two years after infection (Frieden *et al*., 2003). *Mycobacterium tuberculosis* is a highly virulent pathogen and produces disease in immunocompromised individuals and throughout the course if HIV infection (Davis *et al*., 1993).

Tuberculosis has many manifestations, affecting bone, the central nervous system (CNS), and many other organ systems, but it is primarily a pulmonary disease that is

initiated by the deposition of tubercle bacilli contained in aerosol droplets, onto lung alveolar surfaces (Smith, 2003). Consequently, infection may lead to pulmonary or extra-pulmonary TB. The pulmonary TB (PTB) may be smear-positive PTB or smear-negative PTB (IUATLD, 1996). Individuals who do not develop effective cell mediated immune (CMI) responses following primary infection may develop progressive primary TB. This form of disease is most common in young children, the immunocompromised, and the elderly. Miliary or meningeal TB may result after wide spread haematogenous dissemination of the tubercle bacilli (Proudfoot *et al.*, 1969). Low numbers of dormant, slowly metabolising tubercle bacilli persist in small, walled-off fibrocaseous lesions in the lung and other organs seeded during initial *M. tuberculosis* bacteraemia. Such foci may breakdown years later in the event of waning CMI to produce active local or disseminated TB (Johnson *et al.*, 1999). Children usually acquire TB infection from repetitive close contact with sputum smear-positive adult living in the same household. The younger the child, the more is the risk of infection (Johnson *et al.*, 1999).

Mildly immunocompromised patients may present with typical reactivation TB. However, severely immunocompromised patients are at high risk of developing progressive primary TB if recently infected, and for reactivation of latent TB (Davis *et al.*, 1993; Johnson *et al.*, 1999). In early stages of HIV infection, the immunity is less impaired and disease features are consistent with typical reactivation TB (Mukadi *et al.*, 1993). However, as HIV infection progresses, features of TB becomes more atypical, and in severely immunocompromised HIV-infected individuals TB is characterized by

increased disseminated and extra-pulmonary forms (Small *et al*., 1991).

The common forms of extra-pulmonary TB (EPTB) include tuberculous lymphadenitis (Lau *et al*., 1991), tuberculous pleurisy (Wallgren, 1948), TB of bone and joints, intestinal or abdominal TB (Johnson *et al*., 1999), TB meningitis (Ramachandran *et al*., 1986), genitourinary TB (Simon *et al*., 1977) and tuberculous pericarditis (Johnson *et al*., 1999). Most EPTB cases represent reactivation TB. However, some forms of EPTB such as tuberculous pleurisy and phlyctenular conjunctivitis represent hypersensitivity reactions to mycobacterial proteins, and occur following primary infection. In developing countries, EPTB frequently occurs in children and young adults. Conversely, in developed nations EPTB is now more frequent in the elderly probably due to reactivation of dormant TB following weakening immunity due to aging. Some forms of EPTB such as tuberculous meningitis are increased among HIV-infected individuals (Johnson and Ellner, 1999).

Tuberculous pleurisy results from direct extension from lungs, haematogenous seeding or rupture of a sub-pleural caseous focus into the pleural space (Wallgren, 1948). Tuberculous lymphadenitis is the most common EPTB manifestation of TB in developing countries with *M. tuberculosis* as the major cause. However, in developed nations with low TB prevalence, atypical mycobacteria including *M. avium-intracellulare* (MAI) complex, *M. scrofulaceum* are frequent isolates from paediatric cases (Wolinsky, 1995). Tuberculous lymphadenitis may also be a complication of

primary infection, reactivation of contiguous spread, and is more common in young children and women (Lau *et al*., 1991).

Bone and joint (skeletal) TB affects mainly large weight-bearing joints (Gorse *et al*., 1983). Most skeletal TB in infants and children is associated with progressive primary TB. However, in older children and adults, it usually results from reactivation of a previously haematogenously seeded focus (Berney *et al*., 1972) or by haematogenous seeding after breakdown of a latent focus in the kidney, lymph nodes or other organs (Gorse *et al*., 1983).

The term miliary TB is used to describe disseminated TB resulting from haematogenous spread associated with characteristic lesions on chest radiography. Once the bacteria reach the left side of the heart and enter the systemic circulation, the result may be to seed organs such as the liver and spleen with the said infection. Alternately the bacteria may enter the lymph node(s), drain into a systemic vein and eventually reach the right side of the heart. From the right side of the heart, the bacteria may seed or re-seed the lungs, causing the eponymous "miliary" appearance (Kumar *et al*., 2007). Miliary lesions are diffuse 1-2 mm rounded opacities scattered throughout the lung fields. These lesions are named for their resemblance to millet seeds (Johnson *et al*., 1999).

Tuberculous meningitis involves the central nervous system (CNS). It may lead to tuberculomas and tuberculous spondylitis. However, the most common form of CNS

TB is tuberculous meningitis, the life threatening EPTB. It is still the leading cause of mortality and chronic disability in infants and children with TB (Ramachandran *et al*., 1986). Infants and children develop tuberculous meningitis in the setting of progressive primary infection or miliary TB. In developed nations, tuberculous meningitis is frequently seen in adults and the elderly in association with reactivation or late generalised TB. The HIV pandemic has also been associated with increased cases of CNS TB (Small *et al*., 1991).

Genitourinary TB is a disease of young and middle aged adults. Most cases of renal TB arise from secondary haematogenous spread of the tubercle bacilli to the renal cortex from pulmonary lesions either at the time of initial TB infection or due to the breakdown of old caseous focus. Any portion of the urinary tract can be involved secondarily by antitrade infection from the infected urine (Simon *et al*., 1977).

Tuberculous pericarditis results from direct involvement from adjacent pulmonary lesions, from haematogenous seeding and most frequently by spread from involved mediastinal lymph nodes (Johnson *et al*., 1999). Tuberculous pericarditis is the most common pericardial disease in sub-Saharan Africa and may present as pericardial effusion, constrictive pericarditis, or effusive-constrictive pericarditis (Mayosi *et al*., 2005; Reuter *et al*., 2005a). However, pericardial effusion is the most common clinical manifestation (Johnson *et al*., 1999). Tuberculosis accounts for less than 5% of cases of pericardial disease in the developed world, yet it is the cause of 50-70% of cases in

sub-Saharan Africa (Reuter *et al*., 2005a; Reuter *et al*., 2005b; Cegielski *et al*., 1994). The prevalence of tuberculous pericarditis in HIV-infected individuals with pericardial disease is even higher, with TB accounting for 96-100% of cases in this group (Reuter *et al*., 2005b; Cegielski *et al*., 1994). Interestingly, the presence of HIV infection has not been found to significantly alter the clinical, radiologic, echocardiographic, or diagnostic characteristics of tuberculous pericarditis (Reuter *et al*., 2005a; Cegielski *et al*., 1994).

Gastro-intestinal TB (GITB) is a major health problem in many underdeveloped countries. However, a recent significant increase has occurred in developed countries, especially in association with HIV infection. Tuberculosis can also involve the gastrointestinal tract at all levels, although the ileum and colon are common sites (Anand *et al*., 2007; Johnson *et al*., 1999). The routes of GIT infection include ingestion of infected sputum in patients with active pulmonary TB and especially in patients with pulmonary cavitation and positive sputum smears, haematogenous spread from tuberculous focus in the lung to sub-mucosal lymph nodes. It also includes local spread from surrounding organs involved by primary tuberculous infection, for instance renal TB causing fistulas into the duodenum or mediastinal TB lymphadenopathy involving the esophagus (Anand *et al*., 2007). The GITB may also result from drinking of raw milk from infected animals, usually involving *M. bovis*. *Mycobacterium tuberculosis* and atypical mycobacteria frequently involve abdominal lymph nodes and viscera in patients with advanced AIDS (Johnson and Ellner, 1999).

2.11 Anti-tuberculosis drug resistance and risk factors

The increasing global spread of multi-drug resistant TB (MDR-TB) and extensively drug resistant TB (XD-RTB) due to emergence of multi-drug resistant strains of *M. tuberculosis* complex is a major health threat, which is negating TB control efforts (Schwoebel *et al.,* 2000; WHO, 1997; Park *et al*., 1996). Multi-drug resistant TB (MDR-TB) refers to infection with *M. tuberculosis* strains resistant to at least isoniazid and rifampicin. Multi-drug resistance has been clearly identified as the most severe, with a strong impact on morbidity, mortality and long duration treatment at much higher costs (Schwoebel *et al.,* 2000). Some parts of the world including many countries in Africa, Latin America and the Caribbean, South-East Asia and former Soviet Union states, have already reported MDR-TB rates of up to 50% (Davies, 2003).

The WHO (2009) estimates that there are half a million new cases of MDR-TB, which is about 5% of the total 9 million new TB cases worldwide each year. In surveys done in 2002-2006, the highest rates have been recorded in Azerbaijan, where nearly a quarter of all new TB cases (22.3%) were reported as MDR, followed by Moldova with 19.4%. These proportions were 16% in Ukraine, 15% in the Russian Federation and 14.8% in Uzbekistan. Surveys in China also suggest that MDR-TB is widespread in that country. About 8.32% of pulmonary TB patients in China suffer from MDR-TB and 0.68% from XDR-TB. It is estimated that there are 120,000 new cases of MDR-PTB in China each year (WHO, 2009). However, in settings in which only susceptible TB is effectively treated while MDR-TB is not well tackled, case-load with the latter

strains will increase. Untreated MDR-TB may create a series of "fast" sub-epidemic MDR-TB outbreaks in susceptible populations involving households, factories, schools, prisons, military barracks, clinics and hospitals (Farmer *et al*., 1998a).

Extensively drug resistant TB (XDR-TB), defined as MDR-TB with additional resistance to fluoroquinolones and at least one of the three second-line injectable drugs, capreomycin, kanamycin and amikacin, has recently been identified in many countries including South Africa, USA and former Soviet countries. At least 17 countries have reported XDR-TB cases among which mortality is extremely high. The XDR-TB leaves patients virtually untreatable using currently available anti-TB drugs. Majority of XDR-TB cases are HIV co-infected. However, the extent and amount of XDR-TB is unknown (NICHD, 2007; NIH, 2007; NIMH, 2007; WHO, 2009). This is because few countries are equipped at present to diagnose it, limiting the availability of data (WHO, 2009).

The emergence of resistance to anti-TB drugs may be due to either factors related to patient non-compliance (Frieden *et al*., 1993) or the transmission of already resistant strains (Shafer *et al*., 1995). Defaulting from treatment in developing countries is largely as a result of patient's lack of motivation, the impression of being completely cured in the course of chemotherapy, drug side effects, poverty associated problems and socio-psychological factors (Ramachandran *et al*., 1992). Development and transmission of MDR-TB is fanned by many factors, which include HIV infection

(Agerton *et al*., 1997; Poggio *et al*., 1997), inappropriate chemotherapy such as failure to detect resistance to anti-TB drugs, extensive prophylactic use of the anti-TB drugs on TB infected HIV/AIDS patients, non-compliance with chemotherapy by TB patients (Frieden *et al*., 1993; Pfyffer, 2000; Youmans *et al*., 1946) and increasing poverty and its associated factors such as overcrowding and malnutrition (Ramachandran *et al*., 1992).

The MDR-TB of today is to a large extent the result of the yesterday's ineffective TB programmes and practices. Unwise therapeutic decisions in TB treatment have been common, such as the addition of one agent to a failing regimen. However, MDR-TB is not necessarily an incurable disease. It is a failure to treat, and not treatment failure, that accounts for the vast majority of MDR-TB deaths. Elsewhere, there is evidence that with appropriate treatment of young patients without HIV infection, cure rates of even highly resistant TB are appreciably high (Telzak *et al*., 1994).

There are delays in the diagnosis of MDR-TB, running into months or even years (Tenover *et al*., 1993). In some developing countries, persistently smear-positive patients simply receive multiple re-treatment regimens until they die. Rigidly algorithmic approaches commonly lead into some patients faithfully adhering to first-line regimens even though they are contacts of known MDR-TB patients, and they remain smear-positive throughout multiple DOTS. Enrolment of patients with primary resistance to isoniazid (INH) and rifampicin (RIF) into standard algorithms such as

DOTS may make the organisms resistant to ethambutol (EMB) and pyrazinamide (PZA) as well. Since empiric re-treatment is often based on the same drugs plus a short course of streptomycin, the organisms initially resistant to INH and RIF may become resistant to as many as five anti-mycobacterials. This amplifier effect of DOTS has been documented to contribute to MDR-TB outbreaks (Farmer *et al*., 1998a).

In the earlier 1990s, the WHO (1997b) warned: "Once the MDR-TB is unleashed, we may never be able to stop it". However, the MDR-TB has already been unleashed, and the only effective means to interrupt its transmission is prompt identification and effective therapy of the MDR-TB cases (Farmer *et al*., 1998b). The DOTS-plus, a new strategy that targets for treatment of all active TB cases, including those with MDR-TB is aimed to achieve this (Farmer *et al*., 1997). However, the DOTS-Plus is not intended as a universal strategy, and is not required in all settings. It should be implemented in selected areas with moderate to high levels of MDR-TB in order to combat an emerging epidemic (WHO, 2006).

2.12   Multi-drug resistant tuberculosis and human immunodeficiency virus infection

The accompaniment of the resurgence of TB by alarming outbreaks of MDR-TB mainly among HIV-infected patients is worrying. Studies have shown a link between HIV infection and MDR-TB (Poggio *et al*., 1997; WHO, 2009). For instance, MDR-TB strain named 'M' has been reported to cause a nosocomial outbreak in HIV/AIDS cases

in Buenos Aires, Argentina, with the infection spreading to nearby health centres (Poggio *et al*., 1997). In the USA, the highly resistant strain 'W' has been responsible for several nosocomial outbreaks in New York, with mortality rates above 80%, majority (73%) being HIV/AIDS cases (Agerton *et al*., 1997). In this same city, MDR-TB was diagnosed in 241 patients in 1995 through 1997, majority (90%) having no prior treatment history. More MDR-TB patients (53.1%) had HIV infection compared with non-MDR-TB patients (31.2%) (Munsiff *et al*., 2002). In Russia, MDR-TB due to the Beijing strain family is prevalent in prisons a significant number of the affected being HIV-infected (Drobniewski *et al*., 2002). The Beijing family of isolates was first reported in Beijing area, China, in 1995. The highly drug-resistant W strain, first identified in New York, USA, is a member of the Beijing family (Agerton *et al*., 1999). Surveys in Latvia and Ukraine indicate the level of MDR-TB among TB patients living with HIV to be almost twice compared with TB patients without HIV (WHO, 2009).

Munsiff *et al*. (2002) suggested three possible reasons for the observed greater prevalence of HIV infection in MDR-TB patients compared to non-MDR-TB patients. They are of the opinion that the initial outbreaks during which these strains were transmitted, mostly involved HIV-infected persons; a large number of HIV infected patients were likely infected in those outbreaks; and HIV infected patients progress from infection to TB disease at a much higher rate than non-HIV persons. However, studies by Brindle *et al*. (1993) indicate that HIV infection does not affect the microbiological response to anti-tuberculous therapy.

2.13 The biochemical basis and the determination of anti-mycobacterial drug resistance

Multi-drug resistant (MDR) strains of *Mycobacterium tuberculosis* complex have emerged worldwide, constituting a serious threat to the efficacy of TB control programmes. Understanding of the drug targets and molecular basis of resistance to the anti-TB drugs including isoniazid, rifampin, streptomycin, ethambutol, and pyrazinamide is important in gaining control of this epidemic (Somoskovi *et al.*, 2001). Isoniazid, ethambutol, pyrazinamide and ethionamide interfere with enzymes involved in cell wall synthesis. Streptomycin and other aminoglycosides interfere with protein synthesis. Rifampicin interferes with transcription while quinolones interfere with DNA replication (Pfyffer, 2000; Somoskovi *et al.*, 2001). Resistance of *M. tuberculosis* complex to anti-TB drugs is the result of spontaneous genetic event (mutations) that occur at a frequency of approximately 10^{-5} to 10^{-8}. Resistance in *M. tuberculosis* complex is always a result of mutation exclusively confined to chromosomal DNA, without involvement of mobile genetic elements such as plasmids seen in other bacteria and mycobacteria (Pfyffer, 2000).

Resistance to rifampicin (rifampin) is associated with a short core region consisting 27 amino acids in the *rpoB* gene, which codes for the β-sub unit of RNA polymerase (Telenti *et al.*, 1993), the enzyme involved in RNA transcription (Cole and Telenti, 1995; Ramaswamy and Musser, 1998; Pfyffer, 1999). More than 97% rifampin-resistant *M. tuberculosis* isolates carry a mutation in this core region, where a total 15

distinct mutations have been identified. The ethambutol resistance-determining region (ERDR) has been proposed as a mutational hot spot in the *embB* gene (Telenti *et al*., 1997; Alcaide *et al*., 1997). The *embB* gene codes for arabinosyl transferase involved in arabinan polymerization. Mutation in the *gyrA* gene coding for DNA gyrase sub-unit involved in DNA replication is responsible for resistance to fluoroquinolones (Cole and Telenti, 1995; Ramaswamy and Musser, 1998; Pfyffer, 1999). The situation with pyrazinamide is however, not clear (Scorpio and Zhang, 1996).

However, drug resistance in mycobacteria is often associated with more than a single gene mutation. Resistance to isoniazid appears to be the complex result of single or multiple mutations (Cole and Telenti, 1995; Ramaswamy and Musser, 1998; Pfyffer, 1999) in the *katG*, *inhA*, *oxyR-ahpC* and/or *kasA* gene(s). The *katG* gene codes for catalase-peroxidase which protects the organisms from reactive oxygen species, whereas the *inhA* gene codes for enoyl-acyl carrier protein involved in mycolic acid biosynthesis. The *kasA* gene codes for β-ketoacyl acyl carrier protein synthase involved in mycolic acid biosynthesis. The *oxyR-ahpC* gene codes for alkyl hydroperoxide reductase whose function in the mycobacteria is yet to be established (Ramaswamy and Musser, 1998).

Pyrazinamide, a nicotinamide analog that is believed to target an enzyme involved in fatty-acid synthesis (Zimhony *et al*., 2000), is a pro-drug that is converted to its active form (pyrazinoic acid [POA]) by the mycobacterial enzyme pyrazinamidase. It has been

observed that the pyrazinamide-resistant *M tuberculosis* isolates usually lose their pyrazinamidase activity (Konno *et al.*, 1967). After cloning and sequencing the gene that encodes pyrazinamidase (*pncA*), it was found that 72–97% of all pyrazinamide-resistant clinical isolates tested carry a mutation in the structural gene or in the putative promoter region of the gene (Zhang and Telenti, 2000; Scorpio and Zhang, 1996). However, the involvement of other mechanisms (those that involve pyrazinamide uptake, *pncA* regulation, or POA efflux) is indicated by the existence of isolates that exhibit a high level of pyrazinamide resistance without mutations in the *pncA* gene (Raynaud *et al.*, 1999). However, *M bovis* and *M bovis* BCG, are naturally resistant to pyrazinamide, and pyrazinamide resistance in them is due to a unique C to G point mutation in codon 169 of *pncA*. In contrast, mutations in pyrazinamide-resistant *M tuberculosis* have been found scattered throughout *pncA* (Scorpio and Zhang, 1996).

Mutations in the *rpsL* and/or *rrs* gene(s) correlate with resistance in about 80% streptomycin resistant *M. tuberculosis* strains (Pfyffer, 2000). The *rpsL* gene codes for the ribosomal protein S12 involved in translation, whereas the *rrs*gene codes for the 16S rRNA involved in translation.

2.14 Prevention and control of tuberculosis and non-tuberculous mycobacterioses

The HIV/AIDS is dramatically fuelling the spread of TB, and TB is a major killer among people living with HIV/AIDS. For instance, MDR-TB and XDR-TB are highly

lethal in people living with HIV/AIDS, with some studies showing case fatality rates of over 90% in sub-Saharan Africa. Drug-resistant TB is therefore a major threat to the effectiveness of both TB treatment and anti-retroviral treatment programmes (WHO, 2009). Consequently, the control of HIV is key for TB control as well. The realization that it will be impossible to curb the spread of TB without tackling HIV/AIDS and vice versa has led to the upsurge of joint research, surveillance and treatment initiatives into the two diseases that are feeding off each other to devastating effect (Nullis-Kapp, 2005). However, the three principal strategies for the prevention and control of TB are primary preventive measures including BCG vaccination and interruption of transmission, case finding, and chemotherapy (Johnson *et al*., 1999). Preventive therapy is the administration of drugs to prevent development of active TB from reactivation of latent and recent primary infections. It is an approach targeted at secondary prevention of new cases (Ferebee, 1970; Cosmstock *et al*., 1979).

Great efforts are being directed to the TB-HIV patients to interrupt on-going TB transmission through identification and treatment of such cases to reduce the duration of infectiousness and prevent transmission of new infections. Isoniazid (INH) has been widely used in preventive therapy, although the current outbreaks of drug resistant TB have spurred new interest in other agents and regimens (Pape *et al*., 1993; Johnson *et al*., 1999). However, TB control programmes in developing countries are yet to effectively address the problem of nosocomial TB where transmission of MDR-TB is pervasive (Harries *et al*., 1997).

Environmental and administrative measures aimed at reducing exposure of uninfected persons to those with active TB and vaccination with the Bacille Calmette-Guerin (BCG) are being applied (De Cock *et al.*, 1999). The BCG is a living attenuated strain of *M. bovis* derived by serial (*in vitro*) passage by Calmette and Guerin between 1908 and 1918. The BCG vaccines are safe and widely used for the prevention of TB. The BCG is a component of the United Nations Expanded Programme on Immunization (UNEPI) and is the world's most widely used vaccine. It is usually given as a single intradermal injection shortly after birth (Johnson and Ellner, 1999). Complications of BCG vaccination are infrequent and consist mainly of local lymphadenopathy or lymphadenitis. Disseminated disease is rare. However, the rate of complication may be slightly higher in HIV infected persons but is still very low (Ten Dam, 1990). The WHO currently recommends BCG vaccination of infants early in life, even when the mother is known or suspected to be HIV-infected. However, BCG vaccination should be withheld from persons with symptomatic HIV infection (WHO, 1989).

Prompt and correct treatment of active cases before transmission is essential for TB control (ACETB, 1995). The short course chemotherapy (SCC) or directly observed therapy short course (DOTS) is the most effective TB treatment currently. It consists of 6-8 months regimen; an intensive 2 months phase, and continuation phase of 4-6 months. First-line anti-TB drugs include isoniazid (INH), rifampicin (RIF), streptomycin (STR), ethambutol (EMB), pyrazinamide (PZA) and thiazetazone (TAZ) (ATS-CDC, 1994). Para-aminosalycylic acid (PAS), ethionamide, kanamycin,

capreomycin, amikacin, viomycin, cycloserine and fluoroquinolones are the second-line anti-TB drugs. They are used for re-treatment of TB and the treatment of known or suspected multi-drug resistant TB cases. However, these drugs are more toxic, less well tolerated and less effective for TB treatment. Their use is indicated only for drug-resistant TB and substitute agents when the patient cannot tolerate first-line drugs. Treatment with these drugs must also be given for long durations of 12 to 24 months (Johnson and Ellner, 1999).

Cases of multi-drug resistant TB should be managed by specialised units backed with good laboratories and under specialized consultants, for treatment requires access to more expensive and toxic drugs. Re-treatment or treatment failures, relapses and cases of suspected drug resistant TB should be carefully monitored and administered as DOTS (Enarson *et al*., 1996).

Patients with acquired drug resistance to INH, or INH and STR can still be cured by re-treatment regimens such as the standard 8-month International Union Against Tuberculosis and Lung Disease (IUATLD) regimens consisting of 2 months STR, EMB, INH, RIF, and PZA, followed by 1 month of EMB, INH, RIF, and PZA and concluding with 5 months of daily or three-times-weekly INH, EMB and RIF (Enarson *et al*., 1996). However, treatment of MDR-TB lasts for at least 24 months. In Kenya for instance, the regime for MDR-TB consists of five drugs in the intensive phase (capreomycin, ofloxacin, prothionamide, cycloserine and pyrazinamide) for a minimum

of six months, while the continuation phase lasts for a minimum of 18 months with the three drugs, ofloxacin, prothionamide, and cycloserine (DLTLD, 2009).   Drug susceptibility testing should be performed in patients with treatment failure or relapses especially after unsuccessful treatment (Iseman, 1993; Johnson and Ellner, 1999).

The NTM, also known as atypical mycobacteria, have varying patterns of natural drug resistance. The effectiveness of drug therapy is highly variable even with combination therapy, typically manifesting resistance to INH and PZA, with varying susceptibility to RIF, EMB and STR. This heterogeneous resistance makes therapy less predictable than with disease caused by *M. tuberculosis* (Iseman, 1998). *Mycobacterium kansasii* is sensitive to RIF and EMB. However, these drugs give poor results against MAC, *M. malmoense* and *M. xenopi* (Pozniak, 1997).

Mycobacterium marinum infection limited to superficial tissue can be treated with doxycycline, trimethoprim-sulfamethoxazole, or ciprofloxacin. Deeper or extensive infections require combination therapy with RIF, EMB, and possibly STR. Sulfonamides cefoxitin, amikacin, ciprofloxacin and imipenem are active against *M. fortuitum* (Johnston and Ellner, 1999; Wolinsky, 1992).

Treatment of *M. avium- intracellulare* (MAI) complex, *M. scrofulaceum* and *M. kanasii* infections require prolonged multiple drug therapy [MAI complex is highly resistant to anti-TB drug therapy. Pulmonary disease due to MAI complex requires prolonged

treatment and side effects are frequent] (ATS, 2007; Johnson and Ellner, 1999). The new macrolides, clarithromycin and azithromycin are highly effective against MAI complex. One suggested initial regimen is clarithromycin, ethambutol, clofazimine and amikacin for 2-4 months or streptomycin, followed by clarithromycin, ethambutol and clofazimine for 20-22 months (ATS, 2007; Iseman, 1996). Rifabutin, ciprofloxacin, ethionamide and cycloserine are alternative agents (ATS, 2007; Johnson and Ellner, 1999).

In Kenya, TB treatment success rate has been stable above 80% since the introduction of DOTS in 1993 (NLTP, 2004; DLTLD, 2009). The treatment success rates in 2007 and 2008 were 85.2% and 85.43%, respectively, slightly above the 85% success rate recommended by the WHO (DLTLD, 2009). The DLTLD (formerly NLTP) seems to be satisfied with the above 85% treatment success rate considering the high rate of HIV/AIDS among the TB patients in the country. The TB-HIV co-infected patients are at high risk of dying from non-TB opportunistic infections during TB treatment. The re-treatment failure rate stands at about 0.5%. However, an 8% out of control cases may be worrying as this group is a potential source of MDR-TB. The high rate may also be attributed to under reporting of cases that had died. The death rate among the TB patients in 2008 was 6% (DLTLD, 2009).

2.15   Factors that hamper the control of tuberculosis and other mycobacterioses

The standard TB smear microscopy test was developed a century ago and is very imprecise to detect active TB in HIV/AIDS patients with atypical symptoms (Bhargava *et al.*, 2001; Nolte *et al.*, 1995; Generozov *et al.*, 2000; Nullis-Kapp, 2005). This does delay patient entry into treatment under TB protocols. Its replacement with better diagnostic tools would be appropriate. Results involving sputum cultures can take up to eight weeks, a delay which can be fatal. Given the continuing stigma of HIV/AIDS, the majority of TB patients are reluctant to be tested for HIV (Nullis-Kapp, 2005).

Diagnosis and treatment of TB among TB-HIV patients is even more problematic. It requires highly skilled personnel to administer medication. For instance, the WHO (2005) and many national guidelines recommend that priority should be given to treating patients with the six-month course of TB treatment and then move to antiretrovirals (ARVs). For patients with advanced clinical symptoms of AIDS, the alternative approach is to give a TB-HIV patient two months of TB treatment, and then start ARVs. But for really severely immunosuppressed patients (with CD4 count less than 50) the only option is to begin TB and HIV treatments simultaneously (Nullis-Kapp, 2005; WHO, 2005). However, there is no sufficient information on toxicity and other interaction reactions between ARVs and anti-TB drugs which may reduce the efficacy of the drugs.

The long duration and high bill count of current anti-TB and non-tuberculous

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mycobacterioses drug regimens, coupled with the spectre of increasing drug resistance is also hampering efforts to control TB and NTM disease (Nullis-Kapp, 2005). The monitoring and evaluation systems and training capacity for the two diseases in resource poor countries is also inadequate (Nullis-Kapp, 2005; WHO, 2005).

Anti-TB drug susceptibility testing is an expensive exercise and requires relatively high technical skills unaffordable in many health facilities in resource poor countries. Where drug susceptibility testing (DST) facilities are available, inconsistent results is a significant problem. Mixed TB infections have been proposed as the possible explanation of inconsistent DST results (Glynn *et al*., 1995). Such infections could also be confused with exogenous re-infection or laboratory cross-contamination (Yeh *et al*., 1999).

The extent and amount of MDR-TB and XDR-TB, and the actual global burden of childhood TB are unknown. This is because TB diagnosis in children is complicated (NICHD**,** 2007**;** NIH, 2007; NIMH, 2007; WHO, 2003) and many health facilities in developing countries are incapable of achieving this due to lack of specialized facilities and health personnel, and meagre allocation of resources to the health sector (Hanson, 2002; Nullis-Kapp, 2005). Childhood TB is also often accorded low priority by many National TB Control Programmes, due to among others, the said diagnostic difficulties, over-emphasis on BCG, childhood TB being rarely infectious, and lack of data on treatment. Moreover, with the increase of HIV prevalence among children, this

situation has worsened significantly (NICHD**,** 2007**;** NIH, 2007; NIMH, 2007).

Factors that determine TB diagnosis in children include history of contact with TB case especially in the same household, chest X-ray showing unilateral lymphadenopathy and or shows shadows in the lung fields indicating infiltration and positive mantoux test (WHO, 2003). However, the usefulness of the tuberculin test and chest X-ray are further reduced in malnourished or HIV-infected children, yet these are common conditions that often need to be differentiated from TB (NICHD**,** 2007).

The HIV co-infection makes diagnosis and management of TB in children more difficult because other HIV- related diseases such as lymphocytic interstitial pneumonitis may present in a similar way to PTB or miliary TB. Interpretation of tuberculin skin testing and chest X-ray is less reliable, and when TB-HIV co-infection is common in adults a positive contact history is less specific if the contact is the child's parent. The child is at risk of transmission of either or both diseases, and children with TB and advanced HIV/AIDS may not respond as well to TB treatment. Testing for HIV infection can be helpful, especially if the result is negative, as it increases the likelihood of a diagnosis of TB. However, a positive HIV result clearly does not exclude the possibility of TB (WHO, 2003).

Lack or poor coordination between the TB and the HIV/AIDS programmes has been reported to frustrate TB control efforts. It has been observed that once a patient is

diagnosed with TB, they are sent to a TB clinic with little further interaction with the HIV/AIDS specialists even in the same health facility (Nullis-Kapp, 2005). However, the problem faced by high burden countries such as Kenya has spurred the drive to tackle the dual epidemic with coordinated response intended to draw on the strength of the DOTS strategy. Strengthening the DOTS strategy seems to be essential but not sufficient by itself. Implementation of additional existing interventions to prevent TB such as prophylaxis and intensified case finding are necessary (Nullis-Kapp, 2005; WHO, 2005).

The chief priority of TB control programmes is early identification, and prompt and appropriate treatment of active cases before further transmission (ACETB, 1995). However, the current TB case finding strategies in many developing countries like Kenya (NLTP, 2004) are based on passive case finding, where people with symptoms of TB must come to healthcare facility to be 'found'. If they do not go to the healthcare facility, they will not be 'found', and therefore will not be treated. This way Kenyan healthcare system "finds" about 50% of infectious cases and is able to successfully treat about 85.4% of them. Hence for every case found and treated by the healthcare system, there may be one other 'unfound' case. This individual does not receive treatment. Many of these cases with TB but are 'unfound' remain untreated and eventually die from the disease. Their deaths could have been prevented if only they had been 'found'. Many cases suffering from TB may not be reaching healthcare facilities, and if they are, healthcare systems may miss to pick them up (NLTP, 2004). The passive

case finding strategy is therefore ineffective in TB control necessitating the adoption of a more proactive approach by the healthcare systems to find people suffering from TB, coupled with health education.

Limited funding for TB activities remains a major challenge in TB control. Many low-income countries are unable to carry out drug resistance surveys, making it difficult for WHO and other relevant organizations to estimate the true burden and trends of XDR-TB and MDR-TB globally, regionally and nationally. It is likely that many outbreaks of drug resistance are going unnoticed. The WHO (2009) estimates that US$4.8 billion were needed for overall TB control in low- and middle-income countries in 2008, with US$1 billion for X/MDR-TB. But there was a total financing gap of $2.5 billion, including a US$ 500 million gap for XDR-TB and MDR-TB.

CHAPTER THREE

3.0   MATERIALS AND METHODS

3.1   Study design

This was hospital and laboratory based cross-sectional study carried out between September 2007 and September 2009. It was carried out to provide a snapshot (one point in time measurements) description of the prevalence of TB, HIV and TB-HIV and the significance of the NTM in causing TB-like disease in western Kenya. The study was conducted to describe the relationship between HIV co-infection and anti-TB drug resistance.

3.2   Study site and population

The study was done at Nakuru Provincial General Hospital, Kisii Level 5 General Hospital and Busia, Bungoma, Kisumu, Migori, Narok, Kericho, Uasin Gishu and Lodwar district hospitals. These hospitals were selected because they serve populations at high risk for TB due to high HIV prevalence, or socio-cultural practices that favour TB transmission, and the role of NTM in TB-like morbidity in these populations has not been determined. These hospitals like all others at their levels have chest and paediatric clinics where adult and child TB patients obtain healthcare respectively. The numbers of TB suspects enrolled into the study from each hospital are shown in Table 4.1, page 67. Western Kenya includes the expansive former Rift Valley, Nyanza and Western Provinces, with a cumulative population of about 19.8 million people. This constitutes about 52.1% of the Kenyan population (Kenya Census, 2009).

3.3   Sample size

The 2006 national average of TB-HIV/AIDS co-infection rate in Kenya was 40% (NLTP, 2006). Using this as a reference for a single proportion calculation with an error of 5% at 95% power, employing Fischer's exact formula as applied by Mugenda and Mugenda (1999), the required sample N was thus:

$$N = \frac{z^2\,pq}{\delta^2} = \frac{(1.96)^2\,0.4 \times 0.6}{(0.05)^2} = 369$$

Where:

N = sample size

z = standard normal deviate   = 1.96

p = prevalence proportion   = 0.4

q = 1-p   = 0.6

δ = level of precision   = 0.05

At least 369 suspected TB cases were required for this study. However, this study took the advantage of the ease of getting specimens and enrolled a total of 872 TB suspects in order to obtain more *Mycobacterium* isolates and increase the power of the study.

3.4   Sampling frame and patient characteristics

3.4.1 Sampling frame

Purposeful sampling was employed to recruit suspected TB cases into the study between September 2007 and September 2009 as they sought healthcare services at the chest and paediatric clinics. Patients were suspected of having TB if they had a cough of more than two weeks not responding to antibiotic treatment (NLTP, 2003).

3.4.2   Inclusion and exclusion criteria

Subjects suspected of having TB and resident in western Kenya for at least six (6) months, not on anti-TB chemotherapy and consented to participate in the study were recruited. Patients who had not lived in western Kenya for the last 6 months, those already on anti-TB chemotherapy, and those unwilling to participate in the study and not meeting the above inclusion criteria were excluded. Children under 18 years were enrolled into the study after assent from parent / guardian.

3.4.3   Collection of samples and demographic data

Three sputum specimens (spot, early morning, second spot) were collected from participants suspected of having TB under the supervision of trained and competent medical staff. The suspects were requested to cough so that the expectoration came from deep down the chest as possible, and spit into a sterile 50 ml blue cap tubes. For children less than 5 years of age and those less than 10 years of age unable to expectorate sputum had sputum induction performed at the Nakuru provincial and Kisii level 5 hospitals. The samples were refrigerated at $4^{o}C$ awaiting transportation in cool boxes to the Mycobacteria Reference Laboratory, Moi University School of Medicine (MRL, MUSOM) weekly for analysis. At the MRL, MUSOM, the samples were refrigerated at $4^{o}C$ till processing. However, most samples were processed within 7 days of collection in order to minimize loss of viability of the mycobacteria.

Consenting 695 TB suspects were voluntarily counselled before they underwent

phlebotomy for HIV testing. The blood was delivered into Vacutainer Brand STERILE interior EDTA (K3) tubes and stored at -20°C awaiting processing. The samples were transported in cool boxes to MRL, MUSOM, Eldoret, and screened for HIV infection within two weeks (Appendix 2).

A questionnaire was used to collect demographic and medical history data by the designated Nurse or Clinical Officer in-charge of the chest and paediatric clinics. Data collected included age, gender, previous anti-TB treatment, HIV status, and ART.

3.5   Testing for human immunodeficiency virus infection

Whole blood obtained from the consenting 695 TB suspects was allowed to clot. Serum was screened for anti-HIV antibodies using Trinity Biotech Uni-Gold^{TM} test and positives confirmed with the enzyme linked immunosorbent assay (ELISA), following manufacturers' instructions (Appendix 3). The HIV positive cases later diagnosed with TB were considered to TB-HIV co-infected. The HIV positive cases were advised to go for post-test counselling and enrolment to HIV/AIDS Programme.

3.6   Microscopic examination of sputum specimens

Sputum smears were examined for acid-fast bacilli (AFB) after staining following the Ziehl-Neelsen (ZN) method (Find, 2006).

3.7 Identification of recurrent tuberculosis cases

A questionnaire was used to obtain information on subjects who had history of previous TB treatment. Those who indicated to have been previously treated for TB and declared cured and re-notified at least 12 months from the date of the initial notification were considered recurrent TB cases; after verification with their records held at the hospital.

3.8 Isolation of mycobacteria

Sputum specimens were processed (digestion, decontamination and concentration) using the N-acetyl-L-cysteine sodium hydroxide (NaOH-NALC) method. The processed specimens were cultured for isolation of mycobacteria using the Mycobacteria Growth Index Tube 7 ml (BBL^{TM} MGIT^{TM}) supplemented with BACTEC^{TM} MGIT^{TM} Growth Supplement, BBL^{TM} MGIT^{TM} PANTA^{TM} Antibiotic Mixture (BD BBL MGIT Package inserts, 2008) (Appendix 4).

Briefly, a lyophilised vial of BBL MGIT PANTA (Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim, Azlocillin) Antibiotic Mixture was reconstituted with 15 ml of BACTEC MGIT Growth supplement. Using a sterile pipette, 0.8 ml of the Growth supplement/ MGIT PANTA Antibiotic Mixture was aseptically added to the BBL MGIT 7 Ml Tubes (Growth supplement /MGIT PANTA Antibiotic Mixture made just prior to specimen inoculation). Using a sterile transfer pipette 0.5 ml of the concentrated specimen suspension was the added to appropriately labelled MGIT

Tubes. A separate pipette was used for each specimen. Each tube was immediately recapped tightly and the contents mixed by inverting the tube 4 to 6 times. The tube and caps were wiped with 5% phenol (mycobactericidal disinfectant) and allowed to stand at room temperature for 30 minutes.

All the inoculated tubes were then scanned and entered in the BACTEC MGIT 960 instrument for continuous automatic monitoring of growth for up to 42 days, by which time the instrument flagged the tubes as positive or negative. The instrument maintained $37\ ^{o}C \pm 1\ ^{o}C$ temperature. When the instrument signaled a tube for positive growth, and an indicator green light showed the tube location of the positive tube in the drawer of the instrument, the tube was removed and scanned outside the instrument. The tube was then observed visually for growth. Growth of *M. tuberculosis* complex settled at the bottom of the tube. The ZN smears were prepared from positive tubes for AFBs.

In addition to BACTEC MGIT 960, 0.2 to 0.3 ml (2-3 drops) of the concentrated specimens was inoculated onto LJ slants for maximum recovery of the mycobacteria. The bottles were incubated at $37\ ^{o}C$ and examined on a weekly basis until growth was detected. Cultures were reported as negative if no growth occurred after 8 weeks (BD BBL MGIT Package inserts, 2008). Positive tubes (both MGIT and LJ) were processed for identification of mycobacterial isolates, anti-TB drug susceptibility testing (DST) to determine susceptibility of *M. tuberculosis* complex to first-line anti-TB drugs.

3.9   Identification of mycobacteria

The mycobacterial isolates were identified as *M. tuberculosis* complex or species of NTM using Hain's GenoType® Mycobacterium CM and GenoType® Mycobacterium AS Molecular Genetic Assays, techniques based on the DNA•STRIP. Briefly, DNA was isolated from the cultured material. This was followed by multiplex amplification with biotinylated primers and reverse hybridization. Hybridization included chemical denaturation of amplification products, hybridization of single-stranded biotin-labeled amplicons to membrane bound probes, stringent washing, addition of streptvadin/alkaline phosphatase (AP) conjugate, and an AP mediated staining reaction. Identification of *M. tuberculosis* complex from NTM was done by interpretation of banding patterns using a standard temple following manufacturer's instructions (Hain lifescience, GmbH, Nehren, German, Package inserts, 2008) (Appendix 5).

3.10   Primary anti-tuberculosis drug susceptibility testing

3.10.1 GenoType® MTBDR*plus* drug susceptibility testing

The isolates underwent drug susceptibility testing (DST) using the Hain's GenoType® MTBDR*plus* Molecular Genetic Assay for Identification of Resistance to RIF and / or INH. The GenoType® MTBDR*plus* test is based on the DNA•STRIP® technology and permits the molecular genetic identification of *M. tuberculosis* complex and its resistance to RIF and / or INH from cultivated or pulmonary smear - positive direct patient material (Hain lifescience, GmbH, Nehren, German, Package inserts 2008) (Appendix 6).

The identification of resistance to RIF was enabled by the detection of the most significant mutations of the *rpoB* gene (encoding for the β-subunit of the RNA polymerase). For testing of high level INH resistance, the *katG* gene (encoding for the catalase peroxidase) was examined and for testing of low level INH resistance, the promoter region of the *inhA* gene (encoding for NADH enoyl ACP reductase) was examined.

Briefly, DNA was isolated from the cultured material. This was followed by multiplex amplification with biotinylated primers and reverse hybridization. Hybridization included chemical denaturation of amplification products, hybridization of single-stranded biotin-labeled amplicons to membrane bound probes, stringent washing, addition of streptvadin/alkaline phosphatase (AP) conjugate, and an AP mediated staining reaction. The banding patterns obtained were easily interpreted using a standard template following manufacturer's instructions (Hain lifescience, GmbH, Nehren, German Package inserts, 2008).

3.10.2 BACTEC MGIT 960 anti-tuberculosis drug susceptibility testing

The BACTEC MGIT 960 susceptibility testing for STR, INH, RIF, and EMB, called SIRE was done to establish the susceptibility of *M. tuberculosis* complex to the drug concentrations of 1.0 µg/ml, 0.1µg/ml, 1.0 µg/ml, and 5.0 µg/ml, respectively. Standard MGIT^{TM} procedure manual of the SIRE kit was followed (FIND, 2006; BD BBL MGIT Package inserts, 2008). Briefly, each critical concentration of drug vial was

reconstituted with 4 ml of sterile distilled / de-ionized water in 50 ml sterile falcon tubes. The contents were mixed thoroughly to ensure complete dissolution of the drug. A volume of 0.1 ml (100 µL) of each reconstituted drug solution was added into each of appropriately labeled BACTEC MGIT 960 tubes. This resulted into the following critical concentration of drugs in the medium: STR, 1.0 µg/ml of medium; INH, 0.1 µg/ml of medium; RIF, 1.0 µg/ml of medium; EMB, 5.0 µg/ml of medium (FIND, 2006; BD BBL MGIT Package inserts, 2008).

Inocula for DST were prepared from day 1 to day 5 MGIT tube positive by instrument (The day a MGIT tube was instrument positive was considered day 0). Day 1 and day 2 positive tubes were vortexed to break up clumps. The tubes were left undisturbed for 5-10 minutes for the unbroken clumps to settle. A volume of 0.5 ml of the supernatant was used for inoculation. For day 3 to day 5 instrument positive tubes, the tubes were vortexed as described and large clumps left to settle for 5-10 minutes. A volume of 1.0 ml of the supernatant was diluted with 4.0 ml of sterile normal saline (1:5 dilution). A volume of 0.5 ml of the dilution was used for inoculation (FIND, 2006; BD BBL MGIT Package inserts, 2008).

Five (5) MGIT tubes were labeled for each test culture (isolate). One was labeled GC (growth control, without drug), one for STR, one for INH, one for RIF, and one for EMB. A volume of 0.8 ml of BACTEC 960 SIRE supplement was aseptically added to each MGIT tube. This was followed by the addition of 0.1 ml (100 µL) each of each

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reconstituted drug to respective labeled tubes. A separate micropipette was used for each drug. No drug was added to the GC tube. A volume of 0.5 ml of well mixed inocula (culture suspension) was added into each drug containing tube (This culture suspension was not added to the control). For the control, the test culture suspension was first diluted to 1:100 by adding 0.1 ml of test culture suspension to 10 ml of sterile saline. Mixing was done by inverting the tubes 5-6 times. A volume of 0.5 ml of this diluted suspension was added into the growth control tube. Caps of tubes were tightened and contents mixed by gently inverting the tubes 6 times. The labeled tubes were placed in the set carrier in the sequence GC, STR, INH, RIF, EMB. The susceptibility Set Carrier was entered into the BACTEC MGIT 960 instrument using the susceptibility test entry feature (FIND, 2006; BD BBL MGIT Package inserts, 2008).

The instrument monitored the entered susceptibility test set automatically. Once the test was complete (4 to 21 days), the instrument indicated the results were ready. The susceptibility Set Carrier was scanned and the report printed. The instrument printout indicated qualitative susceptibility results for each drug: Susceptible (S), resistant (R) or indeterminate (X). Isolates resistant to at least both rifampicin and isoniazid were to be designated as multi-drug resistant, MDR (FIND, 2006; BD BBL MGIT Package inserts, 2008) (Appendix 7).

3.11   Data management and analysis

Demographic data were confidentially obtained from the TB suspects by clinicians / nurses running the chest and paediatric clinics and confidentially sent to the investigator through courier services. Results of ZN smear microscopy, culture, anti-TB drug susceptibility, and HIV tests were also confidentially sent to the respective clinicians / nurses by the investigator through the courier services. Provisions of these data were made available to the clinicians / nurses for the purpose of managing the patients.

Data was recorded on questionnaires, register books, ELISA reader print-outs, MGIT incubator print-outs, species evaluation sheets, and drug resistance evaluation sheets. The data was entered in MS Excel 8.0 and analysed using Epi Info version 3.5.1. Descriptive statistics were used to summarize data and proportions compared using Chi-square (χ^2) testing. Univariate odds ratio (OR) with 95% confidence intervals (CI) were calculated to assess how risk factors are related to outcomes. Logistic regression was used to analyze multivariate data. P-value < 0.05 was considered as statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Study population distribution per hospital

A total of 872 TB suspects were enrolled into the study at the 10 study sites, of which

479 (54.9%) were males and 393 (45.1%) were females (Table 4.1).

Table 4.1 Study population distribution per hospital

Study site	Males (%)	Females (%)	Total
Bungoma D.H.	74(8.5)	82(9.4)	156 (17.9)
Busia D.H.	48 (5.5)	59(6.8)	107(12.3)
Kericho D.H.	17(1.9)	11(1.3)	28(3.2)
Uasin Gishu D.H.	36(4.1)	39(4.5)	75(8.6)
Nakuru P.G.H.	11(1.3)	10(1.1)	21(2.4)
Narok D.H.	193(22.1)	128(14.7)	321(36.8)
Kisumu D.H.	17(1.9)	19(2.2)	36(4.1)
Kisii L 5 G.H.	31(3.6)	23(2.6)	54(6.2)
Migori D.H.	20(2.3)	10(1.1)	30(3.4)
Lodwar D.H.	32(3.7)	12(1.4)	44(5.1)
Total	**479(54.9)**	**393(45.1)**	**872(100)**

Key: D.H. = District Hospital; P.G.H. = Provincial General Hospital; L 5 G.H. = Level

5 General Hospital.

4.2   Study population and gender-age distribution

The ages of the TB suspects enrolled into the study were between 9 months and 80 years, the median age being 32 years. The majority (33.1%) of the suspects were in the 25-34 age-group, followed by those in the 35-44 (21.8%) and 15-24 (18.7%) age-groups, respectively. Children in the 0-14 age-group constituted 4.6%, with the under fives (<5 years) contributing 0.6% (Table 4.2)

Table 4.2 Study population and gender-age distribution

Age-group	Males (%)	Females (%)	Total (%)
0-14	22(2.5)	18(2.1)	40(4.6)
15-24	80(9.2)	83(9.5)	163(18.7)
25-34	162(18.5)	126(14.4)	288(33.0)
35-44	108(12.4)	82(9.4)	190(21.8)
45-54	53(6.1)	36(4.1)	89(10.2)
55-64	29(3.3)	25(2.9)	54(6.2)
> 64	25(2.9)	23(2.6)	48(5.5)
Total	**479(54.9)**	**393(45.1)**	**872(100)**

4.3   Ziehl Neelsen microscopy, culture and human immunodeficiency virus infection

Sputa from 39.1% (341/872) of the TB suspects were ZN smear positive, of which 53.1% (181/341) were culture positive. Sputa from 3.8% (20/531) of the ZN smear negatives suspects were culture positive. In total 41.4% (361/872) suspects were diagnosed with mycobacterial disease, of which 55.7% (201/361) were culture positive and 44.3% (160/361) were culture negative. The culture positives yielded 92.5% *M. tuberculosis* complex and 7.5% NTM. Five of the NTM isolates were identified as *M. intracellulare* (3 isolates), *M. fortuitum* and *M. peregrinum* one isolate each. The remaining 10 NTM isolates could not be identified to species level (Fig. 4.3). The 46.9% (160/341) smear positive but culture negative cases were treated as TB cases. No cultures yielded tuberculous and non-tuberculous mycobacteria co-infection.

The HIV test was accepted by 695 out of the 872 TB suspects enrolled into the study. Sputa from 36.7% (255/695) were ZN smear positive, 109 from HIV positive and 146 from HIV negative suspects. Out of the 440 ZN smear negatives, 163 were HIV positive and 277 were HIV negative. Sputa from 24.2% (168/695) of the suspects were culture positive, 78 from HIV positive and 90 from HIV negative) suspects. Out of the 527 culture negative suspects, 194 were HIV positive and 333 were HIV negative (Table 4.3). The culture positivity rate was significantly lowered among HIV positive cases [χ^2 = 4.550; P = 0.033], while there was no significant difference in ZN smear positivity rate between HIV positive and HIV negative cases [χ^2 = 1.969; P = 0.160].

Fig.4.3 Identification of mycobacteria by Hain's GenoType Mycobacterium CM/AS

Table 4.3 Ziehl Neelsen microscopy, culture and human immunodeficiency virus infection

N = 695	ZN smear microscopy		culture	
	ZN smear positive	ZN smear negative	Culture positive	Culture negative
HIV positive	109 (42.7%)	163 (37%)	78 (46.4%)	194 (36.8%)
HIV negative	146 (53.3%)	277 (63%)	90 (53.6%)	333 (63.2%)
Totals	**255 (100%)**	**440 (100%)**	**168 (100%)**	**527 (100%)**

Key: HIV = human immunodeficiency virus; N = Number of tuberculosis suspects tested HIV infection; ZN = Ziehl Neelsen.

4.4   Tuberculosis prevalence and gender-and age distribution

A total of 361 (41.4%) out of the 872 TB suspects were diagnosed with mycobacterial disease, 95.8% (346/361) TB and 4.2% (15/361) NTM disease. Hence, 39.7% (346/872) of the TB suspects were diagnosed with TB, 61.6% (213/346) males and 38.4% (133/346) females. The prevalence of TB was significantly higher in males than females [χ^2 = 10.67; P = 0.001]. The majority, 37.0% (128/346) of the TB cases were in the 25-34 age-group, followed by the 35-44 age-group with 22.3% (77/346). The 15-24 age-group ranked third with 20.2% (70/346). Children below 15 years constituted 4.9% (17/346) of the TB cases. Logistic regression analysis for comparing the association of TB with age-group revealed males in the 15-24 and 35-44 age-groups to be most significantly affected compared to females (Table 4.4).

Table 4.4 Tuberculosis prevalence and the logistic regression output for gender and age-group

Age-group	Males (%)	Females (%)	Total (%)	OR	95%CI	P-value
0-14	9(2.6)	8(2.3)	17(4.9)	0.84	0.23-3.05	0.79
15-24	41(11.8)	29(8.4)	70(20.2)	2.02	1.07-3.81	0.03
25-34	78(22.5)	50(14.5)	128(37.0)	1.46	0.91-2.35	0.12
35-44	51(14.7)	26(7.5)	77(22.3)	1.99	1.09-3.64	0.02
45-54	21(6.1)	10(2.9)	31(9.0)	1.51	0.60-3.80	0.38
55-64	8(2.3)	5(1.4)	13(3.8)	1.60	0.44-5.74	0.47
> 64	5(1.4)	5(1.4)	10(2.9)	0.94	0.23-3.83	0.94
Total	**213(61.6)**	**133(38.4)**	**346(100)**	**1.60**	**1.21-2.11**	**0.001**

Key: OR = odd's ratio; CI = confidence interval; P-value = level of marginal significance.

4.5   Tuberculosis recurrence prevalence and gender-age distribution

A total of 155 (44.8%) of the 341 TB cases were recurrences (in agreement with hospital records regarding history of previous TB treatment). Males constituted 54.2 % (84/155) and females 45.8% (71/155) of the TB recurrent cases. A total of 50 (32.3%) recurrent TB cases were ZN smear positive and 105 (67.7%) cases were smear negative. A total of 31 (20%) of the TB recurrent cases were culture positive and 124 (80%) culture negative. A total of 65 (41.9%) of these cases were HIV sero-

positive, 70 (45.2%) cases HIV sero-negative, and the HIV status of 20 (12.9%) cases

was unknown. The majority (35.2%) of TB recurrences were in the 25-34 age-group,

followed by 35-44 age-group with 23.9%. Within age-groups, TB recurrences were

more among males in the 25-54 age-group. There was no significant difference in TB

recurrence between the genders [χ^2 = 0.013; P = 0.909]. However, logistic regression

analysis for comparing the association of TB recurrence with age-group revealed no

significant difference between genders in the various age-groups (Table 4.5).

Table 4.5 Tuberculosis recurrence prevalence and the logistic regression output for
gender and age-group

Age-group	Males (%)	Females (%)	Total (%)	OR	95%CI	P-value
0-14	1(0.6)	4(2.6)	5(3.2)	5.71	0.58-56.67	0.14
15-24	9(5.8)	9(6.8)	18(11.6)	0.96	0.36-2.55	0.93
25-34	32(20.6)	25(16.1)	57(36.8)	1.01	0.56-1.80	0.98
35-44	23(14.8)	17(11.0)	40(25.8)	0.97	0.48-1.96	0.92
45-54	10(6.5	7(4.5)	17(11.0)	1.04	0.35-3.04	0.95
55-64	4(2.6)	8(5.2)	12(7.7)	2.94	0.76-11.34	0.12
> 64	5(3.2)	1(0.6)	6(3.9)	0.18	0.02-1.69	0.13
Total	**84(54.2)**	**71(45.8)**	**155(100)**	**1.04**	**0.73-1.47**	**0.84**

Key: OR = odd's ratio; CI = confidence interval; P-value = level of marginal

significance.

4.6   Human immunodeficiency virus prevalence and gender-age distribution

Out of the 872 TB suspects enrolled into the study, 79.7% (695/872) accepted to undergo HV testing, and 39.1% (272/695) were sero-positive. Females constituted 44.9% (312/695) of which 43.9% (137/312) were infected. Males constituted 55.1% (383/695), with 34.8% (135/388) being infected. Overall females constituted 50.4% (137/272) and males 49.6% (135.272) of the HIV cases. There was a significant difference in HIV infection rate between gender, females being more vulnerable [χ^2 = 5.06; P = 0.020]. The majority (39.3%) of the HIV cases were in the 25-34 age-group followed by the 35-44 (24.6%) and 15-24 (15.8%) age-groups, respectively. The 0-14 year age-group had 2.9% of the HIV cases. Logistic regression analysis for comparing the association of HIV infection prevalence with age-group revealed no significant difference between genders in the various age-groups (Table 4.6). Only 16.9% (46/272) of the HIV/AIDS cases were on antiretroviral therapy (ART), 65.2% females and 34.8% males.

Table 4.6 Human immunodeficiency virus prevalence and the logistic regression output for gender and age-group

Age-group	Males (%)	Females (%)	Total (%)	OR	95%CI	P-value
0-14	3(1.1)	5(1.8)	8(2.9)	0.21	0.03-1.41	0.11
15-24	21(7.7)	22(8.1)	43(15.8)	0.75	0.36-1.57	0.45
25-34	53(19.5)	54(19.9)	107(39.3)	0.71	0.42-1.83	0.19
35-44	32(11.8)	35(12.9)	67(24.6)	0.55	0.29-1.05	0.07
45-54	12(4.4)	12(4.4)	24(8.8)	0.53	0.19-1.46	0.22
55-64	10(3.7)	4(1.5)	14(5.2)	3.86	0.97-15.44	0.06
> 64	4(1.5)	5(1.8)	9(3.3)	0.52	0.11-2.36	0.395
Total	**135(49.7)**	**137(50.4)**	**272(100)**	**0.69**	**0.51-0.94**	**0.020**

Key: OR = odd's ratio; CI = confidence interval; P-value = level of marginal significance.

4.7 Tuberculosis - human immunodeficiency virus co-infection and gender-age distribution

A total of 263 (165 males and 98 females) of the 346 TB cases accepted to be tested for HIV infection and 41.8% (110/263) were co-infected. Males constituted 55.5 % (61/110) and females 44.5% (49/110). There was no significant difference in the TB-HIV co-infection rate between genders [$\chi^2 = 0.01$; P = 0.94]. The majority, (40.9%) of the TB-HIV cases was in the 25-34 year age bracket followed by the 35-44 (24.5%) and 15-24 (19.1%) year age brackets. Logistic regression analysis for comparing the

association of TB-HIV co-infection with age-group also revealed no significant difference between genders in the various age-groups (Table 4.7). However, 24% (83/346) of the TB cases declined to be tested for HIV infection.

Table 4.7 Tuberculosis – human immunodeficiency virus co-infection prevalence and the logistic regression output for gender and age-group

Age-group	Males (%)	Females (%)	Total (%)	OR	95%CI	P-value
0-14	2(1.8)	2(1.8)	4(3.7)	0.80	0.10-6.54	0.83
15-24	13(11.8)	8(7.3)	21(19.1)	1.79	0.69-4.62	0.23
25-34	25(22.7)	21(19.1)	46(41.8)	0.82	0.43-1.56	0.55
35-44	14(12.7)	13(11.8)	27(24.5)	0.87	0.38-1.98	0.74
45-54	4(3.6)	3(2.7)	7(6.4)	0.94	0.19-4.50	0.94
55-64	2(1.8)	1(0.9)	3(2.7)	1.76	0.15-20.75	0.65
> 64	1(0.9)	1(0.9)	2(1.8)	0.78	0.05-13.39	0.87
Total	**61(55.5)**	**49(44.5)**	**110(100)**	**1.01**	**0.67-1.51**	**0.98**

Key: OR = odd's ratio; CI = confidence interval; P-value = level of marginal significance.

4.8   Non-tuberculous mycobacterial disease prevalence and gender-age distribution

Non-tuberculous mycobacteria (NTM) were isolated from 15 cases of TB-like non-tuberculous mycobacterial disease, the majority (66.7%) being males. The majority

(40%) of the NTM disease cases were in the 25-34 year age-group, followed by the 15-24 year age-group with 20% (Table 4.8). Four of the NTM disease cases (3 males and one female) had previously been treated for TB. Six (40%) of cases (3 males and 3 females) were co-infected with HIV, five (33.3%) of the cases (3 males and 2 females) were HIV negative and four (26.7%), all of them males, were of unknown HIV status. Three of the NTM-HIV co-infection cases were on antiretroviral therapy.

Table 4.8 Non-tuberculous mycobacterial disease prevalence and gender-age distribution

Age-group	Males (%)	Females (%)	Total (%)
0-14	0	1(6.7)	1(6.7)
15-24	2(13.3)	1(6.7)	3(20.0)
25-34	4(26.7)	2(13.3)	6(40.0)
35-44	2(13.3)	0	2(13.3)
45-54	0	1(6.7)	1(6.7)
55-64	1(6.7)	0	1(6.7)
> 64	1(6.7)	0	1(6.7)
Total	**10(66.7)**	**5(33.3)**	**15(100)**

4.9   Correlation between human immunodeficiency virus co-infection and anti-tuberculosis drug resistance

The entire 186 *M. tuberculosis* complex isolates (159 from new cases and 27 from recurrences) subjected to anti-TB drug susceptibility testing were susceptible to STR and EMB. Only 6.5% (12/186) of the isolates were mono-drug resistant, nine (75%) to INH, and three (25%) to RIF. The 3 isolates resistant to RIF were from three female new cases co-infected with HIV. Among the 9 isolates resistant to INH, 3 were from recurrences co-infected with HIV, and 6 were from new cases 4 of which were HIV negative, one co-infected with HIV, and one with unknown HIV status. One new case with isolate resistant to INH was on ART (Table 4.9).

Table 4.9 Human immunodeficiency virus co-infection and anti-tuberculosis drug resistance

Patient characteriatics					Drug susceptibility patterns of isolates			
Age (years)	Gender	Type	HIV status	ART	INH	RIF	STR	EMB
20	F	Ne	Unknown	No	R	S	S	S
30	M	Ne	+ve	No	S	R	S	S
32	F	Re	+ve	No	R	S	S	S
43	M	Ne	+ve	No	S	R	S	S
18	F	Ne	+ve	Yes	R	S	S	S
32	M	Ne	-ve	No	R	S	S	S
34	F	Ne	+ve	No	S	R	S	S
56	F	Re	+ve	No	R	S	S	S
39	F	Re	+ve	No	R	S	S	S
40	M	Ne	-ve	No	R	S	S	S
37	F	Ne	+ve	No	R	S	S	S
24	M	Ne	-ve	No	R	S	S	S

Key: F = female; M = male; Ne = New TB case; Re = recurrent case; +ve = human immunodeficiency virus infected; -ve = human immunodeficiency virus uninfected; ART = antiretroviral therapy; R = resistant; S = susceptible; INH = isoniazid; RIF = rifampicin; STR = streptomycin; EMB = ethambutol.

BACTEC MGIT 960

Unloaded AST Set Report

Instrument Number	Current Date/Time	Temperature A	B	C	Software Version	Page Number
1	13/12/08 06.06	36.7°C	37.0°C	36.7°C	V3.06C	1

Sequence No: 439550034833   **TIP:** 7;4   **SOP:** 04/12/08 18.03   **Removed Date:** 12/12/08

Tube Position	Growth Unit	Status	Concentration	Drug Name	
A/L01	400	C		Growth Control	**330/01**
A/L02	0	S	1.00 ug/mL	Streptomycin	
A/L03	0	S	0.10 ug/mL	Isoniazid	
A/L04	0	S	1.00 ug/mL	Rifampin	
A/L05	0	S	5.00 ug/mL	Ethambutol	

Sequence No: 439550034810   **TIP:** 7;19   **SOP:** 04/12/08 18.03   **Removed Date:** 12/12/08

Tube Position	Growth Unit	Status	Concentration	Drug Name	
A/L06	400	C		Growth Control	**346/02**
A/L07	7	S	1.00 ug/mL	Streptomycin	
A/L08	400	R	0.10 ug/mL	Isoniazid	
A/L09	0	S	1.00 ug/mL	Rifampin	
A/L10	0	S	5.00 ug/mL	Ethambutol	

Sequence No: 439550034709   **TIP:** 8;5   **SOP:** 04/12/08 18.02   **Removed Date:** 13/12/08

Tube Position	Growth Unit	Status	Concentration	Drug Name	
A/K06	400	C		Growth Control	**276/02**
A/K07	0	S	1.00 ug/mL	Streptomycin	
A/K08	400	R	0.10 ug/mL	Isoniazid	
A/K09	0	S	1.00 ug/mL	Rifampin	
A/K10	0	S	5.00 ug/mL	Ethambutol	

Sequence No: 439550034831   **TIP:** 8;3   **SOP:** 04/12/08 18.03   **Removed Date:** 13/12/08

Tube Position	Growth Unit	Status	Concentration	Drug Name	
A/K16	400	C		Growth Control	**306/02**
A/K17	0	S	1.00 ug/mL	Streptomycin	
A/K18	400	R	0.10 ug/mL	Isoniazid	
A/K19	0	S	1.00 ug/mL	Rifampin	
A/K20	0	S	5.00 ug/mL	Ethambutol	

Fig. 4.8a: DST on INH, RIF, STR, and EMB by BACTEC MGIT 960; Isolates 346/02, 276/02 and 306/02 show resistance to INH.
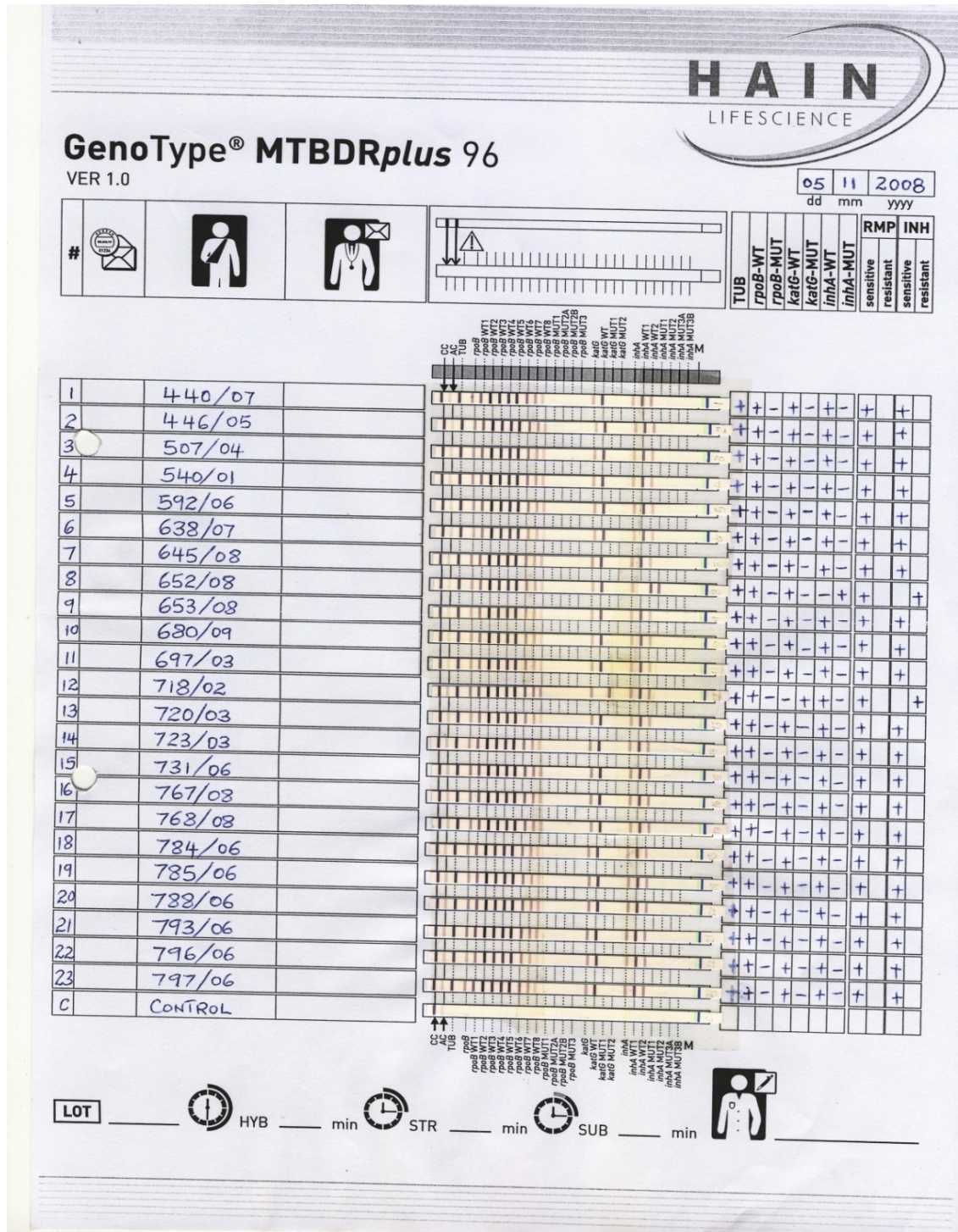
Fig. 4.8b. DST on RIF and INH by Hain's GenoType MTBDR*plus* 96. Isolates 625/08 and 718/02 show resistance to INH.

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CHAPTER FIVE

5.0   DISCUSSION

The present study observed no significant difference in ZN smear positivity rate among HIV positive and HIV negative cases (P = 0.160). This observation contradicts previous studies that have reported HIV infection to lower ZN smear sensitivity with smear positivity rates among TB-HIV cases as low as 31% (Finch *et al*., 1997; Levy *et al*., 1997; Thorntorn *et al*., 1998) and smear negativity rates as high as 82% (Verma *et al*., 2008) having been reported. The plausible explanation for the findings of the present study is that some of the TB-HIV cases may have been in the early HIV infection stage during which the frequency and presentation of TB is similar to that observed in immuno-competent and HIV negative cases (Sharma *et al*., 1997; Verma *et al*., 2008). This explanation may be supported further by the fact that only 16.9% of the HIV cases were on antiretroviral therapy (ART), whose initiation is based on the level of immunity. The high sputum smear negativity tends to be associated with advanced HIV/AIDS disease and progress in immuno-suppression (Verma *et al*., 2008). Due to alteration of the normal host immune response to *M. tuberculosis* in HIV co-infected patients, cavitation and transfer of tubercle bacilli into respiratory secretions is markedly reduced, thereby hampering TB diagnosis by ZN smear microscopy and culture (Caviedes *et al.*, 2000; Lawson *et al*., 2008).

The present study observed HIV infection to significantly lower culture positivity rate. This observation is corroborated with previous studies which report HIV co-infection to

lower TB culture positivity rates (Lawson *et al*., 2008; Elliot *et al*., 1993). As low as 26% TB culture positivity rates have been reported among TB-HIV cases (Mc Shane, 2005). However, the high number of microscopically smear positive but culture negative sputa observed in the current study is worth noting. Only in 51% of the TB cases diagnosed by ZN smear microscopy could *M. tuberculosis* be grown from sputum. This would imply ZN microscopy has in general become inaccurate tool for the diagnosis of TB, yielding a high proportion of false-positive diagnosis. However, the high number of ZN smear positive but culture negative cases observed in the present study could also be attributed to either lose of viability since most sputum samples were cultured on the 7th day after collection, or the presence of fastidious NTM, or the presence of acid-fast non-mycobacteria organisms. Consequently, yet to be found out is which ZN smear positive microorganisms cause this high degree of false-positivity and whether they are emerging infections. Non-tuberculous mycobacteria (Buijtels, 2007), and some other bacterial species including *Nocardia* species which are widespread (Olson *et al*., 1998) yield positive results in ZN smear detection of acid-fast bacilli (AFB).

The present study identified 15 NTM disease cases by culture that would have been put on the entire TB treatment regimen based on ZN smear positivity. It is therefore evident that TB diagnosis and treatment in Western Kenya is not evidence-based, and cases of non-tuberculous mycobacterioses are often put on anti-TB chemotherapy. The contribution of NTM to TB-like diseases in both HIV positive and negative patients in

Kenya may also be underestimated since most health facilities do not perform cultures. However, due to practical reasons cultures are not routinely done and the ZN smear microscopy so far remains the most feasible microbiological method for the diagnosis of PTB in Kenya and other developing countries due to its rapidity, low cost, and high positive predictive value for tubercle bacilli (Conde *et al*., 1999). Cultures are not performed for routine diagnosis and monitoring of TB treatment due to high costs and biosafety infrastructure requirements (Apers *et al*., 2003; Hudson *et al*., 2000). However, Dowdy *et al*. (2008) have reported that TB culture is a potentially sensitive and cost-effective tool for HIV positive TB patients in resource-constrained settings, but integration with existing clinical systems and strengthening of post-analytical processes are required to maximize their impact.

The current study indicated TB to significantly affect males than females. It also revealed the youth to bear the greatest TB burden, constituting 39.3% of the cases. These results compare well with the DLTLD annual reports of 2007, 2008, and 2009, where the 25-34 year age bracket had the highest TB notification. In this age-group, male cases were close to 360 per 100,000 population, and females close to 230 per 100,000 population, giving an average of 295 per 100,000 population (DLTLD, 2007; DLTLD, 2008; DLTLD; 2009).

Children below 15 years constituted only 4.5% of the population in the present study, and 43.6% of them were diagnosed with TB. However, this could be under-estimation

for TB diagnosis in children is difficult using currently available routine methods (Hatherill *et al*., 2010). Up to 95% of children aged less than 12 years are often smear-negative and rarely culture positive. Most young children also present with non-cavitating pulmonary TB and are unable to expectorate sputum for microscopy, due to inability to generate enough tussive force. Invasive procedures such as gastric and broncho-alveolar lavage cannot be carried out in most peripheral health facilities in resource- poor countries. In the present study, sputum induction was performed in two out of the ten hospitals involved. Additionally, most published data on the burden of childhood TB do not reflect or capture the occurrence of extra-pulmonary TB (20-30% of the case load in some settings) which occurs much more in children than in adults with TB. Thus, the true incidence of TB disease in children is significantly underestimated (Hatherill *et al*., 2010; Marais, *et al*., 2006).

In the present study, children below 15 years of age accounted for 4.9% of the TB cases. However, during this period, the National TB Programme reported TB in children below 15 years to account for about 10% of all the TB cases in Kenya. The lower number of childhood TB cases reported in the present study may be attributed to few children having been enrolled into the study. However, since TB detection rate in Kenya is hardly 50% (by microscopy), and diagnosis in children is complicated, these cases may be far more than the 10%. This means that at least some other 50% of paediatric TB cases are never diagnosed in Kenya and go untreated (NLTP, 2006).

The high TB recurrence observed in the present study compared to 2007, 2008 and 2009 national rates reported by the DLTLD in 2007 (3%) and 2008 (5%) is highly discouraging and deserves major attention. In the DLTLD annual report of 2007, retreatment cases contributed 9% of the TB cases, of which 3% were classified as PTB relapse category. The rest were recurrent smear negative PTB and EPTB (4%), treatment failures (0.1%), and return after default (1.4%). However, this totals to 8.5%, leaving another 0.5% of the TB cases unaccounted for. In the 2008 annual report, the DLTLD reported a smear positive relapse rate of 3% and a combined recurrent smear negative pulmonary TB and extra-pulmonary TB rate of 5%. However, in the 2009 annual report, the DLTLD reports a combined retreatment rate of 14%. The suggestion by the present study that the high rate of TB recurrence may be attributed to re-infection compares well with other studies which have reported proportions of re-infection among recurrent TB cases ranging from 0-33% in low-incidence areas (Bandera *et al*, 2001), 12-75% in medium-incidence areas (Jasmer *et al*., 2004), and 23-75% in high-incidence areas (van Rie *et al*., 1999). However, re-infection is an uncommon cause of recurrent TB in low TB prevalence settings (Burman *et al*., 2009; Jasmer *et al*., 2004).

However, previous studies have shown that recurrent TB develops in about 2-5% of the patients after curative treatment with short-course anti-TB chemotherapy (Weis *et al*, 1994). Among TB patients cured by short-course treatment in trial conditions, up to 7% develop recurrent disease needing retreatment within 1 to 2 years (Mallory *et al*., 2000). In settings with a high TB prevalence, a substantial percentage of recurrent TB cases

may be caused by re-infection with a new strain of *M. tuberculosis* complex (Mallory *et al*., 2000; Verver *et al*., 2005).

In the current study males dominated females in TB recurrence, just like in new TB cases. Similarly, the majority of the TB recurrences were in the 25-44 age-bracket, the most productive segments of society. The high TB recurrence observed in the current study requires further investigation to distinguish re-activations from re-infection as the predominant cause. This may lead to consideration of further intensification of the initial regimen or use of secondary prophylaxis (Mallory *et al*., 2000). The findings of the current study are also indicative of the necessity for evaluation of the performance of directly observed therapy short-course (DOTS) in Kenya. Kenya has been under DOTS since 1998 (DLTLD, 2008). However, it cannot be categorically ascertained that DOTS functions as may be expected on basis of its naming. In western Kenya, the TB patients were provided with drugs to take at home for at least two weeks and adherence was not routinely checked. Therefore, it is unknown what the degree of compliance is. However, the findings of the current study suggest that acquired resistance does not seem to play a major role in TB recurrence in western Kenya. The 27 *M. tuberculosis* complex isolates obtained from the recurrent TB cases and subjected to first-line anti-TB drug susceptibility testing were all susceptible to streptomycin (STR), ethambutol (EMB) and rifampicin (RIF). Only 3 isolates from TB-HIV cases out of the 27 isolates from recurrent TB cases were resistant to isoniazid (INH). It therefore becomes likely that there is a very high rate of exogenous re-infection in Kenya. In order to test this

hypothesis, the DNA fingerprints of the *M. tuberculosis* isolates from the two episodes of TB should be compared. However, if the re-infection rate is truly as high as suggested by this study, it complicates the general assumption that an infection generally protects against subsequent infections and this would reduce the hope that vaccination strategies will ever work. Also re-infections may play a significant role in the recurrence of TB. However, the present study could not determine whether the recurrences were due to endogenous re-activations or exogenous re-infection, or even multiple/super infections since there were no *M. tuberculosis* complex isolates from previous disease episodes of the study candidates available. However, DNA fingerprinting studies in South Africa have reported a higher rate of reactivation (74.8%) versus recent transmission or re-infection (25.2%) (Farnia *et al*., 2008). The DNA fingerprinting of *M. tuberculosis* complex is an excellent tool to address this important issue and it is clear this type of molecular epidemiology becomes highly important in determining whether a recurrent TB episode is due to endogenous reactivation, meaning treatment failure, or exogenous re-infection with a different strain (Fine *et al*., 1999; Lambert *et al*., 2003). Different *M. tuberculosis* complex strains can be differentiated using information on genetic markers and their distribution in the genome. Among persons with recurrent TB, if the isolates from two TB episodes have the same genotype, the second episode is a relapse, defined as an endogenous reactivation; otherwise, it is defined as exogenous re-infection (Barnes *et al*., 2003).

The present study observed a high HIV infection among the youth. This was in

agreement with NASCOP (2009) which reports the scourge to continue to afflict the youth and young adults among the poor. This report (2007 national survey) indicates the national HIV prevalence among the 14-49 age-group to be 7.5%. However, the high overall HIV prevalence (39.1%) reported in present study could be attributed to the study having been done among TB suspects, who are HIV high risk group. The HIV prevalence is higher among TB cases compared to the general population, and is currently the greatest risk factor for the progression of latent TB to active TB, and rapid progression of new TB infection. It is also a potential risk for recurrence of TB (Daley *et al*., 1992; Villarino *et al*., 1992). The present study shows that majority (64%) of the HIV infected cases were in the 25-44 age-group, the most productive group in the community, meaning that HIV/AIDS will continue to adversely affect socio-economic development in Kenya for many years to come, and undermine the small gains in economic development that have been achieved.

Females dominated males in the present study with HIV prevalence of 43.9% compared to a prevalence of 35.2% in males. However, in the same period (2007 survey) the NASCOP (2009) reported HIV prevalence of 3.7%, and 6.7% among males and females, respectively. Stigma associated with HIV infection continues to be a significant impediment to HIV/AIDS control as evidenced by 20.3% of study participants declining to undergo HIV testing in the current study.

During the period of the present study, only 16.9% of the HIV cases were put on

antiretroviral therapy (ART), 65.2% females and 34.8% males, respectively. However, the number of HIV cases needing to be put on ART could not be established in the present study since CD4 cell count levels were not determined. Nationally, however, of the 1.4 million Kenyans infected with HIV, 42.6% (243,000/570,000) of those needing ART were on treatment (National AIDS Control Program, 2009). However, in 2008, only 31% (12,426) of the TB-HIV cases were on ART (DLTLD, 2009), which means that access to ART in Kenya still remains a big challenge.

The present study observed a high TB-HIV co-infection prevalence of 41.8% even though this was slightly lower than those reported by the DLTLD. The 2007, 2008 and 2009 DLTLD annual reports gave TB-HIV co-infection prevalence of 48%, 45% and 44%, respectively. The discrepancy with the current study could be attributed to some TB cases declining to undergo HIV testing, or decreasing HIV infection rate. However, the TB-HIV co-infection rate in the present study was higher than the global prevalence of 14.8% during the same period (WHO, 2007). The youth led in TB-HIV co-infection rate at 41.8%. Males dominated females in the TB-HIV co-infection with 54.5% of the cases. However, findings of the present study indicate that HIV infection does not alter the prevalence of TB among the genders. The findings of the present study were also in agreement with the DLTLD's 2007-2009 annual reports, which indicated the increasing TB burden in Kenya. This has been attributed to the concurrent HIV/AIDS epidemic and presents special challenges.

The present study observed that 4.2% of the mycobacterial disease was attributable to NTM. However, the overall prevalence of the NTM disease was 1.72%, and 26.7% of them having been previously treated for TB. Forty per cent (40%) of the NTM disease cases were co-infected with HIV. However, since NTM disease is reported to be more frequent in high HIV prevalence and low income settings (Buijtels *et al*., 2009), some of the ZN smear positive but culture negative cases in the current study could have been NTM disease cases. Similarly, the low prevalence rates of non-tuberculous mycobacterioses (TB-like syndromes) reported in a few high HIV prevalence countries in Africa may be underestimations. Even though NTM disease is being associated with HIV/AIDS, the present study noted five NTM disease cases without HIV infection, which is in tandem with reports by the American Thoracic Society of NTM disease being encountered with increasing frequency in non-AIDS patients (ATS, 2007). However, most of the data reporting high rates of NTM disease among both HIV and non-HIV/AIDS cases come from developed countries (Buijtels, 2007). In Africa, the contribution of NTM to the clinical problem of TB has so far only been examined at a very low scale. In South Africa for instance, two studies have reported prevalence rates of NTM colonization / infection of 1, 400 and 6,700 per 100,000, respectively (Fourie *et al*., 1980). In Zambia, Buijtels (2007) has reported NTM colonization rate of 9% in the patient population with a disease rate of 2%.

The worrying aspect of the NTM TB-like syndromes in western Kenya is their misdiagnosis as TB. The present study identified 15 NTM disease cases by culture and

speciation that would have undergone the entire anti-TB treatment regimen. The ZN smear microscopy invariably used in the diagnosis of TB does not differentiate between TB and NTM TB-like syndromes. Cases of non-tuberculous mycobacterioses are often put on anti-TB chemotherapy, even though the treatment of NTM disease is not directly analogous to TB treatment. Multi-drug regimes are used for NTM TB-like disease treatment, the cornerstone agents being a newer macrolide (azithromycin, clarithromycin) (Griffith *et al*., 2007), ethambutol, and rifamycin, and require prolonged durations of therapy aimed to facilitate clearance of the mycobacteria and minimize the emergence of drug resistance (ATS, 2007; Griffith *et al*., 2007).

During the period of the present study, 44.3% of ZN smear positive but culture negative cases were put on anti-TB chemotherapy without evidence for TB or NTM disease. However, to guide the diagnosis of pulmonary non-tuberculous mycobacterioses, the bacteriological diagnostic criteria established by the American Thoracic Society (ATS) (2007) can provide support; a single NTM culture from bronchial washing fluid or two positive sputum cultures in a symptomatic patient (Buijtels, 2007; Griffith *et al*., 2007). The present study observed these criteria, in which NTM disease was diagnosed based on at least two positive cultures from two separate expectorated sputa from symptomatic patients with chest pain, with or without ZN smear positivity.

While tremendous progress has been made in TB and TB-like syndromes diagnostics in developed countries, techniques for the diagnosis of these diseases have remained

relatively unchanged (ZN smear microscopy) in Kenya and other resource-poor countries. The level of sophistication and cost associated with the new and more sensitive techniques has made their general applicability unfeasible in developing countries (Buijtels, 2007), where the basis for TB diagnosis has continued to be ZN smear microscopy to visualize acid-fast bacilli (AFB), which may capture 50-60% of the cases when done by experienced technicians. However, in most low-income countries, much lower rates of case detection are achieved due to poor quality microscopes, heavy workload and shortage of trained staff. Moreover, ZN smear microscopy is usually negative in advanced disease among HIV/AIDS co-infected patients and extra-pulmonary TB cases. The proportion of cases detected by microscopy in low-income countries is often as low as 20-30% of all cases (Buijtels, 2007; Corbett *et al*., 2003).

In the developed world, molecular detection and speciation of mycobacteria are currently the corner stone of the laboratory diagnosis of TB and NTM disease. Commercial DNA probes (AMPLICOR nucleic acid amplification test – Accuprobe) and the Gene – Probe Amplified *Mycobacterium tuberculosis* Direct Test (MTD) have been available in the developed world for sometime. These tests are based on species-specific DNA probes that hybridize with ribosomal RNA (rRNA) released from the mycobacteria (Buijtels, 2007). The other methods (INNO-LiPA Mycobacteria and the Hain's GenoType Mycobacterium CM/AS) are based on reverse hybridization, in which the mycobacteria 16S-23S internal transcribed spacer region or the 23S gene region are

amplified by polymerase chain reaction (PCR), and the amplicons are subsequently hybridized with probes for several mycobacterial species on membrane strips (Buijtels, 2007; Soini *et al*., 1994).

The present study observed 6.5% of the *M. tuberculosis* complex isolates to be mono-drug resistant to either to isoniazid (75%) or rifampicin (25%). However, this is unlike the 2008 and 2009 annual reports which report MDR-TB cases. In 2008 the DLTLD reported 1.82% (102/5604) of *M. tuberculosis* complex isolates examined to be resistant multi-drug resistant. In 2009, 2.3% (150/6569) of the TB cases reported by the DLTLD had MDR strains of *M. tuberculosis* complex, with one XDR-TB case isolated and initiated on treatment at the Moi Teaching and Referral Hospital (MTRH), Eldoret. At the same time, the WHO (2009) reported global X/MDR-TB cases to be 5.1% of the total cases. The plausible explanation for the much lower resistance levels and no MDR-TB cases observed in the present study compared to the DLTLD report is that the latter deals with retreatment cases (relapses and treatment failures) country wide. However, recurrences can be either relapses (endogenous reactivation), or exogenous re-infections. A relapse (R) is defined as a smear-positive TB patient who has previously been treated and declared cured. Treatment failure (TF) is a patient with a positive smear at the end of five months despite being on anti-TB treatment, hence failing to respond to treatment (IUATLD, 1996). The probability of anti-TB drug resistance is higher in relapses and treatment failures. However, there is a small relapse rate associated with all treatment regimens, even if the treatment has been taken

religiously with 100% compliance (the standard regimen 2HREZ/4HR has a relapse rate of 2 to 3%). The majority of relapses occur within 12 months of completing treatment (Dholakia *et al*., 2000).

However, the findings of the present study compare well with a previous study in Kenya by Githui *et al*. (1998) which reported a national anti-mycobacterials resistance rate of 6.3% among isolates from TB cases without history of prior chemotherapy, and a resistance rate of 37% among those previously treated. However, in another study done three years later on refugee and non-refugee populations in North Eastern Kenya, Githui *et al*. (2000) reported 18% resistance to one or more drugs, and an MDR-TB rate of 2.9% among the isolates. The resistance rate among the neighbouring non-refugee populations was 5.7%, with no MDR-TB causing strains observed.

The present study observes that the drug isoniazid (INH) to be loosing ground in the fight against TB. Among the mono-drug resistant *M. tuberculosis* complex isolates observed in the present study, 75% were resistant to INH. Although INH resistance in general is a negative risk factor for transmission of TB (van Doorn *et al*., 2006)**,** and the virulence of most INH resistant strains may be reduced, multi-drug-resistant *M. tuberculosis* complex strains can be as infectious and virulent as drug-susceptible strains (Devaux *et al*., 2009), and the clinical presentation of MDR-TB is similar to that of drug susceptible TB. However, MDR-TB is difficult to treat and associated with a poor prognosis (Mitchison and Nunn, 1986). Even though drug resistant strains of *M.*

tuberculosis complex isolated in the present study constituted only 6.5%, the majority (66.7%) were from TB-HIV cases, suggesting a positive correlation between HIV co-infection and anti-TB drug resistance.

CHAPTER SIX

6.0   CONCLUSIONS AND RECOMMENDATIONS

6.1   Conclusions

1.   Ziehl Neelsen smear microscopy was inaccurate in TB diagnosis among HIV infected patients compared to culture.

2.   Tuberculosis and HIV prevalence were high in western Kenya.

3.   Tuberculosis recurrence rate was high in western Kenya.

4.   Tuberculosis and human immunodeficiency virus co-infection rate was high in western Kenya.

5.   Non-tuberculous mycobacteria played a significant role in causing tuberculosis-like disease which was misdiagnosed as tuberculosis.

6.   Anti-TB drug resistance was more among *M. tuberculosis* isolates from HIV infected patients.

6.2   Recommendations

1. A more accurate diagnostic technique to augment ZN smear microscopy is needed to improve TB diagnosis among HIV/AIDS patients in Kenya. There is need to explore

new approaches to childhood TB diagnosis in order to increase case detection rate

2.   The pervasiveness of TB and HIV/AIDS in western Kenya underscores the need for more efforts and resources to increase knowledge and access TB-HIV/AIDS healthcare.

3.   The high TB recurrence rate observed in this study calls for studies to determine the proportions of the disease attributable to endogenous re-activation (relapse) and exogenous re-infection.

4.   There is need to strengthen TB and HIV/AIDS collaborative activities to reduce morbidity and mortality associated with HIV infection among TB-HIV/AIDS patients.

5.   A more accurate diagnostic technique and robust scoring system and algorithms for NTM disease need to be developed in order to improve NTM disease diagnosis in Kenya. Large case-control studies are imperative to identify risk factors and determine the contribution of NTM to TB-like disease among HIV/AIDS cases in Kenya.

6.   Since no MDR-TB was observed in this study, continued use and surveillance of resistance trends to the first-line anti-TB drugs would be prudent.

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APPENDICES

Appendix 1: Consent Form

1.0 **Title of the project proposal (SSC No. 837; FAN No. 00092)**

Tuberculosis and Human Immunodeficiency Virus Dual Infections in Western Kenya

Patient's name:…………………………………………………………………………….

Date of birth………………… Age………………….Sex…………………………

Address……………………………………………………………………………...

Telephone……………………Fax…………………..E-mail………………………

2.0 **Purpose of the study**

To evaluate TB, HIV and TB-HIV co-infection rates, and determine the effect of HIV co-infection on the sensitivity of ZN smear microscopy and mycobacterial culture positivity. To determine TB recurrence rate and the effect of HIV co-infection on anti-TB chemotherapy. To determine the role of NTM in TB-like disease morbidity in western Kenya.

3.0 **Procedures to be followed**

In this study the blood and sputum samples that will be taken from you for route diagnosis will also be used for TB and HIV co-infection studies and anti-tuberculous drug susceptibility testing. If you accept to join the study, you will be requested to undergo counselling at the VCT clinic, and if you are infected with HIV you will be

referred to the relevant clinic for follow up.

4.0   **Risks**

The study will not expose you to unusual risks, as trained hospital staff using approved methods will collect the samples.

5.0   **Benefits**

The results of sensitivity testing will be delivered to the clinic to guide your doctor in giving you effective drugs.

6.0   **Confidentiality of the Records**

Numbers will identify your samples to avoid breaching your confidentiality. Drug susceptibility results may be submitted to your doctor to guide her/him in treating you. No identity of any specific patient in this study will be disclosed in any public reports or publications.

7.0   **Basis of Participation**

It is important for you to know that you have the freedom to decline to participate in the study and your refusal will not affect the relationship between you and those treating and caring for you.

Signature

I have read the above information and have had an opportunity to ask questions and all of my questions have been answered. I consent to taking part in the study. I fully understand there are no risks associated with the collection of samples. I have been given a copy of this consent form.

Signature_____Date_____

<div align="center">(Patient)</div>

Signature_____Date_____

<div align="center">(Parent or Legal Guardian)</div>

I, the undersigned, have fully explained the relevant details of this study to the patient named above and/or the person authorized to consent for the patient. I am qualified to perform this role.

Signature:_____**Name**: Henry DN Nyamogoba. **Date**_____

<div align="center">(Investigator)</div>

Signature:_____**Name**:_____**Date**:_____

<div align="center">(Witness)</div>

Address of witness: _____

Appendix 2: Sample collection, storage and transportation

The blood and sputum samples were obtained from TB suspects by competent hospital medical staff. The patient were requested to cough so that expectoration will come from deep down the chest as possible, and spit into a sterile blue capped falcon tubes. The samples were refrigerated at 4°C, awaiting transportation in cool boxes to the Mycobacteria Reference Laboratory (MRL), Moi University School of Medicine (MUSOM) Eldoret, weekly for analysis. With each transport box, there was an accompanying list identifying the specimens it contains and the data for patients from whom the specimens were collected. At the MRL, the samples were refrigerated at 4°C till processing. However, the samples were processed within the next 7 days of collection in order to minimize loss of viability of the mycobacteria.

Consenting patient also underwent phlebotomy to obtain 2-3 ml of venous blood using sterile 5-ml syringes. The blood was delivered into Vacutainer Brand STERILE interior EDTA (K3) tubes and stored at –20°C awaiting transportation to Moi University School of Medicine for processing. The samples were transported in cool boxes to MRL, MUSOM and processed within two weeks.

Appendix 3: Testing for human immunodeficiency virus infection

Testing for HIV infection was done by screening serum by the Trinity Biotech Uni-Gold[TM] test and the positives confirmed with the enzyme linked immunosorbent assay (ELISA) (Murex HIV Ag/Ab Combination, ABBOT murex, Murex Biotech Limited, UK), following manufacturers' instructions.

ELISA procedure

Briefly the Conjugate was reconstituted, and substrate and Wash Fluid prepared following manufacturer's instructions. A volume of 25 µl of Sample Diluent was added to each well. A volume of 100 µl of Samples or 100 µl of Controls were added to designated wells. The wells were covered with the lid and incubated for 60 minutes at 37°C. After incubation, the plate was washed followed by immediate addition of 100 µl of Conjugate to each well. The wells were covered with the lid and the plate incubated at 37°C for 30 minutes. At the end of incubation time, the plate was washed, followed by immediate addition of 100 µl of Substrate Solution to each well. The wells were covered with the lid and the plate incubated at 37°C for 30 minutes, away from direct sunlight. After incubation, 50 µl of Stop Solution (0.5M sulphuric acid) was added to each well. Absorbance was read within 15 minutes at 420 nm.

After calculation of the mean absorbance of the Negative Controls, the Cut-off value was calculated by adding 0.150 to the mean of the Negative Controls.

Interpretation of results: Samples giving an absorbance less than the Cut-off value were considered negative (non-reactive), whereas samples giving an absorbance equal to or greater than the Cut-off value were considered reactive.

Appendix 4: Isolation of mycobacteria

The BACTEC MGIT 960 tubes and Lowenstein-Jensen (LJ) slopes were used in the isolation of mycobacteria. The sputum samples were decontaminated and concentrated using the NaOH-NALC method.

Reagents and supplies

a) N-Acetyl L Cysteine (NALC)

Aliquoted in 2- gram amounts. Store at 2-8°C. This is mucolytic agent. Once dissolved it is unstable when exposed to air. It loses its mucolytic action within 24 hours.

b) 4% Sodium hydroxide (**NaOH**) **(Final concentration 1% for decontamination)**

Aliquoted in sterile 200ml amounts into plastic bottles and stored at 2 – 8°C. NaOH is toxic (bactericidal) at 2%. Mycobacteria will also be killed if exposed to NaOH beyond the time stipulated in the decontamination process.

c) 5% Sodium hydroxide (**NaOH**) **(Final concentration 1.25% for decontamination)**

This is according to the BD protocol for MGIT Culture - used incase of increased contamination.

Aliquoted in sterile 200ml amounts into plastic bottles and stored at 2 – 8°C. NaOH is toxic (bactericidal) at 2%. Mycobacteria will also be killed if exposed to NaOH beyond

the time stipulated in the decontamination process.

d) Sodium Citrate

Aliquoted in sterile 200ml amount, stored at room temperature. Exerts stabilizing effect on the acetyl cysteine because of its ability to bind by chelation of many heavy metal ions that may be present in the specimen.

e) Phosphate Buffer pH 6.8

Aliquoted in sterile 1000ml amounts into plastic bottles and stored at room temperature. Stock solution stored at $4^{\circ}C$ in cold room. Stabilizes pH.

f) Sterile broth Buffer 18ml prepared as follows

A volume of 2ml of 7H9 Broth added to 18ml Phosphate Buffer pH 6.8. This gives approx 0.5% albumen concentration. Dilutes out the effect of the decontamination process. Allows specimen to adhere to the slides (in house method).

Procedure for the decontamination of specimens

Note: All specimens had been collected in sterile 50ml centrifuge tubes marked with patient information.

1. Equal amounts of NALC working solution was added to each specimen. The contents were vortexed for 15 seconds and then mixed. Another vortexing for another

15 seconds followed to ensure that the specimen got liquefied. In case the specimen was still mucoid, some more NALC was added followed by vortexing.

2. After 20 minutes, the buffer was added to 50ml mark of specimen tube. The tube was inverted to mix the contents – this is the neutralizing process.

3. The same buffer was added to the other specimen tubes at 1-minute intervals.

4. The tubes were centrifuged in IEC CENTRA CL3 Centrifuge at 3000g for 20 minutes.

5. The supernatant was decanted into 5% phenol disinfectant bucket.

6. The sediment was re-suspended in buffer broth to a final volume of 2 ml.

7. The tubes were vortexed.

8. Slides were prepared from each of the sediments as follows:

Using a sterile pipette the specimen was spread centrally on the slide: smear size 1 x 2cm. The slides were placed on hot plate (thermostat set at 70°C) under UV light for a minimum of 30 minutes. UV kills aerosols created by the heating of slides.

9. The slides were stained by the Ziehl- Neelsen Stain and examined for AFB.

10. The decontaminated samples were then cultured onto Lowenstein-Jensen (LJ) slopes / slants and BACTEC MGIT 960 incubator.

Inoculation procedures

Note: The Lowenstein-Jensen (LJ) medium slopes were always inoculated before the MGIT tubes.

Inoculation of the LJ slopes

1. The LJ slopes were inoculated with 0.2 – 0.3 ml (2-3 drops) of the decontaminated sputum deposits. The slopes were tilted slightly to cover the entire surface.

2. The slants were then laid out horizontally, with inoculated surface facing upward, for at least 24-48 hours, to allow inoculum to absorb into media, after which they were placed upright in the incubator at 37°C.

3. The caps were left loosened, for at least the first week, to allow for air exchange, and then screwed tight to prevent desiccation.

4. The inoculated LJ slopes were read weekly for 8 weeks. Reading was done the same day each week.

Reporting Scheme:

Colony count	Report
0	No growth
1-9	Actual number
20-100	1+
101-200	2+
201-500 (almost confluent)	3+
>500 (confluent)	4+

Inoculation of BACTEC MGIT 960 tubes

1. The MGIT tubes were labeled with the relevant patient details, randomization number and specimen or control name.

2. A volume of 0.8ml of reconstituted MGIT PANTA was added to each MGIT tube that was to be inoculated using a sterile Gilson pipette tip.

3. Once PANTA had been added the tubes were covered as they are light sensitive.

4. Using a sterile pipette tip 0.5ml of well mixed concentrated specimen was added to the appropriately labeled MGIT tube. The tube was immediately recapped tightly and contents mixed by reverting the tube several times.

5. The tubes and caps were wiped with 5% phenol disinfectant and left at room

temperature for 30 minutes.

6. The tubes were then incubated in the MGIT incubator until the instrument flagged them positive. After a maximum of 42 days, the instrument flagged the tubes negative if there were no growth. (The instrument signals a tube positive for growth, and an indicator green light shows the exact location of the positive tube in the drawer of the instrument).

Work-up of positive cultures

The instrument positive MGIT tubes were observed visually for granular growth.

1. Slides were labeled for each positive MGIT tube as well as a blood agar plate, with the patient details and laboratory number.

2. The MGIT tubes were mixed by vortexing. Using sterile Pasteur pipettes, 1 drop was placed on the slide.

3. The slides were left on a hot plate under a UV light for a minimum of 2 hours. The slides were then stained following ZN method and examined for AFB using 100 X oil immersion lens.

Reading of the ZN stain from a positive MGIT

All the laboratory numbers and patient details were recorded in the MGIT ZN book as follows:

Pos R   =   positive roping

Pos NR   =   No Roping? MOTT.

Pos/C   =   positive mixed with contaminating bacteria.

Neg   =   negative – no organisms seen.

The results were also recorded on the MGIT print out on the right hand side of the page.

Pure positive cultures underwent identification procedures, followed by anti-TB drug susceptibility testing as appropriate.

Work-up of contaminated positive cultures

Contaminated positive culture underwent re-decontamination.

1. The entire MGIT broth was transferred into a 50 ml centrifuge tube.

2. An equal quantity of 4% sterile NaOH solution was added, and the contents mixed and left to stand for 15-20 minutes.

3. Phosphate buffer pH 6.8 was added after 15-20 minutes up to 40ml mark. The

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contents were mixed by inverting the tubes severally.

4. The tube contents were centrifuged at least at 3000x g for 15-20 minutes.

5. The supernatant was poured off, and the sediment re-suspended in 0.5 ml of buffer and mixed well.

6. A volume of 0.5 ml was inoculated into a fresh MGIT tube supplemented with MGIT growth supplement/PANTA.

7. The inoculated tubes were left at room temperature for 30 minutes, and then placed in the instrument and followed for observation of growth. Positive cultures were worked-up as previously described (**BD BBL** MGIT Package inserts, 2008).

Appendix 5: Identification of mycobacteria

Cultures with positive growth underwent definitive ZN stain for AFB. They also underwent Hain's **GenoType® Mycobacterium CM** ("Common Mycobacteria") and **GenoType® Mycobacterium AS** ("Additional Species") molecular genetic assays for detection and discrimination of 13 clinically relevant non-tuberculous mycobacterial species and of *M. tuberculosis* complex as a whole, and to distinguish 16 other non-tuberculous species, respectively.

The **GenoType® Mycobacterium CM** permits the identification of the following mycobacterial species: -
   M. simiae
   M. avium spp
   M. Chelonae
   M. fortuitum
   M. abscessus
   M. gordonae
   M. intercellulare
   M. scofulceum
   M. interjectum
   M. kansasii
   M. malmoense
   M. peregrinum
   M. marinum/ulcerans
   M. tuberculosis complex
   M. xenopi

The **GenoType® Mycobacterium AS** permits the identification of the following mycobacterial species: -
   M. simiae
   M. mucogenicum
   M. goodii
   M. celatum
   M. smegmatis
   M. genavense
   M. lentiflavum
   M. heckeshornense

M. szulgai/intermedium
M. phlei
M. haemphilum
M. kansasii
M. ulcerans
M. gastri
M. asiaticum
M .shimoidei

The **GenoType**® **Mycobacterium CM** and **GenoType**® **Mycobacterium AS protocols are the same the only difference being** the specific probes coated in the membrane strips (STRIPS). The whole procedure was divided into three steps.

i). DNA isolation from cultured material (culture plates/liquid medium) or direct materials (pulmonary, smear – positive, decontaminated).

ii). Multiplex amplification with biotinylated primers.

iii). Reverse hybridization.

The hybridization included the following steps:

i). Chemical denaturation of the amplification products,

ii). Hybridization of the single stranded DNA,

iii). Biotin – labeled amplicons to membrane – bound probes,

iv). Stringent washing,

v). Addition of s streptavidin/alkaline phosphatase conjugate, and an alkaline mediated staining reaction,

vi). A template ensured the easy and fast interpretation of the banding pattern obtained.

DNA Isolation

Bacterial growth on LJ or MGIT tubes was used. Samples were heated in a water bath to 95 ^{0}C for at least 20 minutes in order to inactivate bacteria. The following quick protocol was used to obtain DNA suitable for amplification:

1 (a). When using bacteria grown on LJ, bacteria were harvested with an inoculation loop and suspended in approximately 300 µl of water (molecular biology grade).

1 (b). When using bacteria grown in MGIT tubes, 1 ml of culture growth was directly used. The bacteria were pelleted by spinning for 15 min in a standard table top centrifuge with an aerosol-tight rotor in a class II safety cabinet at approximately 10000 x g. The supernatant was discarded and the pellet (bacteria) re-suspended in 300 µl of water by vortexing.

2. Isolated bacteria from 1a or 1b were incubated for 20 min at 95^{0}C (heating block or boiling water bath).

3. The bacteria were then incubated for 15 min in an ultrasonic bath.

4. The bacteria were spin down for 5 minutes at full speed.   A volume of 5 µl of the supernatant was directly used for PCR. In case DNA solution needed to be stored for an extended time period, the supernatant was transferred to a new tube and stored at -20

°C until use.

The amplification mix (45 µl) was prepared in a DNA-free room (the DNA sample was added in the separated area) as follows:

Per tube mix

35 µl Primer/Nucleotide Mix (PNM)

5 µl 10x polymerase incubation buffer

2.0 µl $MgCl_2$ solution

0.2 µl (2 units) thermostable DNA polymerase

3.0 µl water to obtain a volume of 45 µl (not considering volume of enzyme)

5µl DNA solution (20-100 ng DNA) was added leading to a final volume 50 µl (not considering volume of enzyme).

A negative control sample contained 5 µl of water instead of DNA solution. A master-mix containing all reagents except for DNA solution was prepared and mixed well by pipetting up and down. A volume of 45 µl of the master-mix was aliquoted into each of the prepared PCR tubes.

Preparation of master-mix for polymerase chain reaction

1.	Number of specimen	1	3	5	6	7	12	13	14	24	25
2.	PNM (μl)	35	105	175	210	245	420	455	490	840	875
3.	$MgCl_2$ (μl)	2	6	10	12	14	24	26	28	48	50
4.	Buffer (μl)	5	15	25	30	35	60	65	70	120	125
5.	Molecular grade water (μl)	3	9	15	18	21	36	39	42	72	75
6.	Taq (μl)	0.2	0.6	1.0	1.2	1.4	2.4	2.6	2.8	4.8	5.0

Amplification profile

5 min 95°C   1 cycle

30 sec 95°C

                      10 cycles

2 min 58°C

25 sec 95°C

40 sec 53°C   20 cycles

40 sec 70°C

8 min 70°C   1 cycle

Hybridization

Preparation

The shaking water bath/**TwinCubator® was pre-warmed to 45°C;** the maximum tolerated deviation from the target temperature was +/-1°C. Solutions HYB and STR were also pre-warmed to 37-45°C before use. The reagents were free from precipitates (however, solution CON-D is opaque). Mixing was done when necessary. The remaining reagents were pre-warmed with the exception of CON-C and SUB-C to room temperature. Using a 15 ml falcon tube, Conjugate Concentrate (CON-C, orange) and Substrate Concentrate (SUB-C, yellow) were diluted 1:100 with the respective buffer **(CON-C with CON-D, SUB-C with SUB-D)** in the amounts needed. The contents were mixed to bring to room temperature. For each strip, 10 µl concentrate was added to 1 ml of the respective buffer. CON-C was diluted before each use. (Diluted SUB-C is stable for 4 weeks if stored at room temperature and protected from light).

1. A volume of 20 µl of Denaturation Solution (DEN, blue) was dispensed in a corner of each of the wells used.

2. A volume of 20 µl of amplified sample was added to the solution, pipetted up and down to mix well and incubated at room temperature for 5 minutes.

Meanwhile, strips were taken out of the tube using tweezers and marked with a pencil underneath the coloured marker. Gloves were always won when handling strips.

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3.   A volume of 1 ml of pre-warmed Hybridization Buffer (HYB, green) was carefully added to each well. The tray was gently shaken until the solution had a homogenous colour. Care was taken not to spill solution into the neighbouring wells.

4. A strip was paced in each well.

 The strips were completely covered by the solution and the coated side (identifiable by the coloured marker near the lower end) faced upward. Strips which turned when immersed in the solution were turned over using tweezers. Tweezers were carefully cleaned after each use to avoid contamination. This also applied to all following steps.

5. The tray was placed in shaking water bath/TwinCubator$^{®}$ and incubated for 30 minutes at 45^{0}C.   The shaking frequency of the water bath was adjusted to achieve a constant and thorough mixing of the solution. To allow adequate heat transfer, the tray was dipped into the water to at least one-third ($1⁄3$) of its height.

6. The Hybridization Buffer was completely aspirated using a Pasteur pipette connected to a vacuum pump.

7. A volume of 1 ml of Stringent Wash Solution (STR, red) was added to each strip and incubated for 15 minutes at 45^{0}C in shaking water bath/TwinCubator$^{®}$.

8. Stringent Wash Solution was completely removed.

9. The Wash Solution was poured out into a waste container. All the remaining fluid was poured out by turning the tray upside down and gently striking it on an absorbent paper. This also applied to all other wash steps.

10. Each strip was washed once with 1 ml of Rinse Solution (RIN) for 1 minute on shaking platform/TwinCubator® (pour out RIN after incubation).

11. A volume of 1 ml of diluted Conjugate was added to each strip and incubated for 30 minutes on shaking platform/TwinCubator®.

12. The solution was removed and each strip washed twice for 1 minute with 1 ml of Rinse Solution (RIN) and once for 1 minute with 1 ml of distilled water (e.g. use wash bottle) on shaking platform/TwinCubator® (the solution was poured out each time). Any trace of water was removed after the last wash.

13. A volume of 1 ml diluted substrate was added to each strip and incubated for 15 minutes (protected from light without shaking).

14. The reaction was stopped by briefly rinsing twice with distilled water.

15. The strips were removed from the tray using tweezers and dried between two layers of absorbent paper.

Evaluation and interpretation of results

The strips were pasted and stored protected from light. Evaluation of results was done using an evaluation sheet provided by the manufacturer (Hain lifescience, GmbH, Nehren, German, Package inserts) of the **GenoType® Mycobacterium CM** Kit.

When doing evaluation, the developed strips were pasted in the designated fields by aligning the bands CC and UC with the respective lines on the evaluation sheet.

Positive signals were noted in the last but one column. Species were determined with the help of the interpretation chart. The name of the identified species was entered in the last column.

The supplied template also served as an aid for evaluation was aligned with the bands CC and UC of the strip as well. Each strip had a total of 17 reaction zones as shown below:

Conjugate Control (CC)

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

Universal Control (UC)

This zone detected, as known, all known mycobacteria and members of the group of gram-positive bacteria with a high G+C content. It is only when this zone and the

Conjugate Control zone stained positive that the remaining banding pattern could be assigned respective bacterial species.

Only those bands whose intensities were about as or stronger than that of the Universal Control zone were considered.

Genus Control (GC)

Staining of this zone documented, as known, the presence of a member of genus *Mycobacterium*. The intensity of this band varied depending on the mycobacterial species.

The Genus Control band could drop out in spite of the presence of mycobacterial DNA; as long as a species – specific banding pattern was present, however, the amplification reaction was performed properly and the test result was valid.

When no species-specific banding pattern was present, a pattern indicating the presence of a gram-positive bacterium with a high G+C content could still originate from a mycobacterium that could not be detected by the **GenoType® Mycobacterium CM** kit. In this case the DNA of the isolate underwent identification using the **GenoType® Mycobacterium AS** Kit.

NB: Not all bands of a strip had to show the same signal strength. If a large amount of amplicon was used, additional bands could occur.

Appendix 6: GenoType® MTBDR*plus* anti-tuberculosis drug susceptibility testing

The GenoType® MTBDR*plus* test is based on the DNA•STRIP® technology and permits the molecular genetic identification of *M. tuberculosis* complex and its resistance to rifampicin and / or isoniazid from cultivated or pulmonary smear - positive direct patient material. DNA•STRIP® strips are coated with specific probes which are complementary to the amplified nucleic acid (amplicon). After denaturation, the single-stranded amplicon binds specifically to the probe (hybridization) and is visualized in a subsequent enzymatic colour reaction. As a result, a specific banding pattern develops on the DNA•STRIP®. The interpretation of banding patterns on the DNA•STRIP® is performed by employing the standard template provided by the manufacturer.

In order to validate the correct performance of the test and the proper functioning of reagents, each strip includes 5 control zones:

(i) A Conjugate Control zone to check the binding of conjugate on the strip and a correct chromogenic reaction,

(ii) Amplification Control zone to check for successful amplification reaction,

(iii) Three Loci Control zones (*rpo B*, *katG*, and *inhA*) checking the optimal sensitivity of the reaction for each of the tested gene loci.

DNA isolation

Bacteria grown on culture Lowenstein-Jensen (LJ) slants or MGIT tubes as well as decontaminated smear-positive direct sputum samples were used. The bacteria were heated to 95-105°C for at least 15 minutes to lyse cells and to inactivate vegetative bacteria.

The following quick protocol was used isolated DNA for amplification:

1a. Bacterial growth on LJ was collected using an inoculation loop and suspended in 300 µl of water (molecular biology grade).

1b. A volume of 1 ml of bacterial growth from MGIT tube cultures was directly used. When using direct patient material, 500 µl of a decontaminated sample was applied. Bacteria were pelleted by spinning for 15 minutes in a centrifuge with an aerosol-tight rotor in a class II safety cabinet at 10000 x g. The supernatant was discarded and the bacteria re-suspended in 300 µl of water (for culture samples), or 100 µl of water (for direct patient material) by vortexing.

2. The bacteria from 1a or 1b were incubated for 20 minutes at 95°C (boiling water bath).

3. The bacteria were then incubated for 15 minutes in an ultrasonic bath.

4. The bacteria were spin down for 5 minutes at full speed and 5 µl of the supernatant was directly used for PCR. When the DNA was not immediately used, the supernatant was transferred new tube for storage.

Amplification

The amplification mix (45 µl) was prepared in a DNA-free room. The DNA sample was added in a separated area.

Per tube mix:

 35 µl PNM

 5 µl 10x polymerase incubation buffer

 2.0 µl $MgCl_2$ solution

 0.2 µl (0.2 units) thermostable DNA polymerase

 3.0 µl water to obtain a volume of 45 µl (not considering volume of enzyme)

 5 µl DNA solution (20-100 ng DNA) was added leading to a final volume of 50 µl (not considering volume of enzyme).

A negative control sample contained 5 µl of water instead of DNA solution. A master mix containing all reagents except for DNA solution was prepared and mixed well by pipetting up and down. A volume of 45 µl of the master-mix was aliquoted into each of the prepared PCR tubes.

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	Culture samples	direct patient material
5 min 95°C	1 cycle	1 cycle
30 sec 95°C 2 min 58°C	10 cycles	10 cycles
25 sec 95°C 40 sec 53°C 40 sec 70°C	20 cycles	30 cycles
8 min 70°C	1 cycle	1 cycle

For checking the amplification reaction, 5 µl of each sample was directly applied to a 2% agarose gel without the addition of loading buffer. The amplicons had a length of approximately 63 bp (Amplification Control), 115 bp (*M. tuberculosis* complex), 166 bp (rpoB), 120 bp (*katG*), and 110 bp (*inhA*), respectively.

Hybridization

Preparation

The shaking water bath/TwinCubator® was pre-warmed to 45°C; the maximum tolerated deviation from the target temperature was +/–1°C. The solutions HYB and

STR ware also pre-warmed to 37-45°C before use. The reagents were free from precipitates (however, solution CON-D is opaque). Mixing was done when necessary. The remaining reagents were also warmed (with the exception of CON-C and SUB-C) to room temperature. Using a 15 ml falcon tube, the Conjugate Concentrate (CON-C, orange) and Substrate Concentrate (SUB-C, yellow) were diluted 1:100 with the respective buffer (CON-C with CON-D, SUB-C with SUB-D) in the amounts needed. Mixing was done and the contents brought to room temperature.

For each strip, 10 µl concentrate was added to 1 ml of the respective buffer. The CON-C was diluted before each use. (Note: Diluted SUB-C is stable for 4 weeks if stored at room temperature and protected from light).

1. A volume of 20 µl of Denaturation Solution (DEN, blue) was dispensed in a corner of each of the wells used.

2. A volume of 20 µl of amplified sample was added to the solution. The contents were pipetted up and down to mix well. This was followed with incubation at room temperature for 5 minutes.

Meanwhile, strips were taken out of the tube using tweezers and marked with a pencil underneath the coloured marker. Gloves were always won when handling strips.

3.   A volume of 1 ml of pre-warmed Hybridization Buffer (HYB, green) was carefully added to each well. The tray was gently shaken until the solution had a homogenous colour. Care was taken not to spill solution into the neighbouring wells.

4. A strip was paced in each well.

The strips were completely covered by the solution and the coated side (identifiable by the coloured marker near the lower end) faced upward. Strips which turned when immersed in the solution were turned over using tweezers. Tweezers were carefully cleaned after each use to avoid contamination. This also applied to all following steps.

5. The tray was placed in shaking water bath/TwinCubator® and incubate for 30 minutes at 45 °C.

The shaking frequency of the water bath was adjusted to achieve a constant and thorough mixing of the solution. To allow adequate heat transfer, the tray must be dipped into the water to at least 1/3 of its height.

6.   The Hybridization Buffer was completely aspirated using a Pasteur pipette connected to a vacuum pump.

7. A volume of 1 ml of Stringent Wash Solution (STR, red) was added to each strip and incubated for 15 minutes at 45^0C in shaking water bath/TwinCubator®.

8. Stringent Wash Solution was completely removed.

9. The Wash Solution was poured out into a waste container. All the remaining fluid was poured out by turning the tray upside down and gently striking it on an absorbent paper. This also applied to all other wash steps.

10. Each strip was washed once with 1 ml of Rinse Solution (RIN) for 1 minute on shaking platform/TwinCubator® (pour out RIN after incubation).

11. A volume of 1 ml of diluted Conjugate was added to each strip and incubated for 30 minutes on shaking platform/TwinCubator®.

12. The solution was removed and each strip washed twice for 1 minute with 1 ml of Rinse Solution (RIN) and once for 1 minute with 1 ml of distilled water (e.g. use wash bottle) on shaking platform/TwinCubator® (the solution was poured out each time). Any trace of water was removed after the last wash.

13. A volume of 1 ml diluted substrate was added to each strip and incubated for 15 minutes, protected from light without shaking.

14. The reaction was stopped by briefly rinsing twice with distilled water.

15. The strips were removed from the tray using tweezers and dried between two layers of absorbent paper.

Evaluation and interpretation of results

The strips were pasted and stored protected from light. Evaluation of results was done using an evaluation sheet provided by the manufacturer (Hain lifescience, GmbH, Nehren, German, Package inserts, 2008) of the **GenoType® MTBDR*plus*** kit

When using the evaluation sheet the developed strips were pasted in the designated fields by aligning the bands CC and AC with the respective lines on the sheet. The resistance status was determined and noted down in the respective column. The supplied template also served as an aid for evaluation and was aligned with the bands CC and AC of the strip as well. Each strip had a total of 27 reaction zones. However, not all bands of a strip had to show the same signal strength.

Conjugate Control (CC)

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

Amplification Control (AC)

When the test is performed correctly, a control amplicon generated during amplification will bind to the Amplification Control zone on the strip. A missing band therefore

indicates mistakes during amplification set-up or the carry-over of amplification inhibitors with the isolated DNA. In case of a positive test result, the signal of the Amplification Control zone can be weak. In this case, however, the amplification reaction was performed correctly and the test does not have to be repeated.

M. tuberculosis complex (TUB)

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

Locus Controls (*rpoB, katG, and inhA*)

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

Wild type probes

The wild type probes comprise the most important resistance areas of the respective genes (Appendices 7, 8, and 9). When all wild type probes of a gene stain positive, there is no detectable mutation within the examined regions. The strain tested is sensitive for the respective antibiotic. In case of a mutation, the respective amplicon cannot bind to the corresponding wild type probe. The absence of a signal for at least one of the wild type probes hence indicates a resistance of the tested strain to the respective antibiotic.

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Only those bands whose intensities were about as strong as or stronger than that of the Amplification Control zone were considered. Each pattern that deviated from the wild type pattern indicated resistance of the tested strain. The banding pattern obtained with the *rpoB* probes allowed to draw a conclusion about a rifampicin resistance of the strain tested; the banding pattern obtained with the *katG* probes allowed to draw a conclusion about a high level isoniazid resistance; the banding pattern obtained with the *inhA* probes allowed to draw a conclusion about a low level isoniazid resistance of the strain tested, respectively. (Appendix 7 below shows rifampicin resistance region of the *rpoB* gene).

Mutation probes

The mutation probes detect some of the most common resistance mediating mutations (Appendices 7, 8 and 9). Compared to the other probes, positive signals of the mutation probes *rpoB* MUT2A and MUT2B may show lower signal strength.

Only those bands whose intensities were about as strong as or stronger than that of the Amplification Control zone were considered.

Each pattern that deviated from the wild type pattern indicated resistance of the tested strain. The banding pattern obtained with the *rpoB* probes allowed to draw a conclusion about a rifampicin resistance of the strain tested; the *katG* banding pattern about a high level; and the *inhA* banding pattern allowed conclusion about a low level isoniazid

resistance, respectively.

NB: 1. There was a possibility that the specimen tested contained a heterogeneous strain. If, at investigation, this strain had developed only a partial resistance, one of the mutation probes as well as the corresponding wild type probe could appear.

2. There was a possibility that the tested specimen contained more than one *M. tuberculosis* strain (due to mixed culture or contamination). If at least one of these strains harbored a mutation, one of the mutation probes as well as the corresponding wild type probe could appear.

Appendix 7a: Mutations in the gene *rpoB* and the corresponding wild type and mutation probes (modified according to Telenti *et al*. *Lancet*, 1993; **34**1: 647-650)

Missing wild type probe(s)	analyzed codons	mutation probe		mutation
rpoB WT1	505-509			F505L
				T508A
				S509T
rpoB WT2	510-513			L511P*
rpoB WT2/WT3	510-517			Q513L*
				Q513P
				del514-516
rpoB WT3/WT4	513-519	rpoB MUT1	D516V	
				D516Y
				del515
rpoB WT4/WT5	516-522			del518*
				N518I
rpoB WT5/WT6	518-525			S522L
				S522Q
rpoB WT7	526-529	*rpoB* MUT2A	H526Y	
		rpoB MUT2B	H526D	
				H526R
				H526P*
				H526Q*
				H526N
				H526L
				H526S
				H526C
rpoB WT8	530-533	rpoB MUT3		S531L
				S531P
				S531Q*
				S531W
				L533P

* This rare mutation has only been detected theoretically (*in silico*) yet. It is therefore possible that the mutation cannot be detected *in vitro*.

Appendix 7b: Mutations in the gene *katG* and the corresponding wild type and mutation probes (modified according to Telenti *et al*. *Lancet*, 1993; **34**1: 647-650)

Missing wild type probe(s)	analyzed codons	mutation probe	mutation
katG WT	315	katG MUT1	S315T1
		katG MUT2	S315T2

Appendix 7c: Mutations in the *inhA* promotor region and the corresponding wild type and mutation probes (modified according to Telenti *et al*. *Lancet*, 1993; **34**1: 647-650).

Missing wild type probe	analyzed nucleic acid position	mutation probe	mutation
inhA WT1	−15	inhA MUT1	C15T
	−16	inhA MUT2	A16G
inhA WT2	−8	inhA MUT3A	T8C
		inhA MUT3B	T8A

Appendix 8: BACTEC MGIT 960 anti-tuberculosis drug Susceptibility testing

The BACTEC NGIT 960 susceptibility testing for Streptomycin (STR), Isoniazid (INH), Rifampin (RIF) and Ethambutol (EMB), called SIRE, is a rapid and quantitative procedure for establishing susceptibility of *M. tuberculosis* for the four drugs using critical test concentrations. In addition, higher test concentrations for STR, INH and EMB are also available in case of testing against higher concentrations is indicated.

Reagents

BACTEC MGIT 960 SIRE Kit for critical concentrations contains the following drugs in lyophilized form. Each kit contains one each of STR, INH, RIF and EMB drug vial and 8 vials of MGIT 960 SIRE Supplement.

a). **Drugs**

STR – approximate lyophilized drug per vial ------------- 332 µg

INH – approximate drug per vial -------------------------- 33.2 µg

RIF – approximate drug per vial -------------------------- 332 µg

EMB – approximate drug per vial ------------------------ 1660 µg

b). **SIRE supplement**

The SIRE supplement vial differs from the MGIT Growth Supplement and contains, per liter of purified water, the following:

Bovine albumin ------------------------50.0 g

Dextrose ------------------------------- 20.0 g

Catalase ------------------------------- 0.03 g

Oleic acid ------------------------------- 0.6 g

c). Storage

Upon receipt, the lyophilized drugs were refrigerated at 2-8°C. They were reconstituted prior to use. Once opened and reconstituted, the leftover drug solutions were frozen in aliquots at –20°C or lower and stored for up to 6 months or up to the date of original expiry, whichever came sooner. **Once thawed, the leftover drug was discarded**.

Principle

Isolated cultures from TB patients were subjected to growth in the presence of a known concentration of a test drug. A control was also included with no addition of the drug. If the patient's isolate grew in the control but does not grow in presence of the drug, it was considered susceptible. On the other hand, if it grew in both the tubes, then it was considered to be resistant to that drug; i.e. growth was indicated by the increase in the fluorescence in the sensor measured automatically and designated as growth value (GV). If a drug is added to the medium which is bacteriostatic or bactericidal to the test mycobacteria, it inhibits growth and thus, there is little or no oxygen consumption, therefore little or no fluorescence of the sensor.

Procedures

 a) **Reconstitution of lyophilized drugs**

Each critical concentration drug vial was reconstituted with 4 ml of sterile distilled/deionized water. This was followed by thorough mixing to ensure the drug was completely dissolved.

b) **Addition of a drug to the medium**

A volume of 0.1 ml (100 µL) of reconstituted drug solution was added into each of the labeled BACTEC MGIT 960 tube. This resulted in the following critical concentration of drugs in the medium:

$$STR \text{-----------------------------} 1.0 \text{ µg/ml of medium}$$

$$INH \text{-----------------------------} 0.1 \text{ µg/ml of medium}$$

$$RIF \text{-------------------------------} 1.0 \text{ µg/ml of medium}$$

$$EMB \text{----------------------------} 5.0 \text{ µg/ml of medium}$$

c) **Preparation of the inoculum**

Inoculum from the MGIT tube

It was important to use growth within the following recommended timeframe:

1. The day MGIT tube was positive by the instrument was considered **Day 0**

2. The tube was kept incubated for at least one more day (**Day I**) before being used for the susceptibility testing (incubated in a separate incubator at $37^{o}C\pm1^{o}C$).

3. A positive could be used for drug susceptibility testing up to and including the fifth day (**Day 5**) after becoming instrument positive. A tube that had been positive for more than 5 days was sub-cultured in a fresh MGIT 960 instrument until it was positive. This was used from 1 to 5 days of instrument positivity as previously described.

4. If growth in tube was Day 1 or Day 2, it was well mixed by vortexing to break up clumps. The tube was left undisturbed for about 5-10 minutes to let big clumps settle on the bottom. The supernatant was used undiluted for inoculation of the drug set.

5. If growth was on Day 3, 4, or 5, it was well mixed by vortexing, to break up the clumps. The large clumps were let to settle for 5-10 minutes. Then 1.0 ml of the broth was diluted with 4.0 ml of sterile saline and mixed well. This gave a 1:5 dilution. This well mixed diluted culture was used for inoculation.

Inoculum from Lowenstein-Jensen (LJ) slopes

It was important to have fresh growth on LJ slants (within 15 days of appearance of growth on the medium). Older cultures could result in unreliable susceptibility test results.

1. A volume of 4 ml of BBL Middlebrook 7H9 broth was added to a clean sterile bijou bottle with 8-10 glass beads.

2. As many colonies as possible were scrapped with the help of a sterile loop. Scooping of medium was avoided when removing growth. The growth was transferred into the bijou bottle with broth and glass beads. The cap was tightened and bottle vortexed for 1-2 minutes to break the clumps. The turbidity of the suspension was greater than the McFarland #1.0 standard.

3. The suspension was left to stand undisturbed for 20 minutes. The supernatant suspension was carefully transferred with a sterile pipette into another sterile bijou bottle. Care was taken to avoid taking any growth which had settled at the bottom of the bottle.

4. The bottle was left stand for another 15 minutes undisturbed.

5. The supernatant was carefully taken out with a pipette, without disturbing the sediment, and transferred into another sterile bottle. The turbidity of this suspension was greater than McFarland #0.5 standard.

6. The turbidity of the above suspension was adjusted to McFarland #0.5 standard by adding sterile saline and adjusting by visual comparison with barium sulphate (BaSO$_4$) suspension equivalent to McFarland #0.5 standard.

7. The above suspension was diluted 1:5 by adding 1.0 ml of the suspension to 4.0 ml

of sterile saline. It was well mixed by vortexing and used as the inoculum for drug susceptibility testing.

d) Inoculation and incubation

1. Five (5) MGIT tubes were labeled for each test culture. One was labeled for GC (growth control, without drug), one for STR, one for INH, one for RIF, and one for EMB.

2. A volume of 0.1 ml of BACTEC 960 SIRE Supplement was aseptically added to each of the MGIT tubes.

3. A volume of 0.1 ml (100 μl) of properly reconstituted STR drug was aseptically added in the STR labeled tube using a calibrated micropipette. Similarly, other drugs were added in the other respectively labeled tubes.

A separate micropipette tip was used for each drug. No drug was added to the GC tube.

Preparation of critical drug concentrations

Drug	Concentration of drug after reconstitution*	Volume added to MGIT tube	Final concentration in MGIT tube
STR	83µg/ml	100 µL	1.0 µg/ml
INH	8.3 µg/ml	100 µL	0.1 µg/ml
RIF	83 µg/ml	100 µL	1.0 µg/ml
EMB	415 µg/ml	100 µL	5.0 µg/ml

*The drugs were reconstituted using 4 ml sterile deionized or distilled water to achieve the indicated concentrations.

Calculations of the dilutions factor for MGIT medium:

A volume of 7.0 ml of medium + 0.8 ml of SIRE Supplement + 0.5 ml of inoculum = 8.3 ml. Addition of 0.1 ml of the drug solution in 8.3 ml of the medium = 1:83 dilution.

1. A volume of 0.5 ml of the well-mixed culture suspension (inoculum) was aseptically added into each of the drug containing tubes using a pipette. **Not added to the growth control tube**.

2. For the control, the test culture suspension was first diluted 1:100 by adding 0.1 ml of the test culture suspension to 10.0 ml of sterile saline. The contents were well mixed

by inverting the tube 5-6 times. A volume of 0.5 ml of this diluted suspension was added into the growth control tube.

3. The caps of the tubes were tightened and contents mixed well by gently inverting the tube several times.

The labeled tubes were placed in the correct sequence in the Set Carrier (GC, STR, INH, RIF, EMB).

4. The susceptibility Set Carrier was entered into the BACTEC MGIT 960 instrument using the susceptibility test set entry feature (BACTEC MGIT 960 User's Manual, AST Instructions).

Care was taken to ensure that the order of the tubes in the AST Set Carrier conformed to set Carrier definitions i.e. GC, STR, INH, RIF, EMB for the SIRE standard testing.

Results:

The instrument monitored the entered susceptibility test set. Once the test was complete (within 4 to 21 days), the instrument indicated that the results were ready. Then the susceptibility Set Carrier was scanned and report printed. The instrument printout indicated susceptibility results for each drug. Results were qualitative: Susceptibility (S), Resistant (R) or determinate (X).

The instrument interpreted results at the time when the growth unit (GU) in growth control reached 400 (within 4-13 days). At this point, the GU values of the drug vial were evaluated.

S = Susceptible – the GU of the drug tube is less than 100.

R = Resistant – the GU of the drug tube is 100 or more.

X = Error – indeterminate results when certain conditions occurred which may have affected the test, such as GU of the control reached ≥400 in less than 4 days. In such situations, the test was repeated with pure actively growing culture confirmed to be *M. tuberculosis* complex.

Isolates resistant to at least both rifampicin and isoniazid are designated as multi-drug resistant (MDR).

NB. Certain drug resistant strains grow very slowly in the medium and results may not be achieved within 13 days with the standard inoculum. In such a case, the inoculum is increased by decreasing the dilution of the culture suspension in order to get reportable results.

Appendix 9: Request for form sputum examination & questionnaire

Hospital/treatment unit_____   Date_____

Patient's Name

      Age_____   Sex: (M/F) _____

Address
(precise)_____
_____
_____

Duration of stay in western Kenya: < 1 year [ ]; 1 year [ ]; > 1 year [ ]

Reason for examination: diagnosis_____   follow-up examination _____

Signature of clinician requesting examination_____
_____

HIV STATUS: ELISA negative [ ]; ELISA positive [ ]; Western blot negative [ ];
                      Western blot positive [ ]. other (specify)_____

QUESTIONNAIRE

1.0   Have you been treated for tuberculosis before?   Yes [ ] No [ ]

2.0   If yes, for how long were you treated? Less than 30 days [ ]; For 30 day [ ];
        For more than 30 days [ ]; other (please specify) _____.

RESULTS (to be completed at the MRL, MUSOM)

Laboratory serial No _____

Date	Specimen	Appearance	Neg*	1-9	+	++	+++
	1						
	2						
	3						

Visual appearance of sputum (blood-stained, muco-purulent, saliva)

Date _____   Examined by (Signature) _____

_____