FACTORS AFFECTING THE FREQUENCY OF LIPID BODY POSITIVE TUBERCLE BACILLI IN HUMAN SPUTUM

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Abstract

Mycobacterium tuberculosis (*Mtb*) is the causative agent of tuberculosis (TB). *Mtb* cells contain intracellular lipid bodies (LBs) in sputum and the levels vary between patients. Previous reports showed the essential role of nitric oxide (NO) in killing *Mtb in vivo* and that *Mtb* forms LBs *in vitro* upon NO treatment. These treated populations are tolerant to isoniazid and rifampicin, but the reasons for varing LB levels *in vivo* are not understood. The objective of this study was to determine the association between fractional exhaled nitric oxide (FeNO) concentrations with the proportion of LB^{+ve} tubercle bacilli in sputum.

The majority of TB patients (65%) showed $\leq$ 20.6 ppb FeNO concentration. FeNO concentration was weak but significantly associated with the proportion of LB^{+ve} tubercle bacilli in sputum. FeNO concentration was also significantly associated with the proportion of LB^{+ve} tubercle bacilli among HIV^{+ve} P$^-$ and HIV^{-ve} P$^+$/TB patients. High esosinophil count was significantly associated with FeNO concentration in both HIV^{-ve} and HIV^{+ve} /TB patients infected with intestinal parasite. The CAS and EAL *Mtb* spoligotypes were the dominant *Mtb* spoligotypes in Gondar. FeNO concentration was significantly associated with the proportion of LB^{+ve} tubercle bacilli among TB patients infected with the CAS *Mtb* spoligotype (p<0.01; r^2=0.323) but not among patients infected with the EAL. The anti-microbial susceptibility pattern showed an 11.9% either mono or multi-drug resistance. The average proportion of LB^{+ve} tubercle bacilli among drug resistant TB patients was relatively higher than the corresponding drug sensitive TB patients. The proportion of LB^{+ve} tubercle bacilli was also higher among MDR-TB patients.

The association between FeNO concentrations with the proportion of LB^{+ve} tubercle bacilli raises questions regarding L-arginine supplementation during TB treatment. The association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli among specific *Mtb* spoligotypes may reflect difference in NO tolerance.

Acknowledgements

List of abbreviations

ADMA	Asymmetric dimethylamine
AFB	Acid-fast bacilli
AFS	Acid-fast stain
AG	Aminoguandine
AHR	Airway hyper responsiveness
AHRI	Armauer Hansen Research Institute
AMK	Amikacin
AMs	Alveolar macrophages
ANOVA	Analysis of variance
AP	Alkaline phosphatase
ARI	Annual risk of infection
aRNA	amplified RNA
ASL	Arginine succinate lyase
AST	Anti-microbial susceptibility test
ATCC	AmericanType Culture collection
ATP	Adenine triphosphate
BCG	Bacillus Calmette-Guerin
BMI	Body mass Idex
Bp	base pair
BSC	Biological safety cabinate
CAS	Central Asian strain
CC	Citric acid cycle
CC	Conjugate control
CD	Cluster of differentiation
CDC	Center for disease control and Prevention
cDNA	Complementary DNA
CF	Cystic fibrosis
CFU	Colony forming Unit
CIP	Ciprofloxacin
CLR	Clarithromycin
CON	Conjugate concentrate
CRs	Complement receptors
CSF	Cerebrospinal-fluid
CV	Coefficient of variation
DAG	Diacylglycerol
DALYS	Disability adjusted life years

DDAH	Dimethylarginine Dimethylaminohydrolase
DNA	Deoxyribonucleic acid
DosR	Dormancy survival regulator regulon
DR	Direct repeat
EDTA	Ethylene Diamine tetra acetic acid
EH	Ethambutol, isonaized
ELISA	Enzyme linked Immunosorbant Assay
eNOS	endothelial nitric oxide synthase
ERHZ	Ethambutol, Rifampicin, isonaized, pyrazinamide
FAD	Flavin adenine dinucleotide
FDC	Fixed dose combination
FeNO	Fractional exhaled air nitric oxide
FNA	Fine needle aspiration
GAH	Gondar army hospital
GPC	Gondar poly clinic
GTC	Guanidium thiocynate
GUHOSP	Gondar University Hospital
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
Hgb	Haemoglobin
HIV	Human immunodeficiency virus
HpoA	Protonated pyrazinoic acid
hspX	heat shock protein (a 16 KD α-crystaline protein)
HyB	Hybridization buffer
Icl	isocitrate lyase
IFN	Interferon
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
INH	Isoniazid (Isonicotinyl hydrazine)
iNOS	inducible nitric oxide synthase
IS	Insertion sequence
IUATLD	International Union against Tuberculosis and Lung Disease
IVT	Invitro transcription
KatG	Catalase peroxidase enzyme
Kg	Kilogram
KM	Kanamycin
KMnO4	Potassium permanganate

LB^{+ve}	Lipid body negetive
LB^{+ve}	Lipid body positive
LBs	Lipid bodies
LCFA	Long chain fatty acid
LJ	Lowenstein-Jensen
LJG	Lowenstein-Jensen glycerol
LJP	Lowenstein-Jensen pyruvate
LPS	Lipopolysaccharide
LSP	Large sequence polymorphisms
LZD	Linezolid
MCP	Macrophage chemo-attractant protein
MDR	Multi-drug resistance
MDR-TB	Multi-drug resistance TB
MGIT	Mycobacteria Growth Indicator Tube
MHC	Major histo-compatability complex
MIRU	Mycobacterial interspersed repetitive units
MOH	Ministry of Health, Ethiopia
MR	Manose receptor
MRC	Medical research council
mRNA	messanger ribonucleic acid
Mtb	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NaCl	Sodium chloride
NADH	Nicotinamide-adenine dinucleotide
NALC	N-acetyl-L-Cyseine
NaOH	Sodium hydroxide
NIAD	National Institute of Allergy and Infectious disease
NMDA	N-methyl-D-aspartae
NMMA	N^G-monomethyl-L-arginine
nNOS	neuronal nitric oxide synthase
N-ntW	N-nitrosotryptophan
NO	Nitric oxide
NO$_2$	Nitrite
NO$_3$	Nitrate
NOHLA	N^G-hydroxy-L-arginine
NOS	Nitric oxide syntahse
NRA	Nitrate reductase activity
NRP	Non- replicating persistence

NTC	Negative control
NTP	National tuberculosis program
OADC	Oleic acid dextrose catalase
PAS	Para-amino salicylic acid
PBS	Phosphate buffer saline
PC	Positive control
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoates
PHA_{mcl}	Polyhydroxyalkanoates medium length chain (C_6 to C_{14})
PHA_{scl}	Polyhydroxyalkanoates short length chain (C_3 to C_5)
PHB	Poly-β-hydroxybutrate
PICT	Providing Initiative Testing and counseling
PL	Phospholipid
PNM	Primer nucleotide mix
POA	Pyrazinoic acid
PRMT	Protein-arginine methyltransferase
PYZ	Pyrazinamide
Pzase	Pyrazinamidase
qPCR	quantitative PCR
QQ	Quantile-Quantile plot
RBC	Red blood cell
RD	Region of difference
RFLP	Restriction Fragment Length Polymorphism
RIF	Rifampicin
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
RNS	Reactive nitrogen species
RO	Reactive oxygen species
RPO	Research and publication office
rRNA	ribosomal ribonucleic acid
rRNA	ribosomal RNA
RSNO	S-nitrosothiols
RT	Reverse tyranscription
RT-PCR	real time PCR
SD	Standard deviation
SDS	Sodium dodeecyle sulphate
SIT	Shared International Type number
SLDs	Small lipid droplets

SNP	Single nucleotide polymorphisms
SOP	Standard operating procedures
SPOlDB4	Spoligotype database-4
SUB	Substrate concentrate
TAE	Tris chloride
TAG	Triacylglycerol
TB	Tuberculosis
TG	Triacylglycerol
Tgs	triacyl glycerol synthase
Th	T helper cell
TLR2	Toll like receptor-2
TNF	Tumor necrosis factor
UC	Urea cycle
UOG	University of Gondar
UOL	University of Leicester
VNTR	Variable number tandem repeats
WBC	White blood cell
WEs	Wax esters
WHO	World Health Organization
WS/DGAT	Wax ester synthase/Diacylglycerol Acyltransferase
XDR-TB	Extensively-drug resistant TB
ZN	Zeihl—Neelsen

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CHAPTER ONE: General Introduction

1. BACKGROUND TO THIS STUDY

Tuberculosis is one of the greatest killer diseases worldwide and is caused by the bacterium *Mycobacterium tuberculosis* (*Mtb*) (Lessnau and Luise, 2009)**.** Consumption, phthisis, scrofula (cervical lymphadenitis), Pott's disease (spinal tuberculosis), lupus vulgaris (cutaneous tuberculosis) and the White Plague are all terms used to refer to tuberculosis (Rothman, 1994). The name "Tuberculosis" is drown from the Latin tubercle which means small lump (Dormandy, 1990). In 1882 the German bacteriologist Robert Koch discovered the etiologic agent of tuberculosis; the tubercle bacillus. Later, in 1994 the World Health Organization declared tuberculosis (TB) to be a global health emergency. Currently, TB is robustly associated with poverty and complicated by the upward growing of HIV/AIDS together with the emergence of multi-drug resistance (Cole et al, 2005).

TB is predominantly associated with lung disease, but it can affect other parts of the body such as skin or other organs.There are 12 different types of TB infections categorized in to two: namely pulmonary tuberculosis and extra pulmonary tuberculosis. The predominant primary TB pneumonia mainly affects patient with lower immunity. Miliary TB is distinguished by the presence of small granules in the lungs and TB pleurisy involves shortness of breath, chest pain and accumulation of fluid in the lungs (Lessnau and Luise, 2009).

Cough is the most common symptom of pulmonary tuberculosis. However, at the beginning of the disease TB patients may be nonproductive. Subsequently, when inflammation and tissue death continues, sputum is usually produced. Sputum is the most frequent specimen collected for TB testing. Patients with positive sputum on direct microscopic examination (known as "smear") are more infectious, because they are producing more bacilli,than those positive only on culture (Rieder et al, 2007). However, patients with sputum smear AFB negative but culture-positive can also transmit TB infection (Jensen et al, 2005). For initial diagnosis of pulmonary TB, a series of three sputum specimens (Spot-morning-Spot sputum samples) need to be collected (CDC, 2005) optimally before drug therapy is started.

Recent observations in the Leicester laboratory show that several properties of the tubercle bacillus in sputum are distinct from those found *in vitro* and that the level of expression of these properties varies from patient to patient (Garton et al, 2008). In particular, bacilli containing intra-cytoplasmic lipid droplets, known as lipid bodies (LBs), can be low or high in proportion to the total bacillary population and this may have important implications for the chemotherapy and transmission of TB. Preliminary studies in the Barer's lab have indicated the potential role for a non-replicating bacillary state and exposure to nitric oxide as influencing the occurrence of LBs.

This study is concerned with exploring the basis for variation in LB content of the tubercle bacilli in the sputum of patients with pulmonary TB.

1.1.   Epidemiology and importance of TB

According to the WHO (2012) report, the global incidence of TB was estimated at 8.7 million (13% co-infected with HIV) and 1.4 million people died from TB. Death due to TB was reported as one million among HIV-negative individuals and 430,000 among HIV positives. The burden of disease was reported to be highest in Asia and Africa and about 24% of the world's TB cases are in Africa (WHO, 2012). Previously the Global TB incidence was reported as growing at a 1% per year due to the rapid increase in Africa (WHO, 2005). However, the recent WHO (2012) report showed that the incidence has been declining at a rate of 2.2% between 2010 and 2011. Moreover, the mortality rate was also reported as decreased by 41%. In Africa, HIV was reported as the single most important factor determining the increased incidence of TB in the past years, underlining the synergy between the progress of HIV and TB. Poverty, HIV and drug resistance were also reported as major contributors to the resurging global TB epidemic (Corbett, 2003).

Among the 22 countries with a high TB burden globally, Ethiopia ranks seventh (WHO, 2008). In 2007 the country had an estimated 314,267 TB cases; with an estimated incidence rate of 378 cases per 100,000 population. The 2008 Ministry of Health (MOH), report showed that TB was the leading cause of morbidity, the third cause of hospital admission (after deliveries and malaria), and the second cause of death after malaria (FMOH,

2008).The annual risk of TB infection (ARI) was estimated at 2.2% (FMOH, 2009) and the mortality rate due to TB was estimated at 84 per 100,000 per year (FMOH, 2009). In 2010, the absolute number of new TB cases was reported as 220,000 and 330,000 people living with TB plus 29,000 deaths due to TB (WHO, 2011). Recently Ethiopia conducted a population based national TB study for 2010/2011 and the prevalence of bacteriologically confirmed (smear + culture positive) pulmonary TB infection was reported as 277/100,000 (Alebachew, 2011). Previously, Tesema et al (2009) reported a 16.8% smear positive and 54.9 % smear negative pulmonary TB infection rate in Gondar regardless of HIV/TB co-infection.

1.2. Clinical Tuberculosis

Depending on the main site of infection and the number of factors, TB presents differently. Whether infection goes on to disease depends on the defenses of the person infected (host resistance) (figure 1.1). In some cases, infection may rapidly go on to disease. In others, TB may remain latent with a few remaining and possibly dormant bacilli under control by the immune defenses (Rieder et al, 2007). The interactions of the host and microbe-related factors manipulate the clinical presentation of TB disease. Decreased patient's resistance due to malnutrition, HIV infection, old age and others factors may permit the TB bacilli to reproduce and cause disease (Table 1.1).

The most frequent non-specific indicator of TB infection includes fever, loss of appetite, weight loss, weakness, night sweats, and malaise. However, these symptoms are not distinctly connected with the site of infection or organs involved, but are rather systemic in nature (American Thoracic Society, 2000).

In addition to the generalized effects of tuberculosis, there are remote manifestations that are not a result of the anatomic site of involvement. These include haematological abnormalities, hyponatremia, and psychological disorders. Cameron (1974) reported that the most frequent haematologic manifestations are raised peripheral blood leukocyte count and anaemia.

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Number of infecting bacilli   Disease   Patient's defence

Very large   ++++++ → Very probable ← +   Very poor

Large   +++++ → Probable ← +++   Poor

Small   +++ → Unlikely ← +++++   Good

Very small   + → Very unlikely ← ++++++   Very good
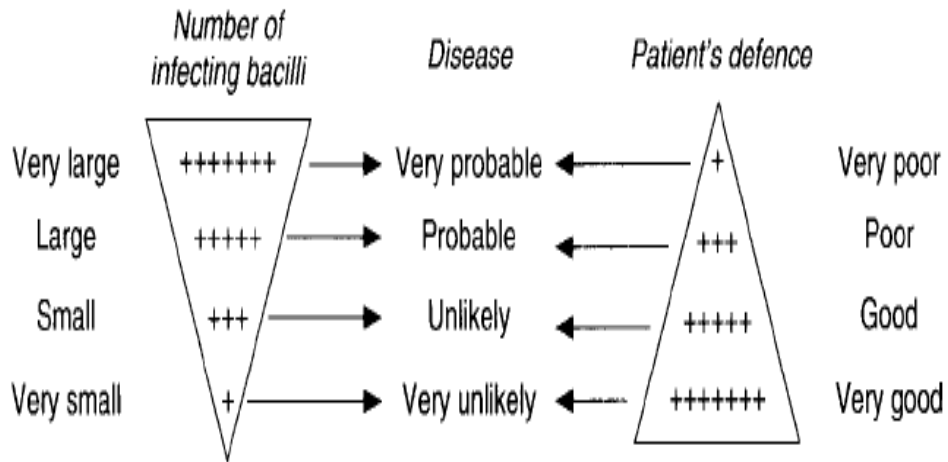
Figure 1.1: Probability of developing tuberculosis

The influence of the number of bacilli (exposure) and the strength of the patients defences on the risk of developing disease following infection (Rieder et al, 2007).

Table 1.1: Factors that influence the clinical features of tuberculosis

The three major factors that influence the clinical features of tuberculosis include host factors, microbial factors and the interaction of the tubercle bacilli with the host (Adapted from American Thoracic society, 2000).

Host factors	Microbial factors	Host-microbial interaction
Age	Virulence of the pathogen	
Immune status	Predilection (tropism)	Sites of involvement
Specific immunodeficiency state	for specific tissues	Severity of disease
Malnutrition		
Genetic factors (not yet defined)		
Co-existing disease		
Immunization with bacillus		
Calmette-Guerin		

Although cough is the universal indicator of pulmonary tuberculosis some patients may present with haemoptysis as a consequence of disease. Haemoptysis is typified by spitting of blood through sputum (Murray et al, 1978).

Lawn and Zumla (2011) documented that pulmonary tuberculosis almost constantly causes abnormalities on the chest film. However, in HIV positive pulmonary TB patients a normal chest pictures is more common compared with TB patients with intact immunity. On the other hand, cavitations may occur when the patient develops cell-mediated immunity and when the primary tuberculosis infection process continues ahead of the time (American Thoracic Society, 2000). Stead et al (1968) illustrated that endogenous reactivation of latent TB causes anomaly in the upper lobes of one or both lungs and cavitation (figure 1.2) is common in this form of tuberculosis.
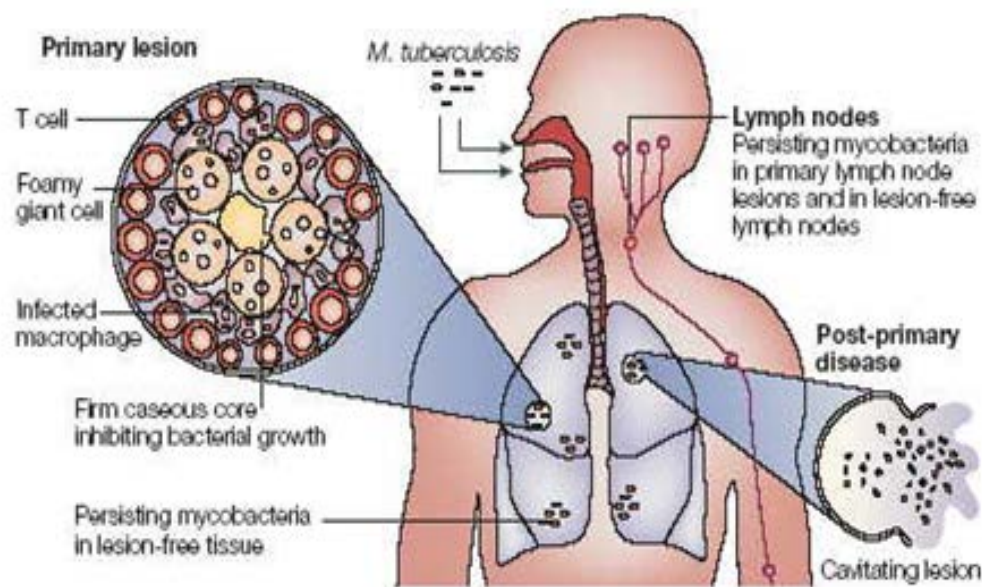
Figure 1.2: the course of *Mtb* infection

After inhalation the tubercle bacilli reach in to the lung and may exhibit granuloma development, persist in lesion free tissue or establish it self in cavitory lesions (Stewart et al, 2002).

1.3. Transmission of tuberculosis

Tuberculosis transmission is mainly through the airborne route where the TB bacilli are suspended in aerosol droplets. The major source is a person suffering from active pulmonary tuberculosis disease and transmission can occur through coughing, sneezing, speaking or laughing (figure 1.3). A cough may release 0-3500 tubercle bacilli, sneezing about 4500 to 1 million and talking 0-200 organisms (CDC, 2005). According to Nicas and colleagues (2005), the infectious dose of TB may be less than 10 bacilli and therefore each one of the aforementioned routes may transmit the disease.

During coughing, sneezing and perhaps talking, aerosol droplets of different sizes may be produced. Large droplets (> 20µm) fall straight to the ground, where as intermediate sized particles (5-20µm) fall at a slow rate or remain temporarily suspended in air. Droplets less than 5 µm in diameter remain suspended in air for longer periods of time (Papineni and Rosenthal, 1997). They may then be breathed in by someone else (airborne transmission). The mycobacteria are swiftly killed by ultraviolet rays (day light). Transmission is therefore predominantly indoors, and this explains the familial clustering of cases, and also the cluster in various areas such as the class room, the work place, cafes, and airplanes.

Small or diminished droplet nuclei (0.5-5µm in size) get to an alveolus in the lung leading to TB infection. Cells of the immune system such as macrophages and lymphocyte migrate to the site and able to arrest the TB bacilli. However, when the immune system fails to eliminate the invading bacteria, the patient develops primary TB (FMOH, 2009). During TB infection only fresh droplet nuclei that hold viable bacteria are valuable for transmission. Therefore, the regularity and dynamism of cough together with the aeration of the environment manipulates the transmission (CDC, 2000). In general, TB transmission may depend on the number of organisms suspended in the air, the volume of room and aeration, the duration of exposure to the contaminated air, and the immune status of the exposed individual (American Thoracic Society, 2000).
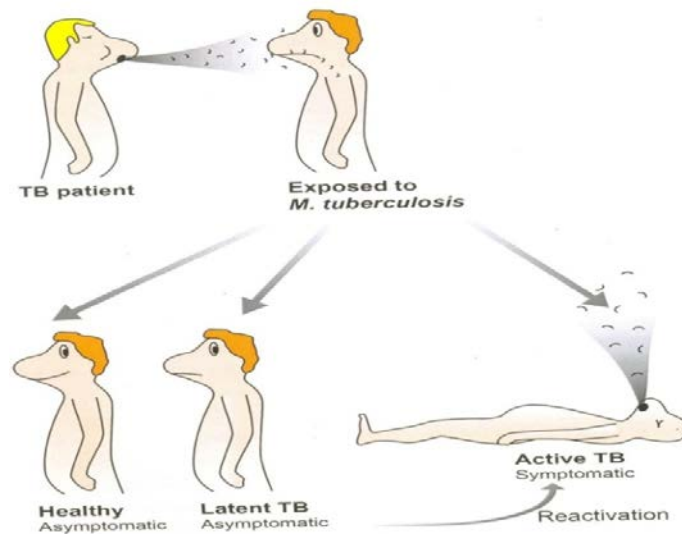
Figure 1.3: Transmission of *Mtb*

When a particular patient is infected with TB there are two probable circumstances. In conditions where the patient has intact immunity and able to contain the infection through effective cellular immune response, there will be no symptoms of disease, and this stage is called latent TB. On the other hand, when the host is incapable to contain the TB bacilli as a result of inadequate immune response, the bacilli undergo replication and subsequently the patient may develop active pulmonary TB disease (Image adapted from Idh et al, 2012).

1.3.1. Risk factors for tuberculosis

The risk of TB transmission is rather higher among households living with TB patients or people that had contact with TB patients (Wood et al, 2010). Moreover, Pienaar et al (2010) reported that members of larger families transmit the TB bacilli more than people living within smaller families. There are also reports that documented outdoor transmission of TB in some areas such as public transport (Pienaar et al, 2010).The risk of developing TB after exposure to *Mtb* is greatest in the youngest children (Marais, 2011) and the elderly are at high risk due to dysfunction of the cellular immunety (Donald and Small, 1998). Immunocommpromized people as a result of HIV infection and/or other factors such as malnutrition are more vulnerable to *Mtb* infection than peoples with intact immunity

(Horsburgh, 1996). In general, peoples infected with non-drug resistant tubercle bacilli are not infectious after two weeks of treatment (Griffith and Kerr, 1996).

1.4. Primary and Secondary TB disease

Primary TB is where the person is exposed to the bacilli through the respiratory tract and *Mtb* is ingested by macrophages then either killed or lies dormant.There will be development of an acquired immune response through a delayed type of hypersensitivity reaction. This immune response creates further activated cells and lymphocytes. All the while, the macrophages are taken into lymph nodes and retained there. The immune system creates a fencelike structure around the foci with the bacilli in them. If for some reason, the immune system is not active enough, then an active primary TB occurs with the nocturnal fever with sweats, and chronic cough. If not, overtime the infected lymph nodes coalesce and retain calcium to form a Ghon focus (Dinusha, 2011). Primary TB is not contagious in this early stage.

Secondary TB is where the patient either reactivates infection from a previous exposure to the bacilli sometime back or acquires a new (super) infection. The immune system gets compromised due to another infection, drugs or immune compromise, leading to the breach in the immunological barricade around the contained bacilli. Because of this, the immune reaction against the bacteria can wreak havoc in the respiratory system leading to blood streaked purulent sputum with a chronic cough, loss of weight, and fever at night with night sweats, etc (Dinusha, 2011).

1.5. Symptoms and signs of pulmonary TB

The most prominent symptom for pulmonary TB infection is persistent cough for two weeks or more. Expectoration of sputum during pulmonary TB infection can be with or without blood stained and might become together with other clinical indications such as weight loss, chest pain, shortness of breath, intermittent fever, night sweats, loss of appetite, fatigue and malaise, breathing difficulty, and Wheezing (FMO, 2008).

1.6.   Sputum production

The respiratory system is responsible for drawing air in to the lungs in order to facilitate gas exchange between the circulatory system and the atmosphere (Richardson, 2003). There are minute hair-like structures identified as cilia that trap larger debris and transport them out of the airways. On the other hand, small particles are removed from the respiratory tract through sneezing and coughing (Richardson, 2003). Small particles such as bacteria are trapped by sticky mucus. Cough is typically involuntary and follows on from sputum production as a result of irritation of the mucosa (Jeffrey and Zhu, 2002; Maestrelli et al, 2001). The nature and consistency of sputum may present evidence about the health status of the patient regarding respiratory abnormalities. Sputum can be mucoid, purulent, mucopurulent, frothy, and viscous or blood stained (Law, 2000). The presence of many of polymorphnuclar cells particularly neutrophiles may produce greener sputum which many doctors take as an indication for treatment with antibiotics. Blood stained sputum samples are indicator of TB infection or cancer (Richardson, 2003).

1.7.   Latent TB infection

In a person with intact immunity, TB infection is usually contained within the lung without causing overt TB infection. This is latent TB infection and people with latent TB are not infectious. When the patient's immunity is debilitating as a result of immunocompromize, the patient may develop active TB (Comstock and Livesay, 1974). Very little is known about the physiology of *Mtb* bacteria during the latent stage of infection or even the location it may inhabit (Boshoff and Barry, 2005). However, given that isoniazid, which is only active against replicating cells, is effectively used to treat latent TB infection, it suggests that these bacteria must be at least intermittently metabolically active (Wayne and Sohaskey, 2001). The prolonged period needed to treat latent TB infection suggests that replication is in a state of flux, possibly due to ongoing dynamic interactions with immune system in which bacteria are currently being released by macrophages through apoptosis, while at the same times others are taken up by macrophages. This may allow periods of replication, which is arrested upon uptake by activated macrophages (Wayne and

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Sohaskey, 2001). The risk of developing active TB infection from latent TB was estimated at 10% per year for HIV positive patients compared with a 10% life time TB infection in the general population (Kawamura, 2013).

1.8. Diagnosis of TB

TB infection can be diagnosed clinically using sign and symptoms or by laboratory tools. Laboratory diagnosis involves the detection and isolation of mycobacteria, speciation and drug sensitivity tests. Different types of clinical specimens can be submitted to laboratory depending on the site of TB infection. However, during pulmonary TB infection sputum is the specimen of choice.

1.8.1. Sputum microscopy

Specimen Collection Methods for Pulmonary TB Disease

There are two well known methods of sputum collection, namely coughing and sputum induction methods. Coughing is the most frequent method used to collect sputum sample from patients suffering from respiratory diseases such as pulmonary TB. Sputum induction is another method of sputum collection which is mainly used when the patient is not capable to produce enough sputum samples. Administration of an aerosol of lukewarm and sterile hypotonic physiological saline at a concentration of 3% to 5% may produce deep coughing that result in the production of induced sputum. Generally, induced sputum is watery and bears a resemblance to saliva (WHO. 2008).

Currently, collection of at least three sputum samples, spot-morning-spot, are recommended to detect acid-fast bacilli (AFB) from sputum (WHO, 2001). The first and third sputum specimens are collected in the health facility (Spot specimens) and the second is generally an early morning specimen, usually collected at home (WHO, 1998).

Conventional sputum Smear microscopy to detect TB bacilli

Sputum direct microscopy is still used as principal method to detect TB. The method is rapid, very economical and specific for *Mtb*. The main drawback of the method is its comparative low sensitivity particularly when patients are co-infected with HIV. There are two main procedures that are commonly used to detect the tubercle bacilli: the Ziehl–Neelsen (ZN) method that uses carbolfuchsin as a primary stain and Auramine-O or Auramine-rhodamine staining method.

Different studies recognized that the conventional sputum microscopic methods such as ZN stain will display positive results only when the sputum sample contain 5000 to 10,000 tubercle bacilli per ml of sample (Hobby et al, 1973). However, the patient is potentially infectious and can transmit the disease when sputum sample contained as low as 10 tubercle bacilli per ml of sputum (Yeager et al, 1967). This indicates that smear negative TB patients can be potential sources for TB transmission as long as they spit the tubercle bacilli. The magnitude of the tubercle bacilli in a particular sample can be quantified and graded (Table1.2).

Table 1.2: Quantitation scale for acid-fast bacillus smears

The two most frequently used laboratory method to detect acid-fast bacilli in sputum and other fluids are the ZN stain and the Auramine O Fluorescence stain. The following table demonstrates the standardized quantitation scale for AFB microscopy (Adapted from American Thoracic Society, 2000).

Carbolfuchsin (X1000)	Fluorochrome (X 250)	Quantity reported
NO AFB/300 fields	NO AFB/30 fields	NO AFB seen
1-2 AFB/300 fields	1-2 AFB/30 fields	Doubtful, repeat test
1-9 AFB/100 fields	1-9 AFB/10 fields	Rare (1+)
1-9 AFB/10 fields	1-9 AFB/ field	Few (2+)
1-9 AFB/field	10-90 AFB/field	Moderate (3+)
> 9 AFB/ field	> 90AFB/ field	Numerous (4+)

Fluorescence Microscopy to detect tubercle bacilli

In 1937 Hagemann described the use of fluorescent dyes for the detection of acid-fast bacilli in clinical specimens (Truant et al, 1962). During fluorescence microscopy samples are stained with a fluorescent dye. Fluorescent dyes absorb light at lower wave length, and emit light at a longer wave length. Fluorescence occurs when a molecule's electron cloud relaxes to its ground state after being photonically excited (Ernst et al, 2007).

When conventional ZN light microscopy is used to detect TB in sputum and other samples, at least a 5 minute microscopic examination is required before concluding that sample is negative for AFB (IUATLD, 2000). A systematic review by WHO/IUATLD found that fluorescence microscopy on average increases the sensitivity by 10% over conventional ZN microscopy and specificity was found very comparable. Moreover, reading a fluorescent stained smear takes only 25% of the time taken to read a ZN stained smear (Laifangbam et al, 2009).

1.8.2. Cultivation of mycobacteria

Culture is the gold standard method used for definitive diagnosis of TB infection and its sensitivity ranges between 80-85% with specificity of 100% (Ichiyama et al, 1993). There are four most important rationales for cultivation of specimens taken from patients suspected of TB infection. The first reason is that culture can detect as low as 10 bacilli/ml of sample which means more sensitive than direct methods. The other important rationales includes that tubercle bacilli grown on culture media can be used for species identification, drug susceptibility tests and to link TB genotypes with the epidemiology of TB (Yeager et al, 1967). There are three different types of culture media: egg based (Löwenstein–Jensen), agar based (Middlebrook 7H10, 7H11 medium), and liquid (Middlebrook 7H9, 7H12).

In developing countries, Lowensten-Jenesen (LJ) medium is the most commonly used medium for culture of *Mtb*, which is also recommended by the WHO (WHO, 1998). Growth of *Mtb* on LJ appears in 2 to 6 weeks and negative culture report cannot be given before eight weeks. Considering this problem, substantial improvement in the time to

detect and the recovery rate was realized by using broth-based culture system such as BACTEC 460 TB, Septi-Chek AFB, Mycobacteria Growth Indicator Tube (MGIT) and BACTEC 9000 (Saito, 1998). A novel system is the MGIT, which is a non-radiometric broth method. The MGIT system can accurately detect the presence of *Mtb* in a short time as 2 days (Hanna et al, 1994).

1.8.2.1. Identification of mycobacteria from Culture

Mtb could readily be identified by its rough, non-pigmented, corded colonies on oleic acid–albumin agars; a positive niacin test; generally weak catalase activity, and a positive nitrate reduction test. The colony morphologies of mycobacteria on various egg-based media may be quite similar, but their appearance on Middlebrook 7H10 or 7H11 agar is distinctive (Runyon, 1970). There are also biochemical methods that can distinguish mycobacterial species, but many of them are time-consuming and laborious. Currently, there are new molecular techniques for species identification, such as spoligotyping, polymerase chain reaction (PCR), and DNA sequencing.

1.8.3. Immunological recognition of infection

1.8.3.1. The tuberculin skin test

The tuberculin skin test (TST), also known as the Mantoux test is a technique used to find out whether a person is infected with *Mtb* or not (CDC, 2000). Administration of culture filtrate known as tuberculin or purified protein derivative (PPD) to an individual suspected of TB results in a delayed-type hypersensitivity response in positive cases (Daniel, 1980; CDC, 2000). Typically 0.1 ml of the PPD is administered intra-dermal and induration is observed after 48 to 72 hours. Indurations of $\geq$15 millimeters, $\geq$ 10 millimeters and $\geq$ 5 millimeters are judged positive in any person or individual that had no known risk for TB (CDC, 2003), in recent migrants (<5 years) from high prevalence countries and drug abusers or children less than 4 years of age and HIV positive individuals (CDC, 2005) respectively. There are reports that documented on a decreased or even diminished ability of tuberculin to react with *Mtb* and may result false negative results. Despite that, the test is

very simple to perform and cheap in cost. Another most important drawback of the TST is the reduced specificity among individuals exposed to BCG vaccination and non-TB mycobacteria (NTM) (Farhat et al, 2006).

1.8.3.2. Interferon Gamma Release Assays (IGRAs)

IGRAs are *in vitro* blood tests that detect the amount of interferon gamma (IFN-γ) in response to *Mtb* proteins such as early secretary antigenic target (ESAT-6) (Rv3875) (Nahid et al, 2006). ESAT-6 is classified as a secreted protein on the basis of its being released by *Mtb* after short periods of growth and in the absence of obvious autolysis. The attenuated *M. bovis* BCG strains lack the ESAT-6 gene due to its location within RD1, a region of the *Mtb* and *M.bovis* chromosome that is deleted in BCG (Hsu et al, 2003). Hence, IGRAs are not affected by immunization or environmental mycobacteria (Pai et al, 2008).Moreover, Lalvani et al (2005) reported that IGRAs test is more sensitive than the skin test. ESAT-6 is a potent inducer of IFN-γ and the RD1 region of *Mtb* is responsible for the secretion of ESAT-6. Therefore, ESAT-6 is only recognized by T cells of TB patients but not by T cells of healthy unvaccinated individuals. Therefore, using ESAT-6 as reagent to determine the level of IFN-γ can be used as a marker to identify active TB or latent TB infection (Cole et al, 2005).

1.9. The genus Mycobacterium

The name mycobacteria (fungus-bacteria) indicate slender, slightly curved or straight rod-shaped organisms. The mycobacteria are divisible into two major groups, the slow and rapid growers; although the growth rate of the latter is slow relative to that of most of other bacteria. The mycobacteria show high tolerance to environmental exposures and inhabit various reservoirs such as water, soil, animals and humans and can be both commensals as well as highly successful pathogens, such as *Mtb*, *M.leprae* and *M.ulcerans* (Ducatti, 2006). The *Mtb* complex includes strains of five species: *Mtb, M.canettii, M.africanum, M.microti*, and *M.bovis* and two subspecies *M.caprae* and *M.pinnipedii* (Lawn and Zumla 2011). There are many different strains of *Mtb*, but six main lineages in different

geographical regions have been identified (Gagneux and Small, 2007). Recently, new *Mtb* linage "the Woldiya lineage" was found and reported in Ethiopia (Firdessa et al, 2011). Detailed molecular characterization of the genome of *Mtb* complex is documented in chapter five.

1.9.1. Taxonomy

Mycobacterium is a genus of *Actinobacteria* given its own family, the *Mycobacteriaceae* (Table 1.3). The Greek prefix myco means "fungus", alluding to the mycobacteria have been observed to grow in mold-like fusion on the surface of liquids when cultivated (Ravan, 2004).

Table 1.3: Taxonomy of *Mtb* (Todar, 2012).

Kingdom: Bacteria	Family: Mycobacteriaceae
Phylum: Actinobacteria	Genus: *Mycobacterium*
Order: Actinomycetales	Species: *Mtb*
Suborder: Corynebacterineae	

In 1896 Lehmann and Neumann anticipated that the genus *Mycobacterium* comprises the tubercle and leprosy bacilli and later a number of *Mycobacterium* species were portrayed by different investigators (Goodfellow and Magee, 1998). There are well known minimum standards to include a bacterium in the genus *Mycobacterium*. These includes the acid-fastness of the bacteria, the presence of mycolic acids containing 60–90 carbon atoms and a guanine + cytosine content of the DNA of 61 to 71 mol % based on the 16S rRNA sequences (Shinnick and Good, 1994; Tortoli, 2006). There are more than 100 *Mycobacterium* species that are divided in to rapid and slow growing based on the rate of their growth on culture media. The slow growing mycobacteria are frequently disease causing and require more than 7 days while the rapidly growing mycobacteria are generally judge non-disease causing and require less than 7 days to grow and multiply (Shinnick and Good, 1994). The different slow growing mycobacteria are listed in table 1.4. Mycobacteria are also categorized under *Mtb* complex (MTBC) and non-tuberculos

mycobacteria (NTM). Members of the MBTC and the global TB lineage are documented in detail in chapter five.

1.10. Classification of slow growing mycobacteria

The slow growing mycobacteria are classified in to 3 groups based on their pigment production when exposed to light or in the dark (table 1.4). There are also non-chromogenic slow growing mycobacteria.

1.11. Genome and Genotypes

The sequence of the *Mtb* H37Rv, comprises 4, 411, 532 bp. Its circular chromosome is about the size of that of *Escherichia coli* and is almost 10 times larger than *Mycoplasma genitalium,* the smallest bacteria (Thomson et al, 2001). The *Mtb* genome has a high G+C content of 65.6%, a parameter which is associated with an aerobic lifestyle (Naya et al, 2002). The discovery of the first genome sequence of *Mtb* was undertaken and offered by the year 1998. This discovery and the expansion of DNA microarrays presented novel break through as well as opportunity to take in hand the complete genome sequence of the different *Mycobacterium* species particularly the multiple strains of the MTBC (Tsolaki et al, 2004). Moreover, there are study reports that documented the promising relationship between the mycobacteria genomic content and pathogenesis or disease severity particularly in human hosts (Kato-Maeda et al, 2001).

Table 1.4: The different slow growing mycobacteia

The slowly growing mycobacteria are classified based on the rate of growth, production of pigment and whether this pigment was produced in the dark or only after exposure to light (Rogall et al, 1990; Brown-Elliott and Wallace, 2002).

Group	Classification by pigment production	Mycobacterium species	
		Rough colonies	**Smooth colonies**
I	**Nonchromogenic**: Slow growing and do not produce pigment	• *M. africanum* • *M. bovis* • *M. laprae* • *M. lacus* • *M. lepraemurium* • *M. microti* • *M. pinnipedii* • *M. shottsii* • *M. tuberculosis*	• *M. branderi* • *M. heidelbergense* • *M. intracellulare* • *M. malmoense*
		Smooth to rough • *M. gastri* • *M. haemophilum*	**Small and Transparent** • *M. avium avium* • *M. avium paratuberculosis* • *M. avium silvaticum* • *M. genavense* • *M. montefiorense* • *M ulcerans*
II	**Photochromogenic:**produce yellow-orange pigment when exposed to light	**Yellow and smooth** • *M asiaticum* • *M. marinum*	**Yellow and rough** • *M. kansasii*
III	**Scotochromogenic:** Produce yellow-orange pigment in light and in the dark	**Yellow** • *M. conspicuum* • *M. botniense* • *M. farcinogenes* • *M.heckeshornense* • *M. interjectum* • *M. kubicae* • *M. lentiflavum* • *M. nebraskense* • *M.nebraskense* • *M. palustre*	**Yellow-Orange** • *M. cookii* • *M. flavescens* • *M. gordonae*

1.12. Morphology and staining of Mtb

The morphology of the tubercle bacillus is rod shape when observed from stained smears prepared from samples such as sputum. There is also V or Y-shape tubercle bacilli where the V-shape is mainly caused by snapping post fission movements (Farnia et al, 2010). *Mycobacterium* is known to form "Y-shaped" cells with branches more interior to the cells and of greater length (Velayati and Farnia, 2012). However, according to growth conditions and age of the culture, bacilli may vary in size and shape from cocco-bacilli to long rods. Advances in microscopic techniques, revealed that the TB bacillus does not always manifest itself in the classical rod shape (Velayati and farina, 2012). They become shorter in older cultures, filamentous within macrophages and ovoid during starvation (Young, 2006; Farina et al, 2010; Shleeva et al, 2011) and they may produce buds (Chauhan et al, 2006) and branches in extensively drug resistance strains (XDR-TB) (Velayati et al, 2010; Farnia et al, 2010).

The cell wall of a *Mycobacterium* resembles the Gram positive bacteria cell wall structure. *Mycobacteria*, however, have a high content of mycolic acid associated with the cell wall which resists staining by the traditional Gram stain method. For this reason the Gram stain is not routinely used to visualize mycobacteria. Alternative stains are employed such as the Ziehl-Neelsen, or Rhodomine-Auromine stains. The mycobacteria are stained using these stains and the high mycolic acid content resists decolorization using a mild acid-alcohol solution. Detailed Ziehl-Neelsen and Auramine staining procedures or principles are documented in chapter two.

1.13. The *Mtb* Cell envelope

Mycobacterial envelopes have been objects of chemical and biochemical study for many decades. The base of the envelope is peptidoglycan; a structure common, with minor variants, to most bacteria. Mycobacterial peptidoglycan belongs to a family of structures possessed by almost all eubacteria but no other type of living organism (Bessler et al, 1997). The mycobacterial cell wall (figure 1.4) consists of a core complex of peptidoglycan, arabinogalactan and mycolic acids, with an outer complex of lipids

associated with polysaccharides, which exist free in the mycolic acid layer (Brennan, 2003). Peptidoglycan is made of alternating subunits of N-acetly-glucosamine (NAG) and N-acetyl-muramic acid (NAM) bound with a β- (1, 4) glycosidic bond. Strands of NAG-NAM are held together by short peptide bonds between two NAM molecules (Brennan, 2003). In mycobacteria, this structure can vary. Instead of the peptidoglycan being made entirely on NAG-NAM, the backbone contains some N-glycoyl-muramic acid (NGM) that forms NAG-NGM. The reason for this variation in peptidoglycan structure is un-known, although it is proposed that the NAG-NGM might increase the number of hydrogen bonds, making the whole structure more rigid (Hett and Rubin, 2008).

Mycolic acids are α-branched-β-hydroxy fatty acids with variable chain lengths up to 90 carbons in length (figure 1.5) (Stodola, 1938). They were first isolated from *Mtb*, but since then have been found in other *Actinobacteria* such as *Corynebacterium* where they are known as corynomycolic acids. Mycolic acids from mycobacteria can be between 60 and 90 carbons long, whereas corynomycolic acids are only 20-36 carbons in length. In the mycobacteria, α-mycolates are the predominant species and have long chain fatty acids that are β-hydroxylated with α-alkyl side chains.
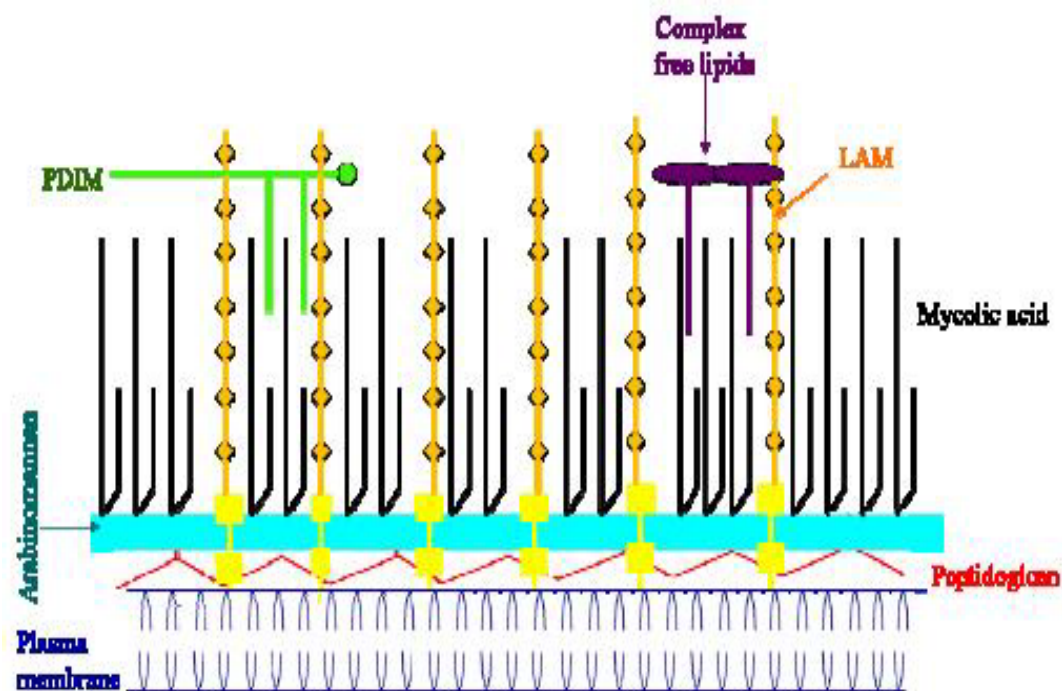
Figure 1.4: The mycobacterial cell wall

The mycobacterial cell wall is composed of the plasma membrane and the outer and inner sections of the cell wall. The core element of the cell wall (inner section) consists of the mycolyl arabinogalactan-peptidoglycan complex, which is linked to the plasma membrane. Lipoarabinomanan is anchored in the plasma membrane by its base phosphatidylinositol mannosides (PIMS). Interspersed between the mycolic acid chains are the complex freelipids and waxes (PDIM) that comprises the upper section of the wall (adapted from Brennan, 1988).

Arabinogalactan is a dimer of arabinan and galactan in a furnaose state, which is attached to the peptidoglycan layer. Unlike with peptidoglycan, arabinogalactan is not made of repeating monomers, but is arranged with galactan molecules in 1-5 and 1-6 linkages. An arabinan molecule is bound to the 5th position of the galactan in the 1-5 links. This is repeated for approximately 30 units, and forms a structural motif that is covalently bound by an N-acetylglucosaminosyl-rhamnosyl linked to peptidoglycan (Hett and Rubin, 2008).

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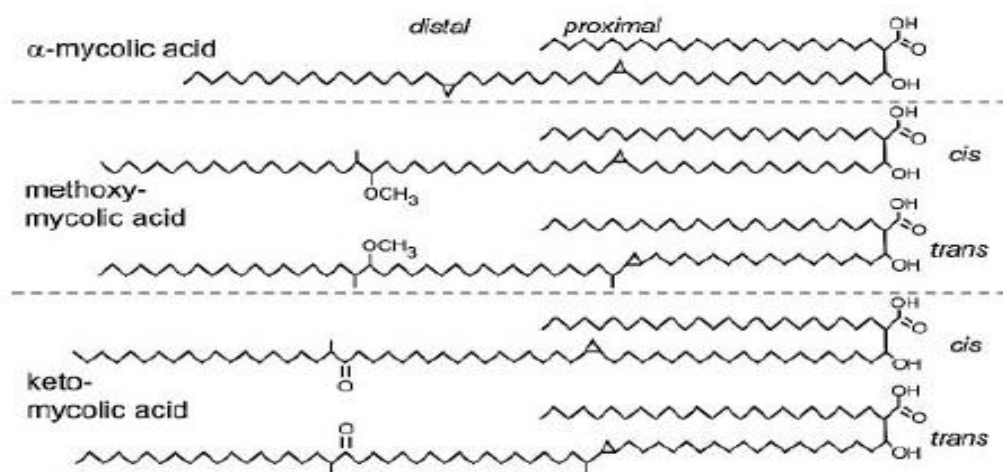
Figure 1.5: The chemical structure of the mycolic acids of *Mtb* (Image taken from Takayama et al, 2005).
Mycolic acids are composed of a long meromycolate chain and a saturated α-chain and can be distinguished by the chemical modification of the former.

1.14. Growth characteristics of *Mtb*

To study the interaction of a single bacterium with a macrophage both *in vivo* and *in vitro* modeling work has been carried out. However, the Wayne model of invitro dormancy is of particular interest. Wayne cultured *Mtb* in a sealed container without agitation to allow an oxygen gradient to form in the vessel. After 14-21 days the *Mtb* cells were recovered into fresh media and demonstrated synchronized replication several hours later. These results suggested that the culture had been in a state waiting to divide. Furthermore, a rapid depletion of oxygen from the culture resulted in the death of the *Mtb* (Wayne, 1977). Following up this work Wayne and Hayes incorporated a slow agitation of the culture to provide a homogenous population. It was found that as oxygen saturation reaches 1% there is increased turbidity of the culture that does not correspond to an increase in colony forming units but due to a thickening in the outer region of the cell wall. At an oxygen saturation of 0.06% the culture is considered hypoxic marking a shift from aerobic to anaerobic respiration (Wayne and Hayes, 1996). The *Mtb* at the two oxygen saturation levels represents different phenotypes known as NRP1 and NRP2. In addition to this Wayne and Hayes noted that during the shift from NRP1 to NRP2 there was increased phenotypic tolerance of the *Mtb* to isoniazid and rifampicin (Wayne and Hayes, 1996).

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One of the most important invivo modeling for *Mtb* is the Cornel Model that describes the *Mtb* latent infection (Gomez and Mckinney, 2004). This models the latent state in humans when few bacteria can be detected in the lung (Cosma et al, 2003). In the stationary phase models, *Mtb* subjected to an extended stationary phase has also been used to model transcriptional changes. In this model, unknown factors become limiting, which results in a non-proliferating culture (Voskuil et al, 2004). It has been suggested that *Mtb* may encounter nutrient depletion *in vivo*. The importance of fatty acid metabolism in *Mtb in vivo* indicates that carbohydrates may be limited in this environment. Betts et al (2002) developed a model for nutrient depletion in which a 7 day culture of *Mtb* was transferred to PBS and incubated statically for six weeks. Oxygen was shown to be depleted after 9 days of incubation of control culture rich media but not in nutrient depleted model. Colony forming unit counts of nutrient depleted cultures remained constant throughout incubation, indicating that unlike NRP cells in Wayne model, nutrient depleted cells entered a dormant-like state before oxygen reached a limiting level (Betts et al, 2002).

1.15. Physiology and metabolism of *Mtb*

The metabolism of *Mtb* within the human host is flexible (Cook et al, 2009). Advances in molecular methods allowed investigators to discover the complex regulatory networks within the *Mtb*. The attribute of *Mtb* contagion is facilitated by the adjustment of the pathogen to host milieu followed by synthesis of molecules that intervene with the immune response of the infected cell (Hatzios and Bertozzi, 2011).

1.16. Nitrogen and carbon source

Many bacteria including mycobacteria exploit and utilize amino acids as sources for nitrogen. Half a century ago, Yabu reported that mycobacteria utilize D-amino acids while the L-forms are very rarely or not used at all (Yabu, 1967). However, there are reports that showed unlike saprophytic mycobacteria, *Mtb* cannot make use of amino acids to support metabolism (Edson, 1951). Nevertheless, there are other reports that explained some amino acids are taken up by *Mtb* and *M. bovis* BCG (Seth and Connell, 2000). For example

nowadays the metabolism or uptake of arginine is under study because arginine is the substrate for inducible nitric oxide synthase (iNOS) that generates the toxic nitric oxide that kill *Mtb* in macrophages (Nathan and Shiloh, 2000; Chan et al, 2001). Amino acids such as asparagine, glutamate and aspartate are the main nitrogen sources for *Mtb* (Ratledge, 1976).

Among the most essential survival adjustment of *Mtb* within the host cell is its ability pertaining to carbon metabolism (Schnappinger et al., 2003). They have the ability to utilize carbohydrates, fatty acids and lipids for carbon source (Wheeler and Blanchard, 2005). However, fatty acids are considered as the major sources of carbon for mycobacteria particularly during persistent infection (Ho¨ner zu and Russell, 2001; Schnappinger et al, 2003). There are reports that showed *Mtb* catabolizes carbon source through the glycolytic, pentose phosphate and/or tricarboxylic acid pathways to distinct metabolic fates (Luiz Pedro et al, 2010).

1.17. Lipid metabolism

Isocitratelyase (icl) is an enzyme that catalyzes the conversion of acetyl-CoA to succinate in the glyoxylate cycle. It is mainly used by bacteria that are metabolically dependant on fatty acids (Kornberg and Beevers, 1956). There are reports that acknowledged the critical role of icl for pathogens that persisted in a host cell as critical source of fatty acids for *Mtb* (Munoz-Elias and McKinney, 2005). There are two *Mtb* icl homologus, icl1 and icl2. The exact sources of fatty acid utilized by *Mtb* are unknown; however, there are a number of possibilities (Munoz-Elias and McKinney, 2006). Fatty acids may be acquired from the lipid rich host-cell debris in mature granulomas (Bentrup and Russel, 2001). Lung surfactant is also rich in fatty acids and can be internalized by macrophages or *Mtb* may be able to utilize macrophage triacylglycerol stores mobilized during phagocytosis (Munoz-Elias and McKinney, 2006). Alternatively, *Mtb* may metabolize fatty acids stored as TAG (Daniel et al, 2004).

The β-oxidation cycle is the principle pathway for the degradation of fatty acids in bacteria and eukaryotes. Successive rounds of β-oxidation yield acetyl-CoA that is channeled into

the citric acid cycle (Munoz-Elias and McKinney, 2006). The β-oxidation cycle generates energy in the form of one molecule of Flavin adenine dinucleotide (FADH2), Nicotinamide-adenine dinucleotide (NADH) and acetyl-CoA (Gurr and James, 1980). The acetyl-CoA can be directed into the glyoxylate shunt pathway and lead the flux of carbon into gluconeogenesis. This allows the organism to acquire and conserves carbon from fatty acid (Russell, 2001).The glyoxylate shunt pathway is a metabolic pathway in which acetate is oxidized to produce ATP. It is a pathway that by passes the two decarboxylation steps of the citric acid cycle and incorporates two carbon molecules, such as acetate from β-oxidation, into the cycle (Wall et al, 2005). The glyoxylate shunt pathway may help intracellular pathogens to reallocate their primary carbon source from carbohydrate to fatty acid (Bentrup and Russel, 2001).

1.18. Responses to external stimuli and stress

In the intracellular environment *Mtb* face hostile environments. However, the bacteria have extraordinary capacity to adapt environmental changes. Nutrient depletion, hypoxia and various exogenous stress conditions are among the expected adverse situation for the bacteria *in vivo*. In general terms mycobacteria are aerobes that require oxygen for their metabolic activity. When mycobacteria are exposed to oxidative stress, they respond to the stressing factor which is extremely important for the pathogen to establish infection. In many mycobacteria, the oxidative stress response is mediated by the transcriptional regulator OxyR gene (Christman et al, 1985). However, there are evidences reported that in some mycobacteria the oxyR orthologous genes found inactivated by several mutations (Deretic et al, 1995). Moreover, the oxidative stress response was also found different by different mycobacteria. For example, *M. smegmatis* was found to mount a protective oxidative-stress response, while *Mtb* and *M. avium* could not (Sherman et al, 1995).

Another intracellular factor that may influence the survival of *Mtb* in the intracellular milieu is the pH. Many of the pathogenic mycobacteria are sensitive to acidic pH which may encounter upon entry in the phagosome (Amer and Swanson, 2002). On the other hand, environmental mycobacteria are likely to encounter acidic pH in their soil habitat

(Iivanainen et al, 1999). When *Mtb* is exposed to change in pH, it demonstrated differential expression of a large number of genes (Fisher et al, 2002; Saviola et al, 2003). On the other hand, Rao et al (2008) documented that*M. Smegmatis* and *M. bovis* BCG show evidence of intracellular pH homeostasis over a large range of external pH values.

Another evidence of environmental adjustment by *Mtb* comes when it was exposed to heat shock. The response of the *Mtb* against heat-shock involves the up-regulation of more than 100 genes. The most important heat-shock proteins that are expressed by *Mtb* includes the ECF sigma factors (SigH andSigE), the general stress sigma factor (SigB), and conserved heat-shock proteins such as the Hsp60/GroE family and Hsp70 (DnaK) (Stewart et al, 2002).

It has been reported that during transition into latency a 48 gene regulon is up-regulated (Parket al, 2003). The DosR and gene expression patterns are documented in chapter six.

1.19. Pathogenesis of TB

1.19.1. Initial infection

Mtb enters the human host through aerosol droplets and is believed to reach alveolar macrophages. The first location of *Mtb* infection is the lungs and the bacilli are internalized by macrophages. Within the lung macrophages, *Mtb* replicate to form the preliminary lesion called the *Ghon focus* (Goodman, 1996). At the same time, some bacteria may migrate to the hilar lymph nodes and the *Ghon focus* jointly with the distended hilar lymph nodes forms the primary complex. There are established reports that the matrix metalloproteinases (MMPs) are involved in dissemination of the TB bacilli to the blood during the development of granuloma (Izzo et al, 2004). Detailed role of the MMPs during TB infection is documented in section 1.21. On the other hand, some small numbers of the bacilli may reach the circulatory system and disseminate through the body. Upon spread the *Mtb* bacilli may reach to different parts of the human body such as Kidneys, brain or bone (DaveManriquezrn, 2009).

The macrophage is thought to be the principle location within which pathogenic mycobacteria are able to survive and replicate (Cosma et al, 2003). In the airways, alveolar

macrophages take up the bacteria. Chemokines produced by immune cells particularly macrophages will recruit neutrophils and monocytes to the site of infection (Petres and Ernst, 2003). The monocytes will differentiate into macrophages or dendritic cells within the tissue, and dendritic cells migrate to the draining lymph nodes, where mycobacterial antigens are presented to T cells (Russell, 2011; Modlin, 2012). When T lymphocytes are activated, they produce cytokines such as interferon gamma (IFN-γ) that can activate macrophages to produce antimicrobial substances (Iseman, 2000; Dheda et al, 2010).

Russel (2007) reported that *Mtb* has adapted strategies to survive in the naïve macrophage through mechanisms that result in the modulation of the host cell function and prevent macrophage activation by arresting the development of a localized immune response. The normal maturation process of the phagosome into digestive, bactericidal organelle involves progressive acidification, accumulation of hydrolytic enzymes and fusion with lysosomal compartments (Amer and Swanson, 2002; Honer zu and Russell, 2001). However, *Mtb* is able to arrest the maturation of the phagosome and prevent its fusion with lysosomal compartments, thereby maintaining the pH at 6.4 (Cosma et al, 2003). *Mtb* is also able to modulate the adaptive immune response by subverting the MHC class II presentation pathway (Voskuil et al, 2003). The ability of *Mtb* to avoid this response is likely to aid the survival and persistence of *Mtb* in the macrophage as MHC class II processing and presentation is required for the priming of CD4^{+} cells which release the macrophage activating molecule IFN-γ (Tufariello et al, 2003).

1.19.2. Development of granuloma

Mtb, after reaching in to a macrophage, resides in the endocytic vacule called the phagosome. The antigen presenting cells begin antigen processing and presentation after 10 days of infection. The processed components of the *Mtb* antigen termed adjuvant are presented to antigen-specific T lymphocytes which undergo clonal proliferation, cytokine release, recruitment of cells and finally granuloma formation (figure 1.7) (Saunders and Copper, 2000)**.** The granuloma is the classical hallmark of TB. Initially thought to be exclusively beneficial to the host, the role of the granuloma is now found to be more

complex (Ramakrishnan, 2012). A granuloma is constantly remodeled, due to the balance between pro-and anti-inflammatory immune signals at the site of infection (Russell et al, 2010; Flynn et al, 2011).

Expression of protective immunity in the lung

(a) Cell recruitment   (b) Cell migration   (c) T cell–macrophage interaction   (d) Granuloma formation Bacterial containment
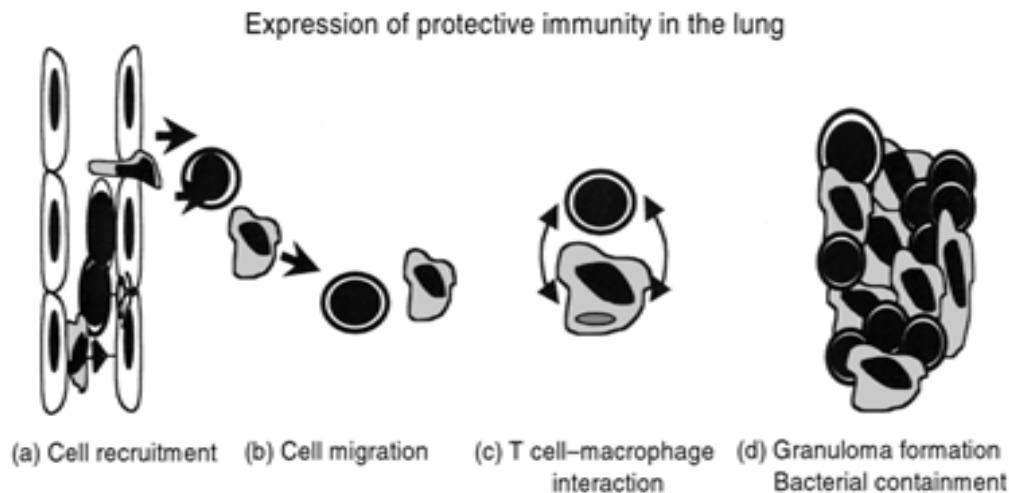
Figure 1.6: Expression of protective immunity against *Mtb*

Protective immunity against *Mtb* infection includes: (a) recruitment of mononuclear phagocytes and activated T lymphocytes. These cells must then (b) migrate to the site of infection where (c) T cells interact with infected macrophages to activate their antimycobacterial mechanisms and (d) enclose infected cells within the developing granuloma, thereby preventing further bacterial dissemination (Saunders and Copper, 2000)**.**

At the onset of adaptive immunity, more cells are recruited to the site of infection, the granuloma then acquires a more organized, stratified structure with a macrophage-rich center surrounded by lymphocytes that in turn may be covered with a fibrous cuff (Russell et al, 2010). The classically described caseous granuloma indicates a central necrotic region (figure1.8) rich in foamy macrophages (Flynn et al, 2011) that may become hypoxic and induce a non-replicative state of the bacteria (Via et al, 2008).
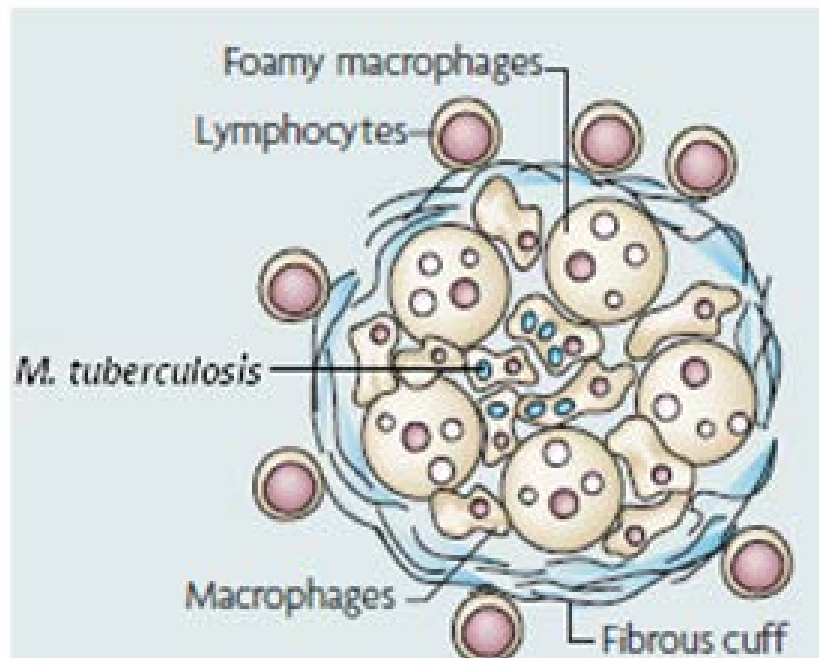
Figure 1.7: *M. tuberculosis* **in a granuloma**

Mtb inside lung macrophages and surrounded foamy macrophages and lymphocytes enclosed in a fibrous cuff (Image adapted from Russell, 2007).

1.20. Latency, reactivation/re-infection to produce secondary disease

The term latency refers only to the disease state and not the responsible bacteria. Given the assumed nature of these bacilli, they will be consigned the designation "dormant". The bacterial context of dormancy has been defined as "a reversible state of low metabolic activity in a unit that maintains viability (Kaprelyants and Kell, 1993; Keep et al, 2006). Latency may last for the patient's life time or the patient may develop reactivation disease. In one study, the IS6110 insertion sequence fingerprint of *Mtb* isolated from a patient with TB was matched to an isolate taken from his father over 30 years previously, demonstrating that reactivation disease is due to long term infection and not the acquisition of a new infection (Boshoff and Barry, 2005). Associated risk factors include people who are immune-compromised either through co-infection or through malnutrition and age.

Individuals with Human Immunodeficiency Virus (HIV) often develop an *Mtb* co-infection either through reactivation of a latent infection or direct from a primary infection.

The progression of latent *Mtb* infection to an active infection occurs when the granuloma starts to degrade and expand, bringing oxygen and other nutrients to the bacteria in the granuloma (Dye et al, 2008). Once the granuloma structure collapses, *Mtb* and infected macrophages spread to other foci in the body, notably the central nervous system and bones. At each focal point lesions are formed that destroy local tissue.The importance of re-infection as a cause for recurrence of tuberculosis is unclear and has potential public-health implications. Causes of recurrence/relapse and re-infection might essentially independent (Lambert et al, 2003). Re-infection is 'the main cause' of drug-resistant TB (Talent Ngandwe, 2007).

1.21. Breakdown of caseous lesions, erosion into bronchi and TB infection

In TB infection, the strength of cell mediated immunity determines whether the tubercle bacilli are arrested or the infectious process continues (Issar Smith, 2003). Liquefaction of granuloma through unknown process but possibly involving proteinase enzymes facilitate for the bacteria to replicate in uncontrolled manner and escape from the granuloma and then spread throughout the lungs. Subsequently, the bacilli may reach and disseminate to other tissue and organs through the circulatory and lymphatic system that may lead to extra pulmonary tuberculosis infection (Issar Smith, 2003). Moreover, such extensive pathology may spill the bacilli into the airways resulting in the transmission to new hosts (Iseman, 2000).

Matrixmetalloproteinases (MMPs) are enzymes that are induced during pulmonary TB infection (Jennifer et al, 2006). These enzymes degrade the extracellular matrix (ECM) components such as collagen and proteoglycan and also contribute for leukocyte migration and tissue modeling (Jennifer et al, 2006). The two most important MMPs are MMP-2 (gelatinase A) and MMP-9 (gelatinase B), which degrade type IV collagen, a major component of the basement membrane within the lung (Parks and Shapiro, 2001). MMP-9 particularly involves in various disease manifestations such as cavitary TB (Chang et al,

1996; Hrabec et al, 2002), meningitis (Matsuura et al, 2000; Lee et al, 2004), and pleuritis (Hoheisel et al, 2001; Hrabec et al, 2004). The ECM is involved in leukocyte trafficking in the granuloma during TB infection (Gonzalez-Juarrero et al, 2001).

Caseation may be regarded as a defense mechanism as bacilli are still viable and embedded in the area of coagulative necrosis. *Mtb* may remain viable for long periods in caseous material, though its calcification is probably their final burial. The caseous material liquefies and dormant bacilli may now proliferate and disseminate. It seems likely that the softening is brought about through the activation of a host protease rather than through the activity of the contained bacilli. One enzyme that is capable of digesting solid caseous *in vitro* is plasmin (Lack, 1953). .

1.22. Treatment and control of TB

The major rationales of TB treatment are to cure the individual patient and to control transmission of the disease. Treatment of TB requires 6 to 12 months to complete and two or more anti-TB drugs that work differently are administered to prevent bacteria drug resistance. There are two phases during the treatment of TB namely the intensive and continuation phases (Nardell, 2008).

1.23. Antimicrobials

1.23.1. Anti-TB drug regimens

The first anti-TB drug in clinical use, around 1945 was streptomycin (Iseman, 2000). However, streptomycin mono-therapy resulted in the emergence of bacterial resistance and treatment failure (McKinney et al, 2000). Isoniazid (INH), Rifampicin (RIF), Pyrazinamide (PYZ), and Ethambutol (ETB) are the drug of choice used during the intensive phase administered orally and daily for two months. In the continuation phase, INH and RIF are given for a further 4 months, either daily or 3 times per week. Vilcheze and Jacobs (2007) documented that INH plays a critical role in the initial killing of replicating bacteria and has been in use since 1952. INH requires activation by catalase-peroxidase enzyme (*KatG*) for commencement of its activity during TB treatment. On the other hand, RIF and PYZ

are believed to eradicate dormant or non-replicating bacteria which make them important to eradicate the tubercle bacilli from the lesion (Heifets and Lindholm-levy, 1989; Garg, 1999). TB treatment always requires a combination of anti-TB drugs. Accordingly, the fixed dose combination formulation therapy (FDC) is developed (Blomberg et al, 2001) were patient are given FDC therapy adjusted to their body weight whenever self-administration of anti-TB drug is permitted (WHO, 2003). The mode of actions of anti-TB drugs are documented in Chapter seven.

1.24. The Directly Observed Treatment, Short -course (DOTS)

In 1994 the WHO began new TB treatment method called the Directly Observed Treatment, Short Course (DOTS) (Raviglione and Pio, 2002). Since then the DOTS management strategy has become the internationally recommended approach for TB control programs (WHO, 1997; WHO, 2002). The WHO TB control program is based on 3 main strategies: DOTS, DOTS-Plus and ProTest (Cole et al, 2005). The five strategies of the DOTS includes Government commitment to sustainable TB control, diagnosis through sputum smear microscopy, standardized short-course chemotherapy, a functioning drug supply system, and a recording and reporting system allowing assessment of treatment results (Cole et al, 2005).

In Ethiopia, the DOTS strategy was started as a pilot in Arsi and Bale zone, Oromia region, in 1992 with gradual expansion to all parts of the country. The DOTS program has been initiated in Tigray, one of the administrative region in Ethiopia, in 1995 (Mesfin, 2005) and in the Southern Ethiopia Regional state in 1996 (Estifanose and Lindtjorn, 2005). Moreover, the DOTS strategy has been subsequently scaled up in the country and implemented at national level.

1.25. Drug resistant TB infection

TB drug-resistance can take place when the anti-TB drugs are not correctly taken such as incomplete the course of therapy or when the dose is inappropriate or the length of time of treatment is unnecessarily shortened or when the drugs are of poor quality (CDC, 2009).

The WHO defines drug resistance as a decrease in susceptibility of sufficient degree to be distinguishable from a wild type strain that has never been exposed to the drug (WHO, 1997). Generally, when one percent or more of organisms in an isolate are found resistant to anti-TB drug, therapeutic success is less likely to occur. It is then that the strain is considered resistant to the drug (Rom and Garay, 1996). Drug-resistant TB was recognized shortly after the introduction of anti-TB chemotherapy (Pyle, 1947). In 1948, TB drug resistance was first reported by the British Medical research Council (MRC) that revealed the treatment outcome of streptomycin found very comparable rather similar among treated and untreated patients (Medical Research Council, 1948). AlthoughTB drug resistance continued little attention was given until the 1990s (Nachega and Chaisson, 2003).

TB drug resistance could be one of the following: (1) Primary anti-TB drug resistance is a type of resistance that occurs in patients who have not had prior treatment with anti-TB drugs (when patient has not had previous treatment). When the history of treatment is doubtful it is called initial resistance which is a mixture of primary resistance and undisclosed acquired resistance. On the other hand acquired resistance is a type of resistance among patients that had previous treatment (more than one month) (WHO, 1993). Recent recommendations for the different types of TB drug resistance includes drug resistance among new cases instead of primary resistance and drug resistance among previously treated patients instead of acquired resistance (Somoskovi et al, 2001).

TB drug resistance could be one of the following (FMOH, 2008): (1) Mono-resistance: patients are resistant to one first line anti-TB drug; (2) Poly-resistant: resistance to more than one first-line drug, other than Isoniazid and Rifampicin; (3) Multi-drug resistant (MDR-TB): resistance to at least both Isoniazid and Rifampicin; (4) Extensively-drug resistant (XDR-TB): resistance to at least Isoniazid and Rifampicin, in addition to any Fluoroquinolone, and to at least one of the three following injectable drugs: Capreomycin, Kanamycin and Amikacin.

Global TB drug resistance prevalence data shows that isoniazid and streptomycin resistance was found in 35 countries in 1994. The overall prevalence of TB drug resistance

was 9.9% with a median of 1% MDR-TB by the same year (WHO, 1997). Later the prevalence of tuberculosis drug resistance to more than one drug was reported 1.7% in Uruguay and 36.9% in Estonia (IUATLD, 2000). In some developing countries such as Mozambique, CoteD'Ivoire, Cameron, Argentina, the Dominica Republic, and Mexico the prevalence of MDR-TB among new cases was reported more than 3% by the year 2000 (WHO, 2000).

Different reports in Ethiopia have shown that drug resistance is on increase. Primary MDR-TB was first reported as 2% in samples taken from Addis Ababa and Harer (Wolde et al, 1986). The incidence of MDR-TB was estimated 2.3% by the year 2003 (WHO, 2003). However, the 2003-2006 national TB survey estimated a 1.6% incidence of MDR-TB and a prevalence of 11.8 % MDR-TB among retreatment cases (FMOH, 2008).

1.26. Phenotypic drug tolerance and the persister *Mtb* cells

Antibiotic tolerance is the capacity of a microorganism to resist the killing action of antibiotics and differs from so-called drug resistance in that the latter involves an increase in the minimal inhibitory concentration (Lewis, 2007). While drug resistance is caused through mutation, antibiotic tolerance is the method of survival from adverse environments through transient, dormant and non-dividing state. During antibiotic treatment, the killing pattern of the antibiotic can be considered biphasic in after the first few days so-called 'persister' cells survive (Lewis, 2010). Persister cells are tolerant to antibiotics without having acquired genetic modifications (Keren et al., 2004a). There are reports documented that persister cells have reduced growth rate (Balaban et al, 2004). On the other hand, dormancy refers to reversible, non-replicating state influenced by an external stimulus for recovery or resuscitation (Chao and Rubin, 2010; Zhang,2004). *Mtb* is also found to produce biofilms that share important features with persister cells (Ojha et al., 2008).

1.27. Immunization against tuberculosis

In early 1900s, Albert Calmett and Guerin isolated the causative agent of bovine TB, *M. bovis*. At that time, *M. bovis* was responsible for about 6% of all human TB deaths in

Europe, due to the use of un-pasteurised milk (Lawn, 2011). Calmett and Guerin observed that after growing the strain for some time they had selected an avirulent variant, which gave immunological protection also against *Mtb*. After a decade they had fully developed what we today call the BCG vaccine (Bacille Calmette-Guerin) (Ducati et al, 2006). BCG is an intradermal vaccine that is cheap, safe that protects children efficiently against TB (Iseman, 2000). Unfortunately, efficacy of the vaccine against pulmonary TB in adults is highly variable and protection can decline fast. Large and well-controlled vaccine trials have estimated the protection to range from 0 to 80% (Lawn, 2011).

Currently, four strategies can be identified in vaccine development: a replacement vaccine for BCG, a booster vaccine, a post-exposure vaccine or a therapeutic vaccine (Agaard et al, 2009). However, no vaccine candidate that today is in a Phase I or Phase II trial is estimated to be available earlier than 2020 (WHO, 2011).

1.28. Lipid bodies in Bacteria

In 1945, Knaysi reported the formation of fat droplets from the cytoplasm membrane in living cells of *Bacillus cereus* (Knaysi, 1945). Latter, Burdon (1946) confirmed the value of Sudan Black B for demonstrating intracellular fatty material in bacteria. The genus *Mycobacterium* is characterized by their high lipid content, which is largely attributable to their cell envelopes (Schweizer and Hofmann, 2004). The cell envelope is composed of unusual lipids derived from long chain fatty acids (LCFA) and the high requirement for LCFA of these organisms is reflected by their ability to synthesize medium and long chain fatty acids via the enzyme systems fatty acid Synthase I and II respectively (Takayama et al, 2005). It was previously believed that the entire lipid that was synthesized or taken up by the cell was located in the cell envelope. However, recent work has revealed that lipid in the form of triacylglycerol (TAG) is also present as intracellular droplets within the mycobacteria cell cytoplasm (Garton et al, 2002).

Lipid bodies were demonstrated by the Leicester laboratory in tubercle bacilli in sputum obtained from a patient withTB infection but they were not observed in *Mtb* cells grown in the laboratory at that time. This difference is indicative of *Mtb* adopting an altered

physiology within the host (Garton et al, 2002). More recently, the occurrence of lipid bodies has been associated with arrest or slowing down of bacterial growth and with bacterial dormancy (Garton et al, 2008, Garton personal communication). Lipid bodies may store the fatty acids that supply abundance of exogenous fatty acids (Munoz-Elias and Mckinney, 2005). Two distinctive characteristics of human TB are the frequent occurrence of latent infection and the extended period required for chemotherapy. It is unknown whether the physiological states of the groups of bacteria underpinning these disease patterns are equivalent or entirely discrete. Exposure of the tubercle bacilli to nitric oxide *in vitro* stimulated LB formation and LB positive cells were tolerant to the cidal action of refampicin (Sherratt, 2008). However, the relationship between the proportion of lipid body positive tubercle bacilli in sputum and concentration of nitric oxide measured as fractional exhaled nitric oxide (FeNO) was not previously reported and this is the main focus of this study.

1.29. Lipid storage in prokaryotes

Lipids, in addition to their role in membranes and envelopes of bacteria can also act as storage material in prokaryotes. Currently, it is well documented that many prokaryotes are able to accumulate large amounts of lipophilic compounds as inclusion bodies in their cytoplasm (figure 1.8) (Waltermann and Stenibuchel, 2005). Among the reported lipophilic compounds found in different microorganisms polyβ-hydroxybutrate (PHB), triacylglycerols (TAGs) and wax ester (WE) lipid bodies are only found within afew prokaryotes (Alvarez and Steinbuchel, 2002).

Polyhydroxybutrate (PHB): is sometimes considered to be a carbohydrate; however, it has solubility characteristics of a lipid (Waltermannnand Stenibuchel, 2005). Accumulation of PHB usually occurs in the presence of an excess of carbon source when another nutrient, such as nitrogen, phosphorous, sulphur or oxygen are limiting (Anderson and Dawes, 1995). Polyhydroxyalkanote (PHA) storage and metabolism is best characterized in *Ralstoni aeutropha* (Figure 1. 8A), in which PHA is stored as spherical intracytoplasmic inclusions accounting for 90% of the cellular dry weight (Anderson and Dawes, 1995).

Lemoigne (1926) documented that accumulation of PHA was reported in *Bacillus megaterium* (Stenibuchel and Valentine, 1995). Almost 150 different hydroxyalkanoic acids have been described as constituents of PHAs (Stenibuchel and Valentine, 1995). In *Ralstoni aeutropha* almost sphere-shaped intracytoplasmic PHA inclusion body accumulation was reported (Anderson and Dawes, 1995).

Triacylglycerol inclusions (TAGs): are found as the principle lipid storage in different eukaryotic cells like moulds, yeasts and algae. In one study 87% of the lipid bodies were composed of TAGs. The other components of the TAG lipid bodies include diacylglycerols (DAGs) (5%), free fatty acids (5%), phospholipids (1.2%), and proteins (0.8%) (Alvarez et al, 1996). Triacylglycerol is fatty acid tri-esters of glycerol and represents a convenient and highly efficient form of storing fatty acids (Alvarez and Stenibuchel, 2002). Triacylglycerol inclusions can almost completely fill the cell and are thought to act mainly as energy and carbon storage bodies. However, there have been a number of other functional roles suggested for TAG bodies, such as deposits for toxic or surplus fatty acids from phospholipid biosynthesis. Triacylglycerol bodies may also be utilized to maintain a water supply during periods of dehydration through oxidation of fatty acid and hydrocarbon chains (Waltermannnand Stenibuchel, 2005).

Wax esters (WE): synthesis in Gram negative bacteria particularly in the genus *Acinetobacter* were reported about 39 years ago (Fixter and Fewson, 1974). Latter, accumulation of WEs were also illustrated in *Moraxella*, *Micrococcus*, and *Fundibacter* (Bredemier et al, 2003). On the other hand, WE synthesis among the member of the *Actinomycetes* such as *Corynebacterium*, *Mtb* and *Nocardia* were also described (Waltermannn and Stenibuchel, 2005). It was also indicated that WE bodies were found extracellular in the case of some bacteria such as some strains of *Acinetobacter* and the marine bacterium *Fundibacterium jadensis.* The major function of TAG and WEs is to provide energy and carbon. They are also used for removal of poisonous fatty acids during the growth of bacteria (Alvarez et al, 1996).
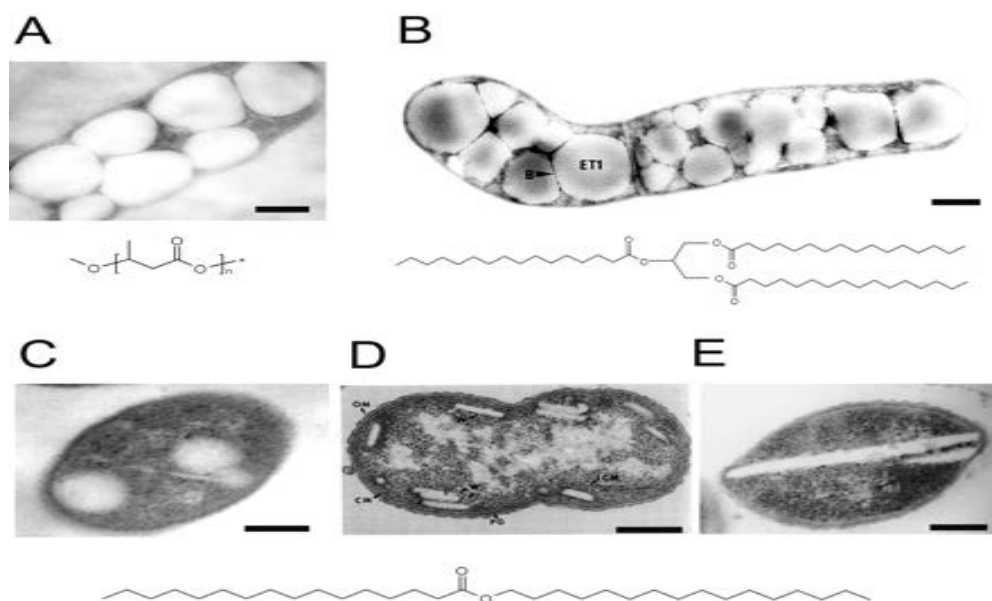
Figure 1.8: Intracellular lipid inclusions in prokaryotes and general structures of the lipids

(A) Cell of *R. eutropha* H16 accumulating PHB inclusions; (B) cell of *R. opacus* PD630 accumulating large amounts of TAG inclusions; (C) cell of *A. calcoaceticus* ADP1 with three spherical WE inclusions; (D) *Acinetobacter* sp. strain HO1-N accumulating small rectangular WE inclusions; (E) *Acinetobacter* sp. strain M1 accumulating large WE inclusions (Adopted from Waltermann and Stenibuchel, 2005).

1.30. Formation of prokaryotic lipid bodies

Although the major lipid inclusions of the Gram negative bacterium *Acinetobacter calcoaceticus* ADP1 were found WEs, small amounts of TAG accumulation was also found. The most important enzyme for TAG synthesis in this bacterium was the Wax ester synthase /Diacylglycerolacyltransferase (WS/DGAT) (Kalscheuer et al, 2004). *In vitro*, WS/DGAT showed broad capability of utilizing a large variety of fatty alcohols (Kalschever et al, 2003). Waltermannn and Steinbuchel (2005) documented that there are three categories of enzymes that are implicated in TAG formation. In eukaryotes, two enzymes that have no resemblance of each other ($DGAT_1$ and $DGAT_2$) have been identified. $DGAT_1$ gene families were found in animals and plants (Makula et al, 1975; Cases et al, 1998), whereas members of the $DGAT_2$ gene family were found in animals, plants and *Saccharomyces cervisiae* (Case et al, 2001; Sandager et al, 2002) .There are

other reports that showed TAG biosynthesis in some plants mediated by phospholipids (PLs). The third alternative mechanism of TAG synthesis in both animals and plants is through the DAG- DAG- transacylase that yield in TAG and monoacylglycerol (Lehner and Kuksis, 1993). Other bacteria such as *Streptomyces coelicolor* synthesizes mainly TAG and no WE accumulation reported in this bacterium (Olukoshi and Packter, 1994).

1.31.   Lipid bodies in mycobacteria

Schafer and Lewis (1965) demonstrated that uptake of oleic acid or its ester Tween resulted in notable accumulation of LBs and increase in optical density in culture. Microscopic study of *Mycobacterium* species including *M. avium* (1950), *M. leprae* (1956), *M. kansasii* (1965), *M. smegmatis* (1972) and *Mtb* (1977) confirmed the occurrence of lipid bodies (Sherratt, 2008). It was also reported that *Mycobacterium bovis* bacillus Calmette- Guerin (BCG) induced lipid bodies within the cytoplasm of the cell (D'Avila et al, 2006). Bacillus Calmette – Guerin (BCG) was reported to be capable of adapting to an aerobiosis *in vitro* by shifting down to a non- replicating persistent state similar to *Mtb* (Lin et al, 2009). Christensen et al (1999) observed the interactions between fluorescent lipid probes and mycobacteria and reported that a substantial proportion of the cell contained intracellular lipophilic inclusions. The principal candidate compounds were poly-β-hydroxybutrate (PHB), triacylglycerols (TAGs) and wax esters and the latter were shown to be a major component (Garton et al, 2002). Recent observations demonstrated that lipid body positive acid-fast bacilli (figure 1.9) are observed almost in every sputum sample taken from patients infected with pulmonary TB (Garton et al, 2008).
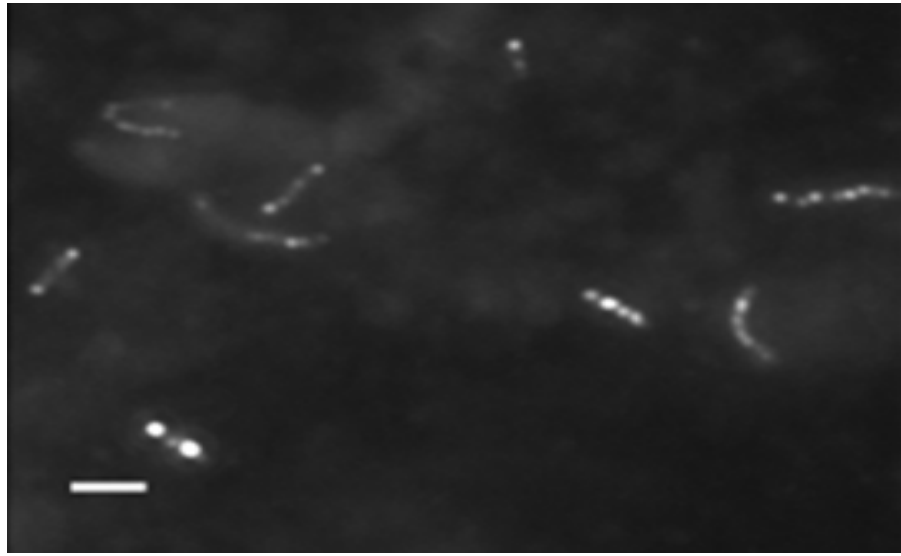
Figure 1.9: Lipid bodies within the AFB

Lipid bodies are composed of the neutral lipid triacylglycerol (TAG). Accumulation of TAG/LB associated with transcription of *tgs1*, a triacylglycerol synthase and TAG accumulation induced under conditions of stress, low O_2, NO, low pH. *tgs1* is member of the DosR regulon, strongly induced in Wayne model of NRP (Garton, unpublished source).

1.32. Lipid body formation in mycobacteria subjected to growth arresting stimuli

Garton et al (2002) reported that lipid body (LB) formation in *M.smegmatis* was environmentally regulated and manipulation of the growth condition affects the occurance of LBs. Sherratt (2008) reported that *M.smegmatis* grown under low nitrogen broth formed LBs. LBs are also formed in *M.tuberculosis* subjected to Wayn's shift-down model of hypoxia and nutrient limiting conditions. Cultures of *M. tuberculosis* H37Rv subjected to pH shock formed lipid bodies. The morphology and pattern of LB occurrence varied according to the conditions under which the cells were grown in *M.smegmatris*. Stored LBs may be available for utilization when conditions become more favourable for growth. LBs are mobilized upon carbon starvation, suggesting that the lipids stored in the LB are broken down and catabolised by β-oxidation during periods of carbon starvation. *Rhodococcus opacus* has previously been shown to mobilize stored TAG in the absence of carbon source (Waltermann and Steinbuchel, 2005). Another important growth arresting stimuli for

M.tuberculosis is exposure to nitric oxide (NO). *M.tuberculosis* subjected to NO produced LBs and the formation of LBs was dependant on the time course (Sherratt, 2008). The relation between NO and LBs is documented in detaile in subsequent chapters.

1.33. Exhaled Nitric oxide

Nitric oxide was initially described in the 1980s as endothelial derived relaxation factor. The detection of NO in exhaled air was first reported in 1991. For many years it has been supposed that the production of an excess of NO play a major role in inflammatory diseases (Herman et al, 2003). NO plays a major role in the pulmonary host-defence mechanism in response to infections and is implicated in bacteriostatic as well as bactericidal processes (Wang et al, 1998). Endogenous NO has been implicated in host defense mechanism against TB and enhanced NO production was also reported during pulmonary tuberculosis infection (Kuo et al, 2000).

1.34. The principal sources of nitric oxide in the airways

Nitric oxide is produced at different sites in the human airways (Tornberg et al, 2002). However, in normal human breath, most NO production takes place in the uppermost portion of the respiratory tract namely the nose and paranasal sinus (Dillon et al, 1996). There are reports that documented high levels of NO in the nasal cavity which may have effect upon gas exchange and pulmonary vascular tone (Tornberg et al, 2002). The concentration of nasal NO declines during hypoxia and nasal NO output is a positive function of lumional gas flow rate. Lundberg et al (1995) reported that NO levels have been several-fold higher in the sinuses than in the nose suggesting that the paranasal sinuses are the main site for NO production within the airways. On the other hand, the potential sources of NO in the lung were found the macrophages, neutrophils, alveolar type-II cells (tumor-cell line from a human lung), endothelial cells and airway cells (Lang et al, 2002). There are also Non enzymatic routes of NO production. These include spontaneous reduction of tissue nitrite to NO under acidosis conditions and reactions between L-or D-arginine and some arginine derivatives with H_2O_2 to form NO. Moreover,

Non-enzymatic NO production has been observed in the stomach, on the surface of the skin, in the ischemic heart and in infected nitrite containing urine (Weitzberg and Lunberg, 1998).

Nitric oxide can be formed from dietary nitrate which in the oral cavity is reduced by bacterial reductases to nitrite (McKnight et al, 1997). Anaerobic bacteria in the colon produce NO, using nitrite and nitrate as substrate (Goretski et al, 1990; Brittain et al, 1992).

1.35. Factors that can affect level of NO

Nasal NO contamination: Current thinking is that NO is formed in both the upper and lower respiratory tract (Lundberg et al, 1994). Nasal nitric oxide concentrations are high relative to the lower respiratory tract in humans (Alving et al, 1993), with the highest levels reported in the paranasal sinuses (Kimberly et al, 1996). The issue of the relative contribution of nasal NO to exhaled NO has been addressed in many publications. Nevertheless, it was documented that orally-exhaled air reflects NO levels from the lower airways (Kharitonov and Barnes, 2006). Moreover, Dweik et al (1998) found that mouth measures of NO accurately reflected lower airway levels.

Eosinophilic inflammation versus NO: The relationship between exhaled nitric oxide and mucosal eosinophilic inflammation in children with difficult asthma has been reported. It was reported that FeNO concentration was associated with eosinophilic inflammation in children with difficult asthma (Berry et al, 2005; Payne et al, 2001).

NO and other lung diseases: NO appears to be important in neural bronchodilator and vasodilator mechanisms, in the regulation of airway and pulmonary blood flow, and in immune defence. When produced in low concentrations by constitutive NOS it appears to have a generally beneficial role, but when produced in large amounts by inducible NOS it may result in increased inflammatory responses and tissue damage, suggesting that nitric oxide may be involved in the pathophysiology of several pulmonary diseases (Barnes and Belvisi, 1993).

Altitude and NO: Exposure to high altitude elicits integrated physiological responses to permit survival during hypoxia. Natives of Tibet living at high altitude have adapted in part

through the generation of high levels of nitric oxide and circulating nitrogen oxide species that enable greater blood flow and oxygen delivery to offset hypoxia. Studies of acutely exposed visitors to high altitude were compared with Native Tibetans. Tibetans had NO levels in the lung, plasma, and red blood cells that were at least double and red blood cell-associated nitrogen oxides were more than 200 times higher (Beall et al, 2012).

NO and smoking: Cigarette smoking is associated with an increased risk of cardiovascular disease through increased hypertension and platelet aggregation. Both symptoms of which may be caused by decreased endogenous nitric oxide (NO) formation (Patrick Wong, 1999).). Higman et al (1996) reported that the release of NO from saphenous veins of nonsmokers was significantly higher than that from veins of heavy smokers.

1.36. Nitric oxide, lipid bodies within *Mtb* and antibiotic tolerance

The entry of *Mtb* in the macrophage is followed by the activation of the cell by IFN-γ and make the intercellular environment more harsher for the survival of the bacteria by lowering pH, stimulating the fusion of phagosome with lysosome and the production of nitric oxide (NO) (Schnappinger et al, 2003). Idh (2012) documented that NO in the host is part of the immune defense in TB. High levels of NO up on exposure to *Mtb* may clear the bacteria even before infection occurs and intermediate levels of NO may lead to latent infection, where the cell mediated immunity (CMI) and NO control the infection for a life time. In case of insufficient levels of NO production by a poor CMI, bacterial replication will continue and cause disease.

On the other hand, Sherratt (2008) demonstrated that *in vitro Mtb* formed lipid bodies after 4 hour NO treatment and this treated populations of bacteria was significantly more tolerant of INH and RIF than cultures treated without NO (control groups). Voskuil et al (2003) reported that NO can trigger *Mtb* to switch from active cell division to a state of non-replicating persistence. Demonstration of lipid body formation in non-replicating cells strengthened the hypothesis that lipid bodies are associated with antibiotic tolerance (Sherratt, 2008). *Mtb* cultivated under hypoxic conditions demonstrated lipid body formation (Garton, communication). Tubercle bacilli were also reported more infectious to

guinea pigs than aerobically grown cultures (Bacon et al, 2004). *Mtb* is likely to encounter NO *in vivo*, as it is released by the activated macrophage (Boshoff and Barry, 2005) and acts as a eukaryotic signaling molecule (Martin et al, 2000). However, the *in vivo* nitric oxide concentration and its association with the proportion of lipid body positive tubercle bacilli in sputum are not fully elucidated. Therefore, it could be important to understand whether endogenous NO contributes to the accumulation of lipid bodies within the tubercle bacilli in sputum.

1.37. Hypothesis, Aims and Objectives

The central hypothesis for this work was that fractional exhaled air NO (FeNO) is a major determinant of the LB content of *Mtb* cells in sputum. The overall aim of this study was therefore to determine FeNO concentration and *Mtb* LB frequencies in sputum from TB patients. Because many other factors could affect both host FeNO levels and the tendency of *Mtb* to form LBs, several of these factors were also investigated.

Specific objectives

To count the proportions of LB[+ve] tubercle bacilli in sputum from these patients and determine any relationship between these and FeNO levels.

To determine the levels of FeNO among smear positive TB patients in Gondar.

To study additional host, environmental and bacterial factors that may impact on this proposed relationship:

Host factorsto be studied include: Nutritional status, sex, age, smoking status, history of asthma, and other host factors reported to affect FeNO concentration.

Environmental factors include: HIV status and intestinal parasite infestation.

Bacterial factors include: *Mtb* spoligotypes, expression of selected genes in sputum samples and drug resistance patterns.

To study relationships between of FeNO levels, LB[+ve] rates and the corresponding proportion of LB[+ve] tubercle bacilli in sputum on treatment outcomes in selected patients.

CHAPTER TWO: Sputum processing and staining procedures

2.1. Introduction

Staining is a technique that helps the identification of different cells and the components of a cell during microscopic examination as many of the bacterial cells are colorless by nature. In the 1940s, Sudan black B stain was used by Burdon to envisage LBs in bacteria (Burdon, 1946). Later, LBs were examined and reported as universal features of tubercle bacilli in sputum (Garton et al, 2008). Purulent sputum is formed by liquefaction of the necrotic internal tissue and caseum contains white blood cells, dead tissue, serous fluid and viscous liquid (mucus) (LeBlond, 2005). Visualizing LBs within the tubercle bacilli after staining the organism in sputum preparations with Auramine O/Nile red stain is an established method in this laboratory (University of Leicester) as documented in Chapter one but the method suffers from a number of problems. During fluorescence microscopy for demonstration of *Mtb* LBs in sputum a high background of staining is often observed due to other lipophilic materials present in the preparations; these may render LB analysis difficult or even impossible.

Reducing the effect of lipophilic materials other than LBs on Auramine O/Nile red stained sputum preparation was explored in this section using detergents, solvents and digesting enzymes. A detergent is a surfactant or a mixture of surfactants with cleaning properties in dilute solution that potentially aids in the removal of loosely associated material by enhancing solubility in water. Detergents are amphipathic molecules, meaning they contain both a non-polar "tail" having aliphatic or aromatic character and a polar "head". The ionic character of the polar head group forms the basis for broad classification of detergents; they may be ionic (charged, either anionic or cationic), nonionic (uncharged) or zwitterionic (having both positively and negatively charged groups but with a net charge of zero). Common non-ionic and zwitterionic detergents include Tween-80 and Triton X-100 (Smulders et al, 2002). A detergent can be denaturing or non-denaturing. Non-denaturing detergents such as Triton X-100 have rigid and bulky non-polar heads that do not penetrate into water-soluble proteins; consequently, they generally do not disrupt native interactions and do not have cooperative binding properties. The main effect of non-denaturing

detergent is to associate with hydrophobic parts of membrane proteins, thereby conferring miscibility to them. Tween 80 also known as polysorbate 80 or Polyoxyethylene-sorbitan-20-monooleate is a nonionic surfactant and emulsifier (Coors et al, 2005).

Denaturing detergents can be anionic such as sodium dodecyl sulfate (SDS) or cationic such as ethyl trimethyl ammonium bromide. Denaturing detergents such as SDS bind to both membrane (hydrophobic) and non-membrane (water-soluble, hydrophilic) proteins. SDS binding is cooperative (the binding of one molecule of SDS increases the probability that another molecule of SDS will bind to that protein) and alters most proteins into rigid rods whose length is proportional to molecular weight.

A solvent, usually thought of as a liquid, is a substance that is capable of dissolving or dispersing other substances and forming a uniform mixture called a solution (Burke, 2011). The substance dissolved is called the solute and is usually considered to be the component present in the smallest amount. Propanol, butanol, formic acid and formamide are examples of polar solvents. Dipolar solvents which contain a C-O double bond without O-H bond include acetone and carbon tetrachloride whereas benzene and diethyl ether are non-polar solvents. The solubility of ethanol differentiates it from other alcohols. Solubility refers to the chemical property of a substance that enables it to dissolve in a solvent. Ethanol has a non-polar carbon chain connected to a polar hydroxyl function group. Thus, due to its polar OH group, ethanol is miscible and soluble in water

2.1.2. Aims and objectives

The major aim of this study was to optimize a method for demonstrating LBs within the tubercle bacilli in sputum samples through microscopy.The specific objectives were the following:

1) To evaluate the efficancy of Sudan Black and Oile red O stains for counting LBs in sputum samples in Gondar.

2) To evaluate methods used to reduce back-ground fluorescence signals in the established Auramine O/Nile red method.

2.2. Materials and Methods

All chemicals were obtained from Sigma-Aldrich Company Limited (Poole, Dorset, UK) or Fisher Scientific (Loughborough, Leicestershire, UK), unless otherwise stated.

2.2.1. Preparation of Reagents

SUDAN BLACK STAIN: 0.3g Sudan Black (Sigma) powder was dissolved in 100 ml 70% ethanol.

OIL RED O: 0.35 g of Oil red O dissolved in 100 ml isopropanol (2-propanol), allowed standing overnight at room temperature and filtered. The working solution was prepared by mixing 6 ml of the stock with 4 ml of sterile distilled water and after 20 minutes re-filtered.

NILE RED STAIN: Nile Red stock solution, 0.5 mg/ml in 95% ethanol, filtered (0. 2µm, Acrodisc), was wrapped in foil and stored at -20 ^{o} C. To prepare the working Nile red solution, the stock was diluted in PBS to 10µg/ml solution.

AURAMINE O STAIN: Solution 1: 0.1 g of Auramine O was dissolved in 10 ml 95% ethanol. Solution 2: 3.0 g of phenol crystal dissolved in 87 ml distilled water. The two solutions were mixed and stored in a tightly stopper amber bottle protected from light by aluminum foil.

ZEIHL-NEELSEN (ZN) STAIN:(i) Carbolfuchsin: a saturated solution of basic fuchsin (3 g of basic fuchsin in 100 ml of 95% ethyl alcohol), 10ml; 5% weight/volume (w/v) aqueous solution of phenol, 90 ml. (ii) Acid –alcohol: 3 ml of HCl with 95% ethyl alcohol to bring the volume to 100 ml. (iii) Counter stain; methylene blue, 0.3% w/v with water**.**

N-ACETYL-L-CYSTEINE (NALC):50mM solution of Sodium Citrate in distilled water was prepared. Immediately before use, 0.5 g NALC powder was added to 50ml sodium citrate solution.

N-ACETYL-L-CYSTEINE /SODIUM HYDROXIDE (NALC/NAOH):50mM sodium citrate (50 ml), 4% (w/v) sodium hydroxide (50ml), NALC (0.5g).

PHOSPHATE BUFFER: was prepared by combining 134 mM NaH_2PO_4 and 134 mM Na_2HPO_4 at a ratio of 51:49 to give a 67 mM solution at pH 6.8.

PHOSPHATE BUFFER SALINE: Phosphate buffered saline (PBS) was prepared using PBS tablets (Sigma-Aldrich). One tablet was dissolved in 200 ml distilled water to give a solution of 0.01M phosphate buffer, 0.002 M potassium chloride and 0.137 M sodium chloride at PH 7.4.

SODIUM HYDROXIDE: 4% (w/v) solution was used for this study.

ACID ALCOHOL: 0.5 ml HCl in 70% (v/v) ethyl alcohol.

POTASSIUM PERMANGANATE: 0.5% (w/v) aqueous solution.

MOUNTING MEDIA: 10% (v/v) glycerol in PBS.

2.2.2. Sputum sample collection and decontamination

Sputum samples were collected using dry, clean, leak proof, translucent and screw-capped plastic containers with a capacity of 30 ml. The leftover sputum samples from routine laboratory investigations for AFB that were collected from three consecutive sputum samples (spot-morning-spot) were pooled together. Depending on the amount of the pooled sputum sample available, $3/4^{th}$ of each (2 to 5 ml) was used for smear preparation and the other $1/4^{th}$ was kept for culture. Two to five ml samples were deposited in a 15 ml Falcon tube (Helena Biosciences, Sunderland, UK), decontaminated and homogenized through treatment with N-acetyl-L-cysteine-Sodium hydroxide (NALC/NAOH) solution. NALC was freshly prepared shortly prior to use as it is readily inactivated by oxidation. An equal volume of digestant was added to the sputum and gently mixed for no longer than 30 seconds. The mixture was allowed to stand at room temperature for 15 minutes with occasional agitation followed by neutralizing by adding 67 mM phosphate buffer to within 1 cm of the top of a centrifuge tube. The bacterial pellet was harvested after centrifugation at 1258 xg for 20 minutes (International equipment company, IEC, Needham Heights, NA 02194, USA, 1998). The pellet was re-suspended with either 250 µl or 500 µl PBS for sputum samples less than 2 ml and greater than 2 ml by volume respectively. Seventy percent of each sputum preparation was directly kept at -20°C for lipid body examination and 30% of it used for DNA assay (see chapter five).

Previously, to confirm the AFB result of each laboratory at the study site, a portion of each sputum sample was also decontaminated as indicated earlier. Ten µl of the digested material was deposited on a clean microscope slide was spread over an area of approximately 2 cm^2 using the side of the pipette tip. Smears were dried and heat fixed by passing the preparation through flame 2 to 3 times and re-fixed with formaldehyde. Each slide was placed inside a

slide holder and placed in a metal jar alongside cotton soaked in 23% (w/v) formaldehyde and sealed with plaster. The slides were fixed overnight. From each sample a slide was stained with Ziehl-Neelsen (ZN) method and the AFB score determined. The slides, both stained and unstained, and unprocessed sputum samples were stored at -20 ^{o}C and finally transported to University of Leicester using STYRO-FOAM with ice box.

2.2.3. Bacterial strains and culture

Table 2.1: Bacterial Strains used in this study

Strain	Description	Source
M. tuberculosis H37Rv	Virulent laboratory strain	Laboratory Stocks
Mycobacterium bovis BCG Glaxo	Laboratory strain	Laboratory Stocks
Mycobacterium bovis BCG Pasteur	Laboratory strain	Laboratory Stocks
Mycobacterium smegmatis mc^{2}155	Laboratory strain	Laboratory Stocks

2.2.4. Growth Media

All culture media were obtained from Difco Laboratories (Detroit, USA), unless otherwise stated. Media were sterilized by autoclaving at 120ºC for 20 minutes unless otherwise stated.

Middlebrook 7H9 Broth

Middlebrook 7H9 broth was prepared by dissolving 4.7g broth powder in 900ml double distilled water containing 2.5g glycerol and 0.5g Tween-80. This was autoclaved at 121°C for 17 minutes. Broth was supplemented with sterile Albumin-dextrose-catalase (ADC) at a concentration of 10% (v/v) prior to use.

Middlebrook 7H10 Agar

Middlebrook 7H10 agar was prepared by dissolving 19g of agar powder in 900ml distilled water containing 6.25g glycerol. The agar was boiled for 30 minutes to allow the powder to dissolve and then kept at 50°C until sterilization by autoclaving at 121 ^{o}C for 17 minutes.

Agar was supplemented with oleic acid dextrose catalase (OADC) at a concentration of 10% (v/v) prior to use.

ADC Supplement

ADC was prepared from the following reagents in 150ml distilled water:

Bovine Serum Albumin fraction V (BSA)	7.50 g
D-Glucose	3.00g
Sodium Chloride	1.28g
Catalase	6.0mg

The solution was centrifuged at 6000 rpm for 30 minutes in a Sorvall RC-5B refrigerated super speed centrifuge. The supplement was then filter sterilized (0.2µm filter) and stored at 4 ^{o}c.

OADC Supplement

The supplement was prepared as above (ADC), with the addition of 8.63 ml Oleic Acid solution (1% (w/v) in 0.2M sodium hydroxide and before centrifugation the solution was sonicated (Decon FS 100, Ultrasound Ltd, UK) for 30 minutes to allow emulsification of the oleic acid.

2.2.5. Cultivation of *M. smegmatis*, BCG Pasteur and BCG Glaxo and preparation of smears

M. smegmatis was cultured on Middlebrook 7H10 agar with OADC and incubated for 48 hours at 37°C. A loop full colony of each culture were taken and deposited in Middlebrook 7H9 broth supplemented with ADC and Tween-80 and incubated at 37°C for 2-3 days. On the other hand, *Mycobacterium bovis* BCG Glaxo and BCG Pasteur taken from Middlebrook 7H10 agar (a loop full) supplemented with OADC were deposited in Middlebrook 7H9 broth supplemented with ADC and Tween-80 and incubated at 37°C for 8, 16 and 24 days. From each culture 1 ml broth was taken and centrifuged at 7558xg for 1 minute. The supernatant was removed and pellet suspended with 1 ml Phosphate buffer saline (PBS), centrifuged at 7558xg for 1 minute and repeated twice. After removing the supernatant, the pellet was re-suspended with 500µl PBS and smears were prepared by depositing 10µl of the suspension on a microscope slide and allowed air dry.

The proportion of LB^{+ve} tubercle bacilli was enumerated at 0, 8, 16 and 24 days of incubation at 37°C.

2.2.6. Alternative processing and staining methods studied

2.2.6.1. Oil Red O / ZN dual staining

Oil red O stain alone was performed by flooding smears with Oil red O for 10 minutes followed by washing with distilled water and allowed to air dry then examined independently by brightfiled microscopy. Another set of oil red O stained slides were flooded with xylene for 10 minutes and washed with tap water. This was followed by the addition of carbolfuchsin and gently heated from the underside with a Bunsen burner and allowed to steam for 5 minutes. Slides were washed with tap water then decolorized with acid alcohol, washed again with tap water and flooded with methylene blue for 30 seconds. Slides were rinsed with tap water and allowed to air dry then mounted with PBS, sealed with nail varnish and examined by Microscopy.

2.2.6.2. Sudan Black/ZN dual staining

In this experiment, staining smears with Sudan black stain alone (without ZN stain) was performed by flooding slides with 0.3% Sudan black for 15 minutes then rinsing with distilled water, air drying and examined microscopically. Another set of Sudan black stained slides were flooded with xylene for 10 minutes and washed with tap water. Slides were then covered with carbolfuchsin, gently heated from the underside with a Bunsen burner and allowed to steam for 5 minutes. Slides were washed with tap water, decolorized with acid alcohol, washed with tap water again and finally flooded with methylene blue for 30 seconds before rinsing with tap water and air drying and mounting with PBS, sealed with Nile varnish and examined microscopically.

2.2.7. Detergents, solvents and lipase

Table 2.2: Detergents, solvents and lipase enzyme used in this study

Treatments	Concentration
SDS	0.025%, 0.05%,0.5%
Tween 80	0.01%, 0.1%
Triton	0.01%, 0.1%
Acetone	0.1%, 1%, 5%, 10%
Ethanol	0.1%, 1%, 5%, 10%
lipase in 100 mM Tris chloride PH 7.7	50µg/ml
lipase in 100 mM Tris chloride PH 7.7 with 0.5% SDS	50µg/ml

2.2.7.1. Detergent and solvent wash on BCG mixed in AFB negative sputum

One ml of the BCG culture was mixed in AFB negative sputum, decontaminated with NALC/NaOH and centrifuged at 7558*xg* for 15 minutes. Pellet was suspended in PBS and 10 µl of the suspension deposited on a slide, heat fixed and rinsed with different detergents and solvents (table 2.2), stained by Auramine O/Nile-red stain, allowed air dry then mounted with PBS, sealed with Nile varnish and examined under fluorescent microscope.

2.2.7.2. Detergent washes followed by lipase treatment as a method for reduction of lipophilic eukaryotic-derived background material in sputum

Duplicate thick sputum smears prepared from two smear positive TB patients were investigated. One slide of each set was soaked in a solution of TritonX-100 (0.05% w/v) 3 times for 10 minutes using a fresh solution for each soak. After briefly rinsed with water, the slides were transferred in to a 9 mg/ml lipase solution and incubated at 37°C for 4 hours. The second two slides of each set were incubated in water as a control for the Triton X-100 and in 10mM NaCl, 100mM tris.HCl PH 7.2 as a control for the lipase treatment.

On the other hand, four sputum pellets each with 4+ AFB score (3 of them with very difficult back ground) were selected to determine the effect of detergent wash and lipase treatment on the background of Auramine O/Nile red stain. The decontaminated sputum pellets were centrifuged at 7558xg for 2 minutes, supernatant removed and re-suspended in 0.5 ml PBS. The suspensions were split in to two 1.5ml tubes and centrifuged at 7558xg for 2 minutes. After removing the supernatant, pellets were re-suspended in 250µl of 0.05% w/v Triton X-100 and the other pellet batches from the same 4 samples were re-suspended in 250µl distilled water (controls). The Triton and distilled water were mixed with the pellets by up and down of the pipette tips; vortex mixed and allowed to stand for 10 minutes at room temperature. These were centrifuged at 7558xg and the Triton wash step was repeated twice. After the last centrifugation step, the test pellets were re-suspended in 500µl of lipase solution and the control pellets in 500µl of the corresponding buffer and incubated at 37°C for 4 hours. This was followed by centrifugation of samples at 7558xg for 2 minutes and pellets were re-suspended in 250µl of Phosphate buffer saline (PBS). From each sample, duplicate smears were prepared using 10µl of the suspension and briefly heat fixed then immobilized overnight in an atmosphere of 23% formaldehyde. The preparations were stained with Auramine O/Nile Red stain and examined by fluorescence microscopy.

2.2.7.3. Extended treatment of sputum pellets and *M. bovis* BCG with lipase

Four previously decontaminated sputum pellets with difficult Nile Red stain background were chosen to assess lipid bodies within the tubercle bacilli. Once thawed and vortex mixed duplicate smears were prepared from each sample using 10 µl of suspension, briefly heat fixed and immobilized overnight in an atmosphere of 23% v/v formaldehyde vapour. This is a further control, a time zero time point control. Sputum pellets were centrifuged at 7558xg for 2 minutes and re-suspended in 0.5ml PBS. The suspensions were split into two using 1.5ml tubes and centrifuged again at 7558xg for 2 minutes and supernatant decanted. One pellet from each sample was treated with detergent and lipase, the other with water and buffer as control. The test pellets were re-suspended in 250µl of 0.05% w/v Triton X-100 and the other pellet from the same sample (control) in 250µl distilled water. The

contents were mixed well by up and down of the pipette tip, vortex mixed and allowed to stand for 10 minutes at room temperature.The samples were centrifuged at 7558xg for 2 minutes, supernatant removed and Triton X-100 washing step repeated twice more. After the last centrifugation step and supernatant removed, pellets were re-suspended in 500µl of lipase solution and the control pellets in 500µl of the corresponding buffer. These were incubated at 37°C for 4 hours, 24 hours, 48 hours, and 96 hours. 125µl of the test and control pairs were withdrawn at each time point and the digests were returned to the incubator and incubated at 37°C for further time. Samples were centrifuged at 7558xg for 2 minutes, supernatant removed and pellets re-suspend in 125µl of 0.05% w/v Triton X-100 and centrifuged, supernatant removed and re-suspended in 125µl PBS. The detergent washing step at this point is expected to remove released fatty acid/glycerides which may still contribute to the background of Nile red staining of sputum smears. Duplicate smears from each sample were prepared by depositing 10µl of suspension on a microscope slide, dried, briefly heat fixed and fixed overnight in an atmosphere of 23% v/v formaldehyde vapour. The residual samples (both treated and control) at each time point in the lipase digestion were stored in the freezer. All the smears from one sample set (control and lipase treated samples from the same patient) were stained together and examined on the same day. The proportion of acid fast bacilli was also determined by comparing the number of tubercle bacilli available on the images of Auramine O compared with the phase image.

The same experiment, extended treatment with TritonX-100 and lipase digestion was undertaken on *Mycobacterium bovis* BCG and *Mtb* H37Rv taken from culture. One ml of each culture was deposited in an eppindrof tube and centrifuged at 7558xg for 2 minutes. The supernatant was removed and pellets were treated similar to the previous experiment.

2.2.7.4. Evaluation of the effect of formaldehyde evaporation, Phosphate buffer saline, 70% ethanol, and acid alcohol rinse on the background of Auramine O/Nile red stain

These experiments were carried out on *Mycobacterium bovis* BCG, *M.smegmatis* cultures and AFB positive decontaminated sputum samples. One ml of broth culture were taken and centrifuged at 7558xg for two minutes and washed with PBS two times and re-diluted with 0.5 ml of PBS. Smears were prepared by depositing 10µl of the suspension on a slide and were

allowed air dry. The preparations were immobilized with 23% formaldehyde overnight and the effect of formaldehyde evaporation and KMnO$_4$ concentration were evaluated. Three slides were taken from the overnight formaldehyde fixation and immediately stained with Auramine O/Nile red stain. One slide was finally stained with 0.5% KMnO$_4$, another with 0.1% KMnO$_4$ and the other with no KMnO$_4$. The effect of formaldehyde was evaluated by taking 3 slides for each experiment after 15 minutes, 30 minutes and 1 hour formaldehyde evaporation, after an overnight immobilization of smears with 23% formaldehyde, and stained with Auramine O/ Nile red stain.

Another experiments dedicated to determine the effects of PBS, 70% ethanol and acid alcohol wash on the background of Auramine O/Nile red stain were undertaken after the formaldehyde was evaporated for 1 hour. Smears were stained by Auramine O for 15 minutes and washed with tap water then decolorized with acid alcohol for 15 minutes and washed with tap water. The preparations were stained with Nile red for 10 minutes and washed with tap water. At this step, separate slides were rinsed with phosphate buffer saline, 70% ethanol and acid alcohol for 5 minutes and washed with tap water. Using the same procedure the effect of PBS, 70% ethanol and acid-alcohol were also evaluated on tick sputum smears.

2.2.8. Finalized Staining methods

2.2.8.1. Ziehl-Neelsen (ZN) stain

After gentle heat fix, each smear was flooded with carbolfuchsin solution and heat applied until steaming and allowed to stand for 5 minutes. After washing with tap water, smear was decolorized with acid alcohol for 1 minute and washed with tap water and counter stained with methylene blue for 30 seconds, washed and air dried. The slides were mounted in PBS and sealed with nail varnish. Slides were examined under 100x oil immersion objective with bright field illumination.

2.2.8.2. Lipid body stain (Auramine O / Nile Red stain)

Smears were flooded with Auramine O solution for 15 minutes, washed in de-ionized water and decolorized by flooding with acid alcohol for 15 minutes. The slides were washed with de-

ionized water, covered with 10μg/ml Nile Red solution in phosphate buffer saline (PBS) for 10 minutes and subsequently washed in de-ionized water. Finally, slides were covered in 0.5% potassium permanganate solution for no longer than 1 minute to reduce non specific background, washed and allowed to air dry. Slides were mounted in PBS and sealed with colorless nail varnish and examined under fluorescent microscope.

2.2.9. Examination of slides

Smears to detect acid-fast bacilli by acid-fast stain were examined by light microscopy. Microscopy results were recorded following the Centers for Disease Control (CDC) methods of report as negative (no bacilli per 100 fields), 1-2 bacilli per 300 fields (±), 1-9 per 100 fields (+), 1-9 per 10 fields (2+), 1-9 per field (3+) and greater than 9 per field (4+). Counts less than 3 bacilli per 300 fields were not considered positive.

2.2.10. Fluorescence Microscopy

Lipid bodies were viewed under fluorescence microscopy. Each sealed microscope slide was examined using a standard epifluorescence Nikon Diaphot inverted microscope with 100 W high pressure mercury vapour lamp. The microscope was fitted with a high speed Peltier-cooled charged couple device camera (Photonic Science) controlled by Image-Pro Plus v4.1.0.0, and all images were taken using a 100x oil immersion objective with a numerical aperture of 1.4. For all images a phase image were taken prior to the fluorescence image being taken, and all images were stored as 12 bit tagged image files (*tif) and converted to 8 bit images for analysis.

2.2.11. Lipid body enumeration (Manual method)

All samples from the same experiment were subjected to the same conditions and all images were taken on the same day. Nile Red images were displayed alongside the corresponding Auramine O images to enable to decide if an object was or was not a single cell. Bright fluorescence spots inside the confines of a cell were decided to be lipid bodies, but not if they were at the very tip of the cell. All the images for lipid body counting were taken with the same

exposure time (500 milliseconds for Nile Red and 1 second for Auramine), and were all opened in Image-Pro Analyser (without applying any image manipulation) on a LCD screen with resolution 1280 x 1040 pixels.

Fluorescence images were displayed alongside the corresponding phase image to enable the user to decide if an object was or was not a single cell. Only single cells but not clumps of tubercle bacilli were included during enumeration of the proportion of lipid body positive tubercle bacilli.

2.3. Results

Numerous experiments were done to find improved staining methods applicable to the enumeration of *Mtb* LBs in sputum smears. These were largely unproductive and the finalized method was similar to that previously established in this laboratory. Nonetheless, the outcome of the experiments done is briefly described here to illustrate some of the problems encountered.

2.3.1. Staining with Oil Red O and Sudan Black

The experiment on BCG Galaxo, BCG Pasteur and *Mycobacterium smegmatis* cultures stained by Nile red stain showed more better back ground contrast than Oil red O stain alone or Oil red O combined with ZN stain. The contrast on Nile red stained images were also by far better than Sudan black stain either alone or combined with ZN stain. In addition, relatively higher proportions of lipid body positive tubercle bacilli were seen when smears were stained by Nile red stain alone. For example, when BCG Galaxo preparations from a single culture were examined, the proportion of LB^{+ve} cells was 82% when stained by Nile red alone but 71 % when stained by oil red O stain. When Oil red O stain was combined with ZN stain, the contrast was relatively good but LBs were not seen clearly. Sudan black stain alone and combined with ZN stain demonstrated similar proportions of LB^{+ve} tubercle bacilli on *M.smegmatis*. The proportion of LB^{+ve} tubercle bacilli was found relatively similar when smears prepared from *M.smegmatis* were stained by Nile red stain alone and Sudan black stain either alone or combined with ZN stain (93% and 80% respectively).

2.3.2. Effects of detergents, solvents and lipase treatment on Auramine O/Nile Red stain on BCG cultures and sputum samples

An example of the established procedure for Auramine O/Nile Red staining is shown in figure 2.1. Both LB positive and negative cells are clearly visible.
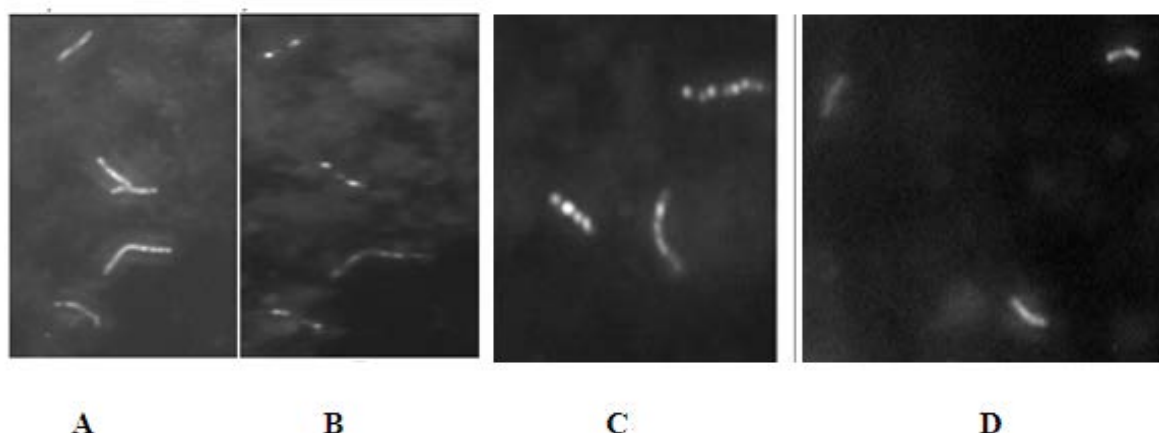
Figure 2.1: Micrograph illustrating standard Auramine O/Nile Red staining of a sputum sample demonstrating acid fast LB^{+ve}and LB^{-ve}tubercle bacilli.

A: Auramine O filter view; B and C: Nile red view on LB^{+ve} AFB cells; D: Nile red view on LB^{-ve} AFB cells.

Detergent wash on *M. bovis* BCG added to an AFB negative sputum sample resulted with no significant background improvement during Auramine O/Nile red stain. On the other hand, application of solvents such as acetone and ethanol on smears prepared from BCG mixed with AFB negative sputum showed by far reduced proportion of LB^{+ve} tubercle bacilli. Reduction of LB^{+ve} tubercle bacilli was also observed upon treatment with 50µg lipase (table 2.3).

Table 2.3: Detergent wash and solvent treatment reduces the proportion of LB[+ve] tubercle bacilli on *M. bovis* mixed with AFB negative sputum

The proportion of LB[+ve] tubercle bacilli on BCG mixed with AFB negative sputum was reduced after smears rinsed with different detergents and solvents. The proportion of LB[+ve] tubercle bacilli at zero point in time was 60%. Smears from BCG mixed with AFB negative sputum were prepared and air dried. Smears were rinsed in the respective detergent and solvent, allowed air dry followed by heat fixation and stained with Auramine O/Nile red stain. The proportions of LB[+ve] and LB[-ve] cells were visualized under fluorescent microscope.

Treatment	Proportion of LB[+ve] cells (%)
10% acetone	15
5% acetone	30
1% acetone	Poor contrast
0.1% acetone	30
10% ethanol	35
5% ethanol	32
1% ethanol	30
0.1% ethanol	10
0.05%, 0.25% and 0.5% SDS	Poor contrast
0.1% Tween 80	Poor contrast
0.01% Tween-80	26
50µg lipase in 100mM Tris	40%

The effects of different detergents, solvents and lipase on the background of Auramine O/Nile red stain among smears prepared from BCG contaminated with AFB negative sputum were also investigated. Treatment of smears with SDS, different concentration of acetone and ethanol had no significant improvement on the background of smears prepared from BCG contaminated with AFB negative sputum. Rather, the proportion of LB[+ve] tubercle bacilli was by far reduced upon the application of the aforementioned detergents and solvents. The proportion of LB[+ve] tubercle bacilli was reduced to as low as 10% and 15% when smears were treated with 0.1% ethanol and10% acetone respectively. The proportion of LB[+ve] tubercle bacilli was 60% prior treatment at zero point in time. On the

other hand, Triton-X100 followed by lipase treatment brought significant background improvement on Auramine O/Nile red stain but the number of AFB was reduced significantly both on sputum samples and *M. bovis* decontaminated AFB negative sputum samples.

2.3.3. Digestion of purulent sputum samples, BCG and *Mtb* H37Rv cultures with lipase enzyme and TritonX-100.

Demonstration of lipid bodies within the tubercle bacilli in sputum samples showed varied back- ground intensity. In some sputum samples the back ground intensity was high and even very difficult to observe the tubercle bacilli itself (figure 2.2 B) and in some others lipid bodies within the tubercle bacilli were observed clearly (figure 2.2D).
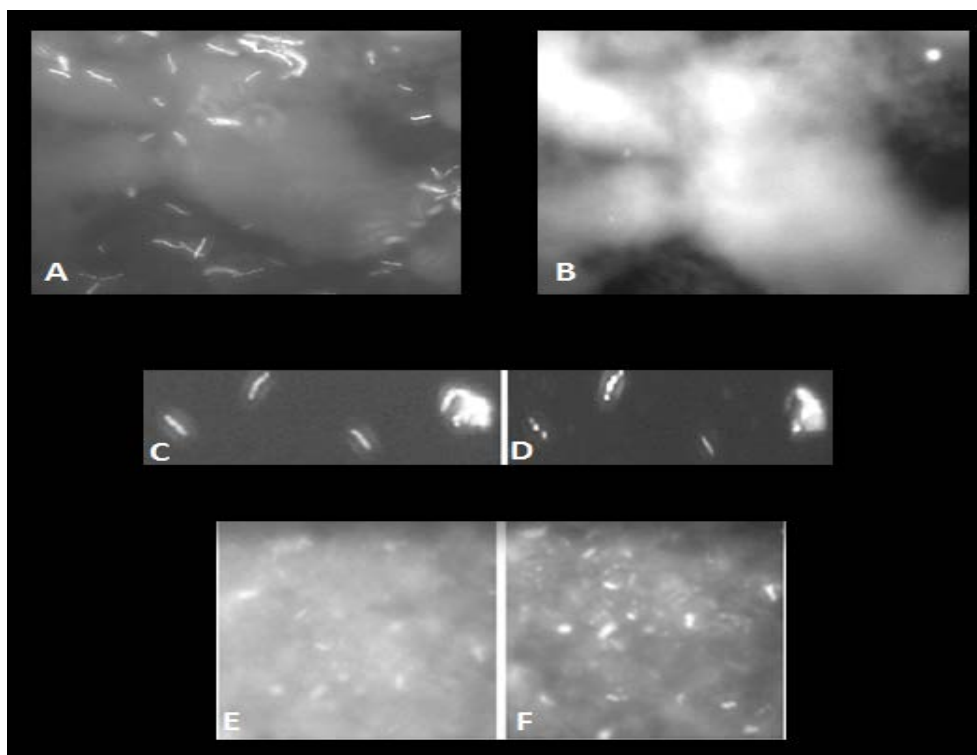
Figure 2.3: Examples of Auramine O/Nile red stained sputum samples presenting difficulties for assessment

A: Assessable Auramine image; B: Un-assessable Nile red image; C and D: Respectively, Auramine and Nile Red views of a readily assessable sputum sample; E and F: Auramine and Nile Red views of a poorly assessable sputum sample.

The initial experiment undertaken by incubating a sputum smear for 1 hour at 37°C with lipase (50µg/ml) was judged to have no effect on the back-ground of Nile red staining. However, 50µg is equivalent to only 55U of lipase. Daniel et al (2011) used much higher lipase activity, 10,000U which is equivalent to 9mg of the lipase with similar buffer concentration. Latter, treatment of sputum smears with Triton X-100 and lipase (4.6 mg/ml expecting a volume of 500µl to contain 10,000U) digestion at 37°C for 4 hours brought a significant improvement of Nile red images background. Moreover, extended TritonX-100 wash and lipase enzyme digestion of purulent sputum samples showed significant improvements on the back-ground of Nile red stained images. However, the number of AFB per image was reduced significantly and even after 96 hour lipase digestion followed by TritonX-100 wash, one of the 4+ sputum sample showed scanty acid-fast bacilli per image.

Because extended TritonX-100 wash and lipase treatment of sputum samples showed a reduction in the number of acid fast bacilli in sputum samples, the same experiment was conducted on *M.bovis* BCG. The extended Triton X-100 wash and lipase digestion on *M. bovis* BCG also resulted in a decrease in the number of acid-fast bacilli. When *M. bovis* BCG was subjected to extended TritonX-100 wash and lipase treatment, the number of AFB was reduced by half upon incubation of the preparation at 37°C for 24 hours (figure 2.3).
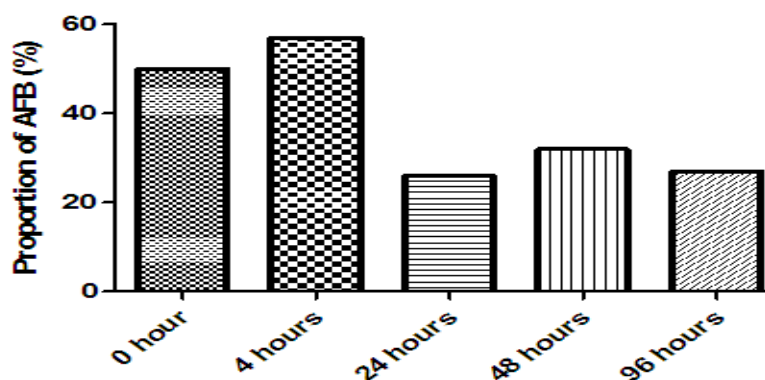
Figure 2.4: Extended Lipase treatment leads to a reduction in acid fastness in *M. bovis* BCG

The proportion of AFB when *M. bovis* BCG was subjected to extended TritonX-100 wash and lipase digestion and incubated at 37°C for 4 hours, 24 hours, 48 hours and 96 hours. Smears were stained with Auramine O/Nile red stain.

The phosphate buffer saline, 70% ethanol and acid alcohol rinse after Nile red stain were not successful in improving the back ground of Auramine O/Nile red stain both on BCG contaminated AFB negative sputum preparations and patient sputum samples. Smears stained with Auramine O/Nile red stain immediately, without evaporating the formaldehyde, showed decreased contrast on both Auramine O and Nile red images. The Nile red image was also poor when the smears were stained with Auramine O/Nile red stain after evaporating formaldehyde only for 15 minutes. On the other hand, both the Auramine O and Nile red images were significantly improved when the formaldehyde was evaporated for 30 minutes and a one hour evaporation of formaldehyde resulted in even clearer images. The effort made to evaluate the effects of different concentrations of $KMnO_4$ on the background of Auramine O/Nile red stain was unsuccessful (unable to observe difference in the background of Auramine O/Nile red stain when 0.5%, 0.1% and no $KMnO_4$ were applied).

2.4. Discussion

The Nile red stain was found to be relatively superior to Oil red O stain or Sudan black both in contrast and determination of the proportion of lipid body positive tubercle bacilli. Although the image contrast on Oil red O stain was good enough, the background was not satisfactory when combined with the ZN stain. Since determination of the acid-fastness of the mycobacteria is a central requirement for this study and back ground intensity reduced when Oil red O combined with ZN stain, the combined Oil red O/ZN stain method was determined unsatisfactory and not used in any of the investigations in this study.

Nile red is a phenoxazone dye that fluoresces intensely, and with different spectra in organic solvents and hydrophobic lipids (Fowler and Greenspan, 1985). Nile red, 9-diethylamino-5H-benzoalphenoxazine-5-one, was reported previously as an excellent stain for the detection of lipid droplets by fluorescence microscopy because it is very soluble in lipid (Greenspan et al, 1985). Hence, Nile red was used to stain lipid inclusions in bacteria. However, demonstration of lipid bodies within the tubercle bacilli in some sputum samples was found difficult during fluorescence microscopy. To produce better back ground during fluorescence microscopy, different detergents, solvents and lipase enzyme were investigated.

Solvents such as acetone and ethanol used to rinse air dried smears before Auramine O/Nile red stain had no significant improving effect on the background of BCG contaminated sputum samples. Rather, the proportion of lipid body positive tubercle bacilli were reduced from 60% to as low as 10% and 15% when smears were rinsed with 0.1% ethanol and 10% acetone respectively. On the other hand, the application of 70% ethanol and acid alcohol rinse after Auramine O/Nile red stain made the Nile red fluorescence reduced so that it was very difficult to observe the bacteria under fluorescence microscope. This experiment pointed out that application of solvents before or after Auramine O/Nile red stain may have significant drawbacks on LBs within the tubercle bacilli. Organic solvents such as alcohols and acetone remove lipids from cells and dehydrate cells during fixation for histological examinations.

Triton X-100 and SDS detergent wash showed also no significant effect on improving the back- ground during Auramine O/Nile red stain both on BCG mixed with AFB negative sputum smears and sputum smears prepared from AFB positive patients. Similarly, the application of lipase at a concentration of 50µg/ml showed no significant effect on improving the back ground of Auramine O/Nile red stain. However, 50µg/ml lipase, which is equivalent to only 55U of lipase, concentration can be considered undersized in its amount when compared with the concentration used by Daniel et al (2011). Daniel et al (2011) used much higher lipase activity, 10,000U which is equivalent to 9mg of the lipase with similar buffer concentration.

Extended Triton X-100 wash and lipase digestion at a concentration of 10,000 U/ml brought a significant reduction of the background of sputum smear preparations during Auramine O/Nile red stain. However, the reduced number of acid fast bacilli observed upon Triton X-100 wash and lipase digestion was a serious concern. In an experiment designed to demonstrate the reduction of AFB upon Triton X-100 wash and lipase digestion, more than half of the proportion of acid fast bacilli in a sputum sample was lost after 24 hour. This was also true when Triton X-100 wash and lipase digestion was applied on *Mycobacterium bovis* BCG and *Mtb* H37Rv. Therefore, it is possible to suggest here that the decrease in the number of acid fast bacilli might be the effect of the lipase enzyme. Although TritonX-100 wash and lipase treatment showed significant improvement on the background of Nile red stain particularly on sputum samples, loss of acid fastness was a serious concern and because of that the conventional Auramine O/Nile red stain was decided as a staining methods of choice to enumerate lipid bodies within the tubercle bacilli in sputum samples.

2.5. Conclusions

1. The Auramine O/Nile red stain was found superior than the Oil red O and Sudan Black stains to visualize lipid bodies within tubercle bacilli.

2. Application of detergents and solvents on sputum smears had not brought significant improvement on the background of Auramine O/Nile red stain used to demonstrate LBs within the tubercle bacilli.

3. Extended Triton X-100 wash and lipase digestion brought significant improvement on the background of sputum smears during Auramine O/Nile red stain but resulted also in reduced number of AFB.

4. Therefore, combined Auramine O/Nile red stain was used as a method of choice to stain mycobacteria and visualize LBs within the tubercle bacilli for all the experiments conducted in this study.

CHAPTER THREE: FeNO levels related to the proportion of LB^{+ve} tubercle bacilli in patients with pulmonary TB

3.1. Introduction

The importance of free radicals was emphasised in the late 1980s after the understanding of nitric oxide as vasodilators. Previous reports documented that free radicals and reactive oxygen species (ROS) were considered toxic by-products of metabolism dependant on oxygen-based respiration (Ford, 2006). Nitric oxide is a non polar gaseous molecule, lipophilic and relatively insoluble in water. Nitric oxide (NO) has been linked with a wide-ranging array of biological activities and pathological processes (Andrew et al, 2002; Moncada et al, 1989).

3.1. 1. Nitric oxide (NO) and its production

In the eukaryotic system, there are two main but not exceptionally different ways of NO production. An enzyme catalyzed oxygenation of the guandine groups of L-arginine that results in the production of NO and L-citrulline is the primary route (figure 3.1). The reduction of nitrite under anoxic conditions via nitrite reductase activity of xanthine oxidase, mitochondrial cytochrome complexes, deoxyhemoglobin and some NOS isoenzymes is the secondary route for NO production (Boudko, 2007). There are three enzymes involved in NO production. Nitric oxide synthase one (NOSI) also called neuronal nitric oxide synthase (nNOS), NOSII (hepatocyte, HE-NOS or inducible, iNOS) and NOSIII (endothelial, eNOS) (Forstermann and Kleinert, 1995). The eNOS and nNOS enzymes are also called constitutive enzymes and their activity is calcium (Ca^{2+})/calmodulin dependant implying that the NO so produced is required for basal physiologic organ function (Ricciardo, 2003).

On the other hand, the activity of iNOS enzyme is not dependant on Ca^{2+} concentration but on pro-inflammatory cytokines such as TNF-α, IL-1β and IFN-γ (Moncada et al, 1991).
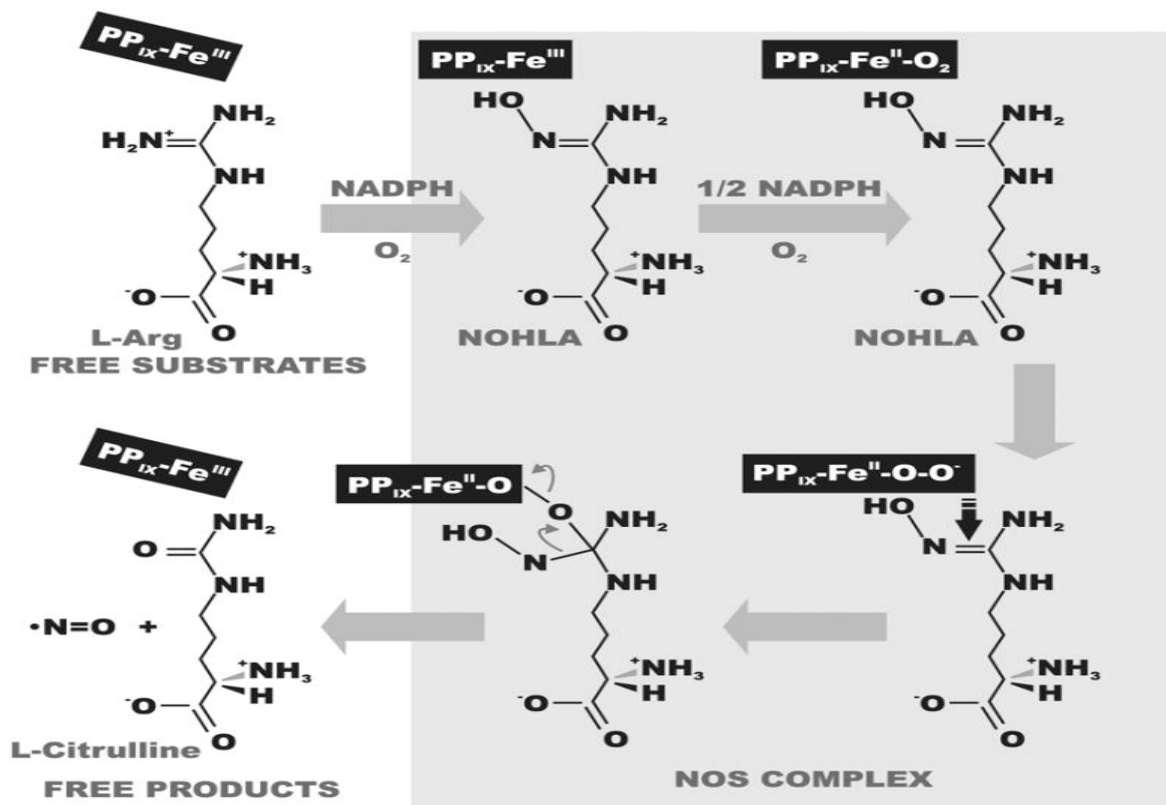
Figure 3.1: Enzymatic NO production by NOS

In this reaction one guanidino nitrogen of L-arginine undergoes a five-electron oxidation via NOHLA (N^G-hydroxy-L-arginine) as the intermediate to yield one molecule of NO. PP_{IX}-Fe, iron coordinated proto-porphyrin XI, a prosthetic group which functions in the turnover of L-arginine (Boudko, 2007).

3.1. 2. Interaction of *Mtb* with Macrophages

Although the environmental conditions that *Mtb* comes across within the macrophage and particularly within the phagosome is expected potentially lethal, the organism replicates and persists within the cell (Mckinney and Gomez, 2003). Schnappiner et al (2003) provides evidence that the phagosome is hostile, poor in conventional nutrients and destructive to bacterial components. More than that, activation of macrophage with IFN-γ makes life in the phagosome even harsher and stimulates the production of NO. The production of NO may trigger *Mtb* to enter a state of non-replicating persistence. This non-replicating or dormant *Mtb* resists killing by antimicrobial drugs (Voskuil et al, 2003).The

existence of iNOS enzyme in the alveolar macrophages of TB patients was verified by Nicholson and co-workers (Nicholson et al, 1996). Alveolar macrophages isolated from TB patients released increase amounts of spontaneous NO compared with normal control subjects (Kuo et al, 2000).

In naïve macrophages, *Mtb* resides in a modified phagosome that intersects the recycling endosome network and does not undergo acidification, maturation or fusion with lysosomes. The macrophage-activating cytokine IFN-γ promotes phagosome acidification and phagolysosome fusion (Figure 3.2). However, *Mtb* containing phagosomes fail to fuse with lysosomes after internalization by macrophages. The ability of the *Mtb* bacteria to inhibit the fusion of phagosome with lysosome can be considered one evasion mechanism that will allow the bacteria to survive in the cell (Hart et al, 1972).

The predominant cytokines and inflammatory mediators involved in iNOS expression and NO production includes TNF-α, IFN-γ, LPS, IL-1β, hypoxia and picolinic acid. *Mtb* cell wall components such as lipoarabinomanan also induce TNF-α and IL-1β which in turn induce NO. Wayne and Hayes demonstrated that *Mtb* also evolved ways to evade the toxic effects of reactive nitrogen intermediates (RNI). They also reported that nitrite (NO_2^-) is reduced to nitrate (NO_3^-) by *Mtb* in hypoxic conditions. It was also documented that the induction of nitrate reductase under hypoxic conditions may serve a respiratory function in supporting the shift of the tubercle bacilli from aerobic growth state to a state of dormancy (Wayne and Hayes, 1998).
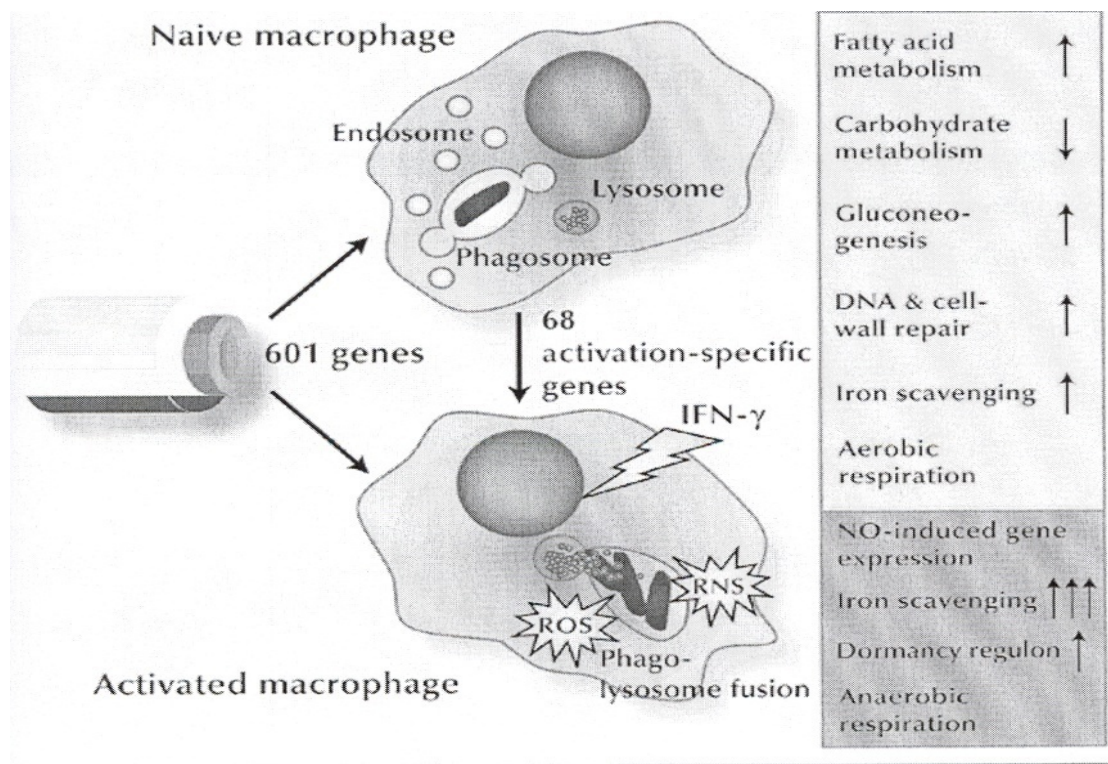
Figure 3.2: *Mtb* gene expression in naïve and activated macrophages

Mtb gene expression patterns both in naïve and activated macrophage reflects environmental stress that can be mimicked *in vitro*. A total of 601 *Mtb* genes are differentially expressed within macrophage phagosomes relative to log-phase axenic cultures. Regulation of a set of 68 activation-specific genes is restricted to activated macrophages and is dependent on the macrophage's ability to produce NO (McKinney and Gomez, 2003).

3.1. 3. Clinical significance of Nitric oxide

Nitric oxide can exert bronchodilator properties and it may afford protection against airway inflammation by inhibiting platelet aggregation and leukocyte adhesion to vascular endothelium (Dupuy et al, 1992; Radomski et al, 1992). NO exerts a variety of pro-inflammatory effects that may be deleterious in airway inflammation (Bernareggi et al, 1997; Rettori et al, 1992). NO induces pathologic vasodilatation and enhances plasma leakage in the trachea (Bernareggi et al, 1997). It also increases the production of pro-inflammatory prostaglandins both *in vitro* and *in vivo* (Salvemini et al, 1995). Ballou et al

(2002) documented that NO synthesized by NO synthase typically acts in a paracrine fashion, where NO synthesized in one cell acts on a target in an adjacent cell. NO is also a well known mediator of blood vessel relaxation that helps to maintain blood pressure (Nathan, 1992). In the central nervous system, NO acts as non-conventional type of neurotransmitter contributing to long term potentiating. In the brain, especially in the cerebellum, NO mediates the ability of the excitatory neurotransmitter glutamate to stimulate cGMP via NMDA (N-methyl-D-aspartate) receptors (Jaffrey and Snyder, 1995). NO may directly influence ion channels, as has been shown with calcium-dependent potassium channels in vascular smooth muscle (Bolotina et al 1994).

3.1. 4. Lipid body formation in response to Nitric oxide

There are several reports that documented about *Mtb* dormancy. The bacteria, very differently from other pathogens, can however persist for years without causing disease. The *Mtb* dormancy has been linked to the hypoxic conditions within the host. *Mtb* expresses genes induced by hypoxic environments which are a transcription factor *RV3133c/DosR* gene (Park et al, 2003). Depletion of oxygen forces the *Mtb* bacteria to shift into non-replicating state that may allow it to persist in human host for decades (Muttucumaru, 2004). Schnappiner et al (2003) showed that IFN-γ and NO intensifies an iron scavenging program that switches the aerobic respiration to anaerobic and induces a dormancy regulator (dormancy survival regulator regulon, DosR). The *Mtb* in the lungs store triacylglycerol (TAG) as inclusion bodies and also accumulate lipids originating from the host. During the reactivation phase these lipids are hydrolyzed and pathology progresses (Cotes et al, 2008).

Sputum has been traditionally thought to contain actively growing tubercle bacilli. This view was recently undermined and the traditional belief was rejected that smear positive sputum may contain heterogeneous groups of *Mtb*. Moreover, survey on clinical sputum samples revealed that lipid bodies are universal features of the tubercle bacilli in sputum (Garton et al, 2008). Accumulation of lipophilic inclusion bodies within the *Mtb* bacteria were influenced by environmental factors such as hypoxia, nitric oxide exposure, pH, heat

and cold shock *in vitro* (Sherratt, 2008). More than that, accumulation of lipid bodies within the tubercle bacilli was associated with phenotypic antibiotic tolerance possibly resulting from transitory growth arrest. Sherratt (2008) reported that *in vitro Mtb* exposed to NO produced lipid bodies after 4 hour NO exposure and these nitric oxide treated populations of *Mtb* were significantly more tolerant of isoniazid and rifampicin than cultures treated without NO (control groups). However, the level of nitric oxide *in vivo* and its association with lipid bodies within mycobacteria has not been elucidated and it would be desirable to understand whether pulmonary NO production contributes to the accumulation of lipid bodies within the *Mtb*.

3.1.4. Aims and objectives

The major aim of this study was to determine whether FeNO concentration was correlated with the proportion of LB^{+ve} tubercle bacilli in sputum.

Specific obijectives

1) To examine the role of host factors such as, sex, age, smoking status, and nutritional status, history of asthma, fasting and postprandial state with FeNO concentration.

2) To determine the association of FeNO concentration with the proportion of the corresponding LB^{+ve} tubercle bacilli in sputum samples.

3.2. Materials and Methods

3.2.1. Study design, study area, study period and study subjects

This cross-sectional study was conducted at the University of Gondar teaching hospital, Gondar Poly clinic and Gondar Army hospital, Northwest Ethiopia from February 16 to September 11, 2009. Gondar town is located 737 kilometers away from the capital city of Ethiopia, Addis Ababa. The town has latitude and longitude $12^0 36^1 N$ and $37^0 28^1 E$ respectively with an elevation of 2100 meters above sea level. It has a projected population of 300,000 (Gondar Zonal statistics office). Gondar College of Medicine and Health Sciences hospital is a referral hospital with 400 beds, serving approximately 6 million people and a teaching center for medical fields since decades. The hospital gives different inpatint and outpatient services to the population in the surrounding area of Gondar town and the adjacent regions.

3.2.2. Patient recruitment and selection (figure 3.1)

Physical examination
of patients at OPD

TB suspected patients
send to the lab for
sputum Microscopy

Smear positive patients (n=116)

Informed consent

Un processed sputum
sample stored at -20 ℃

FeNO
(n=116)

Sputum collection
(n=116)

Socio-demographic
characteristics and
body mass Index

NALC/NaOH
decontamination and
smear preparation

Fluorescence microscopy

Auramine o/ Nile red stain

For LB enumeration (n=44)

Smear stored at -20 ℃

Figure 3.3: Patient requritment and sampling procedure.

Flow chart that summarizes patient requritment, sampling and selection of sputum samples for LB analysis.

Smear positive tuberculosis patients were recruited up on sputum delivery and being positive for acid-fast bacilli (AFB) by ZN method. Socio-demographic data was collected by face-to-face interview using structured questionnaire translated from English to Amharic and then to English language.

Ethical approval was obtained from University of Gondar ethical clearance committee before commencement of the study. Each clinic, laboratory and the outpatient department were communicated through written paper obtained from University of Gondar research and publication office (RPO) with Ref.NO:- RPO/55/138/2001 E.C. Each study subject was requested to participate voluntarily. Soon after the study subject agreed to participate, written informed consent was obtained. Each study subject was informed about the objective of the study that would contribute for the understanding of the TB bacilli existence in sputum and factors affecting the physiology of the tubercle bacilli in sputum samples. Patients were also told that understanding factors that may influence lipid body accumulation within the tubercle bacilli in sputum may next have significant relevance for tuberculosis treatment. The study subjects were told that it was their right to participate or not as well as withdrawal of consent at any time. Voluntary participation and the right to withdraw consent were emphasized. The sputum specimens obtained from the study subjects for routine examination purpose was used for this study. Exhaled air NO was determined only after informed consent was obtained from the study subjects. Volunteers were given adequate and clear information on the study purpose, conduct and expected outcome. No attempt made to get consent from a patient whose sensorium was disturbed or who was in acute pain and all data were kept confidentially. For each confirmed infection case, the responsible clinician of the patient was informed and treatment was started as per the guideline of the hospital in the DOT clinic. Data taken from each study subjects were coded and results obtained from each study subjects were kept always confidential.

3.2.3. Eligible versus included patients

For this particular study, being smear positive for AFB and voluntary to be involved in the study were used as patient selection criteria. One hundred sixty three patients were positive

for acid–fast bacilli (AFB) but only 116 patients were included in this study. FeNO concentration was determined for all 116 patients but the proportion of LB positive tubercle bacilli enumerated on 44 samples that had 3+ and 4+ AFB score.

3.2.4. Measurement of FeNO by NIOX MINO air way inflammation monitor

Fractional exhaled NO (FeNO) concentration was measured using NIOX MINO air way inflammation monitor (Aerocrine AB, SE-171 21, 2005, Sona, Sweden). Prior to measurement the machine was prepared by following the manufacturer's instructions. After explanation of how to use the NIOX MINO machine, the patient empties the lungs by exhaling then inhaled deeply through the filter to total lung capacity. The cloud on the display (figure 3.3) inflated and the top light was turned off while the patient inhaled. The patient then exhaled slowly through the filter and listened to the sound signals and viewed the top light: a continuous sound and steady light indicated that the measurement was valid.

Procedure
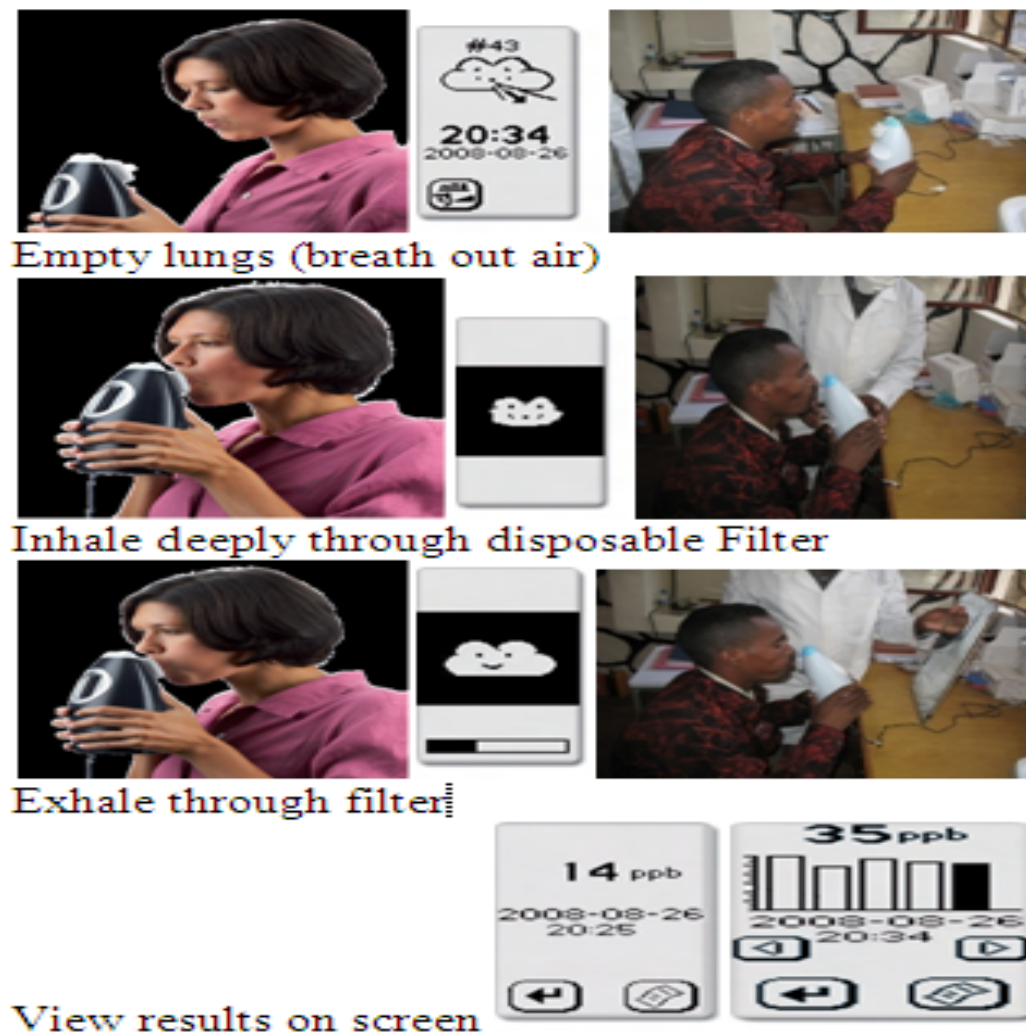
Figure 3.4: Measurement of FeNO using NIOX MINO air way inflammation monitor

FeNO concentration was measured using NIOX MINO air way inflammation monitor (Aerocrine AB, 2005). Patient instructed to breathe out air to empty the lungs first and then inhale air deeply through disposable filter and exhale through the filter. The concentration of FeNO was viewed on the screen and the result determined in ppb.

3.3. Results

A total of 1914 patients delivered sputum samples for acid-fast bacilli examination within six months period of time (February 16 to September 11, 2009) (table 3.2) in 3 Health institutions in Gondar. One hundred sixty three patients were initially reported positive for acid–fast bacilli (AFB) but only 116 patients were included in this study. Forty-seven smear positive patients declined to be involved in the study.

Table 3.1: Study population at different health institutions

Number of patients who delivered sputum samples at University of Gondar Hospital (GUHOSP), Gondar Poly Clinic (GPC) and Gondar Army hospital (GAH); (February 16 to September 11, 2009).

Name of Health institution	Total number of patients delivered sputum sample	Number of Smear positive patients	Number of Patients Recruited
GUHOSP	1431	109	98
GPC	314	42	11
GAH	169	12	7
Total	1914	163	116

All the 116 patients were reported positive for AFB by the three laboratories after conducting three smears and staining the smears with Zeihl-Neelsen stain without either decontaminating the sputum or concentrating the organism by centrifugation. Each sputum sample AFB score was re-examined after the sputum was decontaminated by N-acetyl-L-Cysteine/NaOH. One hundred-three sputum samples were positive for AFB and the other 13 were negative when re-examined. The AFB score of each sputum sample was also determined based on CDC classification. Accordingly, 33 sputum samples were 4+, 20 (3+), 26 (2+), 24 (1+) and the other 13 negative.

The AFB score of 30 sputum samples were compared to the time of sputum collection. Each of the 30 sputum samples were collected at 7 am and 2 pm and examined

independently. Three sputum samples collected in the morning with AFB score of 2+ (2 samples) and 1+ (1 sample) were negative when the sputum collected at 2 pm. On the other hand 2 sputum samples collected at 7 am with 4+ AFB score resulted 1+ when the sputum collected at 2 pm. Six sputum samples collected at 7 am with 4+ AFB score resulted 2+ when the samples collected at 2 pm. The trend shows that AFB score was relatively higher when sputum samples collected in the morning.

The histories of the TB patients revealed that 34 patients were diagnosed and treated for TB previously but the remaining 82 TB patients were newly diagnosed.

3.3.1. Consistency of FeNO concentration measurements using NIOX MINO air way inflammation monitor

Precision of the NIOX MINO air way inflammation monitor was measured on 10 smear positive TB patients by measuring NO three times concecutively from each individual at a five minutes of interval for each measurement (table 3.1).The coefficient of variation (CV) was determined using scale-reliability analysis (one way- ANOVA). The coefficient of variation, CV = 0.016 to 0.000, shows that the NIOX MINO airway inflammation monitor used by this study was highly (99.984% to 100%) consistent. Gill et al (2006) recommends mean acceptable reproducibility to be less than 3 parts per billion (ppb) for FeNO measurements less than 30 ppb, and mean coefficient of variation less than 10% for FeNO measurements more than 30 ppb.

Table 3.2: Precision of FeNO concentration measurement using NIOX MINO air way inflammation monitor

FeNO concentration triply measured on smear positive TB patients (Nitric oxide level <16 ppb n=3, 17-30 ppb n =4, > 31 ppb n=3) (n=10) showed high precision.

Pt.No	FeNO-1 (ppb)	FeNO-2 (ppb)	FeNO-3 (ppb)	Mean	SD	CV
1	12	12	11	11.7	0.58	0.05
2	8	8	8	8	0.00	0.00
3	5	5	6	5.3	0.52	0.05
4	24	24	26	24.7	1.15	0.05
5	18	18	20	18.7	1.03	0.05
6	20	20	22	20.7	1.15	0.06
7	20	20	20	20	0.000	0.00
8	42	42	42	42	0.000	0.00
9	62	62	61	61.7	0.89	0.02
10	33	33	31	32.3	0.89	0.03

3.3.2. FeNO concentration among TB patients

FeNO concentration among TB patients was as low as 5 ppb and as high as 221 ppb. The frequency distribution of FeNO concentration shows that the majority (63.8 %) had ≤ 20.6 ppb (74 patients), 29.3 % (37 patients) 20.7-50 ppb and 6 % ≥ 51 ppb (5 patients). Two patients had 142 ppb and 221 ppb FeNO concentration (figure 3.4). These were considered outliers and excluded from the analysis since the FeNO concentration of these patients were > 5SD from the mean FeNO concentration (Mean=21.37; SD=24.17). The AFB score of the TB patient that had 142 FeNO concentrations was negative after the sputum sample decontaminated with NALC/NaOH. The AFB score of the other TB patient that had 221 FeNO concentrations was 2+. Therefore, the proportion of LB positive tubercle bacillie was not enumerated among these TB patients. The ambient NO concentration was constant throughout the experiments at 5 ppb.
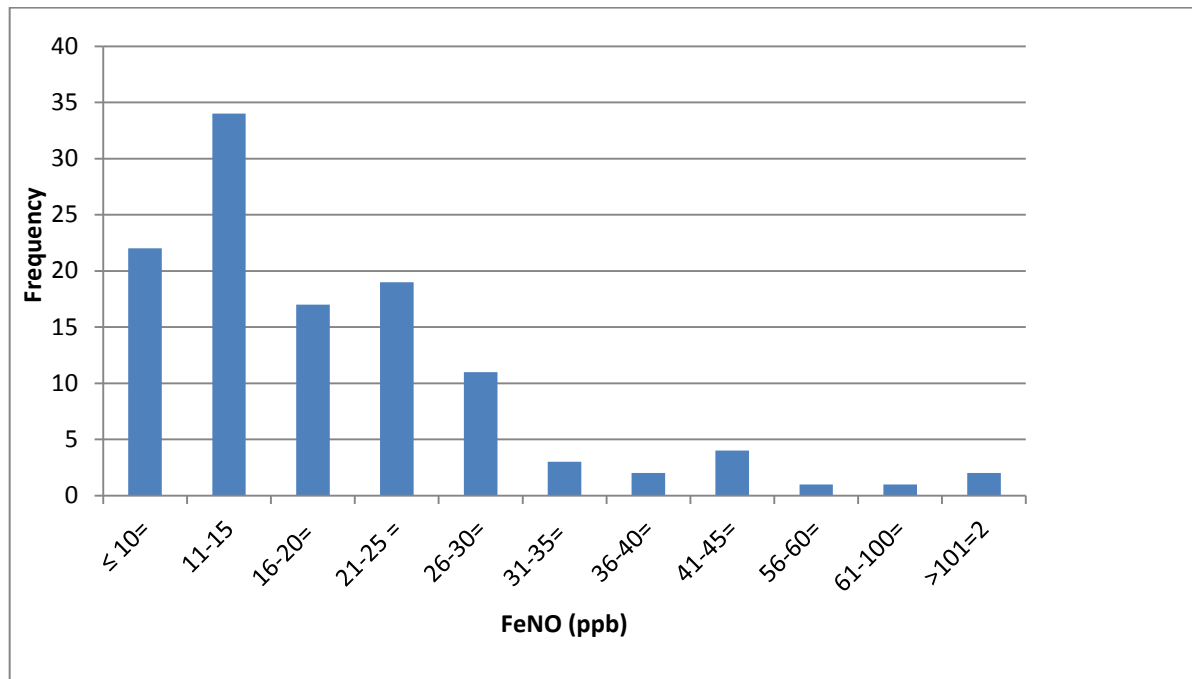
Figure 3.5: Frequency distribution of FeNO

Frequency distribution of FeNO concentration among TB patients in Gondar (n=116). After excluding the two outliers (TB patients that had FeNO concentration of 142 and 221), the average FeNO concentration among TB patients in Gondar was 17ppb (n=114).

The median age of the TB patients was 28 years (range = 10-71 years) and 59.5 % of the TB patients were below 30 years of age followed by 31-50 years (30.2 %) and 51-71 years of age (10.3%). After the two outleirs (tuberculosis patients with FeNO concentration of 141 and 221 ppb) were excluded, the age of TB patients was compared with FeNO concentration. Linear regression analysis showed that age was weakly but significantly associated with FeNO concentration (p=0.004; $R^2 = 0.073$) (Figure 3.5).
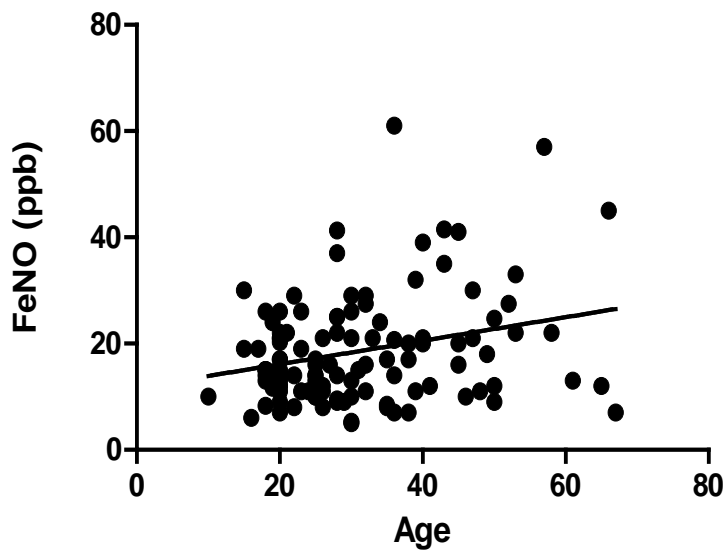
Figure 3.6: FeNO concentration compared with the age of TB patients

Correlation between fractional exhaled nitric oxide concentration and the age of TB patients in Gondar (n=114).

Male TB patients (n= 66) demonstrated relatively higher FeNO concentration (median = 17.50 ppb) than females (n = 48; median = 13.50 ppb) (Figure 3.6). The relation between sex and FeNO concentration was determined by nonparametric t test and there was a weak but significant association between sex and FeNO concentration (p = 0.0073; r^2 = 0.163).
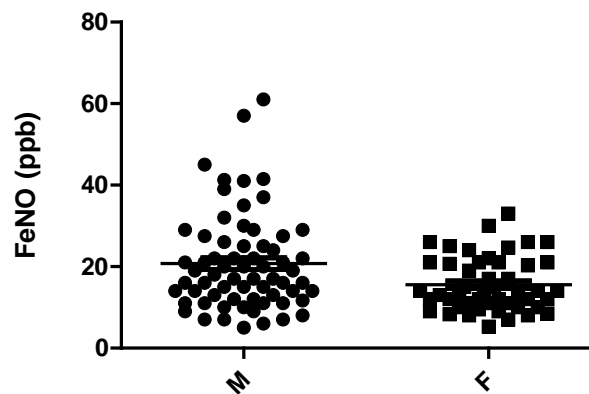
Figure 3.7: FeNO concentration compared with the sex of TB patients

FeNO concentration was weak but significantly associated with the sex of TB patients in Gondar (n=114).

Two patients were currently smoking, 10 smoked previously, the other 102 and the two outliers had never smoked. The average FeNO concentration for the smokers and non-smokers were 20.5 ppb and 21.3 ppb respectively. Only 7 patients had history of asthma. The average FeNO concentrations for the asthmatics and that of non-asthmatics were 25.2 ppb (SD = 11.2 ppb) and 18.22 ppb (SD = 10.06) respectively. None of the TB patients had history of chronic obstructive pulmonary disease, bronchiectasis, pneumonia or diabetes mellitus. Postprandial tuberculosis patients' data shows slight increase in FeNO concentration (mean = 18.86 ppb; SD = 10.95) compared to the fasting once (mean =17.2 ppb; SD =6.14). BCG vaccination was assessed by looking for vaccination scars on the left arm of each patient. Thirteen patients had BCG vaccination and the other 103 were not vaccinated. In this study smoking status, history of asthma, fasting/postprandial state, history of prison and BCG vaccination were not associated with FeNO concentration among TB patients in Gondar.

3.3.3. FeNO concentration compared with the nutritional status of TB patients

The nutritional status of the TB patients was assessed by body mass index (BMI). The World Health Organization (WHO) defines nutritional status by BMI as normal (≥ 18.5), 1 or mild malnutrition (17.0 – 18.49), 2 or moderate malnutrition (16 – 16.99), and 3 or severe malnutrition (< 16.0) (Cole et al, 2007). Accordingly, 41 patients were normal, 14 mildly malnourished, 25 moderately malnourished and 34 severely malnourished (table 3. 2).

Table 3.3: The BMI of TB patients with the corresponding FeNO concentration

Severe and moderate malnutrition were distributed over 34 and 25 TB patients respectively and 63% of either moderately or severly malnourished patients showed FeNO concentration of less than 20 ppb.

BMI	Patients withFeNO ≤ 10ppb	Patients with FeNO 11-20ppb	Patients with FeNO 21-30 ppb	Patients with FeNO 31-40 ppb	Patients withFeNO 41-50 ppb	Patiewnts withFeNO >51 ppb	Total
<16.0	8	16	14	2	1	0	41
16-16.99	1	9	3	0	1	0	14
17-18.49	6	9	6	3	1	0	25
>18.5	5	17	9	0	1	2	34
Total	20	51	32	51	4	2	114

Bivariant linear regression analysis was used to determine the association between BMI with FeNO concentration and data showed a significant association between BMI with FeNO concentration ($p < 0.05$) ($R^2 = 0.015$) (figure 3.7).
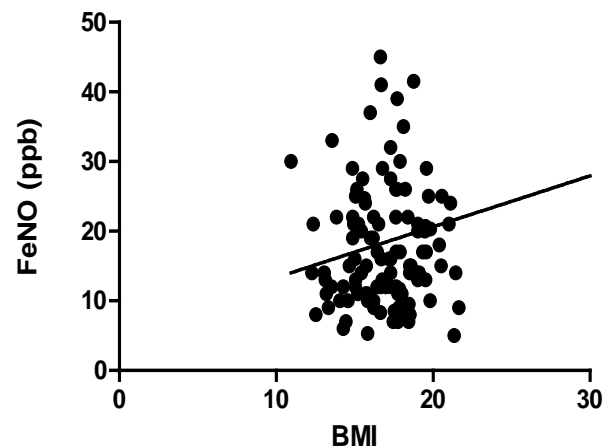
Figure 3.8: Association between BMI with FeNO concentration

Bivariant analysis showed FeNO concentration was weakly but significantly associated with BMI among smear positive TB patients in Gondar (n=114).

3.3.4. Association between FeNO concentration and the proportion of LB⁺ᵛᵉ tubercle bacilli in sputum

The proportion of LB^{+ve} tubercle bacilli was enumerated on 41 sputum samples whose AFB score was 4+ (29 samples) and 3+ (12 samples). The proportion of lipid body positive tubercle bacilli was as low as 2% and as high as 80% (figure 3.8).The median and mean of the proportion of LB^{+ve} tubercle bacilli were 20% and 26.22% respectively with SD of 18.29.
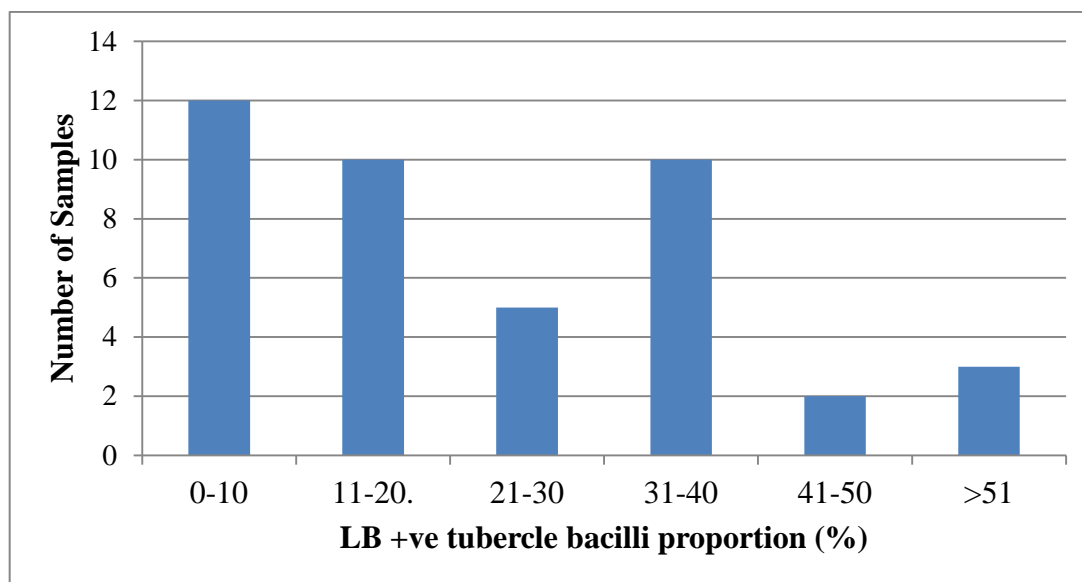
Figure 3.9: The proportion of LB^{+ve} tubercle bacilli among smear positive TB patients

The proportion of LB^{+ve} tubercle bacilli was determined after sputum smears were stained with Auramine O /Nile red solution. Images were taken from each preparation as described previously in section 2 and the proportion of LB^{+ve} tubercle bacilli determined manually (n=41).

The association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli in sputum was determined by linear regression analysis. In this study FeNO concentration was weakly but significantly associated with the proportion of LB^{+ve} tubercle bacilli in sputum (p=0.024; r^2=0.124) (figure 3.9).

LB+ve (%)

Figure 3.10: Association between FeNO concentrations with the proportion of LB^{+ve} tubercle bacilli in sputum.

Once FeNO concentration determined and the proportion of LB^{+ve} tubercle bacilli enumerated the association between FeNO concentrations with the proportion of LB^{+ve} tubercle bacilli in sputum measured and found significant among smear positive TB patients.

The other important observation in this study was the presence of LB^{+ve} TB like bacteria on the Nile red images but not found on the corresponding Auramine O images. A significant number of LB^{+ve} non-acid fast tubercle bacilli were observed in different sputum samples. The number of LB^{+ve}, non-acid-fast but TB like bacilli while enumerating 100 acid-fast bacilli varied from 3 to 70. In addition, poorly acid-fast stained tubercle bacilli which were LB^{+ve} were also observed in some of the sputum samples.

3.3.5. FeNO concentration and the proportion of LB^{+ve} tubercle bacilli among new TB patients compared with re-treatment TB cases

The median FeNO concentration of TB patients that had no previous history of treatment (n=82) was 15.50 ppb (95% CI 15.36-27.13 ppb) and the retreatment TB patients (n=34) had 17.00 ppb (95%CI 16.61-26.76ppb). However, when data from the two groups were compared using unpaired t test, there was no significant difference in FeNO concentration.

The average proportion of LB^{+ve} tubercle bacilli among TB patients that had no history of tuberculosis treatment (n=28) was 22.57% (95% CI 15.72-29.43%). On the other hand, the retreatment tuberculosis patients (n=10) demonstrated an average of 30.73% (95%CI 19.7-41.8%) proportion of LB^{+ve} tubercle bacilli in sputum. In this study FeNO concentration was significantly associated with the proportion of LB^{+ve} tubercle bacilli among TB patients that had no history of tuberculosis treatment (p=0.006; r^2=0.256) and retreatment cases (p=0.01; r^2=0.59) (figure 3.11). The association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli was stronger among retreatment cases.
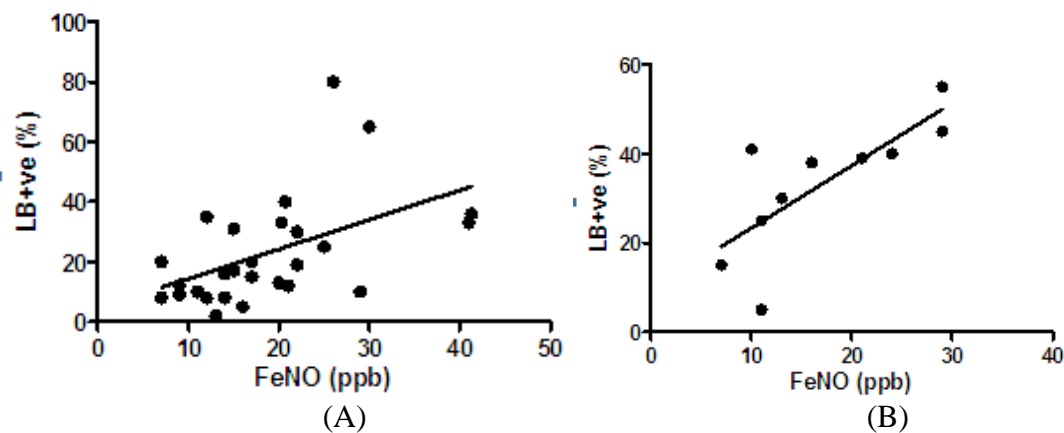
Figure 3.11: The relation between FeNO concentration with the proportion of LB^{+ve}tubercle bacilli among new and retreatment TB cases

(A): Association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli in sputum among TB patients that had no history for TB treatment (n=28) and B among retreatment cases (n=10).

3.3.6. Multivariate analysis

The normal distribution of the FeNO concentration and proportion of LB^{+ve} tubercle bacilli data was checked by Quantile-Quantile (QQ) plot and showed that the two datas were not normally distributed. To maintain normal distribution, data was transformed logarithmically. After datas were transformed logarithmically, the skewness of the FeNO concentration and proportion of LB datas were –0.042 and 0.622 respectively (figure 3.13).

Since the skewness of both FeNO and LB data was less than 1, data was concedered normally distributed after logarithmically transformed.
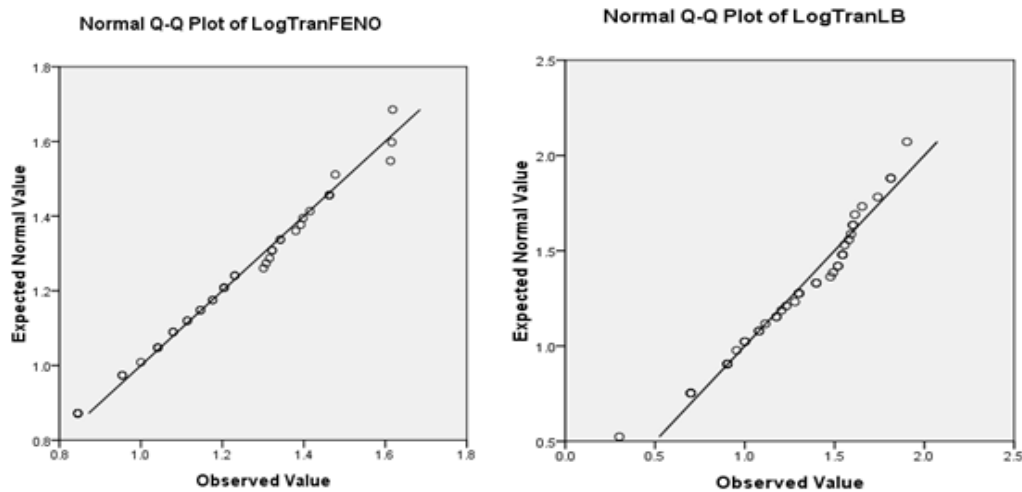
Figure 3.12: QQ plot of FeNO concentration and Proportion of LB[+ve] tubercle bacilli

FeNO concentration and the proportion of LB[+ve] tubercle bacilli datas were normally distributed after logarithmically transformed.

Taking the proportion of LB[+ve] tubercle bacilli as dependant variable and others such as age, sex, BMI, *Mtb* spoligotype and FeNO concentration as independent variables, multivariate regression analysis showed that FeNO concentration was significantly associated with the proportion of LB[+ve] tubercle bacilli in sputum (p= 0.04; r^2=0.24).

3.4. Discussion

Although each of the 3 laboratories did not quantify the AFB score, thirteen sputum samples reported positive for AFB without decontaminating the samples by the ZN method were negative when the sputum samples were decontaminated and concentrated by NALC/NAOH. The exaggerated difference observed in Gondar could be due to interpersonal difference or inappropriate sample selection during direct examination. Concepcion et al showed a 2% false positive result when 696 AFB negative smears were examined by two microscopist (Concepcion et al, 1997). False positive readings could be the result of a carry over from one positive slide to another if the microscopist does not clean the objective after each slide reading, precipitate from unfiltered staining solutions and contaminated tap water. Another possible reason for false negative AFB result could be loss of acid fastness. Loss of acid alcohol fastness of *Mtb* has been documented in the UOL Lab on modeling stress conditions. In laboratory conditions, *Mtb* underwent several thaw cycles, and the proportion of acid-alcohol fast cells was reduced after each cycle when Auramine O staining was applied (Bell, personal communication). Although difficult to determine the exact factors that brought negative AFB result for the 13 samples that had been reported AFB positive by the UOG hospital lab, it is possible to suspect that the harsh effect of the NaOH on the TB bacilli might brought the negative AFB result.

Of the 34 TB patients diagnosed and treated previous to this study, 17 confirmed diagnosis and treatment a year ago. This might indicate treatment failure or inappropriate compliance for anti-TB drugs. Previous to this study, Tessema et al reported an 18.3% and 0.2% treatment default and failure respectively in Gondar (Tessema et al, 2009). Nine and 5 patients had TB infection 2- 5 years and 10-20 years ago respectively. These may have been recurrences of the original infection. TB recurrence after treatment completion can be due to relapse of the infection with the same *Mtb* strain or it could also be re-infection with new *Mtb* strain (Jasmer et al, 2004). In a long term cohort study in Malawi, Amelia et al reported re-infection 1/16 recurrences in HIV negative and 12/23 in HIV positive individuals (Amelia et al, 2010).

Sputum samples collected in the morning showed relatively higher AFB score than the samples collected in the afternoon. This is also supported by other reports. For example, Schoch et al (2007) reported that morning sputum is more sensitive, with a score of 62% for smear- and 48% for culture-positive cases. Other studies also showed AFB score from morning sputa was higher than from specimens collected on the spot (Gopi et al, 2004). In this study there was no significant association between FeNO concentration and sputum AFB score. This is supported by previous report from Gondar which showed that among HIV negative TB patients with FeNO levels > 25 ppb there were no differences with regards to sputum AFB smear score (Idh et al, 2008).

Regardless of the HIV status the average FeNO concentration among smear positive TB patients in the current study was 18.56ppb. A previous report from this location showed an average of 14.2 ppb among HIV[+ve]/TB patients, 17.7 ppb among healthy blood donors and 18.7 ppb among household TB contacts (Idh et al, 2008).

Smoking status, history of asthma, fasting / postprandial state, history of prison and BCG vaccination were not associated with FeNO concentration as indicated in the result section. However, Travers and colleagues found that smoking affected FeNO levels (Travers et al, 2007). An increase in the exhaled air FeNO as a result of eosinophilic inflammation among asthmatic patients was previously reported. Exhaled nitric oxide increases during exacerbation and decreases with recovery in patients with asthma (Ashutosh, 2000). In this study, there was no significant association between FeNO concentrations with fasting or postprandial state. However, Farkas et al (2004) reported that in the fasting stat NO end products such as nitrite or nitrate levels did not differ. On the other hand, excess nourishment and sedentary lifestyle leads to glucose and fatty acid overload. This is followed by the reaction of glucose with plasma proteins and result in complex glycation end products, triggering production of ROS that may initiate a chain reaction leading to reduced nitric oxide availability (Wright et al, 2006).The number of BCG vaccinated patients were very low (13/116) and six of the 13 patients had an AFB score of 4+ (4 patients) and 3+ (2 patients). Previous reports showed that BCG vaccination protection against TB to vary between zero and 80% (Colditzet al, 1994).

3.4.1. FeNO concentration versus gender, age and nutritional status of TB patients

In this study gender and age of the TB patients were mildly associated with FeNO concentration. The relationship between gender and FeNO has been examined previously. In a large randomly selected adult population when both height and gender included in multiple regression model the contribution of gender to FeNO concentration was not significant (Olin et al, 2006). Other studies reported significantly higher FeNO concentration in men compared with women (Olivieri et al, 2006). In addition, Travers and colleagues (2007) reported that sex, atopy and smoking status affected FeNO levels. Another study documented that FeNO levels were nearly 25% less in females compared with males (Taylor et al, 2007). In the current study, we found that women had a significantly lower FeNO concentration than men.

The association of age with FeNO concentration was also reported previously in disease conditions. For example, Franklin et al (2006) reported that FeNO decreased with age in children with cystic fibrosis (CF) but not in the healthy control groups. There is also study report that documented the levels of FeNO concentration were not correlated with age (Olivieri et al, 2006; Tsang et al, 2001).These reports may dictate for the need of the formulation of cut off values for FeNO concentration for healthy people based on sex and age.

3.4.2. Effect of malnutrition and genetic factors on TB infection and FeNO concentration

Although the bivariate regression analysis between FeNO concentration and BMI showed significant association, multivariate regression analysis showed no significant association. This result is not unusual because univariate analysis result may differ with bivariate or multivariate analysis. Univariate analysis is primarily used for descriptive cases while multivariate analysis mainly used for explanation. Moreover, multivariate analysis involves observation and analysis of more than one statistical outcome variable at a time. However, TB has been called "a poor man's disease" indicating risk factors for TB associated with

poverty (Vynnycky and Fine, 1999). Malnutrition certainly contributes to both mortality and morbidity due to tuberculosis infection particularly in resource limited settings (Macallan, 1999). All the indicated deficiencies as a result of malnutrition may facilitate to tuberculosis infection because dietary depletion has a major impact on immune function and depression of lymphocyte function that cannot be desirable in an individual fighting invasive mycobacterial infection. In the present study, 41/116, 15/116 and 28/116 TB patients were severely, moderately and mildly malnourished respectively.In a rat model as a consequence of malnutrition, there were lower numbers of AMs in the bronchoalveolar lavage fluid and NO releases by AMs were impaired (Ferreira-E-Silva et al, 2009). Moreover, monocytes obtained from malnourished adult patients suffering from fibrocaseous TB showed inadequate stimulation even with recombinant gamma interferon. These results suggest macrophage dysfunction to produce NO_2^- in malnourished patients suffering from TB (Bhaskaram, 1995).

There is also evidence that genetic factors might play a role on the genesis of FeNO. Another report demonstrated that female individuals with higher repeats for nNOS enzyme had significantly lower levels of NO than females with fewer numbers of repeats (Graserman et al, 2003). Moreover, Lund and colleagues (2007) found that genetic effects accounted for 60% of variation in FeNO concentration among adult twins. This implies that the Bivariate association result observed between FeNO concentration and BMI maight be confounded by other factors such as nNOS enzyme concentration and genetic factors possibly.

3.4.3. FeNO concentration and its association with the proportion of LB^{+ve} tubercle bacilli in sputum

The presence of LBs as a universal feature of acid-fast bacilli populations in sputum was previously reported (Garton et al, 2008). In the current study, the proportion of LB^{+ve} tubercle cells varied from 2% to 80% of the population of AFB in sputum samples collected from TB patients. This is very similar to the previous report from the Gambia and Leicester which demonstrated a 3% to 80% LB^{+ve} TB bacilli proportion (Sherratt, 2008). In this study FeNO concentration was found significantly associated with the proportion of

LB^{+ve} tubercle bacilli in sputum samples when data was analysed by both bivariate and multivariate regression analysis. Previously, Sherratt (2008) demonstrated that *Mtb* subjected to NO exposure over a 7 hour period produced LBs after 30 minutes and peak LB accumulation occurred after 4-4.5 hours. Daniel et al (2004) demonstrated that expression of tgs1gene is induced by NO treatment, with maximum levels of expression occurring after a concurrent time of 4 hour of exposure. The expression of tgs1 also has been implicated in LB formation in *Mycobacterium smegmatis* and *Mtb* isolated from sputum, indicating a likely role for tgs1 in LB accumulation following NO treatment (Daniel et al, 2004).

Mtb infected macrophages, upon activation release NO and the intracellular environment becomes hypoxic (Bentrup and Russell, 2001), conditions known to induce LB formation. Recently, Daniel et al (2011) reported that lipid-loaded macrophages may provide a fatty acid-rich host environment for *Mtb*. *Mtb* inside the lipid loaded macrophages imports fatty acids derived from host TAG and incorporates them intact into *Mtb* TAG. Previously, Flynn and Chan documented that *Mtb* has a unique ability to persist in the infected host (Flynn and Chan, 2001). This suggests that the tubercle bacillus possesses means by which the anti-mycobacterial effects of reactive nitrogen intermediates including nitric oxide can be evaded and that could possibly be mediated by the accumulation of intracellular lipophilic molecules or lipid bodies. The first check point for *Mtb* to overcome is to prevent itself from being killed by the antimicrobial effector mechanisms of the macrophages which includes acidification of the phagosome, exposure to proteases and antimicrobial peptides and the generation of reactive oxygen and nitrogen species.There are reports documented that the pathogen has evolved strategies to evade and/or tolerate these stresses, and manages to survive in cells that are otherwise effective killers of most microorganisms (Susanna and Maria, 2012).One of the mechanisms that the pathogen might use could possibly be the formation of TAG LBs as LB^{+ve} tubercle bacilli were reported tolerance of antimicrobials and may not be susceptible to the toxic effect of nitric oxide. Ehrt et al (1997) reported that DNAs from a highly RNI-resistant *Mtb* isolate conferred resistance to toxic nitrogen oxides. Moreover, microarray analyses have demonstrated that RNI can

regulate *Mtb* gene expression and the genes that are up-regulated by RNI may contribute to the persistence. Ohno et al (2003) also showed that *in vivo* studies using a murine experimental TB model involving NOS II-deficient mice have provided evidence suggesting that RNI regulate *Mtb* gene expression *in vivo*. Accumulating evidence strongly suggests that *Mtb* is well equipped with anti-RNI activities. Given the existence of multiple and may be redundant mechanisms by which *Mtb* can evade RNI toxicity; the recent demonstration that RNI regulate the expression of *Mtb* genes that may be conducive to survival in the host suggests the possibility that reactive nitrogen species including NO serve as a signal that plays a role in promoting bacterial persistence or dormancy through the formation of intracellular lipid molecules.

The relationship between FeNO concentration and the proportion of LB^{+ve} tubercle bacilli in some TB patients can be considered as representative for the association FeNO concentration with the proportion of LB^{+ve} tubercle bacilli in sputum. For example in a patient that had FeNO concentration of 26 ppb, the proportion of LB^{+ve} tubercle bacilli was 80%. On the other hand in a sputum sample with a 2% proportion of LB^{+ve} tubercle bacilli, FeNO concentration was 13 ppb. These two evidences together with the statistical association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli in sputum may suggest that the higher the concentration of FeNO, the higher the proportion of LB^{+ve} tubercle bacilli and the lower the concentration of FeNO, the lower the proportion of LB^{+ve} tubercle bacilli in sputum.

In this study, the association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli in sputum was by far strong among retreatment TB patients ($r^2 = 0.59$) than TB patients that had no history of TB treatment (new TB cases) ($r^2 = 0.256$). There are *in vitro* reports that showed phenotypic drug tolerance for the first line anti-TB drugs (INH and RIF) as a result of accumulation of LBs within the tubercle bacilli (Sherratt, 2008). It is also possible to speculate that the bacilli accumulate LBs to use as energy source during dormancy or non-replicating persistence.

The limitation of this study was that only sputum samples that had AFB score of 4+ and 3+ were included in the enumeration of the proportion of LB^{+ve} tubercle bacilli and the number of sputum samples investigated for the proportion of LB^{+ve} tubercle bacilli were relatively small. Moreover, the roles of other co-infections such as HIV infection were not investigated in this study.

3.5. Conclusions

1. FeNO concentration was significantly associated with the age and sex of the TB patients.

2. FeNO concentration was significantly associated with the proportion of LB^{+ve} tubercle bacilli in sputum.

3. The association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli was stronger among retreatment patients compared with new TB cases.

CHAPTER FOUR: Influence of HIV, intestinal parasites and blood count status on FeNO and bacillary LBs in sputum

4.1. Introduction

In the mid 1980's Robert Gallo, an American scientist and his team co-discovered the virus responsible for the killer disease known as AIDS (Cichocki R, 2007). Since then, the HIV epidemic remains a major global public health problem. In 2007, 33.2 million people were estimated living with HIV, 2.5 million people became newly infected and 2.1 million people died of AIDS globally. In Sub-Saharan Africa, there were an estimated 1.7 million new HIV infections by the same year, a significant reduction since 2001 (MOH, 2008). HIV was first detected in Ethiopia in stored sera collected in 1984 and the first two cases were reported in 1986 (Lester et al, 1988). The prevalence of HIV among adult people in Ethiopia was estimated to be 2.2 % in 2008 and 2.4% in 2010 (WHO, 2010). About 11.1 million adults are co-infetcted with HIV and TB globally and 70% of co-infected people are living in sub-saharan Africa. In Ethiopia the prevalence of TB infection among HIV positive patients by the year 2005/2006 was reported 41% (MOH, 2008).

4.1.2. HIV/TB Infection and the Immune System

The HIV mainly targets CD4+ T lmphocytes and cells of the monocyte/macrophage lineage (Conner and Ho, 1994). HIV/TB collaborative infection is typified by reduced type one cell mediated immune response perhaps provoked by immunosuppressive cytokines produced by macrophages/monocytes, rather than by type 2 cells (Zhang et al, 1994). An increased pathology caused by HIV/*Mtb* collaborative infection is mainly caused by disturbance and disruption of the granuloma which decreases the ability of the granuloma to contain *Mtb* which might be followed by dissemination and severe pathology (Bezuidenhout et al, 2009).

Kion and Hoffimann (1991) showed that HIV may also trick the immune system into attacking itself. Another method of HIV infection is syncytium formation, which involves massing of healthy T cells around a single HIV-infected T4 cell resulting in loss of immune function (Hoxie et al, 1986). Pantaleo et al (1993) documented that defects in T4 cells caused by HIV infection may produce activation induced cell death or apoptosis. However,

HIV infected cell death could be also due to direct membrane disruption involving mainly calcium channels (Gupta and Vayuvegula, 1987) and/ or phospholipid synthesis (Lynn et al, 1988). Moreover, Bakker et al (1992) reported that infection of monocyte and macrophages by the HIV virus maight equally important to that of CD4+ T cell infection.

4.1.3. Intestinal parasites, HIV and TB co-infection

Intestinal parasitic infections may possibly disturb the sense of balance of the immune response and contribute to HIV replication (Newton, 2005) and this could accelerate progression to AIDS (Karanja et al, 1997). On the other hand the reduced immune response caused by HIV may lead the host susceptible to opportunistic parasite infection (Karp and Auwaerter, 2007; Nielsen, 2007). The depletion of CD4+ T cells, which is a main feature of AIDS, is certainly an important contributor to the increased risk of reactivation of latent TB and susceptibility to new *Mtb* infection (Pawlowski et al, 2012). There are other mechanisms that help *Mtb* infection in HIV positive individuals and these include the up-regulation of *Mtb* entry receptors on macrophages (Rosas-Taraco et al, 2006), HIV manipulation of macrophage's bactericidal pathways (Spear,1990), deregulated chemotaxis (Wahl, 1989), and disturbed Th1/Th2 balance (Havlir and Barnes, 1999).

Ethiopia is one of the highly affected countries by the HIV /TB co-epidemic.The dual epidemics have a number of impacts on the health sector. They increase TB and HIV burden, demand for care and worsen the situation of the already overstretched health care delivery system in the country (FMOH, 2008). There are mounting reports recognizing that intestinal parasitic infections remain an important cause of morbidity and mortality in developing countries (Levy, 1988).

Usually, people at high risk for HIV/TB co-infection live in highly endemic areas for intestinal parasitic infections (Infectious Disease Society of America (IDSA), 2007). There is mounting evidence that helminthic infections influence the HIV disease progression by regulating the host immune response, both at the cellular and humoral level, as the result of a strong T helper 2-type cytokine profile (Sharam and Mohan, 2004).

During TB infections, exhaled NO level is increased in patients with active pulmonary TB. Inducible NOS expression in alveolar macrophages was also found to be upregulated with an enhanced capacity for spontaneous generation of RNI. Cytokines released in the immune response against *Mtb*, such as TNF-α, IL-1β and IFN-γ have been demonstrated to upregulate iNOS expression and enhance NO production in murine macrophages. Nitric oxide synthase has been shown to be induced in human monocytes through an IgE-dependant mechanism or by the sequential treatment with IL-4 and IFN-γ (Cenda et al, 1995). The association between intestinal parasitic infection and elevated levels of IgE, mast cells and eosinophils is well established (Yazdanbakhsh, 1996). Moreover, Zhang et al (1994) reported that endemic helminthoses activate the Th_2 system, particularly at mucosal surfaces to provide a different level of immunological homeostasis. However, the level of FeNO concentration in patients infected with intestinal parasite, HIV and TB is not fully understood. Moreover, although previous reports documented that tubercle bacilli exposed to nitric oxide produced LBs and our previous study showed a significant association between FeNO concentration with the proportion of LB[+ve] tubercle bacilli in sputum, the relationship between FeNO and the proportion of LB[+ve] tubercle bacilli in sputum among patients triply infected with intestinal parasite, HIV and TB is not known.

4.1.4. Aims and objectives

The major aim of this study was to assess the effect of HIV and intestinal parasite infection on the association of FeNO concentrations with the proportion of LB[+ve] tubercle bacilli in sputum.

Specific obijectives

1. To determine the concentration of FeNO in HIV positive and HIV negative TB patients.

2. To compare the FeNO concentration among TB patients with and with no intestinal parasitic infection.

3. To determine the association of eosinophilia with FeNO concentration and the proportion of LB[+ve] tubercle bacilli.

4.2. Materials and Methods

4.2.1. Study design, area and period

This cross-sectional study was conducted at the University of Gondar teaching hospital and Gondar poly clinic, Northwest Ethiopia from May 2010 to April 2011.

4.2.2. Patient recruitment and selection (figure 4.1)
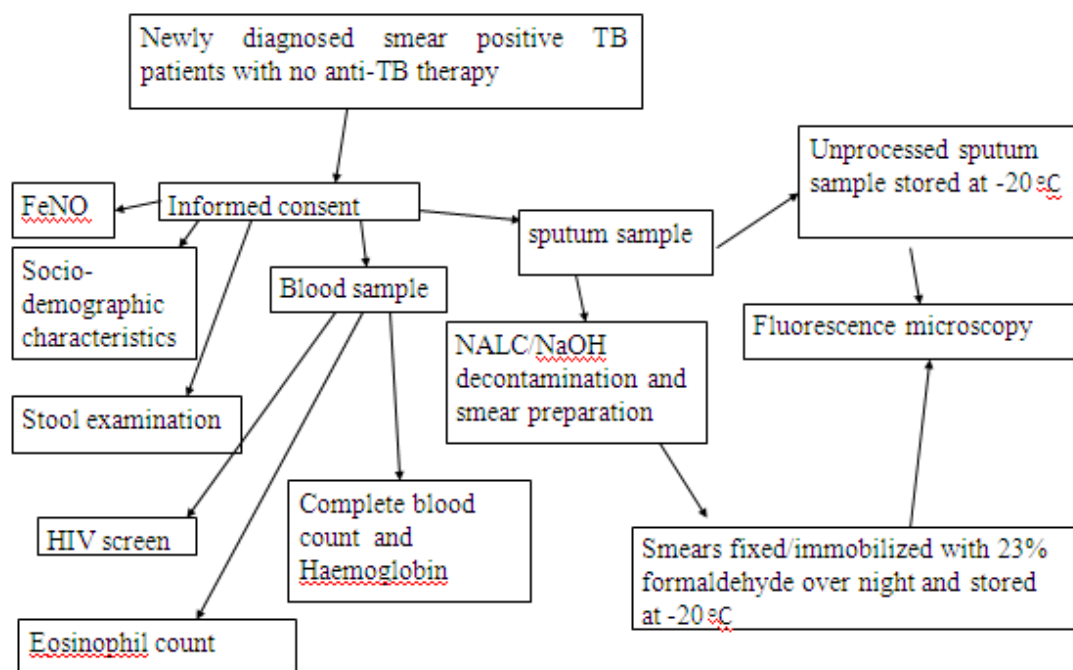
Figure 4.1: Patient selection and recruitment

Smear positive TB patients (n=88) who had not been treated with any of the anti-TB drugs were recruited at the outpatient department (OPD). Patients were recruited upon sputum delivery and being proved positive for acid-fast bacilli.

4.2.3. Eligible versus included patients

Patients suspected of pulmonary tuberculosis were the source population for this study. New smear positive TB patients (n=88) voluntary to participate in the study were recruited at the out patient department of University of Gondar hospital and Gondar Poly clinc. However, FeNO concentration was determined only on 73 TB patients.

4.2.4. Exhaled NO measurement and Laboratory reagents

FeNO concentration was measured using NIOX MINO air way inflammation monitor (Aerocrine AB, SE-171 21 Sona, Sweden) as described in chapter 3. All laboratory reagents used in section three were also used in this experiment.

Five ml of venous blood was collected from each patient. Three ml blood was added to a vial containing tri-sodium citrate anticoagulant. After thoroughly mixed, each blood sample was investigated for total WBC, differential WBC, haemoglobin, RBC and haematocrit using Cell DyN-1800 haematology analyzer (Operator's manual Cell DyN, Abbott Diagnostic Division, 2003). Thin blood films were prepared from each sample and stained with Wright's stain solution. Each stained blood film was examined microscopically and the different white blood cells (Neutrophils, Lymphocytes, eosinophils, basophils, and monocyts) were counted by percentage. A portion of blood sample (2 ml) was deposited in a tube and allowed to stand at room temperature until completely clotted, then centrifuged at 1258xg for 20 minutes (International equipment company, IEC, Needham Heights, NA 02194, USA, 1998) and serum separated. All smear positive TB patients were tested for HIV at the Providing Initiative Testing and Counseling (PITC) clinic by rapid HIV test methods. The serum samples were again tested for HIV by ELISA (Vironostica HIV, bioMerieux) and all ELISA positive samples were included as HIV positive patients.

4.2.5. Stool examination

Stool samples were collected using clean, dry and leak-proof containers. Diarrhoea, dysentery or watery stool samples were examined by direct saline preparation. For formed and hard stool samples, the formol-ether concentration technique was used and the ova of the parasite stained with iodine solution.

Direct saline examination of stool samples: A drop of physiological saline (0.85% w/v NaCl) was placed on a clean microscope slide. With an applicator stick, a small portion of the feces (approximately 2 mg which is about the size of a match stick head) was added to the drop of saline and mixed to homogenize the suspension. The preparation was covered

with a cover slip and examined under the microscope using 10X objective for egg and motile forms and 40X for cyst and oocysts of intestinal protozoa.

Concentration methods for faecal specimen examination: Formalin-Ether Centrifugal sedimentation technique is recommended as the best technique for concentrating the eggs and larvae of helminthes and moderately satisfactory for cysts of protozoa. The formalin is used for fixation and preservation of the morphology of parasites. The faecal debris absorbs ether and become lighter than water.

About 2 gm or 2 ml of stool sample was mixed with 10 ml of normal saline solution and thoroughly mixed. This was filtered through two layers of gauze into a centrifuge test tube and centrifuged for one minute at 1258xg (International equipment company, IEC, Needham Heights, NA 02194, USA, 1998). When the supernatant fluid was very cloudy, the deposit was washed again by mixing it with 10 ml of normal saline. After pouring or removing the supernatant fluid, 10 ml of formaldehyde solution (10% formalin solution) was added to the sediment. After mixing the suspension well and allowed to stand for 5 minute, 3 ml of ether was added and immediately stopper and vigorously shake for 30 seconds. The preparation centrifuged for one minute at 1258xg. There appeared four layers in the preparation; the first layer which is ether, the second layer debris, the third layer formaldehyde solution and the fourth layer which is the deposit containing stages of parasites (cysts, egg and/or larvae). The supernatant was removed by tilting the tube and poured off all the fluid. The sediment mixed with the remaining small fluid and about two drops of the deposit was placed on a slide, to which a drop of iodine solution was added and covered with cover slide. The entire preparation was examined using 10x objective for protozoa eggs and 40x objective for cysts.

4.2.6. Ethical considerations and Informed consent

All the necessary ethical issues were considered and informed verbal and written consent were taken. Ethical clearance and permission were obtained from University of Gondar research and publication office (RPO) with Ref.NO:- RCS/05/505/2002 E.C.

The left over sputum specimens obtained from the study subjects for routine examination purpose were used for the study. FeNO concentration, stool sample for intestinal parasites and blood sample for complete blood cell count were determined only after informed consent obtained from the study subjects. HIV rapid test result was taken from Providing Initiative Testing and Counseling (PITC) clinic and re-tested by ELISA. Volunteers were given adequate and clear information about the purpose, conduct and expected outcome of the study. No attempt was made to get consent from a patient whose sensorial was disturbed or is in acute pain. All data was kept confidentially. All the patients diagnosed during the study received relevant laboratory data.

4.3. Results

The results of the current study are summarised in table 4.1a- 4.1e. FeNO concentration of one HIV^{-ve}/TB patient (83ppb) and another HIV^{+ve}/TB patient (49 ppb) were outside 2SD from the mean of each category (mean FeNO HIV^{-ve}=14.2 ppb; SD=13.0 and mean FeNO HIV^{+ve}= 12.3 ppb; SD=9.2).

Table 4.1a: Summary of FeNO, LB, Parasitology and Blood count results for HIV^{-ve}/Parasite $^{-ve}$ TB patients (n=23).

FeNO (ppb)	LB^{+ve} (%)	WBC	NP	LC	Eo	HGB (g/dl)
5	26	6600	3432	2178	726	8.7
5	19	8800	5192	2464	1056	8.5
6	23	6300	4662	1323	315	13.6
6	28	7500	4725	2025	750	14.5
6	11	7900	6004	1817	79	11.4
7	14	12400	10912	1240	248	12
8	15	6500	3965	1625	845	16.2
8	11	6600	5016	1584	0	6.5
8	21	5500	3795	1650	55	12.7
8	25	5500	2310	3025	55	14.5
8	18	8600	6536	1806	258	13.7
8	40	3900	2730	1092	78	12.6
9	21	7800	4992	2262	546	16.6
10	27	8200	4838	2788	574	12.6
11	27	6400	4224	1856	320	14.3
11	14	6900	4692	2001	69	12.2
13	24	6500	4420	1690	390	10.9
13	17	6600	4620	1716	264	14.3
13	39	7300	6059	511	730	12.4
15	58	7600	5928	1520	152	12.8
16	24	14100	9870	3666	423	14.1
22	10	10300	6901	2266	1030	11.7
83*	38	5800	3538	2146	116	15.9

FeNO=fractional exhaled nitric oxide; ppb=parts per billion; LB^{+ve}=lipid body positive; WBC=White blood cell; NP=Neutrophil; LC=Lymphocyte; EO=Eosinophil; HGB=Haemoglobin.

Table 4.1b: Summary of FeNO, LB, Parasitology and Blood count results for HIV^{-ve}/Parasite $^{+ve}$ TB patients (n=21).

FeNO (ppb)	LB^{+ve} (%)	WBC	NP	LC	Eo	HGB (g/dl)
5	22	8800	6776	1760	264	13.4
5	13	7900	5135	2686	79	10.6
5	15	9800	6860	2058	882	11.9
7	13	3900	2145	1560	195	13.8
8	20	6500	4225	2080	195	13.8
9	45	5900	4071	1652	118	11.9
9	22	4800	2304	1968	480	11.3
11	25	5100	2346	2193	561	14.5
11	73	6600	3762	2244	528	13
12	43	13200	10560	2244	264	11.3
13	9	6100	3172	2196	671	11.5
14	30	14900	12367	1639	745	14.2
17	37	6700	3551	2144	1005	12.5
22	32	6400	1728	3456	1216	9
25	47	5900	2773	1770	1180	7.2
25	33	14900	10281	3576	596	5.6
28	42	11500	8395	1725	1380	11.2
28	42	7100	4331	1846	781	12
29	33	10900	3706	3815	3379*	13.8
36	40	12600	8064	3276	1260	9.3
6	14	13300	9310	3325	665	12.8

Table 4.1c: Summary of FeNO, LB, Parasitology and Blood count results for HIV^{+ve}/Parasite $^{-ve}$ TB patients (n=18).

FeNO (ppb)	LB^{+ve} (%)	WBC	NP	LC	Eo	HGB (g/dl)
5	24	10500	9345	840	315	8.2
5	13	8300	5395	1826	996	9.7
5	41	9200	7728	1104	368	11.9
5	45	7300	4672	2409	219	11.4
5	38	9700	7663	1940	97	6.9
5	26	8800	7480	1144	176	12.7
8	29	7000	4970	1820	210	13.9
9	16	10200	4284	3060	2550*	12.5
10	47	4500	2880	1350	270	10.4
11	31	8400	3108	5040	252	15.3
13	75	5900	3599	2242	59	14
13	42	9900	6336	2970	594	13.9
14	45	10900	9047	1853	0	11.1
14	40	6800	4964	1768	68	9.4
17	41	7800	4680	1560	1248	10.3
22	66	4800	2640	2064	96	13.7
22	50	6600	4950	1320	330	10.2
22	71	7600	3572	3648	380	12.9

Table 4.1d: Summary of FeNO, LB, Parasitology and Blood count results for HIV[+ve]/Parasite [+ve] patients TB (n=11).

FeNO (ppb)	LB[+ve] (%)	WBC	NP	LC	Eo	HGB (g/dl)
5	17	11400	6954	3420	912	12.6
5	65	6400	4928	1344	128	12.7
7	22	8800	6864	1056	0	14.1
7	30	7500	5175	1950	375	13.4
8	18	4400	3784	528	88	7.2
8	35	4100	2788	1230	82	9.8
11	28	6800	3876	2448	408	11.4
12	8	8300	5727	1909	581	11.7
12	58	3000	1470	1110	390	11.4
49*	64	1100	660	363	66	14.6
28	69	7800	4056	2574	1092	13.7

Table 4.1e: Summary statistics for FeNO, LB, Parasitology and Blood count results (n=71).

	N	FeNO (ppb)	LB+ve (%)	WBC	NP	LC	Eo	HGB (g/dl)
O/A	71	13.4	32	7811	5189	2046	519	12.0
Sd		11.7	17	2728	2392	834	553	2.3
HIV-	43	14.2	27	8100	5346	2124	580	12.2
		13.0	14	2891	2573	704	567	2.4
HIV+	28	12.3	40	7372	4952	1927	426	11.8
Sd		9.2	19	2394	2067	988	518	2.1
H-P-	23	13.0	24	7548	5190	1924	395	12.7
Sd		15.4	11	2190	1953	651	319	2.4
H-P+	21	15.5	31	8705	5517	2343	783	11.6
Sd		9.3	15	3400	3104	695	696	2.2
H+P-	18	11.4	41	8011	5406	2109	457	11.6
Sd		6.0	17	1831	2018	1004	597	2.2
H+P+	11	13.8	38	6327	4207	1630	375	12.1
Sd		12.7	21	2806	1925	885	345	2.0

O/A=over all; HIV- =HIV negative; HIV+= HIV positive; SD=Standard deviation; H-P- = HIV negative intestinal parasite negative; H-P+ = HIV negative intestinal parasite positive; H+P- = HIV positive intestinal parasite negative; H+P+ = HIV positive intestinal parasite positive.

Thirty-one of 71 TB patients (43.7%) were co-infected with intestinal parasites and *Ascaris lumbricoids* was the dominant parasite (24%; n=17/71) followed by Hookworm (15.5%; n=11/71) (table 4.2). Regardless of the HIV status and intestinal parasitic co-infection, the mean concentration of FeNO among new pulmonary tuberculosis patients was 13.4 ppb (95% CI 10.70-16.18; SD=11.7). Nine TB patients were co-infected with two or more intestinal parasites.

Table 4.2: Prevalence of intestinal parasite

The overall prevalence of intestinal parasite infection among HIV^{+ve} and HIV^{-ve}/TB patients was high.

Intestinal Parasite	HIV^{+ve}	HIV^{-ve}	Total
Ascaris lumbricoids	4	13	17
*Hook*worm	3	8	11
Schistosoma mansoni	0	3	3
Hymenolepis species	4	5	9
Strongyloides larvae	1	1	2
Entamoeba histolytica	0	2	2
Giardia throphzite	1	1	2
Trichuris ova	0	1	1
Negative for parasites	19	22	41

The AFB score displayed on table 4.3 was determined after the sputum sample decontaminated with NALC/NaOH decontaminantion. One sputum sample result was negative after NALC/NaOH decontamination which was previously reported positive for AFB by ZN method without decontaminating the sputum sample. In this study, similar to the previous result (Chapter 3), there was no significant association between AFB score and FeNO concentration among HIV^{+ve} and HIV^{-ve}/TB patients.

Table 4.3: AFB score among HIV[+ve] and HIV[-ve]/TB patients

The AFB score of smear positive TB patients were determined following the CDC standards.

AFB score	HIV[+ve]	HIV[-ve]	Total
4+	7	14	21
3+	5	12	17
2+	11	19	30
1+	10	9	19
Negative	0	1	1
Total	33	55	88

4.3.1. Lymphocyte count among HIV[+ve] and HIV[-ve] TB patients

The absolute lymphocyte count (LC) of HIV[+ve]/TB (n=33) patients seems lower than the HIV[-ve]/TB (n=55) (Mean ± SEM=1878±166.9 versus 2024±98.35 respectively) patients. However, there was no significant difference on lymphocyte count among HIV[+ve] and HIV[-ve]/TB patients (Figure 4.2).
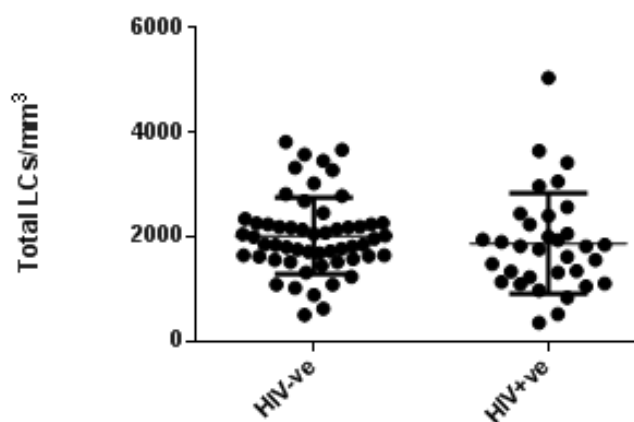
Figure 4.2: The Lymphocyte count of HIV[-ve] and HIV[+ve]/TB patients.

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4.3.2. Association between eosinophil count, intestinal parasite/TB co-infection with FeNO concentration and the proportion of LB[+ve] tubercle bacilli among HIV[+ve]/HIV[-ve]TB patients

An average of 5.62% (95% CI 3.48-7.75%) and 7.6 % (95% CI 5.7-9.5%) eosinophil count was observed on HIV[+ve] and HIV[-ve]/ TB patients respectively. However, the mean absolute eosinophil count for HIV[+ve]/TB patients was relatively higher compared with the corresponding HIV[-ve]/TB patients (Mean± SEM=565.4 ± 71.86 versus 385 ± 88.40 respectively). Nevertheless, there was no statistically significant difference on eosinophil count among HIV[+ve] and HIV[-ve] /TB patients (figure 4.3).

Figure 4.3: The absolute eosinophil count among HIV[+ve] and HIV[-ve]/TB patients.

Taking a 1-4% esinophil count as normal value (Dugale, 2011), the association between FeNO concentration and eosinophil count was also assessed. Accordingly 41/71 TB patients had an eosinophil count of greater than 4% and the other 30 less or equal to 4% (figure 4.4).
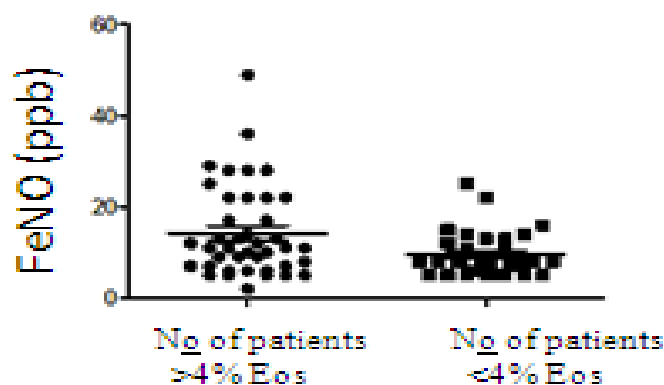
Figure 4.4: Eosinophil score versus FeNO concentration

The average FeNO concentration among TB patients that had greater than 4% eosinophil count was relatively higher compared with those that demonstrated less than 4% eosinophil differential count (n=71)(p=0.031).

The association between eosinophil count, FeNO concentration, HIV status and intestinal parasite co-infection was investigated using linear regression analysis. Among 73 TB patients for whom FeNO concentration determined, 33 were co-infected with intestinal parasites and the other 40 were negative for intestinal parasites. Both HIV[-ve] and HIV[+ve]/TB patients co-infected with intestinal parasite demonstrated relatively higher FeNO concentration (Mean=15.5; SD=9.3 versus Mean=13.8; SD=12.7 respectively) (Table 4.1e and figure 4.3). Intestinal parasite negative TB patients in both HIV[-ve] and HIV[+ve] cases demonstrated lower FeNO concentration (Mean=13.0 ppb versus 11.4 ppb respectively). Moreover, data showed a significant association between eosinophil count and FeNO concentration among H-P+ (p=0.0001) and H+P+ TB patients (p=0.0479) (Figure 4.5). On the other hand, there was no significant corelation between eosinophil counts with the proportion of LB[+ve] tubercle bacilli either with parasite negative or positive TB patients (Data not shown).
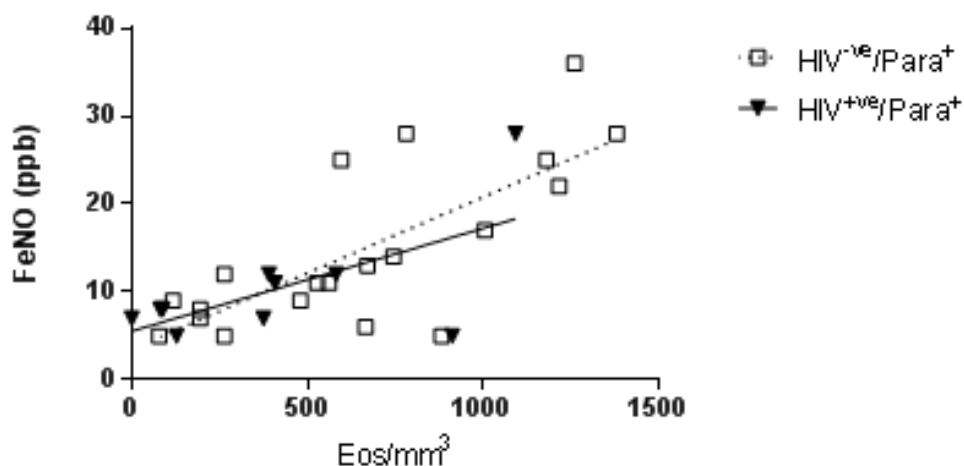
Figure 4.5: Association between eosinophil score and FeNO concentration

Eosinophil count was significantly associated with FeNO concentration among HIV[+ve] and HIV[-ve]/TB patients that were co-infected with intestinal parasites but no significant association were obsereved among patients that are negative for intestinal parasites. The association was stronger in HIV[-ve]/TB patients compared with HIV[+ve]/TB patients.

4.3.3. FeNO concentration and proportion of LB[+ve] tubercle bacilli among HIV[+ve]/HIV[-ve] TB patients

Regardless of intestinal parasite co-infection, the average level of FeNO concentration among HIV[+ve]/ TB patients (n=29) was 12.3 ppb (SD=9.2). The mean level of FeNO concentration among HIV[-ve]/TB patients was 14.2 ppb (SD=13.0). On the other hand, an average of 36.5% and 42.12% LB[+ve] tubercle bacilli proportion was observed among HIV[+ve]/TB patients co-infected with intestinal parasites and HIV[+ve]/TB patients with no intestinal parasite co-infection. The mean proportion of LB[+ve] tubercle bacilli among HIV[-ve]/TB patients co-infected with intestinal parasites was also relatively higher (30.95%) than HIV[-ve]/TB patients with no intestinal parasite (23.27%) infection.

The average proportion of LB[+ve] tubercle bacilli among HIV[+ve]/TB patients (n=29) was higher (39.8%) than HIV[-ve]/TB patients (n=43) which was 27.02 %. However, in HIV[+ve] and HIV[-ve] /TB patients as high as 75% and 73% and as low as 8% and 11% LB[+ve] tubercle bacilli proportion were observed respectively.

The mean haemoglobin (hgb) concentrations for HIV^{+ve} and HIV^{-ve}/TB patients were 11.76 g% (95% CI 10.93-12.59) and 12.13 g% (95% CI 11.39-12.86) respectively. H+P+ and H-P+/ TB patients had an average of 11.73 g% and 11.78 g% hgb respectively. On the other hand, the mean hgb concentrations of H-P+ and H-P-/TB patients were 11.65 g% and 12.58 g% respectively. In this study intestinal parasitosis and/or HIV infection were not associated with hgb concentration. Taking 12 g% as the lower normal range (Gomez and Carrera, 2002), the association of hgb with FeNO concentration was also determined. However, there was no significant association between FeNO and hgb concentrations.

4.3.4. Association between FeNO concentration and the proportion of LB^{+ve}tubercle bacilli in sputum among HIV^{+ve} and HIV^{-ve}/TB patients

Regardless of HIV status, the over all mean FeNO concentration was 13.4 ppb (SD = 11.7 ppb). HIV^{-ve}/TB patients co-infected with intestinal parasite demonstrated relatively higher FeNO concentration (Mean=15.5 ppb; SD= 9.3) followed by HIV^{+ve}/TB patients also co-infected with intestinal parasites (Mean = 13.8 ppb; SD = 12.7). The corresponding overall mean proportion of LB^{+ve} tubercle bacilli was 32%; SD= 17.0. A relatively higher mean proportion of LB^{+ve} tubercle bacilli was observed in HIV^{+ve} patients (mean= 40%) compared with that of HIV^{-ve}/TB patients (Mean=27%). Regardless of the HIV status and intestinal parasitic co-infection, FeNO concentration was significantly associated with the proportion of lipid body positive tubercle bacilli in sputum (P=0.0004; r^2= 0.17). The association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli was relatively stronger among HIV^{+ve} compared with HIV^{-ve}/TB patients (P=0.002, r^2=0.32; P=0.02, r^2=0.12 respectively) (Figure 4.6 A, B, and C).

(A)   (B)   (C)
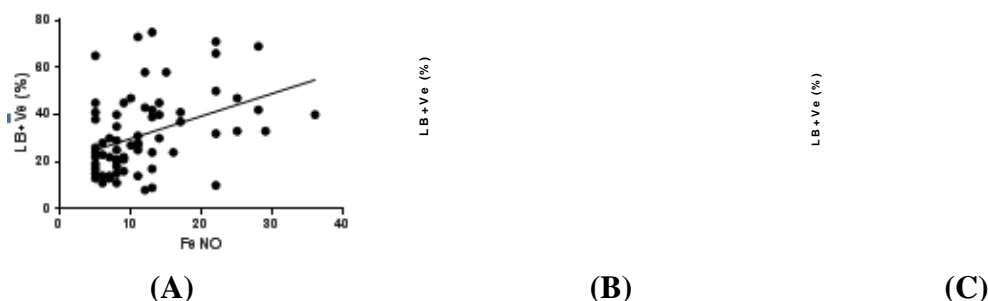
Figure 4.6: Association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli in sputum among new smear positive TB patients

(A)The over all association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli was weakly but significantly associated (n=73); B) FeNO association with the proportion of LB^{+ve} tubercle bacilli among HIV^{+ve}/TB patients (n=29); C) association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli among HIV^{-ve}/TB patients (n=44).

Although FeNO concentration among HIV^{+ve}/TB patients were comparatively lower than HIV^{-ve}/ TB patients, FeNO concentration was significantly associated with the proportion of LB^{+ve} tubercle bacilli among H+P-/TB cases (p=0.0022; r^2= 0.45). H-P+/TB patients also showed an association between FeNO concentration with the proportion of LB^{+ve} tubercle bacilli (p=0.0380; r^2=0.21) (figure 4.7).
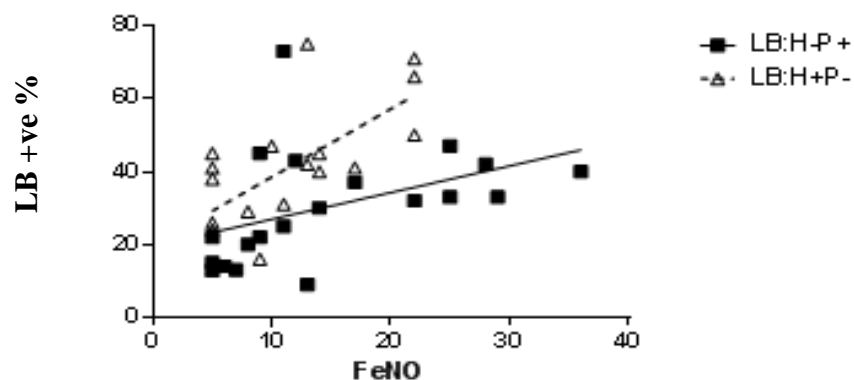
Figure 4.7: The relation between FeNO, LB^{+ve} tubercle bacilli proportion, HIV and intestinal parasitic infecition

The association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli among HIV^{+ve} patients that had no intestinal parasite co-infection was by far stronger than HIV^{-ve} patients co-infected with intestinal parasites (r^2= 0.45 versus 0.21 respectively).

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4.3.5. Multivariate analysis

FeNO concentration and the proportion of LB^{+ve} tubercle bacilli proportion data were
logarithmically transformed and the skewness of each data cheked using the QQ plot
(figure 4.8).
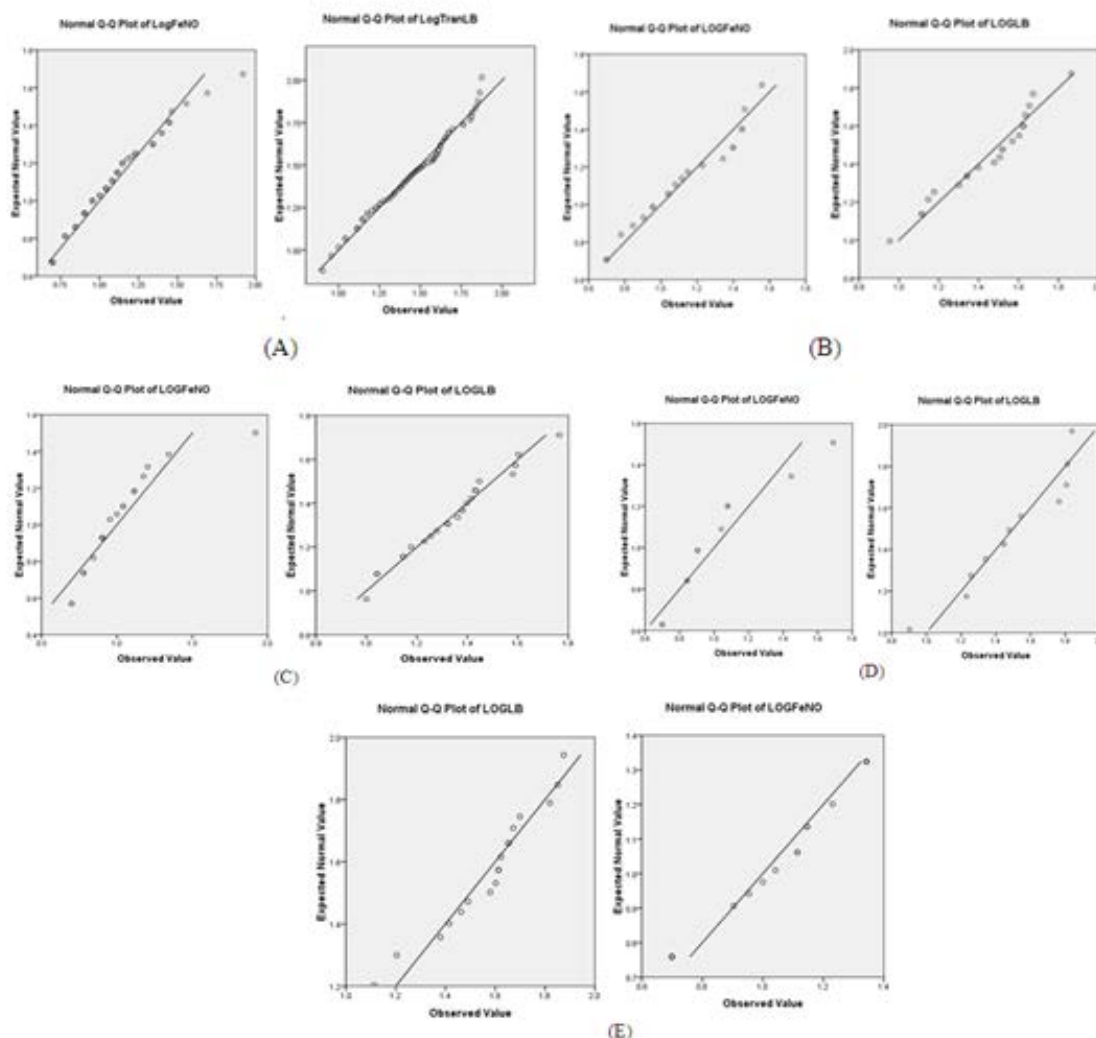
**Figure 4.8: QQ plot of FeNO concentration and proportion of LB^{+ve} tubercle bacilli
among HIV^{+ve} and HIV^{-ve}/TB patinets**

FeNO concentration and the proportion of LB^{+ve} tubercle bacilli datas were normally
distributed after logarithmically transformed. (A) Overall FeNO concentration and
proportion of LB^{+ve} tubercle bacilli; (B) HIV^{-ve} intestinal parasite positive; (C) HIV^{-ve}
intestinal parasite negative; (D) HIV^{+ve} intestinal parasite positive; (E) HIV^{+ve} intestinal
parasite negative.

Once normality of data achived, the over all association of FeNO concentration with the corresponding proportion of LB^{+ve} tubercle bacilli was determined and found significant (P=0.0002; r^2 = 0.32). Taking the proportion of LB^{+ve} tubercle bacilli as dependant variable and others such as age, sex, HIV status, eosinophil count, intestinal parasite infection and FeNO concentration as independent variables; multivariant regression analysis on the association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli were computed on HIV^{+ve} intestinal parasite positive, HIV^{+ve} intestinal parasite negative, HIV^{-ve} intestinal parasite negative and HIV^{-ve} intestinal parasite positive TB patients. Multivariate linear regression analysis demonstrated that FeNO concentration was significantly associated with the proportion of LB^{+ve} tubercle bacilli among HIV^{+ve} intestinal parasite negative (p=0.002; r^2= 0.673) and HIV^{-ve} intestinal parasite positive TB patients (p=0.008; r^2=0.423) but not among HIV^{+ve} intestinal parasite positive and HIV^{-ve} intestinal parasite negative TB patients.

4.4. Discussion

In this section the relationship between FeNO concentrations, intestinal parasitic infection together with leukocyte/haemoglobin concentration among HIV^{+ve}/HIV^{-ve} pulmonary TB patients was investigated. In addition, FeNO concentration of the TB patients was compared with the proportion of lipid body positive tubercle bacilli in each category.

4.4.1. Nitric oxide and its immunological aspects in HIV infection

The mean level of FeNO concentration in both HIV^{+ve} and HIV^{-ve}/TB patients in Gondar was low. Previously Idh et al (2008) reported that HIV^{+ve} and HIV^{-ve}/ TB patients both had lower levels of FeNO concentration compared to healthy blood donors and household TB contact cases in the same area. Our previous work in the same area showed 56 of 116 smear positive TB patients (48.3%) were either moderately or severely malnourished (unpublished source). A previous report (Chandra, 1997) showed that protein energy malnutrition is associated with a significant impairment of cell mediated immunity, phagocyte function; complement system, secretory immunoglobulin concentration, and cytokine production. Therefore, the low FeNO concentration among HIV^{+ve}/HIV^{-ve}/TB patients observed in Gondar could possibly be confounded by malnutrition.

4.4.2. Interactions of intestinal parasites and HIV with tuberculosis infection

The prevalence of intestinal parasite co-infection among smear positive new pulmonary tuberculosis patients in Gondar was high (43.7%). Previously, intestinal helminthes prevalence among TB patients was reported 71% in the same area (Elias et al, 2006).
The patterns of released cytokines during specific immune response are characteristic for CD4+ T helper cells (Th$_1$ and Th$_2$) (Verma and Mahajan, 2008). T helper-1 cells mainly produce IFN-γ but T helper -2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, but not IFN-γ (USAID, 2010). Infection with *Mtb* induces Th$_1$ immune responses where as intestinal parasites, mainly helminthes, elicit Th$_2$ immune responses. The frequency of occurrence of intestinal parasitic infection in Sub Saharan Africa was reported high and

this may elicit Th_1 cytokine production with a dominant Th_2 polarized immune response (Sharma et al, 2005) and has been suggested to increase susceptibility to both *Mtb* and HIV. The discrepancy especially with an increase in Th_2 cells favors IgE production, which may have clinical effects such as poor prognosis in co-infected individuals (FMOH, 2008). Moreover, it was documented that infection with intestinal helminthes results in immune responses involving cytokines produced by T-helper cell type-2 with IgE production, eosinophila and mastocytosis (Degen et al, 2005).

The average FeNO concentration was slightly higher among HIV[+ve]/TB patients co-infected with intestinal parasites compared with that of HIV[+ve]/TB patients with no intestinal parasitic infection in the current study. On the other hand, the mean FeNO concentration was higher on HIV[-ve] intestinal parasite positive TB patients compared with HIV[-ve]/intestinal parasite negative TB patients. Moreover, FeNO concentration was significantly associated with intestinal parasitic infection among HIV[-ve]/TB patients (P=0.014). In addition, FeNO concentration was relatively higher among TB patients infected with mixed intestinal parasite infection. Mixed infection is a common phenomenon in areas where various types of intestinal parasites are encountered (Assefa et al, 2009). The first site of entrance used by invasive intestinal pathogens is the epithelial cells and may supply early signals for the acute mucosal inflammatory response via the release of pro-inflammatory cytokines and inflammatory mediators (Ambriose-Thomas, 2001). Nitric oxide is an important mediator and is also known to have antimicrobial activity (Lebbad et al, 2001). Cytokines such as IL-7, IL-12, IL-15 and IFN-γ come into view to be involved in modulating host resistance against acute parasite infection.The common pathway by which these cytokines increase survival involves the induction of IFN-γ (Baum et al, 2003). The synergistic action of IFN-γ and TNF-α stimulates the production of nitric oxide and the tumoricidal and microbicidal activity of NO against a number of pathogens has been reported (Mosmann et al, 1986). There are several pieces of evidence indicating that helminths induce NO production by host cells (Elias et al, 2006).

 In a hepatic Schistosomiasis model induced by eggs of *S. japonicum*, an increase on the expression of iNOS has been detected in inflammatory cells, neutrophils, macrophages,

Kuppfer cells and hepatocytes (Kasper et al, 1995). Dondji et al (2008) reported that Ancylostoma hookworm infection is associated with reduced lymphocyte proliferative capacity, impaired antigen processing/presentation, depletion of CD4+ and surface IgG+ lymphocyte sub population, and increased production of the immunomodulatory compound nitric oxide.

4.4.3. Eosinophilia versus FeNO concentration during intestinal parasite, HIV and TB co-infection

The eosinophil count data of the current study shows no significant difference between the HIV^{+ve} and HIV^{-ve} TB patients. However, a comparatively high eosinophil count was observed in HIV^{-ve}/TB patients co-infected with intestinal parasites compared with HIV^{-ve}/TB patients with no intestinal parasite (mean 783/mm^3 versus 395/mm^3 respectively). Moreover, the eosinophil count was significantly correlated with FeNO concentration among HIV^{-ve}/TB patients co-infected with intestinal parasites but not among HIV^{-ve}/TB patients with no intestinal parasite infection. Eosinophils are granulocytes that are typically associated with immune response in helminth infection. Eosinophils and mast cells can produce NO and animal studies have shown that NO plays a key role in eosinophil migration and infiltration in rats (Ferreira et al, 1998). Activated eosinophils release NO and may also recruit companion eosinophil migration by other mechanisms such as increasing microvascular leakage (Yate, 2001). It was also postulated that NO produced by eosinophils could have a different function, related to eosinophil accumulation in inflamed tissue as a consequence of adhesive interactions between eosinophils and endothelial cells within the microcirculation. Moreover, Tristao-Sa et al (2002) reported that both eosinophilia and high levels of IgE are consequences of Th$_1$ activation, a preferential response induced by intestinal worms.

4.4.4. Tuberculosis and intestinal parasite induced anaemia

The haemoglobin (hgb) concentration of 29 patients was below 12 g%. More than that 12 TB patients had even below 10 g% hemoglobin concentration. The World Health

Organization defines anaemia as hgb concentration of less than 12 g% for women and less than 13 g% for men (Gomez and Carrera, 2002). The possible mechanisms for the development of anaemia in *Mtb* infection may be due to nutritional insufficiency, impaired iron utilization, mala-absorption, bone marrow granuloma and shortened duration of RBC survival (Berkowitz, 1991). Active pulmonary TB can cause severe auto-immune haemolytic anaemia. There are three basic mechanisms for TB induced anaemia by which infections may result haemolysis on an immune basis: antibodies to RBCs are produced as a result of the infection, antigen-antibody complexes specially related the infectious agent coat RBCs and the infectious agent results in exposure of RBC antigens to naturally occurring antibodies (Chen et al, 2009).

4.4.5. FeNO concentration and its association with the proportion of LB[+ve] tubercle bacilli in sputum among HIV[+ve] and HIV[-ve]/TB patients

The association of FeNO concentration with eosinophil count and the proportion of LB[+ve] tubercle bacilli are summerized on table 4.3. In this study FeNO concentration was significantly associated with the proportion of LB[+ve] tubercle bacilli among HIV[+ve] patients that had no intestinal parasite infection and HIV[-ve]/TB patients co-infected with intestinal parasites. Moreover, a relatively strong correlation between FeNO concentration and the proportion of LB[+ve] tubercle bacilli was observed among HIV[+ve]/TB patients than HIV[-ve]/TB patients. In HIV[+ve]/TB patients, we speculate that the immune pressure against the tubercle bacilli could be reduced as a result of the obliteration of the T helper cells and probably the macrophages too. The T helper cells are potent cytokine producing cells that induce nitric oxide production. We also observed the association of FeNO concentration with intestinal parasite co-infection and the eosinophil count. This can be considered as evidence for the role of eosinophils to induce NO during TB infection. The associations of FeNO concentration with the proportion of LB[+ve] tubercle bacilli in sputum might bring us to be attentive of other factors that may potentially induce NO. For example, IFNs are mainly produced by a virus infected cell and IFN-γ in particular stimulates cells for NO production. However, in the current study in some TB patients with low FeNO concentration a 65% and as high as 75%

proportion of LB^{+ve} tubercle bacilli with the corresponding FeNO concentration of 5 ppb and 13 ppb respectively were observed.

Although the association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli in sputum was significant in both H+P- and H-P+ patients, the high proportion of LB^{+ve} tubercle bacilli in sputum samples observed in some TB patients that had low FeNO concentration may indicate the potential role of other factors in triggering *Mtb* to accumulate lipid bodies *in vivo*.

Table 4.4: Summary table on the influence of HIVand intestinal parasite infection on eosinophil count, FeNO concentration and proportion of LB^{+ve} tubercle bacilii in sputum

	LC	Eos	FeNO/Eos	LB/Eos	FeNO/LBs
H-P-	1924	395	NC	NC	NC
H-P+	2343	783	0.56♦♦♦	NC	0.21
H+P-	2109	457	NC	NC	0.45♦♦
H+P+	1630	375	0.41♦	NC	NC

LC=Lymphocyte count; Eos=Eosinophil count; FeNO=Fractional exhaled air nitric oxide; LB=Lipid body; NC=not correlated; ♦=p<0.05; ♦♦=p<0.005; ♦♦♦=p<0.0005.
The mean of the LC, Eos, FeNO and LB was used to determine the aforementioned relationship.

The limitation of this study was that CD4+ lymphocyte count was not determined. Because this type of cells are mainly important in cell mediated immunity particularly cytokine production, the proportion of CD4+ T lymphocyte and the concentration of FeNO could have been assessed.

4.4.6. Conclusions

1. There was no significant difference in the absolute lymphocyte count among HIV+ve and HIV-ve/TB patients.

2. Both HIV+ve and HIV-ve/TB patients co-infected with intestinal parasite demonstrated relatively higher FeNO concentration and FeNO concentration was significantly correlated with eosinophil count.

3. FeNO concentration was significantly associated with the proportion of LB+ve tubercle bacilli in HIV+ve patients that had no intestinal parasite co-infection but the association was significant in HIV-ve/TB patients that were co-infected with intestinal parasites.

CHAPTER FIVE: Influence of *Mtb* spoligotype on FeNO concentration and the proportion of LB^{+ve} tubercle bacilli in sputum

CHAPTER FIVE: Influence of *Mtb* spoligotype on FeNO concentration and the proportion of LB^{+ve} tubercle bacilli in sputum

5.1. Introduction

The establishment of the genome sequence of *Mtb* H37 Rv in 1998 paved the way for major break through in understanding the biology of tubercle bacilli, in particular and mycobacteria in general (Cole et al, 1998). The MTBC comprises *M. tuberculosis*, *M. bovis*, *M. microti*, *M.africanum*, *M. pinnipedii*, and *M. caprae* species (Gutacker et al, 2002). There is evidence that members of the MTBC are the clonal progeny of a single successful ancestor, resulting from a recent evolutionary bottleneck that occurred 20,000 to 35, 000 years ago (Gutierrez et al, 2005). The genome of *Mtb* complex (MTBC) is highly conserved compared to other pathogenic bacteria and no horizontal gene transfer has been reported in genome (Brown et al, 2010). However, three major groups of MTBC genome alterations have been reported: Single nucleotide polymorphisms (SNPs), large sequence polymorphisms (LSPs), and polymorphisms within repetitive sequences such as variable number tandem repeats (VNTRs). SNPs and LSPs mark irreversible or permanent genetic events and can be used to construct phylogenies for *Mtb* (Brosch et al, 2002; Alland et al, 2007; Gagneux and Small, 2007). An association between geographic region and *Mtb* families, defined by specific polymorphisms, has been demonstrated. The geographic structuring genetically, and perhaps phenotypically, distinct MTBC populations may contribute to difference in clinical features such as severity of disease (Gagneux et al, 2006).

Mtb phenotypic diversity becomes of major interest in epidemiological circles in establishing the relevance of this diversity to human disease. Recent publications suggest that strains belonging to the *Mtb* W/Beijing lineage gain distinctive characteristics that present an increased ability to cause disease and to be transmitted within certain geographic settings (Reed et al, 2009). The most effective means of assigning strains into a small number of un ambiguous lineages is the method based on the detection of large sequence polymorphisms (LSPs) or regions of difference (RDs) that represent a series of well-characterized polymorphisms (deletions) (Gagneux et al, 2006). Using this approach, *Mtb* isolates are currently classified into six major global lineages: Indo-Oceanic, East-Asian,

East-African-Indian, Euro-American, West-African 1, West-African 2 (Gagnex et al, 2006) the majority of which show a high degree of geographic restriction (Reed et al, 2009). Each of the six lineages is defined by a single ancestral LSP common to all isolates within that particular lineage. Sub lineages have also been identified, each possessing its own unique LSP deletion events.

Analysis of the genetic structure and evolution of population of pathogenic microbes is essential for understanding the mechanisms responsible for the ability to escape host immune disease (Liu et al, 2006). Although *Mtb* exhibits relatively little genomic sequence diversity compared to bacteria that experience extensive lateral gene transfer and recombination, there are reports that document phenotypic diversity among clinical isolates (Fleischmann et al, 2002). Definition of sequence diversity in *Mtb* could provide a basis for further understanding pathogenesis, immune mechanisms, and bacterial evolution. There is increasing evidence that the inter strain variation that exists is biologically significant (Filliolet al, 2006). In human infections, molecular epidemiological studies have suggested that certain M*tb* types can be especially prone to drug resistance acquisition or to global dissemination (Drobniewski et al, 2003; Caminero et al, 2001). Supply et al (2003) reported that understanding the structure and dynamics of pathogen populations gives unique insights into crucial public health issues, such as the appearance and persistence of variants escaping immunity or the emergence of resistance to antibiotics. Some related types of *Mtb* also appear to be strongly associated with specific geographic locations (Friedman et al, 1997). Although, there are no well known virulence factors among pathogenic *Mtb* strains, there is evidence that some are more pathogenic than others; for example the W/Beijing lineage. It is worthy to concider that these strains accumulate TAG than others and strain in this lineage have recently shown more lipid body accumulation than *Mtb H37Rv*. However, the degree of accumulation of LBs within the tubercle bacilli associated with specific *Mtb* strains is not reported.

5.1.2: Aims and objectives

The major objective of this study was to determine whether specific *Mtb* spoligotypes are especially prone to accumulate LBs as a result of *in vivo* nitric oxide production in the lung.

Specific objectives

1) To provide an initial and base-line data on the spoligotype pattern of *Mtb* causing pulmonary tuberculosis in Gondar, Ethiopia.

2) To determine the dominant *Mtb* spoligotype causing pulmonary tuberculosis in Gondar.

3) To determine the association between FeNO concentration and the proportion of LB^{+ve} tubercle bacilli in sputum among TB patients infected with different *Mtb* spoligotype.

5.2. Material and Methods

5.2.1. Patient recruitment and selection

In this study DNA were extracted from smear positive sputum samples directly (n=116) and from cultured samples on LJ media (n=88) (figure 5.1).
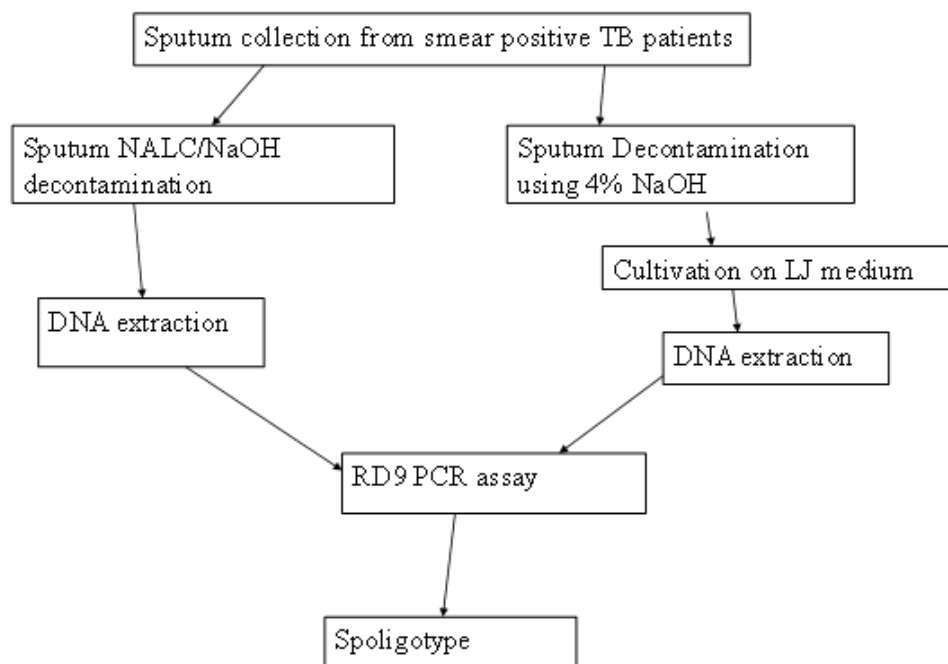
Figure 5.1: Sputum collection, decontamination, PCR and spoligotype assay

5.2.2. Reagents for purification of DNA

Tris-HCl = 20µM

   EDTA = 2 µM

   Triton = 1.2%

Buffers AW1 and AL = guandine hydrochloride

   Ethanol = 100%

   Water =RNase free

Lysozyme solution = 100 µg/ml in TE buffer

TE buffer = 20µM Tris-HCl, 2 µMEDTA, 1.2% (w/v) Triton

5.2.3. Reagents and primers used for RD9 assay

1. Control mycobacteria strains: *Mtb* H37Rv (ATCC25618), *Mycobacterium bovis* Af2122197 (ATCC BAA-935) and nuclease free water (Qiagen) was used as positive and negative controls respectively.

2. Hot Start Tag Master Mix Kit (Qiagen; Prod No 203445). This mix includes DNA polymerase, buffer, $MgCl_2$, and dNTPs.

3. Stock solutions of Oligonucleotide primers.

    3.1. 100µM RD9-Flank FW—5'-AAC ACG GTC ACG TTG TCG TG-3'

    3.2. 100µM RD9-Flank Rev—5'-CAA ACC AGC AGC TGT CGT TG-3'

    3.3. 100µM RD9-Internal Rev—5'-TTG CTT CCC CGG TTC GTC TG-3'

5.2.4. Reagents used for gel electrophoresis (RD9 deletion typing)

    1. DNA Ladder---1 Kilo base

    2. Loading dye---Blue/Orange 6x loading dye, promega.

    3. 1.5% agarose

    4. Ethidium bromide

    5. 1x TAE (Tris-Chloride) buffer

5.2.5. Reagents used for Spoligotype

5.2.5.1. Buffer solutions

    1. 10% Sodium dodecylesulphate (SDS)

    2. 0.5 M Ethylene diamine tetra acetic acid (EDTA)

    3. 20xSSPE (200mM $Na_2HPO_4x2H_2O$ + 10 mM EDTA $x2H_2O$)

All three buffers were stored at room temperature for a maximum of 6 months from the day prepared. The working buffers were prepared freshly on the day of use.

A. Primary buffer (2xSSPE + 0.1% SDS).

B. Secondary buffer (2xSSPE + 0.5% SDS)

C. 2XSSPE (1xSSPE is 0.18M NaCl, 10mM NaH_2PO_4, and 1mM EDTA, PH 7.4)

D. Stripping buffer (1% SDS)

E. 20 mM EDTA X $2H_2O$ PH 8.0

5.2.5.2. Preparation of Stock solutions

A. 10% Sodium dodecyl sulphate (SDS): SDS is a powerful detergent. In Spoligotyping, it is used in conjunction with SSPE to form the primary and secondary buffers, which eliminates contaminations or residues from the membrane during and after the hybridization, thus giving a clearer image when the autorad is developed. One hundred gram SDS was weighed using analytical balance and dissolved with in 900 milliliter distilled water. After ensuring that all SDS has gone into solution, additional distilled water was added up to 1000 ml.

B. 0.5% Ethylene diamine tetra acetic acid (EDTA): EDTA is used as a storage buffer for sorting membranes at 4^oc. The concentration of the EDTA stock solution was 0.5M while the concentration of EDTA used for storing membranes was 20mM. After each usage of a membrane, it was stored in freshly prepared EDTA buffer. At PH 8, it provides the optimum level of buffering to support the oligonucleotides to stay bound to the Biodyne C membrane. Using a fume cupboard, 186.1 g of EDTA was weighed and dissolved into 800 ml distilled water. This was heated in a hotplate at a temperature of ~50^oC for 60 minutes and stirred with magnetic stirring bar. Carefully, pellets of sodium hydroxide were added until the EDTA goes into a clear solution. Slowly, more Sodium hydroxide pellets were added until the pH reaches 8.0, as measured by a calibrated pH meter. AT pH 8.0, distilled water was added to make the volume of the solution 1000 ml.

5.2.5.3. Working buffer solutions

A. Primary buffer: was made up of 2xSSPE/0.1% SDS. 250 ml primary buffer/membrane= 25 ml 20xSSPE + 22.5 ml distilled water + 2.5 ml 10% SDS equilibrate at 60°C.

B. Secondary buffer: was made up of 2xSSPE/ 0.5 % SDS. 1000 ml buffer/membrane = 100 ml 20xSSPE + 850 ml distilled water + 50 ml 10% SDS.

C.1% SDS: 500 ml striping buffer/membrane= 50 ml 10% SDS+450 ml distilled water equilibrated at 60°c.

D. 20mM EDTA: is used for washing and storage of the Spoligotyping membrane. 500 ml 20mM EDTA/membrane= 20ml of 0.5% EDTA PH 8.0 + 480 ml distilled water stored at room temperature.

5.2.6. Equipment used for Spoligotype

Two water baths, 42°C and 60°C, glass bottles, 0.5 and 1 liter; measuring cylinders; Weighing scale, heating plate and magnetic stirrer, stirring bar, pH meter.

5.2.7. The primers used for amplification of the spacer oligonucleotides (figure 5.2)

1. DRa: 5'-GGT TTT GGG TCT GAC GAC-3'
2. DRb: 5'-CCG AGA GGG GAC GGA AAC-3'

Note: DRa is biotinylated in the 5'-end.

1 ATAGAGGGTCGCCGGTTCTGGATCA	23 AGCATCGCTGATGCGGTCCAGCTCG
2 CCTCATAATTGGGCGACAGCTTTTG	24 CCGCCTGCTGGGTGAGACGTGCTCG
3 CCGTGCTTCCAGTGATCGCCTTCTA	25 GATCAGCGACCACCGCACCCTGTCA
4 ACGTCATACGCCGACCAATCATCAG	26 CTTCAGCACCACCATCATCCGGCGC
5 TTTTCTGACCACTTGTGCGGGATTA	27 GGATTCGTGATCTCTTCCCGCGGAT
6 CGTCGTCATTTCCGGCTTCAATTTC	28 TGCCCCGGCGTTTAGCGATCACAAC
7 GAGGAGAGCGAGTACTCGGGGCTGC	29 AAATACAGGCTCCACGACACGACCA
8 CGTGAAACCGCCCCCAGCCTCGCCG	30 GGTTGCCCCGCGCCCTTTTCCAGCC
9 ACTCGGAATCCCATGTGCTGACAGC	31 TCAGACAGGTTCGCGTCGATCAAGT
10 TCGACACCCGCTCTAGTTGACTTCC	32 GACCAAATAGGTATCGGCGTGTTCA
11 GTGAGCAACGGCGGCGGCAACCTGG	33 GACATGACGGCGGTGCCGCACTTGA
12 ATATCTGCTGCCCGCCCGGGGAGAT	34 AAGTCACCTCGCCCACACCGTCGAA
13 GACCATCATTGCCATTCCCTCTCCC	35 TCCGTACGCTCGAAACGCTTCCAAC
14 GGTGTGATGCGGATGGTCGGCTCGG	36 CGAAATCCAGCACCACATCCGCAGC
15 CTTGAATAACGCGCAGTGAATTTCG	37 CGCGAACTCGTCCACAGTCCCCCTT
16 CGAGTTCCCGTCAGCGTCGTAAATC	38 CGTGGATGGCGGATGCGTTGTGCGC
17 GCGCCGGCCCGCGCGGATGACTCCG	39 GACGATGGCCAGTAAATCGGCGTGG
18 CATGGACCCGGGCGAGCTGCAGATG	40 CGCCATCTGTGCCTCATACAGGTCC
19 TAACTGGCTTGGCGCTGATCCTGGT	41 GGAGCTTTCCGGCTTCTATCAGGTA
20 TTGACCTCGCCAGGAGAGAAGATCA	42 ATGGTGGGACATGGACGAGCGCGAC
21 TCGATGTCGATGTCCCAATCGTCGA	43 CGCAGAATCGCACCGGGTGCGGGAG
22 ACCGCAGACGGCACGATTGAGACAA	

Figure 5.2: The 43 spacer-sequences selected for spoligotyping

Each spacer is synthesized with a 5'-terminal amino group (5' amino modifier C6) (Molhuizen et al, 1998). *Mtb* has about 100 spacer sequences but only 43 of them showed sequence variability among different *Mtb* spoligotype.

Table 5.1: Sample orientation on a 96-well Micro-titter plate

Sample orientation on a 96-well micro-titter plate for spoligotyping: O= Unknown samples; X= Empty well; H37Rv=*Mtb* H37Rv; AF2122/97= *Mycobacterium bovis* AF2122/97.

	1	2	3	4	5	6	7	8	9	10	11	12
A	H37Rv	dH₂O	AF2122/9	O	O	O	O	O	O	O	O	O
B	O	O	O	O	O	O	O	O	O	O	O	AF2122/9
C	O	O	O	O	O	O	O	O	O	O	O	O
D	O	O	O	O	O	O	H37Rv	X	X	X	X	X
E	H37Rv	dH₂O	AF2122/9	O	O	O	O	O	O	O	O	O
F	O	O	O	O	O	O	O	O	O	O	O	AF2122/9
G	O	O	O	O	O	O	O	O	O	O	O	O
H	O	O	O	O	O	O	H37Rv	X	X	X	X	X

5.2.8. Purification of DNA

Sputum samples were collected from smear positive TB patients as described in chapter 3 and 4. Following decontamination, seventy percent of each preparation was directly kept at -20°c for LB examination and 30% of it used for DNA assay. For DNA analysis, the pellet was re-centrifuged at 7558xg for 2 minutes, supernatant removed and the pellet re-suspended in 90µl sterile distilled water. This was boiled at 100°C for 30 minutes to inactivate *Mtb* and stored at -20°C.

Lysozyme stock was prepared by dissolving 100 mg lysozyme in 1 ml Tris buffer (20mMTris-HCl pH 8.0, 2 mM EDTA and 1.2% Triton). Nine hundred-sixty µl of the stock was re-diluted with 1440 µl of Qiagen protease to prepare 20 mg/ml lysozyme working solution. To each 90 µl of sample, 90 µl of lysozyme working solution was deposited in a 1.5 ml tube and vortex mixed. The preparation was transferred to a 2 ml safe-lock micro-centrifuge tube. QIAamp Mini spin column and 1.5 ml collection tube were positioned on a rotor adapter. Buffers AW1 and AL (containing guanidine hydrochloride), 100% ethanol, and RNase free water were placed in a reagent bottle rack. Two hundred seventy-nine µl of proteinase K solution was deposited in a 1.5 ml tube to run 12 samples. Samples and reagents were placed into QIAamp DNA Mini cube for automated DNA purification and DNA was purified at an elution volume of 100 µl.

5.2.9. Preparation of unknown samples for RD9 assay

Sputum samples decontamination and DNA purification protocols are documented in the previous sections. DNA samples were lyophilized at University of Leicester in a vacuum dryer at 55°C for 1 hour to enhance preservation during transportation to AHRI, Ethiopia. The lyophilized DNA samples were investigated at the Armauer Hansen Research Institute (AHRI), Addis Ababa, Ethiopia for RD9 deletion typing and Spoligotype. Lyophilized DNA samples were re-constituted with RNase free water, vortex mixed and subjected to PCR.

5.2.10. Preparation of Master Mix and set up of PCR amplification for RD9 assay

The master Mix was prepared on the day of use by mixing water, HotStat Master Mix, and primers in a sterile eppindroff tube (Table 5.2).

Table 5.2: Preparation of master mix for RD9 assay

Corresponding volumes of water, master mix and RD9 primers used to run PCR for RD9 deletion typing.

PCR MIX	Number of Samples		
	10	20	30
7.1µl H$_2$O (Qiagen)	71	142	213
10 µl Master Mix	100	200	300
0.3 µl RD9 Flank-Rev 100 µM	3	6	9
0.3 µl RD9 Flank-FW 100 µM	3	6	9
0.3 µl RD9 Internal-Rev 100 µM	3	6	9
18 µl / tube + 2 µl DNA template	---	---	---
20 µl total reaction volume	---	---	---

In a safety cabinet, 18µl of the PCR mix were aliquot into a PCR tubes and 2µl of the DNA template added to the respective tubes. The tubes were placed in the PCR thermocycler, (Multiplex PCR T3000 thermocycler Biometra, Thistle Scientific) and run with the corresponding PCR program (Table 5.3).

Table 5.3: PCR thermocycler program for RD9 assay

Multiplex PCR thermocycler program used to run RD9 deletion assay.

Termocycle

95°c	15 minutes
95°c	1 minute
55°c	0.5 minute
72°c	1 minute
72°c	10 minute
4°c	10 minute

→ } 35 cycles

5.2.11. Agarose gel electrophoresis

Agarose gel was prepared by weighing out the corresponding amount of agarose to make 1.5% gel. Using an Erlenmeyer flask 1.5 gram agarose was mixed with 100 ml of 1% TAE buffer. To prepare 50% Tris-Chloride buffer, the TAE stock was prepared by dissolving 242 gram Tris base to 750 ml deionized water. Carefully, 57.1 ml of glacial acetic acid was added and then 100 ml of 0.5M EDTA. Finally, the volume was adjusted to 1 liter. One percent TAE buffer (running buffer) was prepared by dissolving 1 ml of 50% TAE in 49 ml of distilled water.

The agarose was heated in a microwave oven for 1 minute and 3µl ethidium- bromide was added to make the final volume of ethidium- bromide concentration 0.3µg/ml. The agarose was allowed to cool to 50-60°C before casting the gel. After assembling the appropriate comb in the gel electrophoresis apparatus, the gel was allowed to polymerize for 30 minutes. The gel was placed in the electrophoresis tank and covered with 1% TAE buffer. To each PCR product 5 µl loading dye was added and mixed thoroughly. Eight micro liter of DNA ladder was deposited to the first well of the gel followed by adding 10 µl loading dye-PCR mix and 10 µl to other wells respectively. The preparation was electrophoresed at 110 volt for 40 minutes. The gel was removed from the tank and placed in EPI Chemi II Dark room, UVP laboratory products and image taken using a 12 bit colored imaging camera and saved in Microsoft Power point.

Quality controls: The positive controls, *Mtb* and *M. bovis* should always give PCR product while the negative control (water) should not generate any product. In case of one or several of the control samples failing, the analysis of the unknown samples was repeated. The PCR result for each sample was judged by comparing with the DNA ladder and the positive controls. A PCR product having a size of 396 bp (RD9- FlankFW + RD9-Internal Rev), was interpreted as that RD9 is present (i.e, *M. tuberculosis*). RD9 (Flank Rev will not contribute to any amplification since the elongation time in the PCR setup is too short. On the other hand, if RD9 is deleted (i.e, *M. africanum* and *M. bovis*), the PCR product will demonstrate a size of 575 bp (RD9-Flank FW +RD9-Flank Rev) and RD9 Internal Rev is redundent.

5.2.12. Spoligotyping of Mycobacterial isolates

5.2.12.1. PCR Amplification of spacers (Performed normally at day 1)

The general procedure and reaction volumes that were used for spoligotype are documented in table 5.4.

Table 5.4: Reaction mixture for PCR during spoligotyping

The water, primers DRa and DRb, and the Quiagen master mix were mixed in a sterile eppindroff tube.

Sample Number	1X	10X	30X	46X	50X	92X
Sterile HPLC water	3.5µl	35 µl	105 µl	161 µl	175 µl	322 µl
Primer 1 DRa	2 µl	20 µl	60 µl	92 µl	100 µl	184 µl
Primer 2 DRb	2 µl	20 µl	60 µl	92 µl	100 µl	184 µl
Qiagenmastermix	12.5µl	215µl	375 µl	575µl	625 µl	1l50µl
DNA template: 5µl of sample						

Twenty micro-liters of the PCR mix was dispensed into each well of a 0.2ml thin walled Plate. DNA samples were vortexes shortly and spin for 30 seconds. Five micro-liters of each DNA sample and the controls (*Mtb* H37 Rv, Distilled water and *M. bovis* AF21222/97) were transferred to the respective well and placed on the thermocycler by selecting the correct PCR program (Table 5.5).

Table 5.5: PCR thermocycler program

Thermocycler programs for a multiplex PCR assay on mycobacterial DNA extracted from sputum samples.

Thermocycler

95°c	15 minutes
95°c	1 minute
55°c	0.5 minute → 35 cycles
72°c	1 minute
72°c	10 minutes
4°c	Soak

5.2.12.2. Hybridization procedures (performed normally day 2)

Initially, the primary, secondary, and stripping buffers were prepared in flasks and allowed to equilibrate to correct temperatures in water bath (42^oC and 60^oC). The stripping buffer was placed at 60^oC. Thirty ml of primary buffer was kept in a diluting tray at room temperature. 2xSSPE and 20mM EDTA were also kept at room temperature. 150µl of the primary buffer was dispensed in to a new 96-well plate. To this, 20 µl of PCR product was added. The plate was tightly closed and placed on a thermocycler for 10 minutes at 96^oC. The membranes, which were placed in 20mM EDTA were washed in 250 ml pre-warmed primary buffer for 10 minutes on a shaking platform in the 60^oC hybridization oven. The miniblotter washed with detergent (Decon) and rinsed thoroughly and dried with paper towel. The support cushion was placed on the smooth bottom plate of the miniblotter and the membrane face down on the grooved plate (figure 5.3).
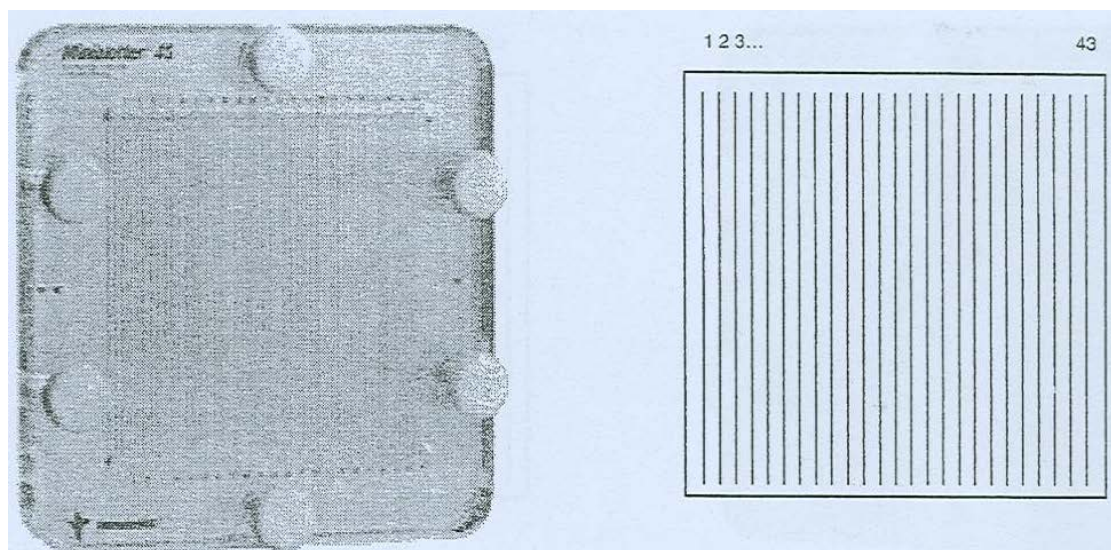
Figure 5.3: the structure of a Mini-blotter

Mini-blotter 45 (Immunetics INC., USA) and a Schematic layout of a typical spoligomembrane with 43 covalently attached spacers (Immage adapted from SOP AHRI by Berg, 2007).

The denatured PCR samples were removed from the thermocycler and placed on ice. One hundred fifty µl of primary buffer were added to lanes 1 and 45 of the miniblotter. One hundred fifty µl of the denatured samples were added to the other lanes of the miniblotter in the order they appear in the 96-well plate. This was hybridized for 60 minute at $60^{o}C$ on a horizontal surface with no shaking. The samples were removed from the miniblotter by aspiration and then the miniblotter was disassembled followed by transferring the membrane to a thermo-resistant container (~20x20cm). The membrane was washed twice with 250 ml secondary buffer at $60^{o}C$ for 10 minutes on a shaking platform. The miniblotter was cleaned with detergent and stored at room temperature in a water container until next usage. The membrane was rolled and transferred to a rolling bottle containing distilled water and allowed cooling. The water was discarded and assured whether the membrane was nicely rolled along the glass in the rolling bottle. 2.5µl of streptavidin-peroxidase conjugate (500 unit/ml) was added to 10 ml of secondary buffer. The streptavidin-peroxidase solution was tipped into the rolling bottle and the lid was placed securely, incubated at $42^{o}C$ for 60 minutes in the hybridization oven. The membrane was removed from the rolling bottle and placed in a plastic container, washed twice with 250 ml of secondary buffer for 10 minutes at $42^{o}C$ on the shaking platform in the oven. The membrane was rinsed twice with 250 ml of 2xSSPE for 5 minute at room temperature. The ECL detection fluid was prepared by adding 10 ml of "solution 1" with 10 ml of "solution 2". The membrane was removed from the 2xSSPE and placed in a specific container marked ECL, and then the ECL solution was added to the membrane. The membrane was immersed two times to ensure that the membrane is completely covered in the solution. In the dark room, the membrane was placed on a covered autorad and wrapped in a clean cling film. In the dark room, the wrapped membrane was placed facing up in an autorad "hypercassette blue' and a new ECL film were positioned on top. The cassette was closed, wrapped in a black sack, and leaved in a drawer to expose for 20 minutes.

5.2.12.3. Developing the autorad

After assuring that the temperature of the developer and fix is approximately 22°C, 100 ml of the film developer, water, and fixer was poured into three separate developing trays (20x25cm); developer in the left, water in the middle, and fixer in the right tray. The infrared safety light was turned on; the autorad was removed from the cassette, and placed in the developer ensuring that it is entirely submerged. The autorad was tipped gently until an image of black squares was visible-approximately 2 minutes. The autorad was rinsed in distilled water for 10 seconds and transferred to the tray containing fixer, agitated gently while incubating for 2 minutes. The autorad was rinsed with water for 2 minutes and allowed to dry.

5.2.12.4. Stripping the membrane

Once satisfied with the autorad, it was necessary to strip the PCR product off the membrane. This was done after setting an oven at 80°C. The membrane was unwrapped and placed in a thermo-resistant plastic container and washed with 250 ml of warm stripping buffer (1% SDS) for 30 minutes at 80°C while shaking. After the second wash, it was left at room temperature for 5 minutes to cool before discarding the liquid, and then washed for another 5 minutes in 250 ml of 20mM EDTA. The liquid was discarded and another 250ml of 20mM EDTA added and stored in a fridge until the membrane was needed.

5.2.12.5. Interpretation of results

The orientation of the autorad was first read using the control samples. The autorad was turned so that the spacers of the first three controls can be read from the top and with spacer 1 to the left and spacer 43 to the right (figure 5.4). When the autorad looks patchy or dark shadowed, it was repeated.
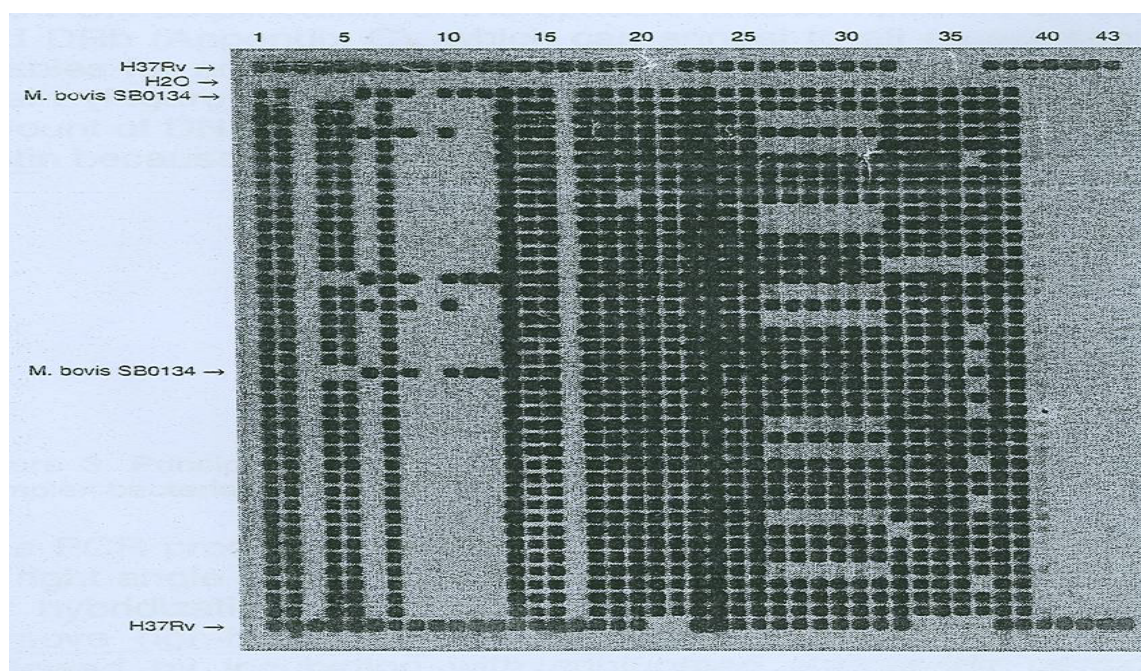
Figure 5.4: A successful autorad

The 43 spacers and the five control samples are marked in the figure. A squared spot indicates of present spacer (Immage adapted from SOP AHRI by Berg, 2007).

The Shared International type number (SIT) of each *Mtb* complex was determined from the SITVIT database, Institute Pasteur using SPolDB4 as entry means in to the international database and recorded in a spread sheet Microsoft excel 2007.The SPolDB4 database is one of the major publically accessible database of *Mtb* complex genetic polymorphism with a collective classification system of spoligotyping (Octal or arbitrary SIT number) as well as a worldwide epidemiological information system (Burdey et al, 2006).

The preparation of culture media, decontamination of sputum samples using the Petroff method, inoculation and incubation of sputum samples, isolation and DNA extraction from mycobacteria grown on culture are documented in chapter seven.

5.3. Results

5.3.1. Eligible versus included patients

A total of 204 DNA samples (116 extracted directly from sputum and 88 from culture) were investigated. Ninty-four DNA samples extracted from sputum demonstrated RD9 intact PCR results. A total of 168 DNA extractes (94 from sputum and 73 from culture) resulted in a successful spoligotype.

5.3.2. RD9 deletion typing on mycobacterial DNA extracted from sputum

All the 116 lyopilized tubercle bacilli DNA samples were subjected to RD9 deletion typing. The result showed that 94 samples were with intact RD9 and the other 21 RD9 deleted (No PCR signal). Previously, when the result of the acid-fast stain (AFS), Zeihl-Neelsen (ZN) stain, delivered by the hospital laboratory in Gondar was compared with the result of another AFS after the sputum samples decontaminated with NALC/NaOH, 13 samples were negative for AFB. The result of RD9 PCR assay on these samples showed that 10 of them were RD9 intact and the other 3 an RD9 deleted (no PCR signal).

The RD9 assay amplification intensity varied from sample to sample (figure 5.6) having the same AFB score. The AFB score of all the mycobacterial DNA PCR product in figure 5.6 were 4+ except lane 1 with a 2+ AFB score.
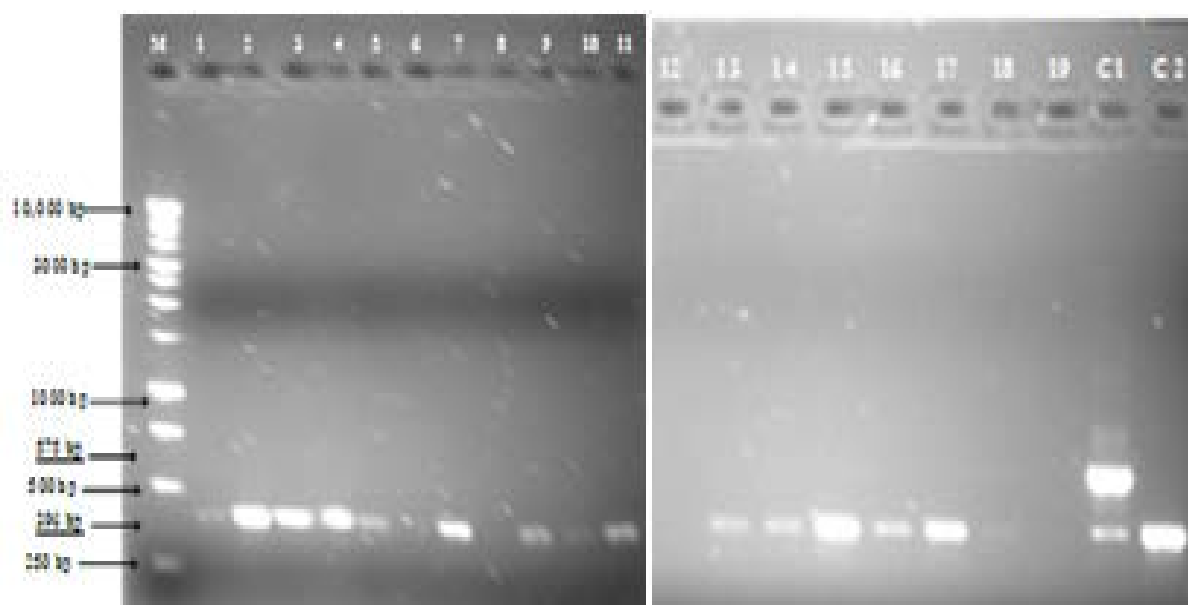
Figure 5.5:RD9 deletion typing

Electrophoresis result (RD9 deletion typing) of some of the mycobacterial DNA extracts obtained directly from sputum. Deletion typing is designed as a multiplex PCR using three primers RD9-flank FW and RD9-InternalRev should amplify a PCR product whil RD9-FlankRev will not contribute to any amplification. RD9 (with a size of about 2.5kb) is deleted in *M.africanum* and *M.bovis* but intact in *Mtb*. *Mtb* is a 396bp bacterium where as *M.bovis* is 575 bp (Berg, 2008). Right image: Lane 19=RNase free water; Lane-C1=*Mycobacterium bovis* AF2122/97; Lane-C2=*Mtb* H37Rv.

5.3.4. The Spoligotye pattern of *Mycobacterium* DNA extracted from sputum and culture

Of 94 *Mycobacterium* DNA extracts obtained from sputum that had RD9 intact PCR result, 72 demonstrated *Mtb* spoligotypes with different Shared International type numbers (SITs). On the other hand, 74 of 88 mycobacterial isolates taken from LJ medium also demonstrated sucessful *Mtb* spoligotypes patterns (figure 5.6 and 5.7). Overall, a total of 53 different spoligotype patterns of *Mtb* were observed among the DNA extracts collected from sputum and culture. The Central Asian Strain (CAS) *Mtb* lineage (CAS) (48.5%; 81/167) and the Euro-American lineage (EAL) (46.7%; 78/167) *Mtb* spoligotype were found the dominant isolates. The spoligotype pattern of one isolate showed W/Bejing (SIT1) pattern. Among the spoligotype pattern of the EAL isolates, the SIT149 and SIT 53

accounted for 19.2% (15/78) and 20.5% (16/78) respectively. The CAS spoligotype pattern showed that the SIT25 (22/81) and SIT289 (17/81) were found dominant (27.2%; 21/81) (Table 5.7).

Table 5.7: The spoligotype pattern of *Mtb* isolates

The distribution of spoligotype pattern of *Mtb* DNA extracts obtained from sputum and culture among pulmonary tuberculosis patients in Gondar.

SIT number	DNA extracted from sputum	DNA extracted from culture	Total
SIT 25	22	-----	22
SIT 26	3	3	6
SIT 289	---	17	17
SIT 53	11	5	16
SIT 149	11	4	15
SIT 118	3	3	6
SIT 50	2	3	5
SIT 21	2	3	5
SIT 523	3	--	3
SIT new	15	10	25
SBO 133	5	---	5
Others	17	26	42
Total	93	74	167

In this study, 25 new *Mtb* spoligotypes (15 isolated directly from sputum that also demonstrated RD9 intact and the other 10 from culture) were identified. The spoligotype patterns of these new *Mtb* spoligotypes were different from each other. Fifteen of the new spoligotypes were clustered within the CAS, 7 within the Euro-American and the other 3 unique to the SPolDB4 database (figure 5.8).

Figure 5.6: The spoligotype patterns of *Mtb* obtained directly from sputum

Mtb DNA was extracted from sputum samples directly using QIAamp DNA Mini cube. The spoligotype colur simply differentiates one type of spoligotype from the other.
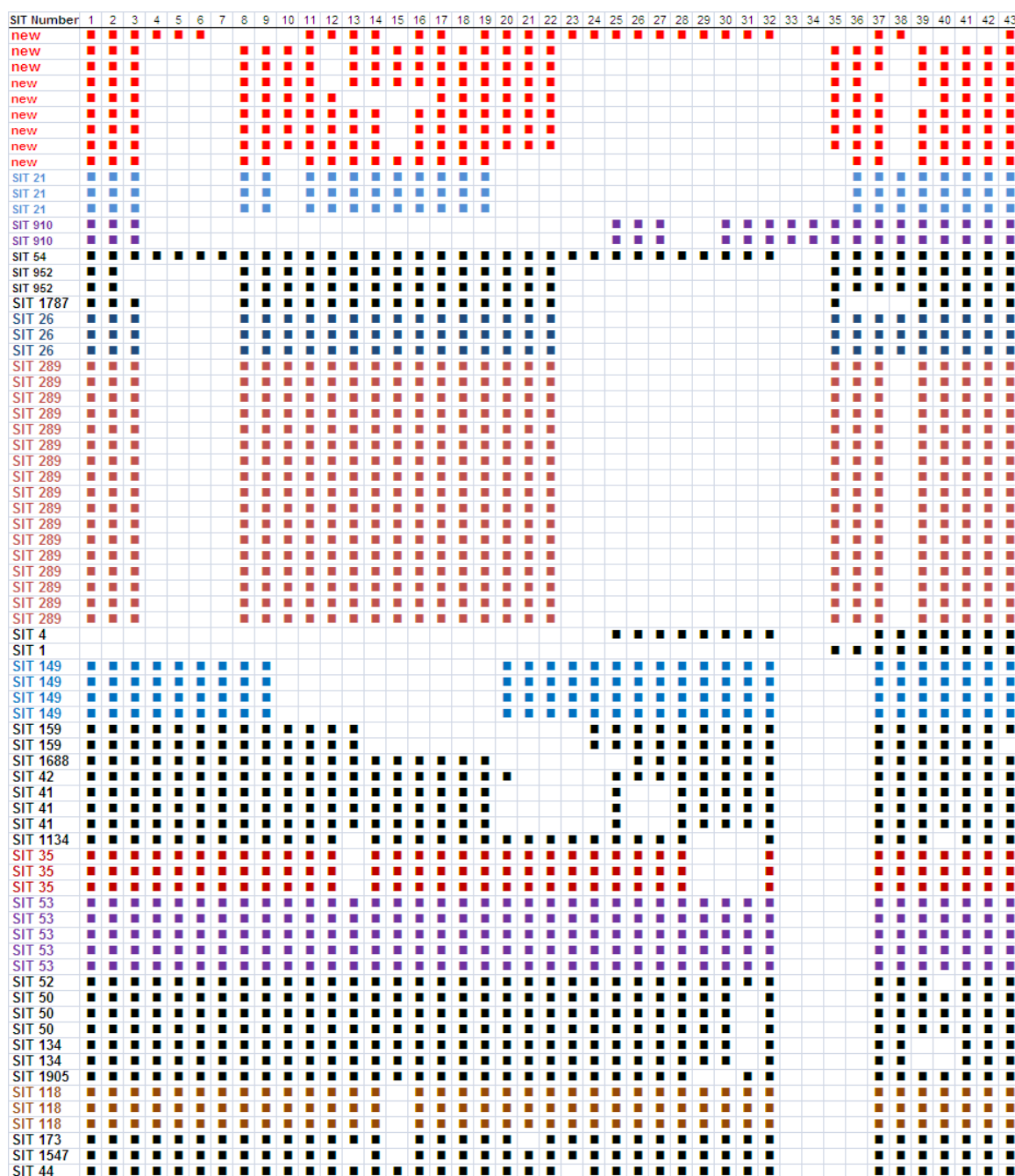
Figure 5.7: The spoligotype patterns of *Mtb* obtained from culture

Smear positive sputum samples were cultured and DNA extracted from the culture as documented previously in the method sections.The spoligotype colur simply differentiates one type of spoligotype from the other.
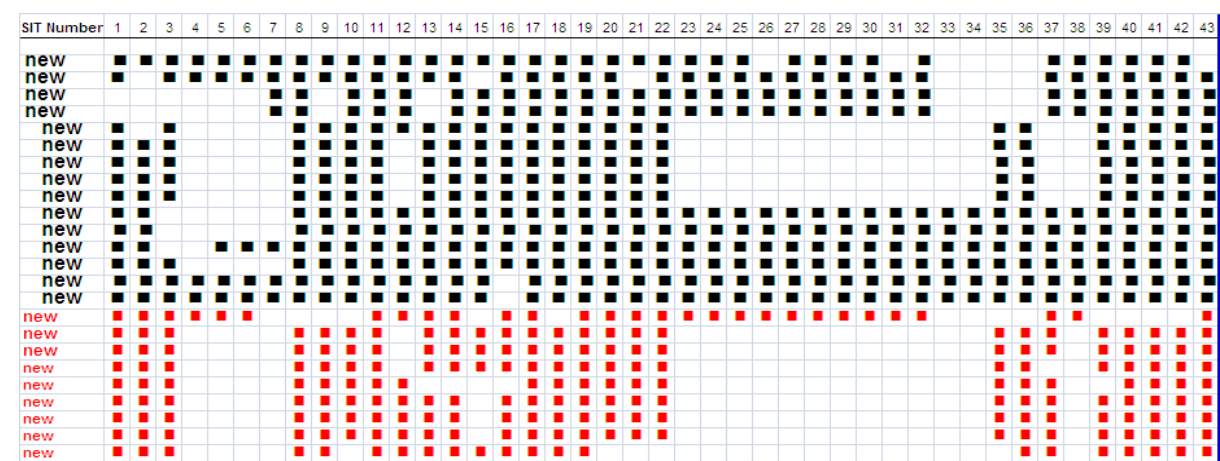
Figure 5.8: The spoligotype patterns of the new *Mtb* isolates

This figure demonstrates the new *Mtb* spoligotypes isolated from pulmonary TB patients in Gondar health institutions (Black=DNA from sputum; Red=DNA from culture).

The spoligotype pattern of 7 *Mycobacterium* DNA extracts obtained directly from sputum showed that five of them were positive for *Mycobacterium bovis*SBO "133" (figure 5.9). However, the RD9 deletion type result for all the seven demonstrated an intact RD9 which is very unusual and the spoligotype pattern in this case may be unreliable. Morover, there was no *M.bovis* isolate observed from cultured sputum samples.
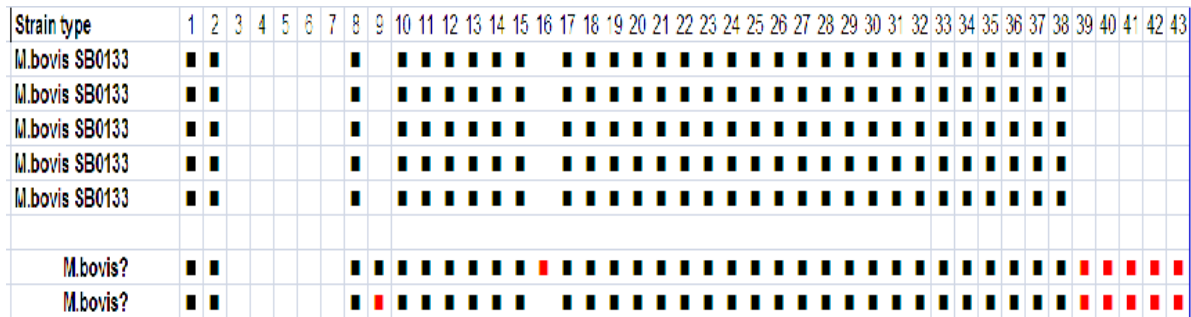
Figure 5.9: Spoligotype that bear a resemblance to *M.bovis*

The spoligotype pattern of mycobacterial DNA extracts demonstrating *Mycobacterium bovis*SBO133spoligotype. All the DNA were extracted directly from sputum and all demonstrated RD9 intact PCR result which is considered very anomalous.

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5.3.5. The Spoligotype profile of *Mtb* among HIV[+ve] and HIV[-ve]/TB Patients

Among 87 smear and culture positive new pulmonary TB patients, 74 resulted in a successful spoligotype pattern. A comparative spoligotype analysis of *Mtb* isolates on 32 HIV[+ve] and 42 HIV[-ve] patients was made. Among HIV[+ve]/TB patients, spoligotyping produced a total of 17 different patterns. In HIV[+ve] and HIV[-ve]/TB patients, 10 spoligotype patterns were new strains that were unique among all patterns in the SITVIT database. Six out of 10 new *Mtb* spoligotypes were found among HIV[+ve]/TB patients. The spoligotyping pattern of the HIV[-ve] patient subgroup resulted in 17 different patterns: 4 corresponding to the new *Mtb* spoligotype (table 5.7). Eight (8/32: 25%) different types of *Mtb* spoligotypes found in HIV[+ve] patients were not observed among HIV[-ve]/TB patients. In HIV[+ve] and HIV[-ve]/TB patients, the dominanant *Mtb* spoligotype was the SIT 289 (CAS) (8/32; 25% and 21.4%; 9/42 respectively).

Table 5.7: Spoligotype patterns of *Mtb* among HIV[+ve] and HIV[-ve]/TB patients

In HIV[+ve] and HIV[-ve]/TB patients, the SIT 289 (CAS) *Mtb* spoligotype was found dominant followed by SIT new and SIT 53 Spoligotypes.

Spoligotype	HIV[+ve]	HIV[-ve]	Total
SIT 53	3	2	5
SIT 289	8	9	17
SIT New	6	4	10
SIT 21	2	1	3
SIT 50	1	2	3
SIT 134	1	1	2
SIT 118	---	3	3
SIT 35	1	2	3
SIT 41	1	2	3
SIT 159	--	2	2
SIT 149	1	3	4
SIT 26	--	3	3
SIT 962	--	2	2
SIT 910	--	2	2
Others	8	4	12
Total	32	42	74

5.3.6. Spoligotype pattern, FeNO concentration and the proportion of LB[+ve] tubercle bacilliin sputum

The spoligotype pattern of *Mtb* isolates in the current study lay down on the CAS and EAL. The average/median FeNO concentration among TB patients infected with the CAS and EAL were 15.9/10.50 ppb and 14.12/ 12.00 ppb respectively. The average/median FeNO concentrations among TB patients infected with *Mtb* spoligotype SIT 50 was relatively higher than others (28.10/28.0ppb) followed by SIT 149 (19/16.5 ppb) and SIT 25 (17.4/16.0 ppb) (figure 5.11). On the other hand, TB patients infected with the new *Mtb* Spoligotypes (SIT new) and the *Mycobacterium bovis* SBO 133 bearing Spoligotype demonstrated an average/median FeNO concentration of 17.2/14.5 ppb and 17.81 ppb respectively. However, when data was analyzed by one way ANOVA (non parametric), there was no significant difference in FeNO concentration among patients infected with different *Mtb* spoligotypes.
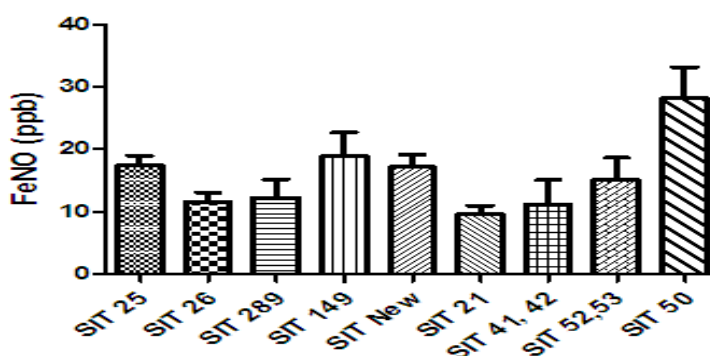
Figure 5.10: FeNO concentration among TB patients infected with different *Mtb* spoligotype

FeNO concentration of pulmonary TB patients was compared with the spoligotype patterns of the *Mtb* that caused the TB disease, but there was no significant difference in FeNO concentration observed among TB patients infected by different *Mtb* spoligotype.

The corresponding mean/median proportion of LB[+ve] tubercle bacilli among TB patients infected with the CAS and EAL spoligotype were 29/24.5% and 31.83/30% respectively. The average/median proportions of LB[+ve] tubercle bacilli among *Mtb* Spoligotype SIT 50,

SIT 149 and SIT 25 were 40.5/41.0%, 28.5/22.0%, and 29.0/20.0% respectively (figure 5.11). However, when data was investigated by one-way ANOVA, there was no significant difference on the proportion of LB^{+ve}tubercle bacilli among the different *Mtb* spoligotypes.
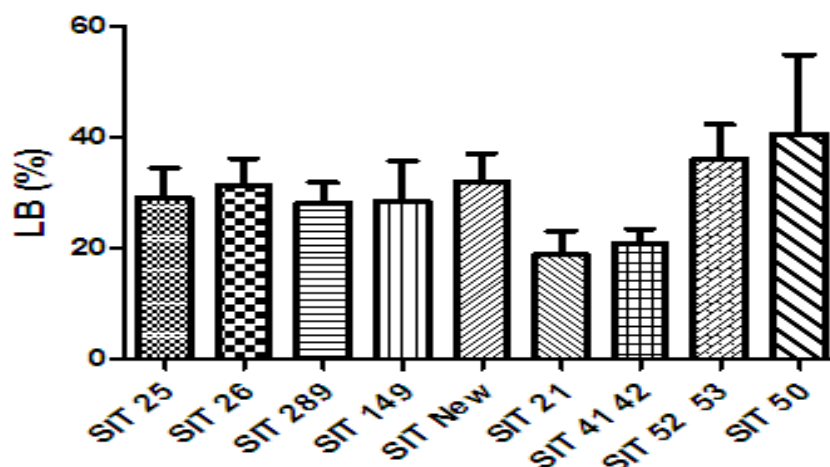
Figure 5.11: The proportion of LB^{+ve}tubercle bacilli among TB patients infected with different *Mtb* spoligotypes

The proportion of LB^{+ve} tubercle bacilli among pulmonary TB patients was compared with the spoligotype patterns of the *Mtb* that caused theTB disease. However; there was no significant difference in the proportion of LB^{+ve} tubercle bacilli among TB patients infected by different *Mtb* spoligotypes.

5.3.7. Association between FeNO concentration with the proportion of LB^{+ve} tubercle bacilli among TB patients infected with different *Mtb* Spoligotypes

The association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli among TB patients infected with different *Mtb* spoligotypes was determined. Linear regression analysis showed that FeNO concentration was weakly but significantly associated with the proportion of LB^{+ve} tubercle bacilli among TB patients infected with the CAS spoligotype (P=0.008; r2=0.14) but not among TB patients infected with the EAL spoligotype (figure 5.12)..

(A)   (B)
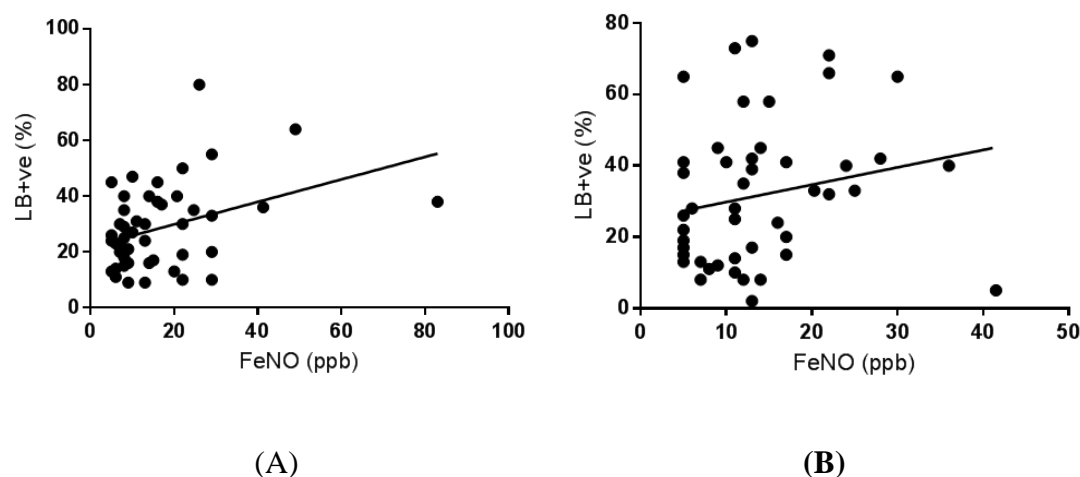
Figure 5.12: FeNO concentration was significantly associated with the proportion of LB^{+ve} tubercle bacilli among TB patients infected with the CAS spoligotype

Association bewteen FeNO concentration with the proportion of LB^{+ve} tubercle bacilli among TB patients infected with the CAS and EAL spoligotype was determined by linear regression analysis. FeNO concentration was weak but significantly associated with the proportion of LB^{+ve} tubercle bacilli among TB patients infected with the CAS spoligotype (p=0.008; r^2=0.14) but not among patients infected with the EAL.

The association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli among TB patients infected with specific *Mtb* spoligotype within the CAS and EAL was also determined. Accordingly, FeNO concentration was significantly associated with the proportion of LB^{+ve} tubercle bacilli among TB patients infected with the SIT 25, 26 and 289 *Mtb* spoligotypes: all members of the CAS (n=32) (P= 0.0145; r2=0.19) (Figure 5.13A). On the other hand, a relatively high proportion of LB^{+ve}tubercle bacilli were observed among patients infected with SIT New *Mtb* spoligotype (an average/median of 32.0/31.0 %). However, there was no significant association between FeNO concentrations with the proportion of LB^{+ve}tubercle bacilli among patients infected with SIT New *Mtb* spoligotype. In addition, there was no significant association between FeNO concentration and the proportion of LB^{+ve} tubercle bacilli among TB patients infected with *Mtb* spoligotype SIT 149 (T3-Ethiopia), SIT 21, SIT (42, 43) (LAM), SIT (51, 53) (T family) and SIT 50 (H3) (figure 5.13 C, D, E, F and G). In this study, *Mtb* W/ Beijing spoligotype

(SIT 1) was isolated from one patient. FeNO concentration on this particular patient was 11ppb and the corresponding proportion of LB[+ve] tubercle bacilli was 27%.

(A)   (B)   (C)   (D)

(E)   (F)   (G)
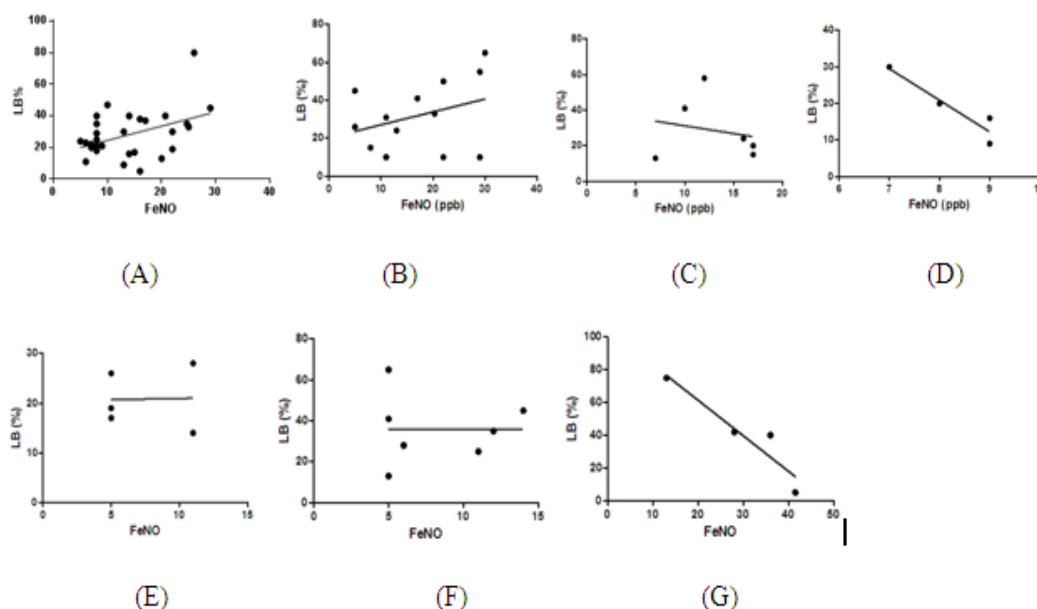
Figure 5.13: Association between FeNO concentration with the proportion of LB[+ve] tubercle bacilli among TB patients infected with different *Mtb* Spoligotype

FeNO concentration was significantly associated with the proportion of LB[+ve] tubercle bacilli among TB patients infected with *Mtb* poligotype SIT 25,26,289 (CAS) (A) but not on others. A= CAS; B=SIT new; C= SIT 149, D=SIT 21; E= SIT 50.

5.3.8. Multivariate analysis

FeNO concentration and proportion of LB[+ve] tubercle bacilli data was not normally distributed. Data was transformed logarithmically and normal distribution checked by the QQ plot. Similar to the bivariate analysis result, multivariate linear regression analysis showed that FeNO concentration was weak but significantly associated with the proportion of LB[+ve] tubercle bacilli among TB patients infected with the CAS spoligotype (p=0.031; r2=0.108) but not among patients infected with the EAL spoligotype.

5.4. Discussion

The AFB score of 13 sputum samples was negative when the sputum was decontaminated by NALC/NaOH. Of these 13 samples, 10 showed RD9 intact results when the DNA extracts were subjected to multiplex PCR. Yajko et al (1995) reported the negative impact of NaOH decontamination on the survival of *Mtb*. The mean number of *Mtb* colony forming unit (CFU) recovered on Mitchison's agar medium after NALC/NaOH treatment was $0.96\log_{10}$ CFU less than the number recovered after treatment with NALC alone, corresponding an average recovery rate of 11% for NaOH treated samples. Therefore, we suspect that the AFB negative result after decontamination of sputum samples with NALC/NaOH decontaminant could be due to the harsh effect of the caustic soda especially among samples with low AFB score. Another possible reason for the AFB negative result could be the effect of freezing as sputum samples were collected daily and kept for some time at -20°C until smears were prepared and stained by ZN stain. Unstained smears were also kept at -20°C prior transported to Leicester for Fluorescence Microscopy. There are reports that documented freezing and repeated thawing brought loss of acid alcohol fastness in *Mtb* (Bell personal communication).

5.4.1. RD9 deletion typing on mycobacterial DNA extracts from sputum samples

In this study, 94 mycobacterial DNA extracts obtained directly from sputum showed an RD9 intact PCR result. The result indicates that the dominant mycobacterium causing pulmonary TB in Gondar was *Mtb*. RD9 deletion analysis discriminates *Mtb* from other *Mtb* Complex (Cadmus et al, 2009). Comparative genomics of the members of the *Mtb* complex (MBTC) identified 18 regions of differences (RDs) that were present in *Mtb* H37Rv and absent in most BCG derivatives and also in other members of the MTBC (Kato-Maeda et al, 2001). Moreover, Behr et al (1999) and Gordon et al (1999) reported that these genomic landmarks can be used as potential markers for understanding the historical origins and genealogy of the present day mycobacterial pathogens.

5.4.2. The *Mtb* spoligotype causing pulmonary tuberculosis in Gondar

In this study the CAS and EAL *Mtb* spoligotype were the domionant isolates. Within the CAS strain the SIT 25 and 289 were the dominant spoligotype causing pulmonary TB in Gondar. The CAS *Mtb* linage is defined by deletion of spacers 4-7 and 23-34 spacers. On the other hand, the EAL *Mtb* linage is defined by deletion of spacer 33, 34, 35 and 36. Previous to this work, Firdessa et al (2011) reported that the Euro-American and the CAS lineages of *Mtb* were predominant in Ethiopia which is very similar to the result of the current study. Within the EAL, SIT 53 and SIT 149 accounted for 9.6% and 8.4% respectively. The SIT 53, SIT 149, and SIT 50 clades are the T1 (supper family T1), T3-Ethiopia, and H3 (Haarlem 3) *Mtb* lineages (Hermans et al, 1995). The SIT 149 (T3-Ethiopia) was previously shown to be frequent in Ethiopia and Denmark among Ethiopian immigrants (Hermans et al, 1995).

Twenty-five of the *Mtb* spoligotypes, 15 clustered within the CAS, 7 in the EAL and 3 unassaigned were new which means they were not registered in the international TB database. The spacer deletion pattern on these *Mtb* spoligotypes varies from each other. Fiften of these spoligotypes demonstrated deletion on spacer 4-7 and 23-34. Spacer deletion patterns of 4-7 and 23-34 are common in the CAS. This may suggest that these new *Mtb* spoligotypes might emerge from the CAS through evolution. The spacer deletion patterns of the Gondar spoligotypes were distinct from the recently reported spoligotypes of the Woldiya lineage (Firdessa et al, 2011). Deletion of spacer 4 to 24 was reported on the *Mtb* isolates of the Woldiya lineage. The Woldiya lineage was identified that sits between the 'Ancient' and 'Modern' *Mtb* branches. The identification of new *Mtb* spoligotype in Gondar and Woldyia strengthens the existence of unidentified *Mtb* lineage that may be circulating in the Amhara National state in particular and in Ethiopia in general.

5.4.3. *Mtb* spoligotype patterns among HIV^{+ve} and HIV^{-ve}/TB patients

The spoligotype profile of *Mtb* strains among HIV^{+ve} and HIV^{-ve} TB patients was investigated. Accordingly, in HIV^{+ve}/TB patients (n=33) 19/33 and 14/33 isolates were within the CAS and EAL linage respectively. In HIV^{-ve}/TB patients, 20/39 and 19/39 *Mtb* isolates were with in the EAL and CAS linage respectively. However, there was no single dominant *Mtb* spoligotype exclusively found in HIV^{+ve} or HIV^{-ve}/TB patients. Nevertheless, there are reports that documented the existence of certain *Mtb* strains dominating in HIV^{+ve} patients. For example, the SIT 373 (T1 lineage) was reported highly prevalent among HIV^{+ve} patients but totally absent from HIV^{-ve} population in Nigeria (Cadmus et al, 2010).

5.4.4. Mycobacterial spoligotype, the concentration of FeNO and the proportion of LB^{+ve} tubercle bacilli

In this study, significant association between FeNO concentration and the proportion of LB^{+ve} tubercle bacilli was observed among TB patients infected with the CAS but not among patients infected with the EAL *Mtb* spoligotype. Earlier studies (Goren et al, 1974) demonstrated variability in lipid profiles among different strains of *Mtb*. *Mtb* synthesizes specific lipids that interact with host and are required for virulence (Jain et al, 2007). The relationship between phenotypic drug tolerance and accumulation of lipid bodies within the tubercle bacilli *in vitro* was mentioned in the previous sections. Moreover, the association between FeNO concentrations and the proportion of LB^{+ve} tubercle bacilli in sputum among patients infected with specific *Mtb* spoligotypes (CAS) but not in others (EAL) is novel. The accumulation of lipid bodies within specific *Mtb* spolygotypes could be a marker for different host parasite relationships. Tubercle bacilli exposed to growth conditions known to enhance LB formation were reported to be more infectious than those grown in conditions unfavourable to LB formation (Bacon et al, 2004 and Garton, personal communication). Therefore, the further infectiousness of particular *Mtb* spolygotype could possibly be related to LB accumulation. But, recent reports indicated that in a cohort study, comparison of IFN-γ responses with the spoligotype of the infecting clinical strains showed

that the "modern" *Mtb* strains like the Beijing and CAS tended to induce lower IFN-γ responses than "ancient" strains like the East African-Indian (EAI) lineage (Niania et al, 2010). On the other hand Reed et al (2007) reported that Beijing strains accumulate large quantities of triglycerides *in vitro* aerobic culture as a result of up regulation of tgs1 which is member of the DosR regulon. In macrophages infected with the east Asian/Beijing and Indo-Oceanic strains, an enhanced expression of TNF-α and IL-1β was observed (Krishnan et al, 2011). The cytokines IFN-γ, IL-1β and TNF-α relation with iNOS, a potentially induced enzyme during inflammatory response, were documented in the previous sections (chapter 3). The enhanced production of inflammatory cytokines by some *Mtb* spoligotypes and the association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli in sputum among patients infected with the CAS in our case might encompass to the significance of LBs in the pathogenesis and transmission of TB caused by specific *Mtb* spoligotype. Therefore, we suggest that further studies are required to confirm the relationship between *Mtb* spoligotype and the corresponding alliance of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli.

The proportions of LB^{+ve} tubercle bacilli were relatively higher among TB patients infected with *Mtb* SIT 50 (H3), SIT 52 and 53 (T family) and SIT new strains. However, there was no significant association between FeNO concentration and the proportion of LB^{+ve}tubercle bacilli among TB patients infected with the SIT 50, SIT 149 and SIT new *Mtb* spoligotypes. The relatively high proportion of LB^{+ve}tubercle bacilli among these spoligotypes in the absence of an association with FeNO concentration may suggest other *in vivo* factors that may force the spolygotypes to accumulate LBs which possibly may vary from patient to patient.

The limitations of this study includes that RD9 deleted samples and spoligotype demonstrating *M.bovis* were not further investigated.

5.4.5. Conclusions

1. The Central Asian Strain and Euro-American variant *Mtb* spoligotypes were dominant in Gondar.

2. New *Mtb* spoligotypes different from the recently discovered Woldiya lineage were observed in Gondar.

2. There was no single *Mtb* spoligotype exclusively found either in HIV^{+ve} or HIV^{-ve}/TB patients

3. FeNO concentration was significantly associated with the proportion of LB^{+ve} tubercle bacilli among patients infected with the CAS *Mtb* spoligotype but not among TB patients infected with the EAL spoligotype.

CHAPTER SIX: Gene expression studies

6.1. Introduction

Reactive nitrogen intermediates (RNIs) and hypoxia are among the expected biologically important signals meet by *Mtb in vivo*. The tubercle bacilli perhaps take advantage of RNI and hypoxia as signals for the regulation of the expression of specific genes that promote survival in the host (Ohno et al, 2003). Immunohistochemical studies have demonstrated abundant NOS2 protein expression as well as in situ production of RNI in the pulmonic granulomas of *Mtb* in mice and humans (Scanga et al, 2000). Indeed, *Mtb* has evolved multiple mechanisms to evade the toxic effects of RNI (Nathan and Shiloh, 2000). NO was also reported to take part in the regulation of bacterial genes that might participate a part in RNI detoxification (Ohno et al, 2003). The expression of hmp gene which encodes a flavor-haemoglobin capable of detoxifying NO as a result of the contribution of RNI in *Escherichia coli* was previously reported (Cruz-Ramos et al, 2002). In *Mycobacteria*, RNI enhance the expression of *hspX* (Garbe et al, 1999), which encodes the 16 kDa α-crystallin necessary for growth of *Mtb* in macrophages (Yuan et al, 1998).

6.1.1. The Dormancy survival regulator (DosR) and *Mtb* dormancy

A number of studies have identified hypoxia as a potential stimulus for triggering the transition of *Mtb* to a non-replicating persistent state analogous to latency *in vivo* (Park et al, 2003). A gradual depletion of O_2 in *Mtb* culture caused the pathogen to reach a non-replicating persistent state that manifested drug sensitivity and structural changes suggestive of dormant state (Park et al, 2003). The DosR regulon encompasses most of the genes known to be strongly regulated by hypoxia (Yuan et al, 1998). Voskuil et al (2003) reported that tubercle bacilli exposed to low, nontoxic concentrations of nitric oxide *in vivo* enter a non-replicating persistent state marked by the induction of a 48-gene regulon under the control of the DosR, suggesting that the DosR regulon may mediate the transition of these bacilli into dormancy. A recent study presented evidence that 49 genes from *Mtb* H37Rv are DosR-dependant, 47 of which lie in nine clusters. Further evidence suggests that most of these DosR-dependent genes and some additional genes associated with the nine clusters are regulated in response to nitric oxide levels (Voskuil et al, 2004). Reactive nitrogen intermediates can serve the function of signaling molecules, there by regulating

the expression of genes that may contribute to the survival of the tubercle bacillus in the infected host (Ohno et al, 2003). As a result, characterization of the association between *Mtb* gene expression patterns and RNI may shed light on the mechanisms by which the tubercle bacillus persists in the host.

6.1.2: Aims and objectives

The objective of this study was to determine the association of *Mtb* gene expression patterns with FeNO concentration and the proportion of lipid body positive tubercle bacilli in sputum among HIV^{+ve} and HIV^{-ve}/TB patients.

Specific objectives

1)   To evaluate the the association of FeNO concentration with *Mtb* gene expression patterns of the TB patients.

2)   To determine the association of the DosR gene expression patterns with the proportion of LB^{+ve} tubercle bacilli in sputum

6.2. Materials and Methods

6.2.1. Reagent preparation

5M guanidium thiocyante solution for *Mtb* RNAstabilisation

5M Guanidiumthiocynate (GTC)

0.5% w/v N-lauryl sarcosine

25 mM trisodium citrate

0.5% w/v Tween 80

0.1M 2-mercaptoethanol

295.4 g GTC, 2.5g N-lauryl sarcosine and 2.5 g Tween 80 were suspended in 250 ml sterile distilled water and incubated at 37°C overnight, to allow for complete dissolution. Sterile distilled water was added to the preparation to make a total volume of 500 ml. Aliquots were prepared into 40 ml amounts, wrapped with aluminum foil and stored at room temperature in the dark. Immediately before use 7µl/ml of 2-mercaptoethanol was added to the 40ml aliquots.

6.2.2. Sputum sample collection and *Mtb* RNA stabilization

Sputum samples for this study were collected early in the morning. Soon after the patient's sputum AFB result was delivered to the outpatient department (OPD), patients were asked for previous history of anti- TB therapy and only new TB patients were recruited. The HIV results of the TB patients were taken from the Providing Initiative Testing and Counselling (PITC) clinic with the hospital director's office permission. Patients were asked for consent to participate in the study and voluntary patients were appointed for another day early in the morning to deliver sputum samples. Patients were requested to expectorate sputum samples into a screw-capped sputum cup for 5 minutes. Immediately, approximately 4 volumes of 5M Guanidiumthiocynate (GTC) solution were decanted into the cup for 1 volume of sputum sample and were gently mixed.

6.2.3. Storage of RNA stabilized sputum samples

The combined sputum samples and GTC solution were further homogenized by drawing the solution with a syringe and dispensing back into the container for about 4 times until the sample-GTC mixture became less viscous. The sample-GTC mix was transferred to 15 ml universal tube and centrifuged at 1761xg for 30 minutes (International equipment company, IEC, Needham Heights, NA 02194, USA, 1998). Carefully the supernatant (~2 ml) was removed from the sediment. The pellet was re-suspended with the remaining 2 ml GTC solution and transferred to a screw capped 2 ml tube. Both the GTC-stabilized and backup sputum samples were stored at -20°C. Frozen GTC-stabilized RNA samples were transported to Leicester with dry ice and stored at -80°C until they were investigated.

6.2.4. RNA extraction from GTC stabilized sputum samples and Reverse transcription of RNA to form cDNA

The GTC-stabilized sputum suspensions were transferred to a 2 ml screw capped tubes and centrifuged at 7558xg for 3 minutes and supernatant was removed. One ml of Trizol LS reagent was added to the pellet followed by the addition of the contents of one tube of Lysing matrix B (MPBio) to each preparation. In a class I safety cabinet, tubes were transferred into a Fastprep-24 and processed for 45 seconds at speed of 6.5 m/s. After allowing the tubes to stand for 10 minutes at room temperature, 200µl chloroform and 200µl RNase free water were added, vortex mixed for 30s and centrifuged at 7558xg for 15 minutes to separate the different phases. The aqueous phase (~ 600µl) was carefully transferred to a fresh centrifuge tube and re-extracted with an equal volume of chloroform, vortex mixed for 30 seconds and centrifuged at 7558xg for 3 minutes. The upper aqueous phase was carefully transferred to a fresh tube and keeping note of the amount (480µl for 600µl), 0.8 volumes of isopropanol and 1µl of Glycoblue (Ambion) were added. The preparations were incubated overnight at -20°C to precipitate the nucleic acids. The next day, the preparations were centrifuged at 7558xg for 20 minutes at 4°C to pellet the RNA. The supernatant was removed and the pellet was washed by adding 1 ml of 70% v/v ethanol and re-centrifuged at 7558xg for 3 minutes at 4°C to remove any salt. After discarding the supernatant, the pellets were washed with 200 µl 95% v/v ethanol and centrifuged at 7558xg for 3 minutes at 4°C to speed up drying of the pellet. The ethanol

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was removed from the pellet which was allowed to air dry (~ 10 minutes) and then dissolved in 50µl RNase-free water. The concentration of the crude RNA was determined by measuring the absorbance at 260 nm of 1µl preparation using a Nanodrop spectrophotometer and the crude RNA was stored at -80°C. *Mtb* RNA was purified by digesting gDNA with DNase. This is followed by second DNase digestion. The RNA samples were purified using the RNeasy® Mini protocol for RNA clean-up (Qiagen). The concentration of the purified RNA was determined by measuring the absorbance at 260 nm of 1 µl of the preparation using the Nanodrop and the RNA was stored at –80 ^{o}C. For reverse transcription of RNA to form cDNA, 0.5µg of RNA was used (Natalie J Garton personal communication) for samples that had relatively high concentration of RNA (table 6.1). However, for samples which initially showed low 16S signal, reverse transcription was repeated with 11µg of the available RNA. For each sample, the following were added in a 200µl PCR tube and all incubation was done in the PCR machine (Peltier Thermal cycler, DNA Engine, DYAD, GRI, MJ Research).

Table 6.1: Reaction volumes used for cDNA preparation

The volume of Genomic directed primers and RNase free water used during denaturation of RNA extracted from mycobacteria.

	RT (µl)	no RT (-RT) control (µl)
RNA	X	X
Genomic directed primers (25 pmol/µl)	1.0	1.0
dNTPs(10mM)	1.5	1.5
RNase free water	15.5-X	15.5-X

In a PCR machine, the preparation was heated at 65°C for 5 minutes to denature RNA and immediately transferred to ice. 6.0µl of 5x Superscript II first strand buffer, 3.0µl 0.1M DTT and 1.5µlRNase Out (Invitrogen) was added to each sample. This was incubated at 25°C for 2 minutes to allow binding of primers. To each RT tube 1.5µl of Superscript II reverse transcriptase (Invitrogen) was added, and to that of the 'no RT' control tubes 1.5µl

RNase free water. The preparation was incubated for reverse transcription of RNA to cDNA followed by inactivation of Superscript II enzyme at 25°C for 10 minutes, 42°C for 50 minutes, 70°C for 15 minutes. The cDNA was stored at -20°C.

6.2.5. 16S cDNA Quantitative Real-Time Polymerase Chain Reaction using SYBR Green

Real-time Quantitative Polymerase Chain Reaction (qPCR) was performed on the cDNA produced during the RT-PCR step. Absolute™ qPCR SYBR Green Master-Mix (ABgene) was used for the reactions. The mix contained SYBR Green, reaction buffer, dNTPs, $MgCl_2$ and a hot-start DNA polymerase (Table 6.2).

Table 6.2: Reagent volumes of reagents used per qPCR reaction

The proportions of template cDNA, forward primer, reverse primer, Master-mix and water in a reaction volume of 25µl.

Template cDNA	1µl
MYCO16SF Primer (10pmol/µl)	1µl
MYCO16SF (10pmol/µl)	1µl
2X Absolute SYBR Green Master-Mix (ABGene)	12.5µl
H_2O	9.5µl
Total	25µl

Forward primer MYCO16SF: 5'-GAAACTGGGTCTAATACC-3'
Reverse primer MYCO16SR: 5'-ATCTCAGTCCCAGTGTGG-3'

Cycling conditions (including annealing temperatures and acquisition temperatures) were common for all the reactions (Table 6.3).

Table 6.3: Cycling conditions in qPCR

Cycling conditions in qPCR and the corresponding incubation time in the PCR machine

Temperature Hold	56°C	2 minutes
Temperature Hold	95°C	15 minutes
Cycling (40X)	95°C	30 seconds
	59°C	30 seconds
	72°C	20 seconds
	82°C	20 seconds
Melting	50-99°C	1°C per 5 seconds

Mtb CDC1551 genomic DNA with graded concentrations was used as a standard. The DNA standards included in each run were: 10^7, 5×10^6, 10^6, 10^5, 10^4, 10^3 and 10^2 genome copy numbers per µl, respectively.

Combined 16S SYBR Green / TaqMan Assay

Reactions were carried out in accordance with the method of Cheah et al (2010). PCRs were completed in 25µl volumes containing 250nM each of primer MYCO16SF and MYCO16SR, 150nM of TaQman probe MYCO16SPr and 1x Absolute QPCR SYBR green mix.

Probe MYCO16SPr: 5' ROX-TCC ACC ACA AGA CAT GCA TCC CGT G-BHQ 3'

Cycling conditions

Tempperature hold	95°C	15min
Cycling (40x)	95°C	15s
	60°C	60s
	72°C	20s
	84°C	20s

6.2.9. Quantitative Real-Time Polymerase Chain Reaction using SYBR Green for *hspX*, *tgs1* and *icl1* genes

For economical utilisation of cDNA products, the cDNA was diluted 1:2 and the *hspX*, *tgs1* and *icl1*genes quantitative real-time polymerase chain reactions were carried out on these diluted samples using the primers described in Table 6.4. PCR master mixes were prepared using specific forward and reverse primers designed for *hspX*, *tgs1* and *icl1*. For each gene prototype, 3 PCR assays were undertaken and the average gene concentration was used for analysis.

Table 6.4: SYBR Green PCR primers for selected *Mtb* genes

GeneName	Primer	Primer Sequence	Amp(bp)	T_m(°C)
icl1	Forward	5' – GCG GTG CGG AGG TGC TGT GG –3'	181	66.4
	Reverse	5' – AGG CTC TGG TCG GGG TAG GTG–3'		66.6
hspX	Forward	5' – ACT CCG GCC CAC CTT CGA CA – 3'	235	63.6
	Reverse	5' – AGC ACC TAC CGG CAG CGA CA – 3'		64.0
tgs1	Forward	5' – AAC GAA GAC CAG TTA TTC GAG–3'		55.0
	Reverse	5' – CTC ATA CTT TCA TCG GAG AGC– 3'		54.9

6.3. Results

A total of 25 smear positive TB patients were included in this study. Thirteen of them were HIV^{+ve} and the other 12 HIV^{-ve}. The majority of the TB patients (11/25) demonstrated 2+ AFB score and 10 TB patients had either 3+ or 4+ AFB score but the other 4 patients were with 1+ AFB score (table 6.5).

Table 6.5: AFB score among HIV^{+ve} and HIV^{-ve}/TB patients

Although the sample size is low, the majority of the TB patients demonstrated low AFB score ($\leq$ 2+ AFB score).

AFB score	HIV+ve	HIV-ve	Total
4+	2	4	6
3+	1	3	4
2+	6	5	11
1+	3	1	4
Total	12	13	25

6.3.1. Crude and Purified RNA concentration

The concentration of the crude and pure RNA was determined by measuring the absorbance at 260 nm of 1 µl preparation using the Nano drop spectrophotometer. The crude RNA concentration was below the limit of detection for two samples and as high as 439 ng/µl. However, the purified RNA concentrations were by far lower than the crude RNA in most of the samples (Table 6.6).

Table 6.6: Crude and Pure RNA concentration

Crude RNA (ng/µl)	260/280	Pure RNA (ng/µl)	260/280	Pure RNA/ Crude (%)
439.2	1.91	412.2	2.13	94
27	1.33	14	1.61	52
217.3	2.03	128.6	2.18	59
108	1.53	17.6	1.61	16
38.1	1.41	13	2.01	34
200.9	1.76	31.3	1.65	16
78.2	1.82	18.1	2.61	23
136.8	1.86	34.8	1.94	25
136.5	1.83	18.1	1.82	13
319.3	1.91	150.8	2.18	47
109.1	1.67	8.6	1.61	8
-53.7	2.54	30.5	1.60	Anomalous
-60	1.82	10.4	1.60	Anomalous
94.4	1.83	96.4	2.19	100
169.5	1.94	77.3	2.11	46
159.5	1.72	12.1	1.70	8
317.8	2.33	8.6	1.64	3
12.5	3.19	75.9	2.05	> 100 : Anomalous
406.0	1.99	22.7	1.77	6
61.2	1.79	44.72	2.16	74
99.8	1.81	10.6	1.77	10
140.7	1.63	10.8	1.80	8
122.1	1.86	58.2	2.09	48
439.2	2.34	262.4	2.09	60
167.3	1.87	99.7	2.02	60

6.3.2. 16S cDNATaQman/SYBR Green assay

The 16S cDNATaQman/SYBR green assay (Cheah et al, 2010) was carried out on all the 25 samples and 22 of them were positive for *Mtb* complex. For the normalization of the hspX, tgs1 and icl1 transcript signals, results from a 16S SYBR green assay were used.

6.3.3. HspX, tgs1 and icl1 gene transcription levels

Reliable signals were obtained for transcripts in thirteen of the twenty five samples. The hspX assays failed in two samples and the tgs1 assay failed in two separate samples. For one sample, only the hspX assay was run. The target gene transcripts numbers were normalized to the 16S gene transcript numbers determined by the SYBR green assay and these results are shown in Figure 6.1.
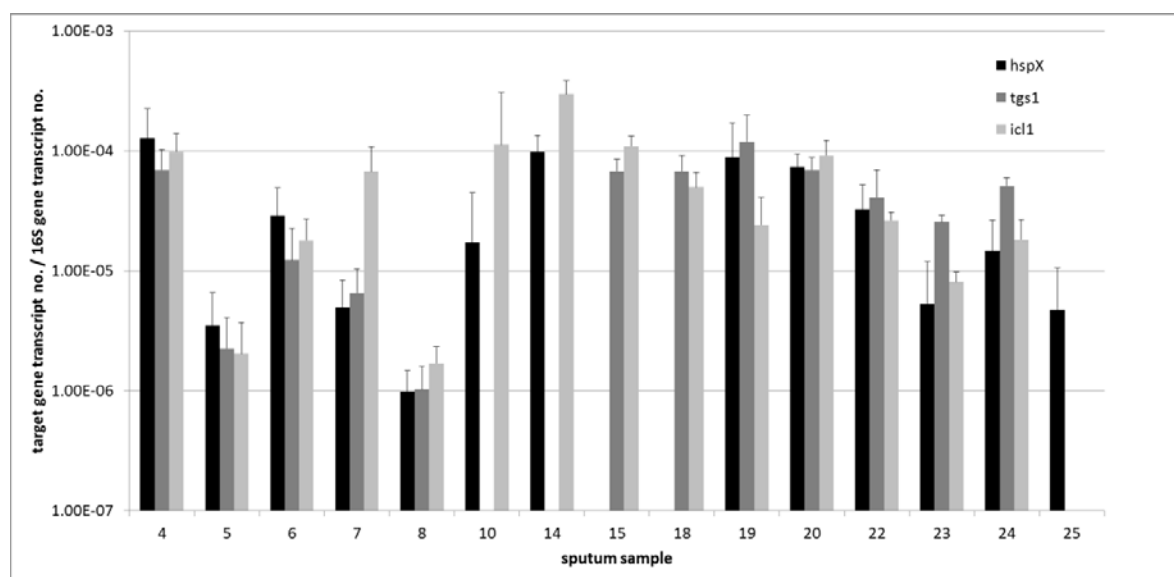
Figure 6.1: Normalised hspX, tgs1 and icl1 transcripts from sputum samples

The hspX assay failed for samples 15 and 18 and the tgs1 assay failed for samples 10 and 14. Samples 6, 14, 19 and 25 were taken from HIV-positive patients.

The normalised levels of expression of hspX, tgs1 and icl vary from sample to sample over two orders of magnitude. For each sample for which the hspX and tgs1 transcript levels are available, these transcript levels are relatively similar. In three out of fifteen samples, levels of icl1 were relatively higher than hspX.

It was an original aim to assay a larger number of genes known to be differentially regulated in sputum. As low amounts of RNA were extracted from the samples, RNA amplification was attempted to enable more genes to be targeted. However, a 16S qRT-PCR assay of the amplified RNA suggested that amplification had not been successful.

Of the fifteen patients producing sputum samples which could be analysed for RNA transcript levels, four were HIV positive and the other eleven were HIV negative. Because the numbers of HIV positive patients were too small, we were unable to compare the gene expression levels between HIV positive and HIV negative patients. However, the correlation between gene expression levels compared with FeNO concentration and LB positive tubercle bacilli proportion was determined. In this comparison, the level of hspX, tgs1 and icl1 transcripts were not significantly correlated with either FeNO or the proportion of LB positive tubercle bacilli (Figures 6.2 and 6.3).

hspX/16S

tgs1/16S

icl1/16S

Figure 6.2: Normalised hspX, tgs1 and icl1 transcript levels compared with FeNO concentration

Although no significant correlation between transcript levels of these target genes and FeNO was found, the correlation between tgs1 and FeNO suggests an association (hspx: r^2 = 0.028, p = 0.582; tgs1: r^2 = 0.017, p = 0.689; icl1: r^2 = 0.080, p = 0.329).

LB%

LB%

LB%

Figure 6.3: Normalised hspX, tgs1 and icl1 transcript levels compared with sample LB positivity.

Although no significant correlation between transcript levels of these target genes and LB positivity was found, the correlation between tgs1 and LB positivity suggests an association (hspX: r^2 = 0.039, p = 0.518; tgs1: r^2 = 0.069, p = 0.410; icl1: r^2 = 0.112, p = 0.242).

6.4. Discussion

The 16S cDNA TaQman/SYBR Green assay result of this study showed that 22 of the 25 TB patients were positive for *Mtb* complex. 16S was used as housekeeping gene to normalize the target gene transcript levels. Genes that have constant expression, traditionally called housekeeping genes have been used as controls without prior validation of their merit (Vandesompele et al, 2009). However, many of the well-known housekeeping genes have been shown to be affected by different handling, treatment, biological processes, and even different tissues or cell types (Wong and Medrano, 2005).

6.4.1. HspX, tgs1 and icl1 gene transcription levels

The *Mtb* sputum transcriptome revealed many signatures of slow or non-growth (Garton et al, 2008). HspX, tgs1 and icl1 were reported to be upregulated in sputum compared with *in vitro* growth of H37Rv. This reflects induction of the DosR regulon and metabolism of fatty acids. In this study, these transcripts were reliably assayed in fifteen of the sputum samples collected for RNA analysis. Variable levels of expression of these genes between patient sputum samples could reflect host variability, for example immunological differences. The low number of samples analysed do not allow us to reach a conclusion on the effect of HIV co-infection on *Mtb* gene expression. In this study, there was no significant correlation between the hspX, tgs1 and icl1 gene transcripts levels compared with the concentrations of the corresponding FeNO or proportion of LB^{+ve} tubercle bacilli among TB patients. Although not statistically significant some evidence of association between tgs1 gene copies with the concentration of FeNO was observed. Voskuil et al (2003) reported that a low and nontoxic concentration of NO induces a 48-gene regulon via the response regulator DosR but at a high concentration it results in cell death. There are also varieties of intracellular signaling functions of NO that regulate essential functions in eukaryotes (Martin et al, 2000). The hspX and tgs1 genes are members of the DosR regulon. The hspX gene is also called the acr gene, and encodes the 16-kDa α-crystalline protein, a well-characterized virulence factor required for Mtb growth in macrophages (Yuan et al, 1998). Since several lines of evidence have linked *Mtb* growth inhibition with hypoxic conditions, these genes may be required for the establishment of a latent state

during TB infection. Shi et al (2003) elegantly confirmed the up-regulation of acr expression in mouse lungs infected with *Mtb*. In hypoxia an exaggerated induction (~80-fold) of the hspX gene has been reported. The acr gene was found as an immunodominant antigen that is recognized by the majority of patients with active tuberculosis (Lee et al, 1991).

Accumulation of LBs within the tubercle bacilli as a result of hypoxia was reported previously and documented in the previous sections of this thesis. Hu et al (2006) established that expression of hspX results in sluggish growth of *Mtb in vitro*, and proposed that hspX plays an active role in slowing the growth of *Mtb in vivo*. In hibernating animals, triacylglycerol was reported to be the commonly used storage form of energy that can be used for long term survival (Alvaraz and Steinbuchel, 2002). Additionally, there are also reports that confirmed the up regulation of some tgs genes, particularly tgs1, during persistent infection and hypoxia, and these same genes were also up regulated upon NO induction of the dormancy genetic program (Daniel et al, 2004). Daniel et al (2011) reported that mycobacterial DosR regulon genes, hspx, tgs1 and icl1 are up-regulated in *Mtb* within hypoxic lipid loaded macrophages.

There is evidence which suggests that genes of the DosR regulon are more actively transcribed by HIV^{-ve}/TB patients than HIV^{+ve}/TB patients, implying that the immune pressure on *Mtb* in HIV^{-ve} individuals is reduced (Bouke de Jong personal communication and the information obtained via Natalie J Garton).

The average proportion of LB^{+ve} tubercle bacilli was relatively higher in HIV^{+ve}/TB patients (29%) compared with HIV^{-ve}/TB patients (18%). However, there was no significant association between the expression levels of the hspX, tgs1 and icl1 genes with the proportion of LB^{+ve} tubercle bacilli in sputum among HIV^{+ve} and HIV^{-ve}/TB patients. Up regulation of genes such as the hspX, nark2 (nitrate/nitrite transporter), and tgs1 genes confirm the up regulation of the DosR regulon (Park et al, 2003). Garton et al (2008) reported that elevated expression of tgs1 in sputum and the presence of LB^{+ve} *Mtb* cells there in suggest a likely direct link between tgsl expression, LB formation, and increased bacillary triacylglycerol content. Garton et al (2008) also documented that in *M.smegmatis*,

both increased triacylglycerol and LB content were significantly associated with tgs1 overexpression.

The limitation for the current study was the small sample size (n=15). Reliable signals were obtained in qPCR assays of cDNA from these 15 samples. During collection of sputum samples for this part of the study, there were continuous power interruptions in Gondar which may have affected the RNA of some samples. We recommend that further study with a larger sample size is required to determine the relationship between *Mtb* gene expression patterns among HIV^{+ve} and HIV^{-ve}/TB patients and their association with FeNO concentration and the proportion of LB^{+ve} baclli.

CAPTER SEVEN: Studies on drug resistance and treatment outcome in relation to LBs and FeNO concentration

7.1. Introduction

In 1998, a working group of the WHO and the International Union against Tuberculosis and lung disease (IUATLD) published recommendations standardizing the surveillance of TB treatment outcomes across Europe (Ditah et al, 2000). The indicators for treatment outcomes are cure, treatment completion; treatment stopped, death, interruption, lost to follow up, transferred out, and not completed (reason unknown). Treatment of TB infection requires taking multiple medications for a minimum of 6 months. Non-adherence to treatment results in treatment failure, relapse, acquired drug resistance, and prolonged infectiousness of patients (Weis et al, 1994). Advance in TB treatment, The Directly Observed therapy (DOT) program, designed to improve adherence to treatment is recommended by several leading international organizations (Enarson et al, 2000).

The world wide rate of treatment success was 82% in DOTS areas, but only 67% in non-DOTS areas by the year 2000 (WHO, 2003). Previous to DOTS implementation, up to half of all patients with TB do not complete treatment (Cuneo and Sinder, 1989). The difficulty experienced by patients following particular treatment regimens has raised awareness of adherence as a complex behavioral issue, influenced by many factors (WHO, 2003).

One of the major challenges facing TB control globally is the HIV epidemic. Tuberculosis is responsible for about 30% of deaths among HIV infected individuals (WHO, 2008). Although increasing frequency of TB/HIV has considerable impact on TB control particularly in Sub-Saharan Africa and other resource poor countries, there are also other factors both from the *Mtb* and the human host that may have significant drawback on the treatment outcome against tuberculosis infection. Among these factors nitric oxide concentration and the accumulation of LBs within the tubercle bacilli or the proportion of LB^{+ve} tubercle bacilli in sputum are relevant to the present study.

In 2007, the WHO estimated an occurrence of about 511, 000 new MDR-TB cases and about 5% of all TB cases world wide harbor MDR-TB *Mtb* strain (WHO, 2009). Moreover, high rates of anti-TB drug resistance have been shown, with values reaching as high as 20% of MDR cases among new TB cases in certain regions (WHO, 2008). In addition,

extensively drug-resistant TB (XDR-TB) cases have been detected in at least 55 countries (WHO, 2009).

Treatment of MDR-TB cases requires second line drugs and drug susceptibility testing may be required (Richer et al, 2009). In resource- rich countries, the standard of care has shifted from standard therapy with first- line drugs to individually tailored regimens based on the susceptibility profile of each isolate (NiazBanaiee et al, 2003). It is well-known that timely delivery of this information has proven to be critical under some settings (Turett et al, 1995). Several reports documented that for proper and effective management of TB, particularly pulmonary TB, drug sensitivity testing is a must. However, several reasons such as lack of technical expertise, cost, and time factors make the standard method out of reach for many clinicians managing TB. So much so many cases are over treated without any benefit and often with unwanted side-effects (Mishra et al, 2009).

The increase in the occurrence of drug-resistant *Mtb* dictates the need for methods that quickly detect *Mtb* and identify drug-resistance cases (WHO, 2008). Failure to recognize and treat drug resistant TB cases leads to an increased mortality, outbreaks and dissemination of drug-resistant bacteria to the community (Farmer et al, 1998).

Drug resistant TB infection was reported at different points in time and places in Ethiopia. For instance, the national TB drug resistance survey conducted in 2005 documented a 1.6% and 11.8%   prevalence of MDR-TB among new and previously treated TB patients respectively (WHO, 2008).

In vitro drug susceptibility tests on lipid loaded tubercle bacilli have indicated phenotypic drug tolerance as documented in the previous sections. Moreover, lipid accumulation within the tubercle bacilli when exposed to nitric oxide *in vitro* was also reported. In addition, our previous data showed a significant association between FeNO concentration and the proportion of LB[+ve] tubercle bacilli in sputum. However, the relation between FeNO concentration and the proportion of LB[+ve] tubercle bacilli to that of treatment outcome and anti-microbial susceptibility pattern is not investigated previously.

7.1.1: Aims and objectives

The major objective of this study was to explore whether sputum LBs or FeNO concentration determined at the time of TB diagnosis might be related to treatment outcome or the anti-microbial susceptibility pattern of *Mtb* isolates from pulmonary TB patients who had no history of anti-TB therapy.

Specific objective

1. To determine the impact of FeNO and the corresponding proportion of LB^{+ve} tubercle bacilli on the treatment outcome of tuberculosis infection.

2. To assess the anti-microbial susceptibility patterns of *Mycobacterium* isolates causing pulmonary tuberculosis infection in Gondar.

3. To determine the possible relation ship of FeNO concentration and the proportion of LB^{+ve} tubercle bacilli to that of anti-microbial susceptibility patterns of *Mycobacterium* isolates.

7.2. Material and Methods

7.2.1. Patient recruitment and selection
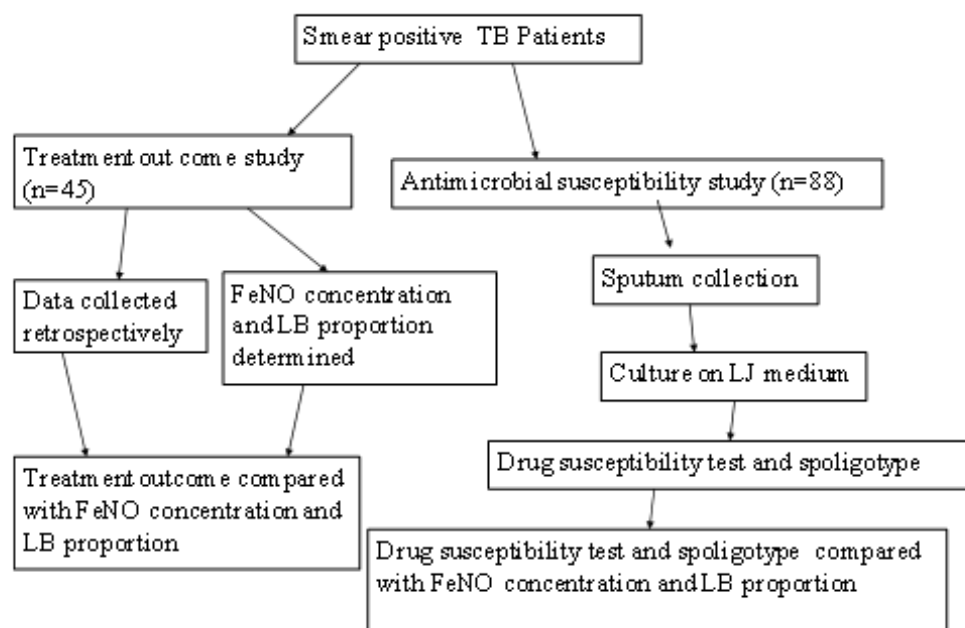
Figure 7.1: Treatment outcome and drug susceptibility study

Treatment outcome was assessed and determined retrospectively on 45 smear positive TB patients. The drug suceptability test was undertaken and its association with FeNO concentration and the proportion of LB^{+ve} tubercle bacilli was also determined.

In the study designed to evaluate treatment outcome among 45 previously diagnosed and treated patients, data was collected from hospital records in the DOTS clinic of University of Gondar hospital, Gondar poly clinic and Gondar Army hospital. The drug suceptability pattern and *Mtb* spoligotype procedures were undertaken on new smear positive TB patients (n-88) at the AHRI research laboratory, Addis Ababa, Ethiopia.

7.2.2. Specimen collection

Sputum samples were collected following standard operating procedures as documented in the material and method section of Chapter two.

7.2.3. Culture media preparation

Solution 1 (Day 1): Mineral Salt solution—750 milliliter

    A. Potassium di-hydrogen phosphate--------3 gram

    B. Magnesium sulfate-------------------------0.3 gram

    C. Magnesium citrate--------------------------0.75 gram

    D. L-Asparagines------------------------------4.5 gram

    E. Distilled water------------------------------750 milliliter

    F. Dissolved by heating at $100^{o}C$ water bath

    G. The solution was kept overnight in the cold room ($+4^{o}C$ to $+8^{o}C$).

Solution 2 (Day 2): Starch Solution

Potatoes starch----37.5 gram

Potatoes starch was dry sterilized at $140^{o}C$ for 15 minutes and solution 1 was allowed to stand at room temperature. In the meantime, the water bath was adjusted at $100^{o}C$. The starch was added to mineral salt solution and shacked continuously (to avoid coagulation) until the solution gets thick. It was allowed to stand in a $56^{o}C$ water bath until solution 3 and solution 4 prepared.

Solution 3: Malachite green solution

Malachite green solution--------1% - 2%

Malachite green was dissolved in 100 ml distilled water and kept in a $56^{o}C$ water bath until it dissolves completely.

Solution 4: Egg solution

Fresh eggs (Possibly 7 days old) were cleaned by scrubbing with brush in distilled water and rinsed thoroughly in running water. The eggs were kept in big dish, wrapped in clean sheet and soaked for 5 minute in 96% ethyl alcohol. The eggs were transferred to a biological safety cabinet (BSC) and broken aseptically into a sterile beaker using sterile scissors and mixed thoroughly. The volume (1750 ml) was measured using sterile measuring cylinder and transferred into a sterile bottle containing sterile glass beads. The egg solution was homogenized for 20 minutes using a shaker and added by filtering (using 4-fold sterile gauze) the egg solution into solution 2. To this malachite green solution was

added, mixed well and the mix was divided into two. 12.5 ml of glycerol (LJG) was added to one and 0.4% w/v pyruvate (LJP) in to the other. The preparations were dispensed into sterile culture tubes with screw caps, ~ 5 ml to~6 ml amount per tube depending on the size of the tube and the screw caps were tightly closed, slanted and coagulated by inspissations at 85°C for 50 minutes. The tubes were allowed to cool at room temperature and sterility was checked by incubating 1 LJG and 1 LJP at 37°C for 4 hours. The medias were kept at +4°C to +8°C (cold room) for three months (Shelf life). The qualities of the LJ culture media were controlled by inoculating *M. gordonae* (fast grower mycobacteria and incubating overnight at 37 ^{o}C (when LJ is good quality growth is seen after an overnight incubation).

7.2. 4. Decontamination of sputum sample

Reagents (Petroff's method)

The decontamination reagent was 4% NaOH autoclaved at 121°C for 15 minutes; the neutralizing reagent was 2N HCl and Phenol red indicator. The decontamination reagent was kept for a week at 37°C and after a week a fresh one was prepared.

An equal volume of 4% NaOH was added to the sputum sample and vortex mixed. One ml of the decontaminated sputum sample was transferred into a centrifuge tube using sterile 1 ml pasture pipette and incubated at room temperature for 10 minute on the shaker to free the bacilli. This was centrifuged at 1761xg for 15 minutes. The supernatant was decanted, the sediment mixed and pellet was re-suspended in 1 to 2 ml (depending on the thickness of the pellet) of phosphate buffer saline (PBS). One drop of phenol red indicator was added followed by neutralizing the sediment by adding 2N HCl drop by drop with continuous shaking until the color changes from red to yellow.

7.2.5. Inoculation of decontaminated sputum samples

Two Lowenstein Jensen base + Glycerol (LJG) and 2 Lowenstein Jensen base + Pyruvate (LJP) culture medias were labeled with AHRI number and date. 0.5 ml of neutralized sputa was inoculated to each culture media using sterile disposable Pasteur pipette and incubated

at 37°C in slant position for 1 week. After one week, the inoculated culture media were kept in an upright position and monitored weekly for growth for 12 weeks. In case of contaminated samples before the 1st growth, the data was recorded and the preparation discarded then the samples repeated.

7.2.6. Molecular Genetic Assay for identification of resistance to Rifampicin and/or Isoniazid of the *Mtb* complex by GenoType MTBDRPlus

The GenoType MTBDRPlus test is based on the DNA-strip technology and permits the molecular genetic identification of the *Mtb* complex and its resistance to rifampicin and/or isoniazid from cultivated sample or pulmonary smear-positive clinical specimens. The manufacturer's instruction demonstrated that the identification of rifampicin resistance is enabled by the detection of the most significant mutations of the rpoB gene (coding for the β-sub-unit of the RNA polymerase). For detection of high level isoniazid resistance, the katG gene (coding for the catalase peroxidase) is examined and for detection of low level isoniazid resistance, the promoter region of the inhA gene (coding for the NADH enoyl ACP reductase) is examined (GenoTypeMTBDRplus, HainLifescience GmbH, 2009, Germany).

DNA extraction

Bacteria were collected with an inoculation loop and suspended in 300µl sterile distilled water. Pellets were prepared by spinning the preparation at 1761xg for 15 minutes. The supernatant was discarded and pellet was re-suspended in 300µl of molecular grade water and vortex mixed. The preparation was incubated at 95°C water bath for 20 minutes. This was spined down at 1761xg for 5 minutes and directly 5µl of the supernatant was used for PCR (GenoTypeMTBDRplus, HainLifescience GmbH, 2009, Germany).

Amplification

The amplification mix was prepared in 50µl total volume according to the following per tube mix.

35µl primer nucleotide mix (PNM)

5µl 10x polymerase incubation buffer

X µl $MgCl_2$ solution

1-2 unit(s) thermostable DNA polymerase

Y µl water to obtain a volume of 45µl

5 µl DNA solution leading to a final volume of 50 µl

Amplification Profile

	Culture samples	Clinical samples
15 minutes 95°c	1 cycle	1 cycle
30 second 95°c 2 minuets 58°c	10 cycles	10 cycles
25 second 95°c 40 second 53°c 40 second 70°c	20 cycles	30 cycles
8 minutes 70°c	1 cycle	1 cycle

Hybridization

A shaking water bath was pre-warmed to 45°C with the maximum tolerated deviation from the target temperature +/-1°C. The solution of hybridization buffer (HYB) and Stringent Wash solution (STR) were also pre-warmed to 37- 45°C before use. After assuring that the reagents are free of precipitates, all reagents were warmed with the exception of CON-C and SUB-C which were kept at room temperature. 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) and mixed well at room temperature.

For each strip, 10µl of concentrate was added to 1 ml of the respective buffer. Twenty µl of denaturation solution (DEN, blue) was dispensed in the corner of each of the wells and 20 µl of amplified sample was added to the solution, mixed by up and downing the pipette and incubated at room temperature for 5 minutes. Meanwhile, strips were taken out using tweezers and marked with pencil on the undernth side with colour marker. Carefully, 1 ml of pre-warmed hybridization buffer (HYB, green) was added to each well and the tray was gently shaked until the solution had a homogenous colour. This was followed by placing the strip in each well. The strips were completely covered by the solution and the coated side was faced upward. The tray was placed in a shaking water bath and incubated for 30 minutes at 45°C.The hybridization buffer was completely aspirated using a Pasteur pipette connected to a vacuum pump. One ml of stringent wash solution (STR, red) was added to each strip and incubated for 15 minutes at 45°C in a shaking water bath and after this step all works was under taken at room temperature. The stringent wash solution was completely removed by turning the tray upside down and gently striking it on an absorbent paper. Each strip was washed once again with 1 ml of rinsing solution (RIN) for 1 minute on shaking platform and 1 ml of diluted conjugate was added to each strip and incubated for 30 minutes on shaking platform. The solution was removed and each strip was washed twice for 1 minute with 1 ml rinse solution (RIN) and once with approximately 1 ml of distilled water on shaking platform. The water was removed after the last wash and 1 ml of diluted substrate was added to each strip and incubated at room temperature by protecting from light without shaking. The reaction was stopped by briefly rinsing twice with distilled water. Using tweezers, the strips were removed from the tray and allowed to dry between two layers of absorbent paper.

Evaluation and interpretation of results

Strips were pasted and stored protected from light. An evaluation sheet was provided with the kit. During evaluation of the results 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 was determined in the respective column (figure 7.1).

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 will bind to the amplification control zone. If this band is developed, mistakes during extraction and amplification setup and the carry-over of amplification inhibitors can be excluded. In case of a positive test result, the signal of the Amplicon control zone can be weak or even vanish totally. This might be due to competition of the single reactions during amplification. A missing AC band in case of a negative test result indicates mistaking during amplification set-up, or carry-over of amplification inhibitors. In this case, the test is not valid and the reactive sample has to be repeated.

Mtb **complex (TUB)**: This zone hybridizes, as known, with amplicons generated from all members of the *Mtb* complex. If the TUB zone is negative, the tested bacterium does not belong to *Mtb* 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 when the TUB zone has documented the presence of *Mtb* strain. If neither the locus control probe nor the wild type or mutation probes of one of the three genes examined are developed, the test can not be evaluated.

Wild type probes: The wild type probes comprise the most important resistance areas of the respective genes. When all wild type probes of a gene stain positive, there is no detectable mutation within the examined regions. Hence, the strain tested is probably sensitive for the respective antibiotics. 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 antibiotics. Only those bands whose intensities are about as strong as or stronger than that of the amplification control zone (AC) are to be considered. Each pattern deviating from the wild type pattern indicates a resistance of the tested strain (Figure 7.1).
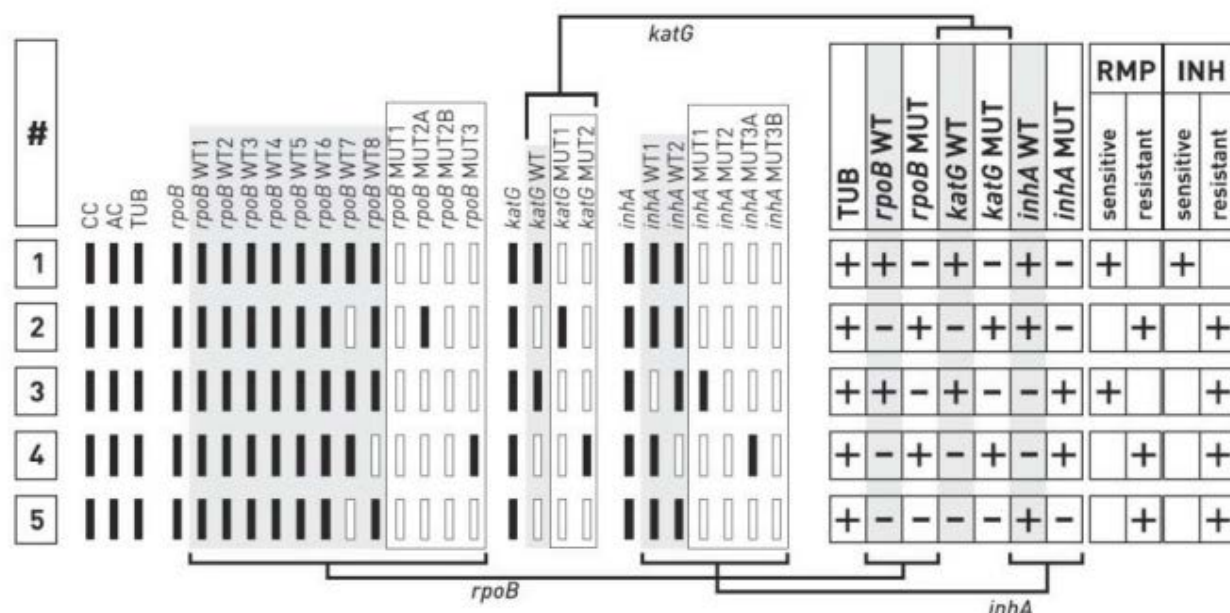
Figure 7.1: Evaluation and interpretation of GenoType MTBDRplus results

Examples for banding patterns and their evaluation with respect to rifampicin and/or isoniazid resistance (Adapted from GenoTypeMTBDRplus, HainLifescience GmbH, 2009, Germany).

Mutation probes: The mutation probes detect some of the most common resistance-mediating mutations. Compared to other probes, positive signals of the mutation probes *rpoB*MUT2A and MUT2B may show lower signal strength. Only those bands whose intensities are about as strong as or stronger than that of the amplification control zone (AC) are to be considered. Band pattern deviating from the wild type pattern indicate a resistance of the tested strain.

If all wild type bands display a signal, this is classified as positive and marked in the WT column of the respective gene as "+". If at least one of the wild type bands is absent, this is classified as negative and marked in the WT column of the same gene as "–". Negative entries are only made to the mutation columns when none of the mutation bands display coloration. If at least one of the mutation bands display coloration, this is classified as mutation-positive. Figure 7.1, example 1 shows the wild type banding pattern. All wild

type probes display a signal, but none of the mutations probes; hence, the evaluation chart has a "+" in the three wild type columns and a "–" in the three mutation columns. Accordingly, the boxes for rifampicin (RIF) and isoniazid (INH) are marked as sensitive. One of the rpoB and the katG wild type probes are missing in example 5; hence, the boxes for rpoB WT and katG WT are marked with a "–". As none of the mutation probes are developed, these boxes are also marked with a "–". The inhA promoter region does not deviate from the wild type pattern. The strain is evaluated as RIF and INH resistant. The INH resistance is caused by a mutation in the katG gene and is therefore a high level isoniazid resistance.

The spoligotype patterns of *Mtb* strains were determined following the AHRI standard operational procedures as documented in chapter five.

7.3. Results

7.3.1: Eligible versus included patients

In the treatmentout comes study, all the 45 smaer positive TB patients were included in the study. Sputm sample collected from 88 new smears positive TB patinets were cultivated and growth of *Mtb* colonies obsereved on 87 samples. Of these 87 culture positive samples, only 84 *Mtb* isolates were tested for drug susceptibility test and 73 of them demonstrated successful spoligotype pattern.

7.3.2: treatment outcome

A total of 45 smear positive pulmonary TB patients treatment outcomes were investigated retrospectively. Ethambutol hydrochloride, Rifampicin, Isoniazid, and pyrazinamide (ERHZ) coated in a single tablet was used for the intensive phase treatment but RIF and INH were used during the continuous phase. Fourteen patients were HIV^{+ve}, 23 HIV^{-ve} and the other 8 patient's HIV status were unknown. Fourty-two patients were new TB cases and the other 3 were retreatment (relapse) cases. Table 7.1 shows the treatment outcome of both the intensive and continuous phase together with the AFB score after completion of the recommended drug regimen within the specific period of time.

The initial weight of the TB patients were compared with the respective weight gained after completion of the intensive and continuous phase of TB treatment (n=30) (figure 7.2). The weight of the TB patients before starting treatment was as low as 28 kg and as high as 60 kg with an average of 46.02 kg (95% CI 43.54-48.51). The corresponding minimum/maximum weight were 33/74, 36/74 and 37/74 kg after completion of the 2 month, 5 month and 7 month treatment.

Table 7.1: Treatment outcome

Thirty-six of the 45 TB patients completed the intensive phase treatment at the health facility they were diagnosed. Of which, 4 patients were positive for AFB after completion of the intensive phase. Two TB patients that were negative for acid fast bacilli after completion of the two month treatment were positive for AFB when the sputum sample examined at the end of the 7th month treatment completion. Data for treatment outcome showed that 29 cured, 2 treatments completed, 1 patient died, 11 transferred out, and 2 were lost. There was no treatment failure and defaulting in the three health institutions during the study period.

Cu=Cured; TC=Treatment completed; Di=Died; Fai=failure; Def=defaulted, TO=Transferred out

	Complete			Incomplete	
	n	%		n	%
Intensive phase	36	80		9	20
Continious phase	29	64.4		16	35.6

	2nd Month			5th Month			7th Month		
	Pos	Neg	Un known	Pos	Neg	Un known	Pos	Neg	Un known
AFB score after treatment	4	30	11	1	31	31	1	28	16

	Cu	TC	Di	Fai	Def	TO	Lost
Treatment outcome	29	2	1	0	0	11	2
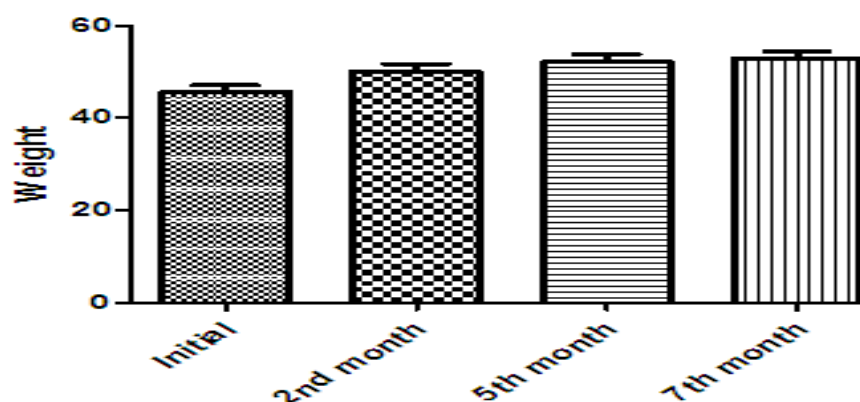
Figure 7.2: Comparison of the initial weight of the TB patients to the corresponding weight after TB treatment

The initial weight of the TB patients compared with the corresponding weight after 2nd, 5th and 7th month treatment of pulmonary TB patients.

The over all average initial weight of the TB patients (n=30) were 45.73 kg (95% CI 42.84 - 48.62 kg). The mean, weight after completion of the two month, five month and seven month treatment were 50.5 kg, 53.00 kg and 54.00 kg respectively (figure 7.2). Regardless of HIV status, data of TB patients analyzed by one-way analysis of variance (ANOVA) demonstrated that the average weight of the TB patients before treatment was significantly different from the average weight after completion of treatment (p=0.0042; r^2=0.107). The mean initial weight of the HIV^{-ve}/ TB patients (n=17) was 44.24 kg (95% CI 40.89-47.58). On the other hand, the average weights of HIV^{-ve}/TB patients after completion of the 2nd, 5th, and 7th month treatment were 47.47 kg, 49.89 kg and 50.76 kg respectively. Moreover, data on HIV^{-ve}/TB patients showed that the mean weight before initiation of treatment was significantly different from the corresponding average weight after completion of treatment (P=0.0221; r^2=0.1386) (figure 7.3A). On the other hand, the mean initial weight for HIV^{+ve}/TB patients was 46.18 kg (95% CI 40.27-52.10) and the average weight after completion of the 2nd, 5th, and 7th month therapy were 51.55 kg, 53.27 kg, and 54.09 kg respectively (figure 7.3B). However, when data from HIV^{+ve}/TB patients was analyzed by one way ANOVA, there was no significant difference between the mean weights of the patients before treatment initiated compared with the average weight after completion of treatment. The average initial weight of TB patients with unknown HIV status (n=7) was 52.14 kg (95% CI 48.09-62.91). The corresponding mean weight after 2 month, 5 month and 7 month treatment were 63.0 kg, 64.00 kg and 64.25 kg respectively.
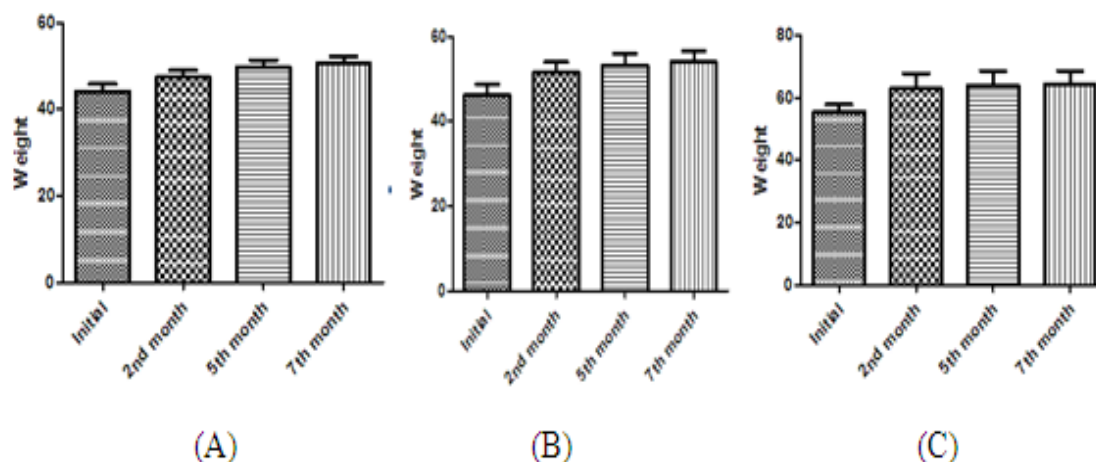
Figure 7.3: Comparison of the initial weight of TB patients with the corresponding weight after treatment initiation stratified by their HIV status

The initial weight of the TB patients was compared with the corresponding weight after 2nd, 5th and 7th month treatment of pulmonary TB patients stratified by HIV status. (A): Comparison of the weight of HIV^{-ve}/TB patients; (B): comparison of the weight of HIV^{+ve}/TB patients; (C): comparison of the weight of HIV status unknown TB patients.

7.3.3. Initial FeNO concentration and the proportion of LB^{+ve} tubercle bacilli compared with the weight gained by TB patients

The average FeNO concentrations among HIV^{-ve}/TB patients (n=21) was 17.30 ppb (95% CI 12.83-21.77 ppb) with minimum and maximum concentration of 5 ppb and 41.30 ppb respectively. HIV^{+ve}/TB patients (n=13) demonstrated relatively lower FeNO concentration ($\bar{x}$=11.94 ppb) than HIV^{-ve} and HIV status unknown (n=4) TB patients ($\bar{x}$=25.93 ppb). When the FeNO concentration data was analyzed by one way ANOVA, there was significant difference in FeNO concentration among HIV^{-ve}, HIV^{+ve} and the HIV status unknown/TB patients (p=0.0099; r^2=0.216) (Figure 7.4).
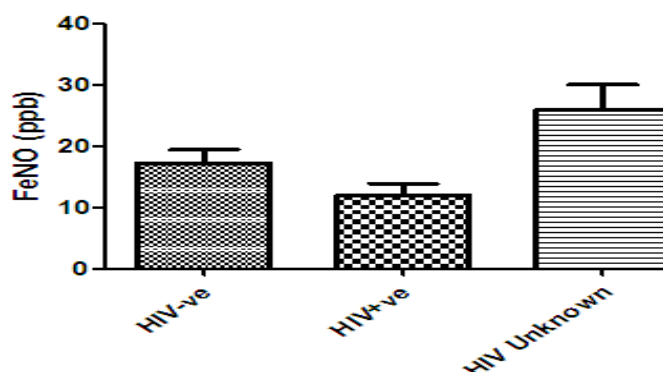
Figure 7.4: FeNO concentration versus HIV status

FeNO concentration among HIV[-ve], HIV[+ve] and HIV status unknown TB patients before initiation of anti-TB treatment (n=38).Data showed significant difference on FeNO concentration between HIV[-ve] and HIV[+ve]/TB patients.

Regardless of the HIV status, the average weight gained by the TB patients after treatment completion was 7.4 kg (95% CI 5.65-9.217 kg) and the corresponding mean FeNO concentration was 16.5 ppb (95% CI 12.63-19.66). The association of FeNO concentration with the weight gained by the TB patients was determined by linear regression analysis and data showed no significant association between FeNO concentration and the weight gained by the TB patients after 7 month therapy (Figure 7.4A). Analysis between FeNO concentrations with the weight gained by HIV[-ve]/TB patients also showed no significant association (figure 7.4B). On the other hand, the mean weight gained by HIV[+ve]/TB patients (n=10) was slightly higher than HIV[-ve]/TB patients (8.7 versus 7.4 kg respectively). In addition, FeNO concentration was weakly associated with the weight gained by HIV[+ve]/TB patients (p=0.048; r^2=0.4026) (figure 7.4C). The mean weight gained by HIV status unknown TB patients was 8.5 kilogram (95% CI 1.44-15.56 kg) and there was no significant association between FeNO concentration and the weight gained by the 4 HIV status unknown TB patients (figure 7.4D).
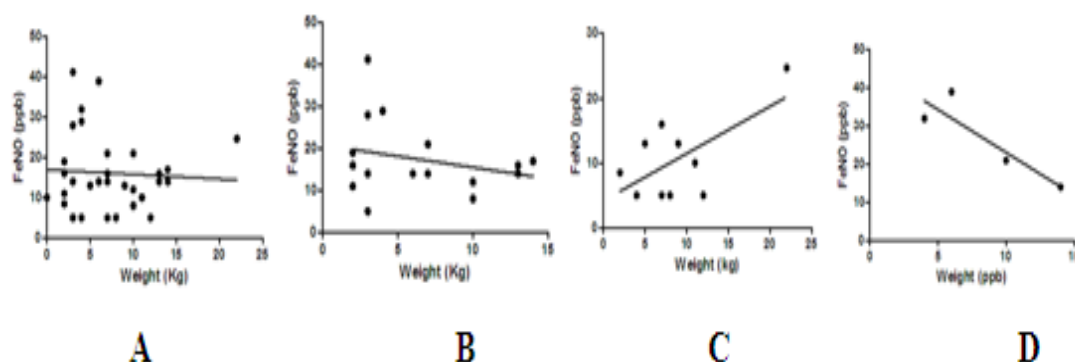
Figure 7.5: Association between the weight gained and FeNO concentration

FeNO concentration taken from TB patient's prior treatment compared with the weight gained by the respective patient after 7 months of therapy. (A) FeNO concentration versus weight gained among TB patients regardless of HIV status; (B) FeNO concentration versus weight gained among HIV[-ve]/TB patients; (C) FeNO concentration versus weight gained among HIV[+ve]/TB patients and (D) FeNO concentration versus weight gained among HIV status unknown TB patients.

The percentage of the weight gained by the TB patients after completion of the two month, 5 month and 7 month treatment was also determined. The mean proportion of weight gained by the TB patients after completion of the intensive phase treatment was 8.4 kg%. On the other hand, the average percentage of weight gained by the TB patients (n=30) after 5 and 7 month of therapy was 12.3kg% and 10.9kg% respectively (figure 7.5).
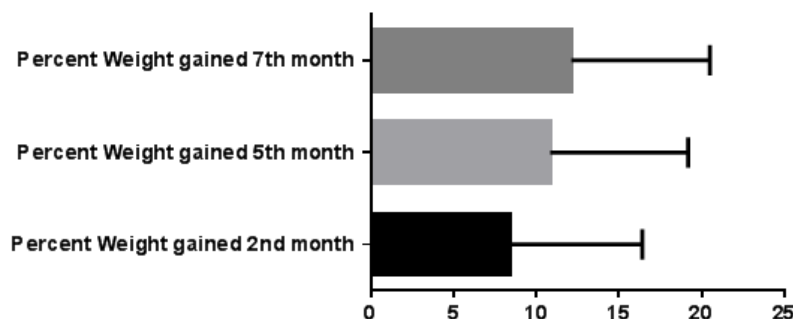
Figure 7.6: Frequency distribution of the percentage of weight gained among TB patients

The percentage of the weight gained by TB patients showed significance difference up on successive treatment.

When the mean percentage of weight gained after completion of the intensive phase was compared with the corresponding weight gained after completion of the 5th and 7th month therapy, the difference was significant (p=0.022 and 0.026 respectively). The mean percentage of weight gained by the TB patients was also compared with the AFB result after completion of the initial phase of treatment. Accordingly, data showed an average percentage weight gained of 9.05 kg% (SD 8.36 kg) (n=26) and 3.96 kg% (SD 3.03 kg (n=4) for patients with AFB negative and positive result after completion of the initial phase of TB treatment.

Regardless of the HIV status of the TB patients (n=18), the mean proportion of LB[+ve] tubercle bacilli was 32.47% (95% CI=23.7-41.3%). The proportion of LB[+ve]tubercle bacilli was compared with the weight gained by the TB patients. Linear regression analysis showed that there was no significant association between the proportion of LB[+ve]tubercle bacilli and the weight gained by the TB patients (figure 7.6A). There was also no significant association between the percent weights gained by the TB patients with the corresponding proportion of LB[+ve] tubercle bacilli in sputum. The average proportion of LB[+ve]tubercle bacilli among HIV[+ve]/TB patients (n=9) were relatively higher than HIV[-ve]/TB patients (24.3%; n=10).

(A)   (B)   (C)
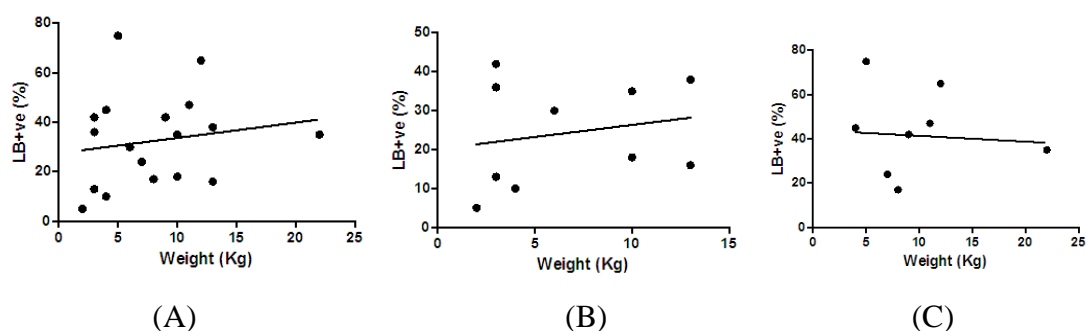
Figure 7.7: Comparison of the weight gained by the TB patients with the proportion of LB[+ve] tubercle bacilli in sputum

The proportion of LB[+ve]tubercle bacilli taken from TB patient's prior treatment compared with the weight gained by the respective patient after 7 months of therapy (n=18). (A) The proportion of LB[+ve]tubercle bacilli versus weight gained among TB patients regardless of HIV status; (B) The proportion of LB[+ve]tubercle bacilli versus weight gained among HIV[-ve]/TB patients; (C)The proportion of LB[+ve]tubercle bacilli versus weight gained among HIV[+ve]/TB patients.

7.3.4. FeNO concentration and the proportion of LB^{+ve} tubercle bacilli compared with AFB score after treatment

Overall, 6 patients were AFB positive after completion of either the intensive phase (n=4) or the contineuation phase (n=2) of TB treatment. Of which, one TB patient was retreatment case and military by profession. Three out of the six patients were HIV negative, one HIV positive and the HIV status of the other two patients were unknown. The average FeNO concentration of the TB patients that dempnstrated positive AFB score after treatment (n=5) was relatively higher than TB patients that demonstrated negative AFB score after completion of the same course of therapy; 27.26 ppb versus 11.91ppb respectively. The corresponding average proportion of LB^{+ve}tubercle bacilli among patients that were positive for AFB after treatment was also comparatively higher than TB patients with negative AFB score; 39.50% versus 28.9 % respectively (table 7.2). However, there was no statistically significant association between FeNO concentration and the proportion of LB^{+ve} tubercle bacilli among TB patients that demonstrated positive AFB score after treatment. Although difficult to draw conclusion from this data due to the small sample size, relatively higher proportions of LB^{+ve} tubercle bacilli was observed among patients that demonstrated positive AFB score after completion of treatment. In addition there was no significant association between FeNO concentration and the proportion of LB^{+ve} tubercle bacilli compared with the weight gained amongst patients that showed positive AFB score after treatment.

Table 7.2: Comparison of FeNO concentration, the percentage weight gained with the proportion of LB^{+ve} tubercle bacilli among AFB positive patients after completion of treatment

The mean proportion of LB^{+ve}tubercle bacilli was comparatively higher (39.50%; range 10-75%) among TB patients that demonstrated positive AFB score after completion of the TB treatment. However, there was no significant association between the percent weights gained to that of the proportion of LB^{+ve}tubercle bacilli.

AFB score	FeNO (ppb)	LB+ (%)	Spoligotype	HIV Status	Percent weight gained		
					2nd	5th	7th
Positive	29	10	SIT new	Negative	7	5.3	5.3
Positive	41.3	36	SIT 952	Negative	0.95	5.5	5.5
Positive	16	38	SIT 25	Negative	6.12	6.12	6.12
Poitive	13	75	SIT 50	Positive	1.8	6.8	8.3
Positive	37	39	SIT new	Unknown	5.6	-----	-----
Positive	32	-----	SIT new	Unknown	-----	-----	-----

The mean percentage weight gained by TB patients that demonstrated negative AFB after completion of the initial phase of treatment was relatively higher than patients that showed positive AFB after completion of the same course of TB treatment; 9.05 kg (SD 8.36 Kg) (=26) versus 3.9 kg (SD 3.03) (n=5) (table 7.3). However, there was no significant association between sputum AFB score and percent weight gain in both cases.

Table 7.3: Comparison of the percentage weight gained with FeNO concentration and the proportion of LB^{+ve} tubercle bacilli among AFB negative patients after completion of treatment

The mean proportion of LB^{+ve} tubercle bacilli was comparatively lower among TB patients that demonstrated negative AFB score (n=19) after completion of the TB treatment. However, there was no significant association between the percent weights gained to that of the proportion of LB^{+ve} tubercle bacilli.

AFB score	FeNO (ppb)	LB (%)	Percent weight gained		
			2nd	5th	7th
Ngegative	29	10	7	5.3	5.3
Ngegative	12	35	15.6	19.2	19.2
Ngegative	41.3	36	0.95	5.5	5.5
Ngegative	16	38	6.12	6.12	6.12
Ngegative	14	16	16.1	21.7	20.3
Ngegative	16	5	0	2.0	3.85
Ngegative	24.7	35	38	38	38
Ngegative	5	65	20	20	21.4
Ngegative	10	47	9.6	20.3	20.3
Ngegative	5	24	8.1	12.8	17.1
Ngegative	13	75	1.8	6.8	8.3
Ngegative	13	42	7.1	7.1	13.8
Ngegative	8	25	3.8	3.8	3.8
Ngegative	28	42	5.6	5.6	5.6
Ngegative	8	18	14.9	16.7	20
Ngegative	5	17	6.6	16.2	11.8
Ngegative	5	45	7.4	7.4	7.4
Ngegative	5	13	0	0.2	6.25
Negative	14	30	2.2	4.35	12

Three out of the six patients that demonstrated positive AFB after treatment was infected by the new *Mtb* spoligotype. The average FeNO concentration of TB patients infected by the new spoligotype was 32.7 ppb which was higher than the average FeNO concentration of patients infected by other *Mtb* strains ($\bar{x}$=23.4 ppb). On the other hand, the weight gained after 7 months treatment among patients infected by the new *Mtb* (n=3) spoligotype

was by far lower than the average weight gained by patients infected with other *Mtb* spoligotypes (n=3) (2.3 kilogram versus 7 kilogram respectively).

7.3.5. The anti-microbial susceptibility pattern of mycobacterial isolates among pulmonary TB patients

The anti-microbial sensitivity pattern of *Mycobacterium* isolates was determined using the Genotype MTBDRplus test method. In this method, the drug susceptibility pattern was tested for rifampicin and isoniazid only. 87 sputum samples showed growth of mycobacteria but the anti-microbial susceptibility test was conducted only on 84 isolates. Ten of the isolates (11.9%) showed either mono or multi-drug resistance.

7.3.6. Rifampicin / Isoniazid mono-resistance

The anti-microbial susceptibility patterns of 84 new smear positive TB patients showed that 5/84 (5.9%) showed rifampicin mono-resistance but only one isolate was resistant to isoniazid. All TB patients that demonstrated mono-drug resistance were HIV negative. In this study, *Mtb* mono-drug resistance (resistance to rifampicin or isoniazid) was 6/84 (7.14%) among new smear positive pulmonary TB patients.

7.3.7. Multi-drug resistance among new smear positive TB patients

The drug susceptibility pattern of 4 TB patients demonstrated resistance both for rifampicin and isoniazid (multi-drug resistance) making the prevalence of MDR TB infection among new smear positive TB patients 4.76 %(4/84). Moreover, 3 MDR-TB patients were HIV positive and the other one MDR-TB patient was HIV negative.

7.3.8. FeNO concentration and the proportion of LB^{+ve} tubercle bacilli in sputum compared with TB drug resistance

Among 10 TB patients that showed either mono or multi-drug resistance, FeNO concentration and the proportion of LB^{+ve}tubercle bacilli were determined only for 7 patients (table 7.3). The average FeNO concentration among rifampicin mono- resistant TB patients (n=3) was 7 ppb and a TB patient with isoniazid mono-resistance showed FeNO

concentration of 6 ppb. The average proportion of the corresponding LB^{+ve} tubercle bacilli among rifampicin mono-resistant patients was 27.3 %. However, there was no significant association between FeNO concentration and the proportion of LB^{+ve}tubercle bacilli among rifampicin mono-resistant TB patients. The average FeNO concentration among MDR-TB patients (n=3), all HIV positive, was 13.3 ppb, but the corresponding mean proportion of LB^{+ve}tubercle bacilli was relatively high (62.33 %). Nevertheless, there was no significant association between FeNO concentration and the proportion of LB^{+ve}tubercle bacilli among MDR-TB patients co-infected with HIV.

Table 7.4: Comparison of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli among TB patients infected with drug resistant tubercle bacilli

FeNO concentration was compared with mono and multi-drug resistance among HIV^{+ve} and HIV^{-ve} TB patients (n=8). Although FeNO concentration was not associated with the proportion of LB^{+ve}tubercle bacilli, higher proportions of LB^{+ve} tubercle bacilli was observed among MDR cases. R= Resistance; S=Sensitive.

FeNO (ppb)	LB+ve (%)	Spoligotype	RIF	INH	HIVStatus
13	75	SIT 50	R	R	+ve
6	11	SIT 289	S	R	-ve
5	41	SIT 53	R	R	+ve
22	71	SIT 1547	R	R	+ve
8	20	SIT 21	R	S	-ve
5	22	SIT 134	R	S	-ve
8	40	SIT 26	R	S	-ve
Unknown	----------	----------	R	R	-ve
Unknown	----------	SIT new	R	S	-ve
Unknown	----------	--------------	R	S	-ve

The mean FeNO concentration among TB patients that demonstrated either mono-drug resistance or multi-drug resistance (n=7) was 9.6 ppb (95% CI 3.9-15.24). New smear positive TB patients (n=66) that were sensitive for both rifampicin and isoniazid had an average of 13.85ppb (95% CI 10.9-16.83) FeNO concentration. When FeNO concentration data from TB patients that showed drug resistance was compared with that of drug sensitive patients, there was no statistically significant difference observed (figure 7.8). The

average proportion of LB^{+ve}tubercle bacilli among TB patients that showed drug resistance (n=7) was 40% (95% CI 16.87-63.13) which was higher than the average proportion of LB^{+ve} tubercle bacilli (31.42%; 95% CI 27.5-35.4) among drug sensitive TB patients (n=66). However, there was no statistically significant difference in the average proportion of LB^{+ve}tubercle bacilli between drug resistant and sensitive TB patients.

(A)   **(B)**
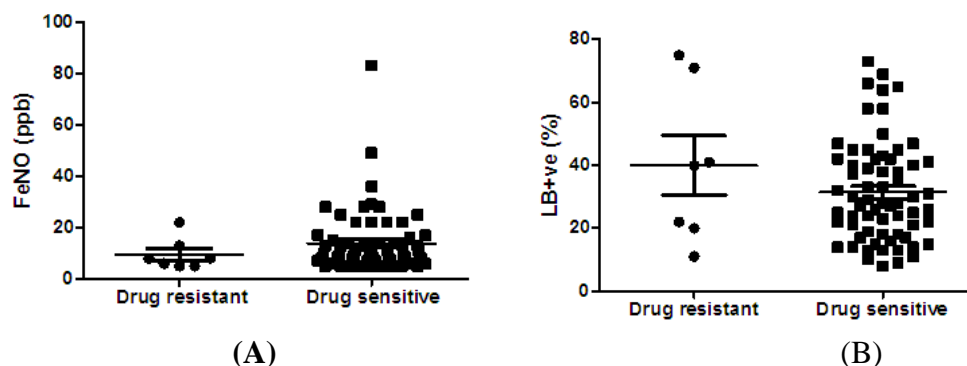
Figure 7.8: Drug resistance compared with FeNO concentration and the proportion of LB^{+ve} tubercle bacilli

FeNO concentration and the proportion of LB^{+ve}tubercle bacilli among TB patients infected with drug resistant (A) and drug sensitive tubercle tubercle bacilli (B).

7.3.9. Multivariate analysis

Taking the proportion of LB^{+ve} tubercle bacilli as dependant variable and others such as age, sex, HIV sttus, intestinal parasite infection, *Mtb* spoligotype, RIF and INH drug sensitivity pattern, eosinophil count and FeNO concentration as independent variables, multivariate linear regression analysis showed a significant association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli (P=0.008; r^2=0.236) among TB patients that were sensitive to both RIF and INH. However, because the numbers of drug resistant TB patints were too small, we can not get sufficient information to compute multivariate regression analysis.

7.4. Discussion

There are six possible outcomes during TB treatment. These outcomes are mutually exclusive and are especially pertinent in countries with high incidence of TB infection. TB treatment outcome categories are cure, treatments completed, failure, death, treatment interrupted, and transfer out (WHO, 1994; Enarson, 1991).The same six categories ought to be used in low-incidence countries, even though more detailed subsets may be chosen. TB treatment outcome is generally expressed as a proportion of the total number of notified cases.When the patient completes the full course of TB treatment and demonstrates a negative culture or two AFB negative sputum smears at the end of the continuation phase, they can be graded as cured.Treatment completed TB patients are patients that completed the prescribed treatment but showed no bacteriological conversion occurred or no smear result available at the end of the continuation phase. Interruption of TB treatment for more than two months for any reason or if the drug intake was below 80% of the prescribed dose, shoud be graded as a default case.TB treatment failure is a case that comes about when the patient's sputum culture fails to show bacteriological conversion within 5 months after treatment initiated or sputum smear becomes positive for AFB. A patient who died of any cause during the course of TB treatment is recorded under death. When the patient disappears and no additional information is available, the case is lost to follow up.Transfers out patients are patients that traveled to another health facility from the health facility that they got diagnosis (WHO, 1994).

Important challenges for successful treatment and TB control include HIV co-infection and drug resistance (Corbett et al, 2003). In this retrospective study, 14 of the 45 smear positive TB patients (31.1%) were HIV positive. HIV infection was reported as independent risk factor for the development of TB and HIV-related immune-suppression as a critical risk factor for mortality (Mukadi et al, 1997).

Treatment outcome of the current study showed that 11.8% of the TB patients (4/34) were positive for AFB after completion of the intensive phase; one HIV[+ve] and 3 HIV[-ve]/TB patients. Moreover, the sputum AFB score of two patients were also positive after completion of both the intensive and contentious phase of TB therapy among the HIV

status unknown patients. The overall sputum conversion rate was 88.2% (30/34) after completion of the 2 month intensive phase therapy; 9 HIV^{+ve} and 21 HIV^{-ve}/TB patients. Previously, Okawera et al (1994) reported that sputum conversion at the end of the 2 month treatment was similar for HIV positive and HIV negative TB patients. On the other hand, Bwire et al (1999) reported that HIV sero positive status is not a principal factor in delaying sputum conversion among patients receiving intensive phase of TB treatment.

Relatively, an average of low FeNO concentration was observed (11.91ppb) among TB patients that demonstrated negative AFB score after treatment. A comparative and relatively high mean FeNO concentration (27.26 ppb versus 11.91 ppb) was observed among TB patients that demonstrated positive AFB score after treatment. The average proportion of LB^{+ve} tubercle bacilli was also relatively higher (39.6% versus 30.4%) among TB patients that demonstrated a positive AFB score after treatment as compared with that of TB patients with negative AFB score after treatment. Although difficult to relate LB accumulation to drug resistance at this level, the result of the current study can be used as a base line for further investigations. The small number of patients with drug resistant TB bacilli found in this study would make difficult to qualify or hinder whether the high proportion of LB^{+ve} tubercle bacilli is associated with drug resistance. However, there are several reports that showed tubercle bacilli when cultured demonstrated no lipid body accumulation. Neverthless, INH and RIF drug tolerance as a result of LB accumulation was reported previously as documented in the previous sections of this thesis.

The role of FeNO during TB infection and the significance of LBs in phenotypic drug tolerance were documented in the previous sections. Besides, FeNO has been implicated as a pulmonary biomarker in various respiratory diseases, including chronic obstructive pulmonary disease (COPD) (Antus et al, 2010). The average proportion of LB^{+ve}tubercle bacilli among HIV^{+ve}/TB patients was relatively higher than the corresponding proportion of LB^{+ve}tubercle bacilli among HIV^{-ve}/TB patients. Comparatively low FeNO concentration among HIV^{+ve}/TB patients than HIV^{-ve}/TB patients was also observed among new smear positive TB patients. This result was different from the result of the previous prospective studies that showed no significant difference of FeNO concentration among HIV^{+ve} and

HIV^{-ve}/TB patients. Therefore, another study is required to determine the exact contribution of FeNO concentration and the proportion of LB^{+ve} tubercle bacilli associated with the treatment outcome of TB infection probably with large sample size.

When the mean initial weight of HIV^{+ve}/TB patients was compared with the mean initial weight of HIV^{-ve}/TB patients, there was no significant difference. However, the average weight of HIV status unknown TB patients, all of them military by occupation, was significantly different from HIV^{+ve}/TB patients (P= 0.01032; r^2=0.213) but not from that of HIV^{-ve}/TB patients. The weight gained among HIV^{+ve}/TB and HIV^{-ve}/TB patients was not also significantly different. This is supported by Elliott et al (1995) report that although HIV^{+ve} patients are more likely than HIV^{-ve}/TB patients to die before completion of TB treatment, surviving HIV^{+ve} patients gained weight and experienced improvement in symptoms at the same rate as did surviving HIV^{-ve} /TB patients.

7.4.1. Drug resistance TB infection

TB successful treatment depends on several factors. Some of the factors include the drug susceptibility pattern of bacterial strain, the anti-TB drug regimen in use and the duration of intake, the availability of the essential drugs, adherence of both the patient and the treatment provider to recommended standards of care. Cure is achieved by an adequate treatment regimen, based on a properly selected combination of drugs; with the correct dosage for a sufficient duration (Veen et al, 1998).The prevalence of rifampicin mono-resistance among new smear positive TB patients in Gondar was relatively high. Earlier reports revealed a primary drug resistance rate of 15 and 12% for Isoniazid and 5 and 9.4% for streptomycin in Ethiopia (Wolde et al, 1986). In Addis Ababa, the overall primary drug resistance was found to be 15.6% and primary resistance to two or more drugs was 7.2% in 1997 (Demissie et al, 1997). In Gondar, a 15.8% resistance to at least one first-line drug and a 5.0% multi-drug resistance were recently reported (Tessema et al, 2012). Rifampicin is an antibiotic that is active against bacteria principally through its ability to interfere with nucleic acid metabolism by inhibiting DNA directed RNA polymerase (Konno K et al,

1973). Previous reports indicated that the most important cause for TB treatment failure and deadly clinical outcome is resistance to refampicin (Mitchison and Nunn, 1986). A mutation in the β-sub unit of the RNA polymerase (rpoB) gene is the central region for refampicin resistance (Telentiet al, 1993). Zhang and Telenti (2000) reported that more than 96% of refampicin-resistant *Mtb* strain contains a mutation in this 81 bp (27codon) region of rpoB. Although, mono-resistance to isoniazid is reported quite common, mono-resistance to rifampicin is infrequent. In many cases rifampicin resistance arise among *Mtb* strains that are also resistant to isoniazid and rifampicin resistance can be used as a surrogate marker of multi-drug resistance (Somoskovi et al, 2000).

The prevalence of MDR TB infection among new smear positive TB patients in this study was 4.76% (4/84). Three out of the 4 MDR-TB patients were HIV positive. The prevalence of MDR-TB infection among new smear positive pulmonary TB cases in Gondar can be graded high. A high level of MDR-TB prevalence was defined previously as >3% overall prevalence (Nachega and Chaisson, 2003). HIV infection was shown to be a risk factor for having drug-resistant TB independent of geographic location, history of prior therapy, age or race. Nolan et al (1965) reported that acquired rifampicin resistance is somehow associated with co-infection due to HIV and TB. This was also supported by Bishai et al's (1996) report which states that HIV-seropositive patients are more likely to develop acquired drug resistance than seronegative cases. Different reports in Ethiopia have shown that drug resistance is on the increase. Primary MDR-TB was first reported as 2% in samples taken from Addis Ababa and Harer (Wolde et al, 1986). In 1997, 0.4% of patients from Harer and 1.2% from Addis Ababa (Demissie et al, 1997) were reported to have primary MDR-TB. In 2003, the percentage of MDR-TB in new cases was estimated to be 2.3% (WHO, 2003). The 2003-2006 nationalTB survey estimated an MDR-TB about 1.6% of new TB cases in Ethiopia (FMOH, 2008). In 2006, 5825 MDR-TB cases (4964 among newly diagnosed and 861 among previously treated TB cases) were estimated to have occurred in Ethiopia. Another survey showed that among 804 newly diagnosed TB cases, 1.6% had MDR-TB. The rate of MDR-TB among specimens from 76 previously treated TB cases was 11.8% (FMOH, 2008).

The concentration of FeNO among TB patients infected with mono-drug resistant *Mtb* spoligotype was lower than the corresponding concentration of FeNO concentration among patients infected with MDR-TB *Mtb* spoligotypes. The average proportion of LB^{+ve} tubercle bacilli was also found relatively higher among patients infected with MDR-TB *Mtb* spoligotypes compared with that of mono-drug resistant TB infection. Our previous work showed significant association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli in sputum and LB^{+ve} tubercle bacilli was reported tolerance of isoniazid and rifampicin previously (Sherratt, 2008). In this study, the drug susceptibility patterns of the mycobacterial isolates were carried out after culturing the organisms. The tubercle bacilli at this state are expected actively replicating but the gene expression pattern is very difficult to understand. Therefore, the exact contribution of *in vivo* nitric oxide and accumulation of lipid bodies in sputum to TB drug resistance remains unanswered which needs further study. However, the presence of relatively high proportion of lipid loaded tubercle bacilli among MDR cases in sputum may direct a sort of relation with drug resistance.

In the treatment outcome study, the small sample size was the main limitation of the current study. The GenoTypeMTBDRplus test only detects those resistances of the *Mtb* complex that have their origins in the *rpoB, katG* and *inhA* regions. Resistance originating from mutations of other genes or gene regions as well as other rifampicin and isoniazid resistance mechanisms will not be detected by this test. Moreover, the presence of multiple bacterial species in the sample to be analyzed might hamper the interpretation of the test.

7.4.2. Conclusions

1. The mean initial weight of HIV^{-ve}/TB patients was significantly different from the corresponding average weight after completion of treatment but the case was not seen on HIV^{+ve}/TB patients.

2. Tuberculosis mono-drug resistance was 7.4% and MDR-TB 4.76%.

3. There was no significant association between FeNO concentration or proportion of LB^{+ve} tubercle bacilli to either treatment outcome or drug susceptibility pattern.

4. Higher proportions of LB^{+ve} tubercle bacilli were observed among MDR-TB patients.

CAPTER EIGHT: General Discussion

8.1. General Discussion

The current study investigated factors that influence the occurrence of LBs within the tubercle bacilli in sputum. Fluorescence microscopy was used to visualize LBs within the tubercle bacilli and the NIOX MINO air way inflammation monitor to determine FeNO concentration. Because some sputum samples had very difficult background after Auramine O/Nile red stain, we investigated the application of detergent wash and lipase digestion for better microscopy. Although detergent wash and extended lipase digestion of sputum samples brought a significant reduction of the background of sputum smears, the reduction of the number of acid fast bacilli as a result of lipase digestion rendered the procedure inapplicable in most cases.

In this study FeNO concentration was as low as 5ppb and as high as 221 ppb among smear positive TB patients. Age, gender, malnutrition, smoking status, history of asthma, history of prison and other factors were investigated for their association with FeNO concentration. We found significant association between FeNO concentration compared with the age and gender of the TB patients. Detailed explanation about the aforementioned factors versus FeNO concentration has been given in the previous sections (Chapter 3 and 4). There are also other factors that may contribute for high or low levels of nitric oxide. High levels of exhaled NO have been demonstrated in chronic bronchial asthma (Alving et al, 1993). Altered levels of exhaled NO have also been found in smokers compared with non smokers. Moreover, Erzurum et al (2007) observed an increased level of NO among peoples living at high altitude. For example, Tibetans who live at altitude around 14, 000 feet have 10 times more NO. Lower levels of FeNO have been linked with altered pulmonary mechanics in numerous pathologic conditions (Brett and Evans, 1998). Decreased levels of NO were also reported in children with cyanotic congenital heart disease (Puthucheary et al, 2006). Reduced plasma level NO have been found in obstructive apnoa which is defined as complete cessation of oronasal flow in the presence of thoracoabdominal breathing movements (Schulz et al,2000).

8.2. Over all association of FeNO concentration with the proportion of LB[+ve] tubercle bacilli in sputum and multiple logistic regression analysis

The minimum and maximum FeNO concentrations regardless of HIV status and intestinal parasite co-infection were 5 ppb and 83ppb respectively with an average/median concentration of 15.32/12.0 ppb among new smear positive TB patients. The minimum and maximum proportions of the corresponding LB[+ve] tubercle bacilli in sputum were 2% and 80%. In the current study, the preliminary finding was the significant association of FeNO concentration with the proportion of LB[+ve]tubercle bacilli among smear positive pulmonary TB patients. On the first 116 TB patients HIV status and intestinal parasite infection were not investigated. Latter, on 88 TB patients the magnitude of HIV/TB and intestinal parasite/TB co-infection were determined. The association of FeNO concentration with the proportion of LB[+ve] tubercle bacilli among intestinal parasite/TB co-infected patients and the absence of significant association of FeNO concentration with the proportion of LB[+ve] tubercle bacilli among intestinal parasite negative TB patients is another notable finding of this research. Intestinal parsitic infection could result a change in immunological responses particularly cytokine induction from Th1 to Th2 cells. CD4+ T cells can differentiate into T helper 1 (Th1), Th2, Th17 and Treg cells depending on the type of stimuli initiated the differentiation. The Th1 cell response results in IFN-γ production and activation of macrophages, with the subsequent killing of intracellular bacteria by phagolysosomal fusion and effector mechanisms of the macrophage. On the other hand, the Th2 cell response produces B cell stimulating cytokines such as Ll-4, IL-5, IL-10 and IL-13 that can suppress the Th1 immune response (Ducati et al, 2006). Depending on the type of stimuli from T cells, macrophages polarize to M1 (classically activated) or M2 (alternatively activated) phenotypes. Stimulation of macrophages with IFN-γ, TNF-α or IL1-β will lead to the M1 phenotype. The M1 polarization induced by Th1 cytokines leads to iNOS expression, NO and ROS production and anti-microbial activity. The M2 polarization is induced by Th2 cytokines and has regulatory properties with up-regulation of arginase and limited antimicrobial activity beneficial for tissue repair but not for bacterial killing (Benoit et al, 2008).

Linear regression analysis of data on 112 TB patients regardless of their HIV status and intestinal parasite-TB co-infection showed that the overall association of FeNO concentration with the proportion of LB^{+ve}tubercle bacilli in sputum was significant (p=0.0003; r^2=0.115) (Figure 8.1).
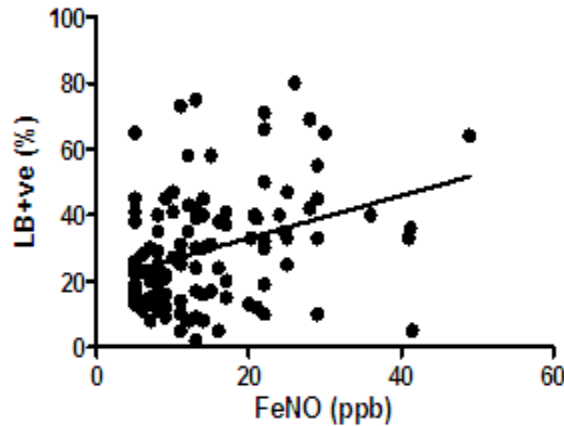
Figure 8.1: Over all association of FeNO concentration with the proportion of LB^{+ve} TB bacilli

The overall association of FeNO concentration with the proportion of LB^{+ve}tubercle bacilli in sputum was significant.

The average LB^{+ve} tubercle bacilli proportion among HIV^{+ve}/TB patients (39.6%) was significantly different (p=0.021) from the corresponding HIV^{-ve}/TB patients (27.65%). Taking the proportion of LB^{+ve} tubercle bacilli as dependant variable, multivariant regression analysis showed a significant association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli. We also observed that among HIV^{+ve}/TB patients, for each additional units of FeNO concentration there can happen twice increase in the proportion of LB^{+ve} tubercle bacilli in sputum (the slope HIV^{+ve} versus HIV^{-ve} = 1.8 and 0.78 respectively) . However, since LB enumeration was made manually throughout the experiments in this study, there might be unceratinity.

The age and sex of the TB patients were also significantly associated with FeNO concentration (p=0.040 and 0.022 respectively). However, multivariant linear regression analysis showed no significant association between the age and sex of the TB patients with

the proportion of LB^{+ve} tubercle bacilli in sputum. On the other hand, in a multivariant regression analysis (n=96), FeNO concentration was significantly associated with the proportion of LB^{+ve}tubercle bacilli in sputum (p=0.017), the age (p=0.010) and sex (p=0.003) of the TB patients.

8.3. Comparison of FeNO concentration and the proportion of LB^{+ve} tubercle bacilli among new smear positive and retreatment TB patients

The association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli was also compared among previously treated and new TB patients. Accordingly, the average FeNO concentration among new smear positive TB patients (15.27ppb; 95% CI 13.61-16.93 ppb) (n=149) was lower than the retreatment cases (21.62 ppb; 95% CI 17.38-25.86 ppb) (n=38). Two research groups have investigated FeNO concentration in pulmonary TB patients and reported inconsistent findings. Wang and colleagues reported that FeNO concentration was correlated with disease stage and severity. They also found that FeNO to be significantly higher in people with newly diagnosed TB (Wang et al, 1998). In contrast, Idh and colleagues (2008) reported inconsistent FeNO concentration results in Ethiopian adults with smear positive pulmonary TB. Therefore, we still lack definite evidence to give remarks about FeNO concentration among new and retreatment TB infection.The mean proportion of LB^{+ve} tubercle bacilli among retreatment cases was 29% (95% CI 17.84-40.16pp) versus 29.75% (95% CI 26.27-33.24%) among new TB cases. The association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli was stronger among retreatment cases compared with new smear positive TB patients (r^2=0.59 versus 0.256 respectively). However, our sample size may not be large enough (n=10) to conclude the role of the proportion of LB^{+ve} tubercle bacilli in tuberculosis treatment.

8.4. FeNO concentration among HIV^{+ve} compared with HIV^{-ve}/TB patients

The average FeNO concentration among HIV^{+ve} and HIV^{-ve}/TB patients was relatively similar in the current study. Previously, Idh et al (2008) reported that HIV^{-ve}/TB patients had significantly lower levels of nitrite and nitrate in urine than household contacts to TB

patients and healthy blood donors in Gondar, Ethiopia. There are reports that recognized HIV infection increases systematic production of nitric oxide (Groeneveld et al, 1996) and macrophages infected with HIV express iNOS and produce NO (Bukrinsky et al, 1995). *In vitro* and *in vivo* over production of nitric oxide has been reported in the presence of HIV-1 infection (Torre et al, 2002). The mechanism of virus infection mediated by NO may be related to: direct antiviral effects of NO, impairment of antiviral defense mediated by T-helper-1 immune response and suppressing T-helper-1 function (Torre et al, 2002). Macrophages play a crucial part in persistence and tissue dissemination in HIV-1 infection and TNF-α could trigger NO production from HIV infected macrophages (Bukrinsky et al, 1995). Blond and colleagues have shown substantial induction of the iNOS gene in primary cultures of human monocyte-derived macrophages, concomitantly with the peak of virus replication and exposure to low concentrations of NO donors results in significant increase of HIV-1 replication (Blond et al, 2000). Moreover, Jimenez and co-workers (2001) reported that NO acts as an autocrine factor and mediates HIV-1 replication.

On the other hand, Reiss and Komatsu reported that nitric oxide may inhibit an early stage in viral replication and thus prevent viral spread, promoting viral clearance and recovery of the host (Reiss and Komatsu, 1998). Akarid and colleagues noted the anti-retrovirus properties of nitric oxide (Akarid et al, 1995). Moreover, Jiménez et al (2001) reported that NO acts as an autocrine factor that mediates HIV-1 replication and several NO-generating compounds at low to moderate concentrations were able to activate HIV-1 replication in normal T cells as well as in human T-cell lines.

8.5. Association between eosinophil score with *Mtb* spoligotype

The average eosinophil score among TB patients infected with the CAS and EAL *Mtb* spoligotype were relatively similar (6.4% versus 6.2% respectively). The corresponding mean FeNO concentration was also relatively similar (13.8ppb versus 12.64ppb respectivel). However, the mean proportion of LB^{+ve} tubercle bacilli among patients infected with the EAL was relatively higher than that of CAS (35.6% versus 28.2% respectively). The median eosinophil score among TB patients infected with the LAM and

T3-Ethiopia *Mtb* spoligotype (mean 12% and 10% respectively) was by far higher than the eosinophil score of TB patients infected with other *Mtb* spoligotypes. In addition, when the eosinophil score of TB patients infected with different *Mtb* spoligotype were compared using Bartlett's statistics (assuming homogeneity of variances of eosinophils among patients infected with different *Mtb* spoligotype), there was significant difference (P=0.039).The eosinophil score observed among TB patients infected with the CAS *Mtb* spoligotype was even lower than the others (Figure 8.2). However, we found significant association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli among TB patients infected with the CAS *Mtb* spoligotype as documented in Chapter 4. This might suggest that eosinophils may not be the central factor or may have little significance to initiate NO production during TB infection by some specific *Mtb* spoligotypes such as the CAS.
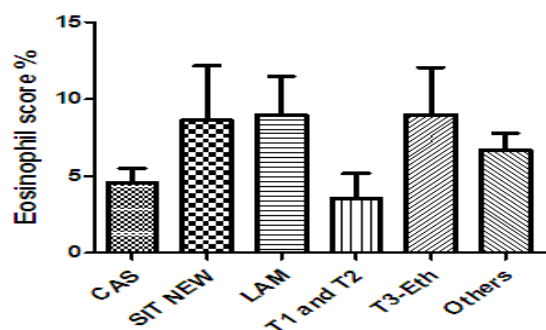
Figure 8.2: Eosinophil score among TB patients infected with different *Mtb* spoligotype

The eosinophil score of TB patients infected with CAS (n=17), SIT New (n=8), LAM (n=5), T1 and T2 (n=7), T3-Ethiopia (n=5) and other (n=24) *Mtb* spoligotye.

Eosinophils are potent effector cells as the result of the release of several cytotoxic mediators upon activation. Eosinophil peroxidase (EPO), one of the most abundant granule protein in human eosinophil exhibits bactericidal activity against *Mtb* H37Rv (Violetta et al, 2003). Eosinophil cytoplasmic granules contain high amounts of cytotoxic proteins, in particular cationic proteins (major basic protein [MBP], eosinophil peroxidase [EPO], and eosinophil derived neurotoxin [EDN]) (Gleich and Adolphson, 1986). Moreover, eosinophils have been evidenced in different mycobacterial infection models and human

eosinophil expression of α-defensins towards mycobacterial species seems strain dependant (Kisich et al, 2002). Alpha-defensins are also expressed by neutrophils and one of the α-defensins of the neutrophils, the human neutrophil pepetide-1 (HNP-1) has been shown to prevent growth of *Mtb* (Miyakawa et al, 1996). Moreover, Zasshi (1993) reported that the HNP-1 had the ability to kill *Mycobacterium avium* complex but the degree of killing varied from strain to strain. However, we believe that our data may not be strong enough to answer questions that can be raised on the influence of eosinophils on *Mtb* spoligotype.

8.6. The influence of *Mtb* spoligotype on FeNO concentration and the proportion of LB^{+ve}tubercle bacilli in sputum

Data from the current study showed no significant difference of the average FeNO concentration among TB patients infected with different spoligotypes of *Mtb*. However, the influence of *Mtb* spoligotype variation on the outcome of infection is an emerging area of interest. In a study designed to investigate the influence of *Mtb* strains outcome of infection (Idh et al, 2012), cultured clinical isolates were exposed to NO *in vitro*. In fifty clinical strains exposed to NO there was a time and dose-dependant susceptibility to NO. The median survival after 24 hours exposure to 1mM DETA/NO was 10%. Previously, Voskuil et al (2011) reported that dormancy regions are up-regulated after NO exposure suggesting that NO rather has a bacteriostatic than a bactericidal effect on *Mtb*. Moreover, it was hypothesised that a host infected with a NO tolerant strain of *Mtb* would present a more severe disease or slower recovery compared to a host infected with a NO susceptible strain (Idh et al, 2012). The mechanism by which the *Mtb* strains resist to NO was not reported. In the present study, a significant association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli was observed among TB patients infected with the CAS *Mtb* spoligotypes. On the other hand we observed an inverse association of FeNO with the proportion of LB^{+ve} tubercle bacilli among TB patients infected with the EAL. Nitric oxide can trigger *Mtb* to switch from active cell division to a state of non-replicating persistence (Voskuil et al, 2003). However, *in vivo* accumulation of LBs within specific *Mtb* strains as a result of NO need to be determined in large scale studies.

8.7. Antibiotic resistance associated with FeNO concentration and the proportion of LB^{+ve} tubercle bacilli among different spoligotypes

Drug resistances to first-line TB drugs (INH and RIF) were distributed over 10 spoligotype patterns. INH resistance was observed on 5 spoligotypes whereas RIF resistance was seen on 9 spoligotypes and multidrug resistance was observed on 4 spoligotype patterns.Three out of 4 MDR-TB patients were HIV positive and had an average of 62.3% proportion of LB^{+ve} tubercle bacilli which was higher than the corresponding mono-resistant TB patients that demonstrated an average of 27.3% LB^{+ve}tubercle bacilli proportion. However, there was no statistically significant difference in FeNO concentration and the proportion of LB^{+ve} tubercle bacilli among patients infected with drug resistant *Mtb* spoligotypes compared with the drug sensitive ones. The recent report by Idh et al (2012) documented that resistance to first-line TB drugs distributed over 6 spoligotype based clusters showed clear association between NO tolerance and resistance to first-line TB drugs, INH in particular. INH-resistant strains showed a median survival of 53% when exposed to NO compared to 10% in INH-sensitive strains. By the same report, it was also documented that reduced NO susceptibility could be linked to antibiotic resistance, and in particular to INH resistance. Although lipid loaded tubercle bacilli were reported tolerant to anti-TB drugs, the contribution of intracellular lipid inclusions to NO susceptibility or resistance remains to be determined in the future. Nitric oxide was proved toxic to the tubercle bacilli and the *Mtb* evolved ways to evade the toxic effects of reactive nitrogen intermediates (Wayne and Hayes, 1998) and exposure of tubercle bacilli to NO reached the organism in the production of LBs. Here, we can speculate that the link between reduced NO susceptibility or resistance to NO which were linked by Idh et al to antibiotic resistance, particularly to INH resistance could be attributed by the intracellular LB accumulation as a result of nitric oxide exposure. This speculation can be explained by our finding on the association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli in sputum samples. It is also possible to imagine that *Mtb* produced LBs and acquired resistance to NO to avoid its toxic effects. Since RNI are potent lipid peroxidising and nitrating agents(Borrell and Gagneux, 2011), it could be also speculated that variation of NO susceptibility could be

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due to modification of the cell wall and compensatory up regulation of alternative defence mechanisms. This requires further investigation to understand the interactions between different drug resistance mutations and compensatory mutations to be able to predict the global epidemics of MDR TB and XDR TB as well as for identification of new drug targets.

The contribution of NO to the killing of *Mtb* in human TB infection still remains controversial. Some studies demonstrated that certain strains of *Mtb* differ in susceptibility to NO, but how this correlates to drug resistance and clinical outcome is not known. However, increased tolerance to NO was associated with INH resistance mediated by *inhA*. *InhA* encodes enoyl-acyl reductase, a NADH-dependent enzyme essential for mycolic acid biosynthesis for the cell wall (Almeida and Palomino, 2011). Collectively, we guess that the NO resistant *Mtb* strains reported by Idh et al (2012) could be loaded with lipophilic intracellular molecules but the other NO sensitive strains might be negative for LBs. However, the degree of accumulation of LBs within different *Mtb* spoligotype needs to be determined at large.

8.8. Significance of FeNO and the proportion of LB^{+ve} tubercle bacilli on treatment outcome against *Mtb* infection

In the present study 6 TB patients demonstrated positive AFB score after completion of treatment. The median FeNO concentration among these TB patients was relatively high (30.5 ppb) and the corresponding median proportion of LB^{+ve} tubercle bacilli was 38%. However there was no statistically significant association between FeNO concentrations with the proportion of LB^{+ve} tubercle bacilli among TB patients that demonstrated positive AFB score after completion of the initial phase of treatment. Moreover, we found no significant correlation between FeNO concentrations or the proportion of LB^{+ve} tubercle bacilli to that of treatment outcome which was measured by sputum AFB conversion and weight gain. Previously, Idh et al (2012) reported no significant correlation between susceptibility to NO and treatment outcome or sputum conversion, but found that 4 TB patients who did not increase in weight were all infected with NO tolerant *Mtb* strains. In

conclusion, we found no convincing evidence on the impact of FeNO concentration and the proportion of LB^{+ve}tubercle bacilli or difference of the infecting *Mtb* spoligotype on treatment response.

8.9. Arginine supplementation and nitric oxide mediated LB accumulation within the tubercle bacilli

Inducible NOS enzyme catalyses the synthesis of NO and citrulline from L-arginine in macrophages activated by cytokines such as IFN-γ and TNF-α (Nathan and Shiloh, 2000). Schon et al (2003) reported that a randomized double-blind study results showed that in HIV negative patients with smear positive TB, arginine supplementation had a significant and favorable effect on weight gain, sputum conversion, and reduction of symptoms like cough. Boshoff and Barry also documented that *Mtb* is likely to encounter NO *in vivo*, as it is released by the activated macrophages (Boshoff and Barry, 2005). Therefore, in addition to hypoxia, nitric oxide may also act as an environmental signal of immune activation and allow *Mtb* to adapt its metabolism to the anticipated change (Voskuil et al, 2003). On the other hand, *Mtb* formed LBs after 4 hours nitric oxide treatment as described previously. This LB^{+ve} population of tubercle bacilli was tolerant to isoniazid and rifampicin. Moreover, restoration of antibiotic susceptibility and decline of LBs occurred when the nitric oxide dissipates (Sherratt, 2008). Phenotypic drug tolerance is thought to be elicited by an environmental signal that induces the cells to enter an antibiotic refractory growth phase (Michele et al, 1999). The result of the current study showed a significant association between FeNO concentration and the proportion of LB^{+ve} tubercle bacilli in sputum samples. Collective evidence raise now question for the benefit of arginine supplementation during TB therapy which was anticipated to increases the bactericidal activities of the anti-TB drugs.

8.10. NO synthase inhibitors versus LBs within the tubercle bacilli

Aminoguandine (AG) and N^{G}-monomethyl-L-arginine (NMMA) are potent nitric oxide synthase enzyme inhibitors. Although information concerning the pharmacodynamics and

metabolism of AG and NMMA is still incomplete, both compounds have been used extensively *in vitro* and *in vivo* to inhibit RNI production (Evans et al, 1993; Beckerman et al, 1993). These two NOS inhibitors differ not only in their chemical structure but also in their effects on the various isoforms of NOS. Thus, AG has been shown to be relatively selective at inhibiting iNOS. The relative inhibitory effect of AG on iNOS compared with that on the constitutively expressed isoforms was reported 40:1 (Corbett et al, 2003), while such selectivity has not been shown for NMMA. In addition, over production of the free radical nitric oxide (NO) has been implicated in the pathogenesis of variety of inflammatory and immunologically mediated disease. Aminoguandine is equivalent to N^G-Monomethyl-L-arginine (NMMA) as an inhibitor of the cytokine-induced isoforms of NO synthase but is 10 to 100-fold less potent as an inhibitor of the constitutive isoforms. Thus, aminoguandine may be useful as a selective inhibitor of the iNOS in the treatment of disease states characterized by the pathological overproduction of nitric oxide (Misko et al, 1993). Considering the effect of NO on the accumulation of LBs within the tubercle bacilli and the resulted drug tolerance as a result of LBs, nitric oxide synthase inhibitors may probably diminish the effect of NO so that the tubercle bacilli will be less likely to accumulate LBs *in vivo*. However, the contribution of RNI inhibitors during TB treatment needs to be determined in the future.

8.11. Accumulation of lipid bodies and loss of acid-fastness of *Mtb*

In the current study, dual Auramine O/ Nile red staining of sputum smears has been used to reveal the acid-fast bacilli and LB accumulation in the same cell. During this microscopy we found a significant proportion of either poorly acid-fast bacilli or TB like non-acid fast bacilli. Bell previously (unpublished data) reported that in one occasion Auramine O staining was applied from a long deep freezed sample and only small proportion of *Mtb* (less than 20%) were acid-alcohol fast. More over, Deb et al (2009) demonstrated that when a young, synchronous culture of *Mtb* was subjected to the multiple-stress condition for increasing periods of time, a steady decrease in Auramine O stained green-fluorescing acid-fast cells with a corresponding increase in Nile red stained red-fluorescing LB

containing cells were reported. In that experiment, initially, in the freshly grown starter culture about 90% of the population was acid-fast positive and after 18 days under multiple-stress, acid-fast positive cells decreased to about 30% of the population while Nile red stained cells with LBs increased from 10% to 70%. The investigators documented that the difference in dual staining property indicated generation of at least three different sub-population in the *Mtb* cultures under multiple-stress conditions: a subset that stained only with Auramine O (probably actively multiplying), a second subset which stained with both Auramine O and Nile red (probably transitioning to non-replicating state) and a third subset that stained only with Nile red (probably non-replicating and dormant). In addition, standard bacteriology tells us that non-replicating bacterial cell take longer to initiate growth than their replicating counterparts (longer lag phase) (Weichart and Kell, 2001; Mukamolova et al, 2002). Threfore, we suggest here the accumulation of LBs within the tubercle bacilli that may be due to NO exposure may possibly encompass for the prominent effect in the diagnosis of the tubercle bacilli in sputum especially in area where the least sensitive Zeihl-Neelsen method is used such as in resource poor settings.

8.12. Conclusions

The work presented in this thesis has fulfilled the major objective of the study which was to determine whether FeNO concentration has an association with the proportion of LB^{+ve} tubercle bacilli in sputum. The specific findings of this study were the following:

►The first part of this study showed that FeNO concentration in some TB patients was moderately and in two patients highly elevated. However, the majority of TB patients (64.7 %) demonstrated relatively low exhaled nitric oxide concentration.

► FeNO concentration was significantly associated with sex, age and the proportion of LB^{+ve} tubercle bacilli in sputum. However, smoking status, history of asthma, fasting/postprandial state, history of prison and BCG vaccination were not associated with FeNO concentration among TB patients.

►The loss of acid-fastness following LB accumulation is another important finding we observed in live sputum samples which may have an impact in the diagnosis of TB particularly in resource poor countries.

► The prevalence of HIV infection among new smear positive pulmonary TB patients in Gondar was 37.5% (33/88).

►The prevalence of intestinal parasite co-infection among smear positive new pulmonary TB patients in Gondar was high (44.3%; 39/88). Intestinal parasite infection prevalence among HIV^{+ve} and HIV^{-ve}/ TB patients was relatively similar (41.4% versus 39.62% respectively).

►The mean level of FeNO concentration in both HIV^{+ve} and HIV^{-ve}/TB patients were low. The mean FeNO concentration for HIV^{+ve}/TB patients co-infected with intestinal parasites was slightly higher (13 ppb) than that of HIV^{+ve}/TB patients with no intestinal parasite infection (11.76 ppb). However, there was no statistically significant association between intestinal parasite infection and FeNO concentration among HIV^{+ve} pulmonary TB patients.

► The mean FeNO concentration among HIV^{-ve}/TB patients co-infected with intestinal parasites was relatively higher (15.48 ppb) than HIV^{-ve}/TB patients with no intestinal parasitic infection (9.82 ppb). Moreover, FeNO concentration was significantly associated with intestinal parasitic co-infection (P=0.015) among HIV^{-ve}/TB patients.

►The mean absolute eosinophil count for HIV^{+ve}/TB patients was relatively higher than the corresponding HIV^{-ve}/TB patients. However, data showed no statistically significant difference on eosinophil count between the HIV^{+ve} and HIV^{-ve}/TB patients. On the other hand, high eosinophil count was observed on HIV^{-ve}/TB patients co-infected with intestinal parasites compared with HIV^{-ve}/TB patients with no intestinal parasite. Moreover, eosinophil count was significantly correlated with FeNO concentration among HIV^{-ve}/TB patients co-infected with intestinal parasite (p=0.031) compared with HIV^{-ve}/TB patients

with no intestinal parasite infection. Conversely, there was no significant association between eosinophil score with the proportion of LB^{+ve} tubercle bacilli.

► The average proportion of LB^{+ve} tubercle bacilli among HIV^{+ve}/TB patients was higher (39.8%) than HIV^{-ve}/TB patients which was 27.02 %. In addition, linear regression analysis showed that in HIV^{+ve} intestinal parasite negative and HIV^{-ve} intestinal parasite positive TB patients, FeNO concentration was significantly associated with the proportion of LB^{+ve}tubercle bacilli in sputum (p= 0.0014 and 0.02; r^2= 0.32 and 0.12 respectively).

► In some TB patients that had low FeNO concentration, high proportion of LB^{+ve} tubercle bacilli in sputum were observed which may indicate the potential role of other factors in triggering *Mtb* to accumulate LBs *in vivo*.

► The dominant *Mtb* spoligotype causing pulmonary TB in Gondar was the CAS and EAL. Twenty-five *Mtb* isolates were new spoligotypes that were not reported previously and the spoligotype pattern of these new *Mtb* were different from the recently reported Woldiya lineage.

► The spoligotype pattern of new smear positive TB patients showed that there was no single dominant *Mtb* spoligotype exclusively found in HIV^{+ve} or HIV^{-ve}/TB patients.

► FeNO concentration was significantly associated with the proportion of LB^{+ve}tubercle bacilli in sputum among patients infected with the CAS *Mtb* spoligotype (P<0.01; r2=0.323; Pearson r=0.57) but not among patients infected with the EAL.

►The other part of this study was dedicated to demonstrate the association of FeNO concentration and the proportion of LB^{+ve}tubercle bacilli in sputum with that of *Mtb* gene expression patterns. However, there was no significant correlation between the hspX, tgs1 and icl1 gene concentrations and the concentration of the corresponding FeNO as well as the proportion of LB^{+ve}tubercle bacilli in sputum both in HIV^{+ve} and HIV^{-ve}/TB patients. In Gondar there were continuous power interruptions during storage of *Mtb* samples for RNA

extraction. Although the RNA samples were stabilised in GTC, the effect of this is unknown.

►Treatment outcome of the current study showed that 11.8% of the TB patients (4/34) were positive for AFB after completion of the intensive phase. A comparatively high mean concentration of FeNO was observed among TB patients that demonstrated positive AFB score after treatment than TB patients that showed no AFB after treatment and the average proportion of LB^{+ve}tubercle bacilli was also relatively higher among AFB positive patients after treatment. However, there was no statistically significant association between FeNO concentration and the proportion of LB^{+ve}tubercle bacilli among TB patients that demonstrated positive AFB score after treatment.

►The anti-microbial susceptibility patterns of 84 new smear positive TB patients showed that 5/84 (5.9%) showed rifampicin mono-resistance but only one patient was resistant to isoniazid. The drug susceptibility pattern of 4 TB patients demonstrated resistance both for refampicin and isoniazid (multi-drug resistance). Moreover, 3 out of 4 MDR-TB patients were HIV positive. However, there was no significant association between FeNO concentration and the proportion of LB^{+ve}tubercle bacilli among rifampicin mono-resistant TB patients and MDR-TB patients.

8.13. Future work

Lipid bodies as candidate virulence factors for *Mtb*

The work presented in this thesis demonstrated a weak but significant association of FeNO concentration with the proportion of LB^{+ve} tubercle bacilli in sputum. The association was also significant among TB patients infected with the CAS spoligotype but not among TB patients infected with the EAL *Mtb* spoligotype. Previous reports showed that some *Mtb* strains are more virulent than oethers. However, the role of LBs as virulence factors during TB infection is not yet confirmed. Therefore, understanding the role of LBs as virulence

factors for *Mtb* infection in general and its connotation in severity and pathogenesis of TB infection among specific *Mtb* spoligotype in particular are planned for future work.

Virulence is the measure of pathogenicity of a microorganism as determined by its ability to invade host tissues and to produce severe disease (Palanisamy, 2008). It remains unclear exactly why *Mtb* is virulent. Various suggestions have been put forward, including the ability to divide (relatively) rapidly in the infected cell, its intracellular niche, the ability to enter dormancy and its possession of a complex cell wall (Sirakova et al, 2002; Smith; 2003). Park et al (2006) reported that rapid intercellular growth rate and induction of necrotic cell death within host macrophages are virulence factors of *Mtb* in the early stages of bacterial infection. Without a doubt, a better identification of *Mtb* virulence factors is essential to understand the pathogenesis of TB and may reveal prominent components of the host defense system.

Virulence of *Mtb* can be determined by the differences in survival in experimentally infected mice or guinea pigs (North et al, 1999). Weight loss and clinical signs are also considered the most common correlates of disease progression (Baldwin et al, 1998). Antigen-specific delayed-type hypersensitivity (DTH) (Smith, 1985) and antigen-induced production of IFN-γ by T cells (Martin et al, 2000) have also been used as the determinants of protective immunity with IFN-γ production generally being considered a better correlate of resistance in mice (McMurray, 2001).

Recent investigations and the current study results clearly showed the association of NO with the proportion of LB^{+ve} tubercle bacilli in sputum. There are also reports that documented the existence of heterogeneous tubercle bacilli in sputum in terms of LB accumulation. This is suggestive for the influence of environmental factors where the tubercle bacilli location may contribute for LB accumulation. Moreover, there is no clear evidence as yet to demonstrate that LB^{+ve} *Mtb* bacilli *in vivo* are in the same physiological state as those observed in laboratory conditions. The critical question that can be raised here is, are LB^{+ve} tubercle bacilli more pathogenic than LB^{-ve} in human host? If lipid loaded tubercle bacilli are more pathogenic than LB^{-ve} tubercle bacilli in humans, it will be very wise to consider LBs within the tubercle bacilli as candidate virulence factors during TB

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infection. In fact, it has been shown that hypoxically grown *Mtb* cultures which were LB^{+ve} were 10-fold more infectious for guinea pigs than their aerobically grown counterparts (Bacon et al, 2004). Therefore, the future study will concentrate to evaluate the potential contribution of LBs to TB disease severity or pathogenesis which can be determined by animal model studies together with clinical presentation of the diseases and treatment outcome comparisons.

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Appendices

Appendix 1: Demographic characteristics and BMI of smear positive pulmonary tuberculosis patients in Gondar, Ethiopia, February to June 2008.

No	Sex	BMI	AFB	TB V	TBT	SMS	AM	COPD	Bro	Pneu	DM	FAS
1	M	16.69	4+	no	no	Stop-6M	no	no	No	No	no	No
2	F	13.12	2+	yes	yes	no	no	no	No	No	no	Yes
3	M	14.29	-ve	yes	no	no	no	no	No	No	no	Yes
4	F	19.81	2+	yes	no	no	no	no	No	No	no	No
5	F	16.99	2+	yes	no	no	no	no	No	No	no	Yes
6	M	20.56	1+	yes	no	no	no	no	No	No	no	No
7	F	16.21	3+	no	no	no	no	no	No	No	no	No
8	F	13.52	2+	Yes	Yes	no	no	no	No	No	no	Yes
9	F	13.06	2+	Yes	no	no	no	no	No	No	no	No
10	M	13.88	2+	no	no	no	no	no	No	No	no	Yes
11	M	17.30	1+	no	no	no	no	no	No	No	no	No
12	F	19.70	4+	no	no	no	no	no	No	No	no	Yes
13	F	15.05	4+	no	no	no	no	no	No	No	no	No
14	F	13.56	2+	yes	yes	no	no	no	No	No	no	No
15	F	18.45	4+	yes	no	no	no	no	No	No	no	No
16	F	21.10	4+	no	no	no	no	no	No	No	no	No
17	M	15.70	4+	yes	yes	Stop-2Y	yes	no	No	No	no	No
18	M	15.50	2+	yes	yes	no	yes	no	No	No	no	Yes
19	M	18.75	3+	yes	yes	no	no	no	No	No	no	No
20	M	15.15	4+	no	no	Stop-2Y	no	no	No	No	no	Yes
21	F	18.75	2+	no	no	No	no	no	No	No	no	Yes
22	M	23.70	3+	no	no	Stop-1M	no	no	No	No	no	Yes
23	M	15.82	2+	yes	yes	no	yes	no	No	No	no	Yes
24	F	16.66	1+	no	no	no	no	no	No	No	no	No
25	M	15.00	-ve	no	no	no	no	no	No	No	no	Yes
26	F	17.53	1+	no	no	no	no	no	No	No	no	No
27	F	10.94	3+	no	no	no	yes	no	No	No	no	No
28	M	14.85	2+	yes	yes	Stop-5y	no	no	No	No	no	No
29	M	18.10	3+	yes	yes	no	yes	no	No	No	no	No
30	M	17.75	4+	no	no	smoking	no	no	No	No	no	Yes
31	M	16.18	3+	no	no	no	no	no	No	No	no	Yes
32	F	17.65	3+	no	no	no	no	no	No	No	no	No
33	M	14.88	2+	no	no	no	no	no	No	No	no	No
34	M	17.63	-ve	no	no	no	no	no	No	No	no	No
35	F	14.95	1+	yes	yes	no	no	no	No	No	no	No
36	F	17.63	2+	no	no	no	no	no	No	No	no	No
37	M	15.06	-ve	no	no	no	no	no	No	No	no	No
38	F	15.24	4+	no	no	no	no	no	No	No	no	No
39	M	19.47	1+	no	no	no	no	no	No	No	no	No
40	M	15.67	3+	yes	yes	no	no	no	No	Yes	no	Yes
41	M	17.30	3+	no	no	no	no	no	No	No	no	No
42	M	17.65	1+	yes	yes	no	no	no	No	No	no	No
43	F	14.28	4+	no	no	no	no	no	No	No	no	No
44	M	19.56	1+	no	no	no	no	no	No	No	no	No
45	M	12.52	1+	yes	yes	no	no	no	No	No	no	No
46	M	15.42	1+	no	no	no	no	no	No	No	no	No
47	F	15.06	-ve	no	no	no	no	no	No	No	no	No
48	M	14.05	1+	no	no	no	no	no	No	No	no	No
49	M	14.28	2+	no	no	no	no	no	No	No	no	No
50	M	13.32	1+	no	no	no	no	no	No	No	no	No

51	M	16.65	-ve	yes	yes	no	no	no	No	No	no	No
52	F	17.69	1+	no	no	no	no	no	No	No	no	No
53	M	14.66	2+	no	no	no	no	no	No	No	no	No
54	M	19.03	4+	no	no	no	no	no	No	No	no	No
55	M	17.65	4+	yes	yes	no	no	no	No	No	no	No
56	F	12.28	-ve	no	no	no	no	no	No	No	no	No
57	F	18.59	4+	no	no	no	no	no	No	No	no	No
58	F	18.22	4+	no	no	no	no	no	No	No	no	No
59	M	17.45	3+	yes	yes	no	no	no	No	No	no	No
60	M	16.22	1+	yes	yes	Stop-1M	no	no	No	No	no	No
61	M	15.99	4+	no	no	no	no	no	No	No	no	No
62	M	12.35	2+	yes	yes	Stop-6M	no	no	No	No	no	No
63	M	16.20	3+	yes	no	no	no	no	No	No	no	No
64	M	18.50	1+	no	no	no	no	no	No	No	no	No
65	M	18.00	1+	no	no	Stop-3W	no	no	No	No	no	No
66	M	17.75	2+	no	no	no	no	no	No	No	no	No
67	M	17.71	2+	no	no	smoking	no	no	No	No	no	No
68	M	18.44	3+	no	no	no	no	no	No	No	no	No
69	M	16.77	2+	yes	yes	no	no	no	No	No	no	No
70	F	21.64	3+	no	no	no	no	no	No	No	no	No
71	M	19.58	4+	no	no	no	no	no	No	No	no	No
72	F	18.67	1+	no	no	no	no	no	No	No	no	No
73	F	19.53	3+	no	no	no	no	no	No	No	no	No
74	F	15.21	1+	no	no	no	no	no	No	No	no	No
75	F	17.83	3+	no	no	no	no	no	No	No	no	No
76	M	17.19	4+	no	no	no	no	no	No	No	no	No
77	M	21.44	4+	yes	yes	no	no	no	No	No	no	No
78	M	19.03	2+	no	no	no	no	no	No	No	no	No
79	F	15.63	4+	no	no	no	no	no	No	No	no	No
80	F	16.65	2+	no	no	no	no	no	No	No	no	No
81	M	17.85	-ve	no	no	no	no	no	No	No	no	No
82	F	17.33	4+	no	no	no	no	no	No	No	no	No
83	F	15.82	4+	no	no	no	no	no	No	No	no	No
84	F	14.45	3+	No	no	no	no	no	No	No	no	No
85	M	20.70	3+	No	no	no	no	no	No	No	no	No
86	F	21.00	4+	No	no	no	no	no	No	No	no	No
87	F	13.20	4+	Yes	yes	No	no	no	No	No	no	No
88	M	16.76	4+	Yes	yes	no	no	no	No	No	no	No
89	F	20.51	4+	No	no	no	no	no	No	No	no	No
90	F	19.50	4+	No	no	no	no	no	No	No	no	No
91	M	17.30	4+	Yes	yes	no	no	no	No	No	no	No
92	M	22.04	-ve	Yes	yes	no	no	no	No	No	no	No
93	M	21.34	-ve	No	no	no	no	no	No	No	no	No
94	M	15.74	1+	Yes	no	no	no	no	No	No	no	No
95	M	17.30	3+	Yes	yes	no	no	no	No	No	no	No
96	F	16.44	4+	No	no	no	no	no	No	No	no	No
97	F	19.82	4+	No	no	no	no	no	No	No	no	No
98	F	15.80	1+	Yes	yes	no	no	no	No	No	no	No
99	M	17.90	2+	No	yes	no	no	no	No	No	no	No
100	F	18.09	2+	Yes	yes	no	no	no	No	No	no	No
101	F	18.51	4+	No	no	no	no	no	No	No	no	No
102	F	16.00	1+	No	no	no	no	no	No	No	no	No
103	M	19.03	-ve	Yes	yes	no	no	no	No	No	no	No
104	M	15.43	3+	No	no	Stop-3M	no	no	No	No	no	No
105	M	18.40	1+	No	no	no	no	no	No	No	no	No
106	F	22.10	2+	No	no	no	no	no	No	No	no	No

107	M	16.52	2+	No	no	no	no	no	No	No	no	No
108	F	14.57	4+	Yes	yes	no	no	no	No	No	no	No
109	M	19.10	-ve	No	no	no	no	no	No	No	no	No
110	M	17.90	4+	No	no	no	no	no	No	No	no	No
111	M	16.42	1+	No	no	no	no	no	No	No	no	No
112	F	18.42	1+	No	no	no	no	no	No	No	no	No
113	M	15.04	-ve	No	no	no	no	no	No	No	no	No
114	M	20.40	2+	Yes	yes	no	no	no	No	No	no	No
115	M	17.90	4+	No	no	no	no	no	No	No	no	No
116	F	19.34	3+	No	no	no	no	no	No	No	no	No

TBV=TB visit; TBT=TB treatment; SMS+Smoking status; AM=Asthma; Bro=Bronchiectasis; Pneu=Pneumonia; DM= Diabetes mellitus; FAs=Fasting status.

Appendix 2: FeNO concentration and the proportion of LB^{+ve} tubercle bacilli together with the spoligotype isolates among smear positive pulmonary tuberculosis patients in Gondar, Ethiopia, February to June 2008.

No	FeNO	LB+ve	LB-ve	Auramin -^{ve}/LB+ve/100AFB	M.tb strain
1	41	33	367	12	Anomalous
2	13	_____	____	_____	SIT 25
3	142	_____	____	_____	SIT 53
4	10	_____	____	_____	SIT 159
5	12	_____	_____	_____	Anomalous
6	25	_____	_____	_____	Anomalous
7	10	_____	_____	_____	SIT 25
8	12	_____	_____	_____	SIT 25
9	14	_____	_____	_____	SIT 53
10	22	30	70	NO	SIT 25
11	27.5	_____	_____	_____	SIT 523
12	25	25	75	70	Anomalous
13	21	_____	_____	_____	SIT 37
14	33	_____	_____	_____	Anomalous
15	9.5	_____	_____	_____	SIT 26
16	24	_____	_____	_____	Anomalous
17	11	5	95	13	Anomalous
18	27.5	_____	_____	_____	SIT 149
19	41.5	5	95		SIT 50
20	26	_____	_____	_____	Anomalous
21	22	_____	_____	_____	SIT 523
22	16	5	95	25	SIT 25
23	10	_____	_____	_____	Anomalous
24	12	_____	_____	_____	SIT 522
25	16	_____	_____	_____	M.bovis?
26	8.5	_____	_____	_____	SIT 53
27	30	_____	_____	_____	SIT 41
28	29	45	55	29	SIT 25
29	35	_____	_____	_____	SIT 25
30	7	20	80	NO	SIT 25
31	19	_____	_____	_____	SIT 53
32	12	_____	_____	_____	SIT 149
33	19	_____	_____	_____	Anomalous
34	22	_____	_____	_____	SIT 50
35	21	_____	_____	_____	SIT 25
36	26	_____	_____	_____	Anomalous
37	25	_____	_____	_____	Anomalous
38	21	_____	_____	_____	SIT 53
39	20	_____	_____	_____	M.bovis SBO133
40	24	40	60	2	SIT 316
41	14	_____	_____	_____	SIT 26
42	57	_____	_____	_____	SIT 149
43	12	8	92	No	SIT 291
44	17	_____	_____	_____	SIT 25
45	8	_____	_____	_____	M.bovis?
46	20	_____	_____	_____	M.bovis SBO 133
47	13	_____	_____	_____	SIT New

48	10	_____	_____	_____	SIT 53
49	6	_____	_____	_____	SIT 910
50	9	_____	_____	_____	SIT 343
51	45	_____	_____	_____	M.bovis SBO 133
52	26	_____	_____	_____	Anomalous
53	15	_____	_____	_____	SIT 21
54	20	13	87	1	SIT 25
55	17	_____	_____	_____	SIT 149
56	14	_____	_____	_____	SIT 25
57	15	17	83	No	SIT 25
58	26	80	20	15	SIT 25
59	7	15	85	NO	Anomalous
60	9	_____	_____	_____	M.bovis?
61	37	_____	_____	_____	SIT New
62	21	39	61	No	Anomalous
63	22	19	81	NO	SIT 25
64	15	_____	_____	_____	SIT New
65	11	_____	_____	_____	SIT New
66	11	_____	_____	_____	SIT 1688
67	39	_____	_____	_____	Anomalous
68	7	8	92	No	SIT 244
69	29	55	45	No	SIT New
70	9	9	91	6	SIT 21
71	29	10	90	3	SIT New
72	14	_____	_____	_____	SIT 291
73	13	2	98	No	SIT 1539
74	11	_____	_____	_____	M.bovis SBO 133
75	9	12	88	No	SIT 35
76	16	_____	_____	_____	SIT 149
77	14	_____	_____	_____	SIT New
78	13	_____	_____	_____	SIT 75
79	24.7	35	65		SIT 25
80	8.3	_____	_____	_____	Anomalous
81	11.7	_____	_____	_____	M.bovis SBO 133
82	12	35	65	5	SIT 53
83	5.3	_____	_____	_____	SIT New
84	7	20	80	No	SIT 25
85	41.3	36	64	No	SIT 952
86	21	12	88	11	Anomalous
87	11	25	75	51	SIT 53
88	13	30	70	NO	SIT 26
89	15	31	69	16	Anomalous
90	20.7	40	60	12	Anomalous
91	16	38	62	_____	SIT 25
92	61	_____	_____	_____	SIT 53
93	5	_____	_____	_____	SIT 159
94	15	_____	_____	_____	Anomalous
95	32	_____	_____	_____	Anomalous
96	17	15	85	_____	SIT 25
97	20.3	33	67	_____	SIT 25
98	11	_____	_____	_____	SIT 53
99	17	_____	_____	NO	SIT 25
100	8	_____	_____	_____	SIT 523
101	14	_____	_____	70	Anomalous
102	19	8	92	_____	SIT 37
103	21	_____	_____	_____	Anomalous

104	14	____	____	_____	SIT 26
105	22	16	84	_____	Anomalous
106	221	____	____	13	Anomalous
107	21	____	____	_____	SIT 149
108	10	____	____		SIT 50
109	14	41	59	_____	Anomalous
110	11	____	____	_____	SIT 523
111	12	10	90	25	SIT 25
112	8	____	____	_____	Anomalous
113	12	____	____	_____	SIT 522
114	18	____	____	_____	M.bovis?
115	30	65	35	_____	SIT 53
116	17	20	80	_____	SIT 41

Appendix 3: Demographic characteristics, blood cell count, intestinal parasite infection among new smear positive tuberculosis patients in Gondar, Ethiopia.

No	Age	Sex	WBC	Neutro	Lympho	Eosinoph	Inte P	RBC	HGB	HCt
1	56	M	5900	47	30	20	pos	2.59	7.2	22.2
2	18	M	7600	78	20	2	neg	4.59	12.8	40.9
3	38	F	4500	64	30	6	neg	3.67	10.4	32.3
4	20	M	7800	64	29	7	neg	6.2	16.6	51.2
5	88	M	6700	53	32	15	Pos	4.43	12.5	39.5
6	28	F	10500	89	8	3	neg	3.83	8.2	27.2
7	21	M	6400	66	29	5	neg	5.83	14.3	45.2
8	37	M	5100	46	43	11	Pos	5.38	14.5	44
9	16	M	6300	74	21	5	neg	6.37	13.6	42.6
10	35	F	5900	69	28	2	Pos	4.63	11.9	38.4
11	28	M	6600	57	34	8	Pos	4.59	13	38.64
12	58	F	8200	59	34	7	neg	4.52	12.6	37.9
13	22	M	7500	63	27	10	neg	5.6	14.5	47
14	39	M	8300	65	22	12	neg	3.1	9.7	29.4
15	50	F	6100	52	36	11	Pos	4.96	11.5	36.6
16	11	M	10300	67	22	10	neg	4.44	11.7	35.4
17	30	F	6600	52	33	11	neg	3.97	8.7	29.2
18	40	F	8800	59	28	12	neg	3.8	8.5	28.8
19	22	F	4800	55	43	2	neg	4.98	13.7	44.3
20	20	M	6900	68	29	1	neg	6.7	12.2	39.2
21	65	M	6500	61	25	13	neg	5.7	16.2	48.1
22	48	M	10900	34	35	31	pos	5.26	13.8	42.3
23	60	M	13300	70	25	5	Pos	5.3	12.8	39.1
24	40	M	5800	61	37	2	neg	5.46	15.9	48.1
25	52	M	5900	61	38	1	neg	5.02	14	42.1
26	18	F	6800	57	36	6	Pos	4.77	11.4	36.3
27	40	M	7800	52	33	14	Pos	5.28	13.7	46.3
28	60	M	7800	60	20	16	neg	4.84	10.3	34.6
29	42	M	8400	37	60	3	neg	5.47	15.3	46.9
30	19	F	7900	76	23	1	neg	4.9	11.4	37.7
31	60	M	6600	75	20	5	neg	4.02	10.2	32.5
32	60	M	1100	60	33	6	Pos	5.1	14.6	42.2
33	35	M	4400	86	12	2	Pos	3.15	7.2	23.4
34	15	F	14100	70	26	3	neg	5.29	14.1	42.8
35	60	M	6400	27	54	19	Pos	3.89	9	29.4
36	25	M	4800	48	41	10	Pos	4.19	11.3	36.1
37	30	F	9200	48	12	4	neg	4.43	11.9	34.6
38	40	M	6500	68	26	6	neg	3.9	10.9	33.4
39	20	M	6600	76	24	0	neg	2.67	6.5	21.9
40	54	F	6600	70	26	4	neg	5.34	14.3	45.2
41	15	M	7000	71	26	3	neg	5.44	13.9	42
42	17	F	8800	78	12	0	Pos	5.04	14.1	41.7

43	15	F	5500	69	30	1	neg	5.19	12.7	39.2
44	19	F	5500	42	55	1	neg	5.12	14.5	43.2
45	22	M	9900	64	30	6	neg	5.27	13.9	41.4
46	23	M	7600	47	48	5	neg	4.83	12.9	39.4
47	20	M	11500	73	15	12	Pos	4.87	11.2	34.6
48	20	F	10900	83	17	0	neg	4.87	11.1	34.6
49	20	M	8600	76	21	3	neg	5.27	13.7	40.4
50	22	M	8300	69	23	7	Pos	4.3	11.7	37.3
51	28	F	11400	61	30	8	Pos	4.69	12.6	37.4
52	31	M	14900	69	24	4	Pos	2	5.6	17.8
53	54	F	7100	61	26	11	Pos	4.23	12	36.9
54	20	F	10200	42	30	25	neg	4.83	12.5	41.1
55	26	M	13200	80	17	2	Pos	5.46	11.3	36.7
56	19	F	7300	64	33	3	neg	4.88	11.4	37
57	22	F	9700	79	20	1	neg	3.54	6.9	24
58	25	M	6800	73	26	1	neg	3.83	9.4	29.8
59	21	M	3900	55	40	5	Pos	5.2	13.8	41.4
60	20	M	6500	65	32	3	Pos	5.02	13.8	42.1
61	25	F	6400	77	21	2	Pos	4.74	12.7	38
62	28	F	8800	85	13	2	neg	4.68	12.7	40.8
63	23	F	8800	77	20	3	Pos	4.83	13.4	39.4
64	32	M	4100	68	30	2	Pos	3.77	9.8	28.4
65	27	F	7500	69	26	5	Pos	3.98	13.4	41.8
66	23	F	7900	65	34	1	Pos	4.26	10.6	33.4
67	26	M	3900	70	28	2	neg	4.85	12.6	40.4
68	40	M	3000	49	37	13	Pos	3.79	11.4	32.9
69	62	M	7300	83	7	10	neg	5.02	12.4	38.2
70	20	F	9800	70	21	9	Pos	4.84	11.9	38.3
71	13	F	12600	64	26	10	Pos	4.39	9.3	31.1
72	27	F	12400	88	10	2	neg	4.47	12	37.9
73	19	M	14900	83	11	5	Pos	5.61	14.2	44.4
74	30	M	9100	72	24	3	neg	4.22	9.4	32.8
75	30	M	4700	80	19	1	Pos	3.8	8.8	28.9
76	18	F	7900	65	26	9	neg	4.63	11.2	38.2
77	27	M	5500	73	27	0	neg	3.91	9.4	31.9
78	52	M	9400	60	30	10	neg	5.92	16.1	49.3
79	32	F	5800	72	28	0	neg	4.5	11.6	37.1
80	38	F	5900	59	34	6	neg	3.39	7.6	25.7
81	23	M	6900	54	34	12	Pos	4.3	11.4	36.9
82	20	M	10900	88	10	2	neg	4.2	10.2	33.4
83	30	F	10500	91	6	3	Pos	4.74	10.5	35
84	22	F	12100	85	12	3	neg	4.01	9.4	31
85	45	M	11400	83	9	8	Pos	4.08	9.3	29.3
86	50	M	7400	63	25	11	Pos	3.96	9.8	31
87	51	F	7600	78	20	2	neg	4.29	9.8	31.3
88	44	M	2400	58	41	1	neg	3.25	10.6	32.8

Appendix 4: FeNO concentration and the corresponding proportion of LB[+ve] tubercle bacilli among HIV[+ve] and HIV[-ve]/TB patients and the spoligotype pattern of *Mycobacterium tuberculosis* strains causing pulmonary tuberculosis among smear positive new patients in Gondar, Ethiopia.

No	FeNO (ppb)	LB (%)		Spoligotype	HIV status
1	25		47	---------------	Negetive
2	15		58	SIT 35	Negetive
3	10		47	SIT 289	Positive
4	9		21	SIT 289	Negetive
5	17		37	SIT 26	Negetive
6	5		24	SIT 289	Positive
7	11		27	SIT 1	Negetive
8	11		25	--------	Negetive
9	6		23	SIT 289	Negetive
10	9		45	SIT 118	Negetive
11	11		73	SIT 118	Negetive
12	10		27	SIT 910	Negetive
13	6		28	SIT 53	Negetive
14	5		13	SIT 1787	Positive
15	13		9	SIT 289	Negetive
16	22		10	SIT new	Negetive
17	5		26	SIT 41	Negetive
18	5		19	SIT 41	Negetive
19	22		66	SIT 134	Positive
20	11		14	SIT 4	Negetive
21	8		15	SIT new	Negetive
22	29		33	SIT 952	Negetive
23	6		14	SIT 910	Negetive
24	83		38	SIT 289	Negetive
25	13		75	SIT 50	Positive
26	11		28	SIT 41	Positive
27	28		69	------	Positive
28	17		41	SIT new	Positive
29	11		31	SIT new	Positive
30	6		11	SIT 289	Negetive
31	22		50	SIT new	Positive
32	49		64	SIT 289	Positive
33	8		18	SIT 289	Positive
34	16		24	SIT 149	Negetive
35	22		32	SIT 1905	Negetive
36	9		22	----------	Negetive
37	5		41	SIT 53	Positive
38	13		24	SIT new	Negetive
39	8		11	SIT 173	Negetive
40	13		17	SIT 118	Negetive
41	8		29	SIT 289	Positive
42	7		22	SIT 289	Positive
43	8		21	SIT 289	Negetive
44	8		25	SIT 289	Negetive
45	13		42	SIT 1688	Positive
46	22		71	SIT 1547	Positive

47	28	42	SIT 53	Negative
48	14	45	SIT 52	Positive
49	8	18	SIT 26	Negative
50	12	8	SIT 35	Positive
51	5	17	SIT 42	Positive
52	25	33	SIT 149	Negative
53	28	42	SIT 50	Negative
54	9	16	SIT 21	Positive
55	12	43	--------	Negative
56	5	45	SIT new	Positive
57	5	38	SIT 1134	Positive
58	14	40	SIT 289	Positive
59	7	13	SIT 149	Negative
60	8	20	SIT 21	Negative
61	5	65	SIT 53	Positive
62	5	26	SIT new	Positive
63	5	22	SIT 134	Negative
64	8	35	SIT 289	Positive
65	7	30	SIT 21	Positive
66	5	13	SIT 53	Negative
67	8	40	SIT 26	Negative
68	12	58	SIT 149	Positive
69	13	39	SIT 35	Negative
70	5	15	SIT 159	Negative
71	36	40	SIT 50	Negative
72	7	14	-----------	Negative
73	14	30	-----------	Negative
74	Not done _____		-----------	Negative
75	Not done _____		----------	Negative
76	Not done _____		SIT 289	Negative
77	Not done _____		SIT new	Positive
78	Not done _____		----------	Negative
79	Not done _____		SIT 54	Positive
80	Not done _____		SIT 44	Postive
81	Not done _____		SIT 159	Negative
82	Not done _____		---------	Negative
83	Not done _____		SIT 952	Negative
84	Not done _____		SIT new	Negative
85	Not done _____		---------	Negative
86	Not done _____		-------	Negative
87	Not done _____		-------	Negative
88	Not done _____		-------	Positive

1.19. **Appendix 5**: The total spoligotype pattern and average FeNO concentration with the corresponding proportion of LB[+ve] tubercle bacilli among smear positive pulmonary tuberculosis patients in Gondar, Ethiopia.

SIT	N	FeNO (ppb) $\bar{x} \pm SD$	N	LB (%) $\bar{x} \pm SD$
SIT 25	22	17.83 ± 7.23	13	29.08 ± 19.37
SIT 26	6	11.58 ± 3.66	4	31.25 ± 9.78
SIT 289	15	12.27 ± 11.28	15	28.13 ± 14.17
SIT 149	12	18.88 ± 13.04	6	28.50 ± 17.56
SIT new	22	17.16 ± 9.35	13	31.92 ± 18.36
SIT 21	5	9.6 ± 3.13	4	18.75 ± 8.77
SIT 41 + 42	6	11.17 ± 9.68	5	20.80 ± 5.97
SIT 52 + 53	15	15.03 ± 13.89	7	36.00 ± 16.64
SIT 50	5	28.10 ± 11.26	5	40.50 ± 28.59
Others	49	14.64 ± 8.48	26	29.42 ± 20.68
Total	**157**		**98**	

Appendix 6: The 16S rDNA, RD750, RD9 and spoligotype pattern of Mycobacterial DNA extracts obtained directly from sputum samples (n=.48).

No	16S rDNA	RD750	RD9	Spoligotype
1	MTBC	Intact	MTB	Anomalous
2	NTM	Negative	MTB	SIT 25
3	NTM	Negative	MTB	SIT 53
4	MTBC	Deleted	OMB	Anomalous
5	MTBC	Deleted	MTB	SIT 25
6	MTBC	Negative	MTB	SIT 25
7	NTM	Negative	MTB	Anomalous
8	NTM	Negative	MTB	Anomalous
9	NTM	Negative	OMB	SIT 50
10	NTM	Intact	OMB	Anomalous
11	NTM	Deleted	OMB	SIT 523
12	NTM	Negative	OMB	SIT 25
13	MTBC	Negati ve	MTB	SIT 25
14	MTBC	Deleted	MTb	SIT 25
15	NTM	Negative	MTb	SIT 53
16	NTM	Negative	MTb	SIT 53
17	Negative	Negative	OMB	SIT 26
18	NTM	INTACT	MTB	SIT 291
19	NTM	Intact	MTB	SIT 25
20	MTBC	Negative	MTB	SIT 149
21	MTBC	Negative	MTB	SIT 25
22	MTBC	Negative	MTB	SIT 25
23	Negative	Negative	MTB	Anomalous
24	MTBC	Negative	MTB	SIT new
25	NTM	Negative	MTB	Anomalous
26	NTM	Negative	MTB	SIT 25
27	MTBC	Negative	MTB	SIT 244
28	NTM	Negative	MTB	SIT 21
29	MTBC	Negative	MTB	SIT new
30	NTM	Deleted	MTB	SIT 1539
31	MTBC	Negative	MTB	SIT 35
32	MTBC	Negative	MTB	SIT 149
33	MTBC	Negative	MTB	SIT new
34	MTBC	Negative	MTB	SIT 25
35	Negative	Negative	MTB	SIT 53
36	MTBC	Negative	MTB	SIT new
37	MTBC	Negative	MTB	SIT 25
38	NTM	Negative	MTB	SIT 952
39	NTM	Negative	MTB	Anomalous
40	NTM	Deleted	MTB	SIT 53
41	NTM	Deleted	MTB	SIT 26
42	NTM	Deleted	MTB	Anomalous

43	MTBC	Intact	MTB	SIT 25
44	NTM	Negative	MTB	SIT new
45	MTBC	Negative	MTB	SIT 149
46	Negative	Negative	MTB	SIT new
47	MTBC	Negative	MTB	SIT 25
48	MTBC	Negative	MTB	SIT 149

MTBC=*Mycobacterium tuberculosis* complex; NTM=None tuberculosis Mycobacteria; Negative=No Taqman nor Sybeer Green Signal; SIT=Shared-Internaztional type number; RD=Region of difference; OMB=Other Mycobacteria.

Appendix7: Treatment outcomes compared with FeNO concentration, the proportion of LB^{+ve} tubercle bacilli in sputum and the anti-microbial susceptibility pattern and spoligotype of *Mycobacterium tuberculosis* strains among pulmonary tuberculosis patients in Gondar.

Spoligotype	HIV Status	2 month treatment		FeNO	LB	Sputum AFB result			Weight gained (kg)	Treatment Outcome	DSP	
		Com	Not			2nd Mon	5th Mon	7th Mon			INH	NIF
SIT 53	Neg	Com	-------	142	----	neg	neg	Neg	Unknown	Cured	------	----
Anomalous	Unknown	-----	Not C	13.5	----	----	------	------	Unknown	Transferred	------	----
SIT 149	Unknown	-----	Not C	27.5	----	----	------	------	Unknown	Transferred	------	----
SIT 53	Pos	Com	-------	8.5	----	neg	neg	Neg	2	Cured	------	----
SIT 41	Neg	-----	Not C	30	----	----	------	-----	Unknown	Transferred	------	----
Anomalous	Neg	Com	-------	19	----	neg	neg	Neg	1.5	Cured	------	----
Anomalous	Neg	------	Not C	26	----	----	------	------	Unknown	Transferred	------	----
SIT 26	neg	Com	-------	14	----	neg	neg	Neg	4	Cured	------	----
SIT new	Unknown	Com	-------	37	----	neg	neg	Pos	Unknown	Transferred	------	----
Anomalous	Unknown	Com	-------	21	39	neg	neg	Neg	Unknown	Transferred	------	----
Anomalous	Unknown	Com	-------	39	----	neg	neg	------	Unknown	Transferred	------	----
SIT new	neg	Com	-------	29	10	Pos	neg	Neg	1	Cured	------	----
M.bovis	neg	Com	-------	11	----	neg	neg	Neg	1	Cured	------	----
SIT new	neg	Com	-------	14	----	neg	neg	Neg	Unknown	Transferred	------	----
SIT 25	neg	Com	-------	24.7	35	neg	neg	Neg	0	Cured	------	----
SIT 53	neg	Com	-------	12	35	neg	neg	Neg	6	Cured	------	----
SIT 952	neg	Com	-------	20.7	36	Pos	Neg	Neg	3	Cured	------	----
SIT 25	neg	Com	------	16	38	Pos	Neg	Neg	0	Cured	------	----
SIT new	Unknown	Com	-------	32	----	neg	Pos	------	Unknown	Transferred	------	----
SIT new	neg	Com	-------	17	----		neg	Neg	7	Cured	------	----
SIT new	Unknown	Com	-------	11	10	neg	------	------	Unknown	Transferred	------	----
SIT289	Neg	Com	-------	10	47	neg	neg	Neg	7	Cured	S	S
SIT 289	Neg	Com	-------	5	24	neg	neg	Neg	5	Cured	S	S
SIT 118	Neg	Com	-------	11	73	neg	neg	Neg	Unknown	Cured	S	S
SIT 50	Pos	Com	-------	13	75	pos	neg	Neg	4	Cured	R	R

Spoligotype	HIV Status	2 month treatment		FeNO	LB	Sputum AFB result			Weight gained (kg)	Treatment Outcome	DSP	
		Com	Not			2nd Mon	5th Mon	7th Mon			INH	NIF
SIT 149	Neg	Com	-------	16	24	----	------	------	Unknown	Transferred	S	S
SIT 1905	Neg	Com	-------	22	32	neg	neg	Neg	6	Cured	S	S
SIT 289	Neg	Com	-------	8	25	neg	neg	Neg	Unknown	Cured	S	S
SIT 1688	Pos	Com	-------	13	42	neg	neg	Neg	4	Cured	S	S
SIT 53	Neg	Com	-------	28	42	neg	neg	Neg	0	Cured	S	S
SIT 26	Neg	Com	-------	8	18	neg	neg	Neg	3	Cured	S	S
SIT 42	Pos	Com	-------	5	17	neg	------	Neg	4	Cured	S	S
SIT new	Pos	------	Not C	5	45	----	------	------	Unknown	Transferred	S	S
SIT 289	Pos	------	Not C	14	40	----	------	------	Unknown	Transferred	S	S
SIT 21	Neg	------	Not c	8	20	----	------	------	Unknown	Transferred	S	R
SIT 53	Pos	Com	-------	5	65	neg	neg	neg	1	Cured	-----	----
SIT 35	Neg	Com	-------	13	39	neg	neg	neg	Unknown	Cured	S	S
Anomalous	Neg	-----	Not c	7	14	----	------	------	Unknown	Lost	S	S
Anomalous	Neg	Com		14	30	neg	neg	Neg	4	Cured	S	S
Anomalous	Neg	-----	Not C	-------	----	----	------	------	Unknown	:Lost	S	S

LB=Lipid body; FeNO=Fractional exhaled nitric oxide; COM=treatment completed; Mon=Month; DSP=Drug susceptibility pattern; INH=Isoniazide; RIF=Rifampicin; S=Susceptible; R =Resistance' Not C= treatment completed not completed.

I the undersigned individual have understood the objectives of the study entitled "Association between exhaled NO in patients with pulmonary tuberculosis and lipid bodies within tubercule bacilli in sputum in Gondar health institutions, North-west Ethiopia " after the study explained to me by the investigator and I agreed to give the necessary information and samples for laboratory investigations. Moreover, it was explained to me that every secret will be kept confidential and I have the full right to participate or not in the study.

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Name----------------------------------Signature----------------------Date---------------

1. Name-- Card number--------------------------------
   -------Diagnosed centre---------------------------Date of sample collection------------

2. Socio-demographic data of patients

   A. Address: Rural------------Urban----------Keble-------------- House number------

   B. Sex: Male------------- Female---------------

   C. Age---------------

   D. Occupation--------------------------------------

   E. Marital status: Married-------Single-----widowed-------Divorced------others----

   F. Educational status: Illiterate--------Primary school---------Secondary school----

      Diploma------Degree--------

3. History of the patient

   A. Does the patient visit any health institution for TB previously? Yes-----No----

   B. Does the patient diagnosed for TB previously? Yes----------No----------

      If yes when? Three months ago---------Six months ago--------One year ago---

         Two years ago-----------Three years ago------- Four years ago----

         Five years ago----------Others---------------

   C. Does the patient treated for tuberculosis previously? Yes---------No------------

      If yes when?

4. Have you ever been in prison? Yes------------No-----------------

      If yes, when? Three months ago---------Six months ago--------One year ago----

         Two years ago-----------Three years ago------- Four years ago-----

         Five years ago----------Others---------------

      For how long did you stayed in prison?

         Less than three months -----Three months----Six months-------

         One year---------Two years-------Three years--------

         Four years-------Five years------------

5. Is the patient in a fasting state? Yes-------------------NO--------------------

   If no, types of food taken on the day of the test----------------------

6. BCG vaccination scars: yes-------------No-------------------

7. Ambient NO during the experiment-------------------

8. Nutritional status of the patient

Body mass------- Height------Triceps skin-fold thickness-------Mid arm circumference-------

9. Laboratory test results

    A. Smear result by AFS

        Positive----------number of bacilli per field------------------

        Negative-------------

B. Fluorescent microscopy result

        Lipid body Positive-------

        Percent of Lipid positive bacilli------------

        Percent of lipid body negative bacilli-------------

    C. NIOX MINO result parts per billion----------------------

10. Number of patients at the OPD level delivered sputum to the Lab per day-------------

       ■ Smear positives-----------------

       ■ Smear negatives-----------------

RNA amplification is a method developed to expand very small RNA samples. *Mtb* RNA extracted from sputum samples was amplified using the MessageAmpTMII-Bacteria prokaryotic RNA amplification Kit (Ambion, Life Technologies Corporation, 2010).

10.1. Polyadenylation of Template RNA

RNA samples were brought to a 5µl volume with Nuclease-free water and vortex mixed briefly, then centrifuged to collect sample at the bottom of the tube. Samples were incubated at $70^{c}C$ for 10 minutes in a thermal cycler. The RNA samples were removed from the thermal cycler and briefly centrifuged (~ 5 seconds) to collect sample at the bottom of the tubes and placed on ice for 3 minutes. While the samples were incubated at $70^{o}C$, a polyadenylation Master Mix was prepared in a Nuclease-free tube at room temperature (Table 1.1).

Table 10.1: The preparation of polyadenylation Master mix

Components of polyadenylation Master mix with the corresponding volume of the reactants per reaction.

Component	Amount
Nuclease-free water	1.5µl
10XPoly (A) Tailing buffer	1.0 µl
RNase inhibitor	1.0 µl
Poly (A) Tailing ATP	0.5 µl

The master mix for all the samples was prepared and was vortex mixed gently to make a homogenous mixture, then centrifuged for~ 5 seconds to collect the master mix at the bottom of the tube. To each RNA sample, 4µl polyadenylation master Mix was transferred, mixed thoroughly by gentle vortex followed by a quick spin to collect the reaction. The preparation was placed in the thermal cycler, equilibrated to $37^{o}C$ and incubated for 15 minutes. Samples were removed from the thermal cycler and briefly centrifuged to collect the reaction at the bottom of the tube and placed on ice.

10.2. Reverse Transcription to Synthesize first strand cDNA

Reverse Transcription master mix was prepared in a nuclease-free tube at room temperature (Table 1.2). The master mix was gently vortex mixed to make a homogenous mixture and centrifuged for ~5 seconds to collect the master mix at the bottom of the tube. 10µl of reverse transcription master mix was transferred to each sample, vortex mixed thoroughly followed by quick spin to collect the reaction. Samples were incubated at 42°C for 2 hours, then centrifuged briefly (~5 seconds) to collect the reaction at the bottom of the tube and placed on ice.

Table 10.2: The preparation of reverse transcription Master mix

Components of reverse transcription Master mix with the corresponding volume of the reactants per reaction.

Component	Amount
Nuclease-free water	3 µl
T7 Oligo (dT) VN	1.0 µl
10X first strand buffer	1.0 µl
dNTP mix	4 µl
ArrayScript	1 µl

10.3. Second strand cDNA synthesis

On ice, a second strand master mix was prepared by mixing the reagents listed in Table 1.3.The master mix was gently vortex mixed to make a homogenous mixture and centrifuged for ~ 5 seconds to collect the master mix at the bottom of the tube. Eighty µl of the second strand master mix was transferred to each sample, vortex mixed gently followed by a quick spin to collect the reaction and incubated at 16°C for 2 hours.

Table 10.3: The preparation of second strand cDNA synthesis Master mix

Components of second strand cDNA synthesis Master mix with the corresponding volume of the reactants per reaction.

Component	Amount
Nuclease-free water	63 µl
RNase H	1 µl
10X second strand buffer	10 µl
dNTP mix	4 µl
DNA polymerase	2 µl

10. 4. Complementary DNA (cDNA) purification

Before beginning the cDNA purification, 20µl nuclease free-water per sample was heated at 55°C and ethanol had been added to the bottle of wash buffer. Two-hundred fifty µl cDNA binding buffer was deposited to each sample and mixed thoroughly by gentle vortex. The cDNA sample/cDNA binding buffer mixture was pipetted onto the center of the cDNA filter cartridge and centrifuged at 7558g for 1 minute. The flow-through was discarded and the cDNA filter cartridge replaced in the wash tube. Five-hundred µl wash buffer was applied to each cDNA filter cartridge and centrifuged at 7558g for 1 minute until all the wash buffer was through the filter. The flow-through was discarded and the cDNA filter cartridge spine for an additional minute to remove trace amounts of ethanol. The cDNA filter cartridge was transferred to a cDNA elution tube and 18µl of preheated (55°C) nuclease-free water applied to the center of the filter in the cDNA filter cartridge. This was kept at room temperature for 2 minutes and then centrifuged at 7558g for 2 minutes. The double-stranded cDNA is now in the elute (~16µl).

10. 5. In Vitro Transcription to synthesize amplified RNA (aRNA)

In vitro transcription (IVT) master mix was prepared at room temperature by adding reagents to a nuclease free micro-centrifuge tube in order listed in Table 1.4. The master mix was mixed gently by vortex, centrifuged briefly (~5 seconds) to collect the IVT master mix at the bottom of the tube and placed on ice. Twenty-four µl IVT master mix was transferred to each sample, mixed thoroughly by pipetting up and down 2-3 times, then

flicking the tube 3-4 times, and centrifuged briefly to collect the contents in the bottom of the tube. The samples were incubated at 37°C for 14 hours in the thermocycler.

Table 10.4: The preparation of in vitro transcription Master mix

Components of in vitro transcription Master Mix used to synthesize aRNA with the corresponding volume of each reactant per reaction.

Component	Amount
T7 ATP	4 µl
T7 GTP	4 µl
T7 GTP	4 µl
T7 10X reaction buffer	4 µl
T7 enzyme mix	4 µl

10. 6. Amplified RNA (aRNA) purification

This purification removes enzymes, salts, and unincorporated nucleotides from the RNA. Before beginning the aRNA purification, a minimum of 150µl per sample nuclease-free water was heated at 55°C. For each sample, an aRNA filter cartridge was placed into an aRNA collection tube. Each IVT reaction was brought to 100µl with nuclease-free water. Three-hundred fifty µl of ACS-grade 100% ethanol was added to each aRNA sample, and mixed by pipetting the mixture up and down 3 times without vortex or centrifugation. Immediately, each sample mixture was pipetted onto the center of the filter in the aRNA filter cartridge and centrifuged at 7558g for 1 minute. The flow-through was discarded and the aRNA filter cartridge replaced in the aRNA collection tube. Ethanol had been added to the bottle of wash buffer and 650µl wash buffer was applied to each aRNA filter cartridge and centrifuged at 7558g for 1 minute. The flow-through was discarded and the aRNA filter cartridge was spanned for an additional 1 minute to remove trace amounts of ethanol. The filter cartridge was transferred to a fresh aRNA collection tube and to the center of the tube; 150µl nuclease-free water preheated to 55°C was added. The samples were incubated in the 55°C heat block for 10 minutes and centrifuged at 7558g for 2 minutes. The aRNA was collected in a collection tube in ~150µl of nuclease-free water. The aRNA filter cartridge was discarded and the concentration of the aRNA was analyzed by measuring the

absorbance at 260nm of 1 µl using the Nanodrop and the purified RNA stored at -70 ^{o}C. The total aRNA available in 150µl volume was also determined and the aRNA samples were stored at -70°C. The aRNA was reverse transcribed to cDNA as described previously and the cDNA stored at -20°C.

10.7. Quantitative PCR for human β2-Microglobin A

Eukaryotic RNA are polyadenylated and more stable than bacterial RNA which are unstable. The potential problem to be answered by this experiment is weather the level of priming would be equivalent for unamplified and amplified RNA. Three amplified cDNA with the corresponding unamplified cDNA samples were selected and subjected to qPCR for human β2-Microglobulin A. PCRs were run in a 20µl volume and PCR master mix was prepared according to Table 1.5. PCR cycling conditions were 95°C for 15 minutes, 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds with acquisition of the SYBR Green signal at 72°C. Dilutions of Monomac 6 monocyte cDNA were used as standards, these contained 10,000,000; 20,000,000; 400,000; 80,000; 16,000; 3,200 and 640 copies of Human β2 microglobin A. The Monomac 6 monocyte cDNA and the primers for the Human β2 microglobin A qPCR were obtained from Bernie Burke, University of Leicester. Quantitative PCR (qPCR) was performed on cDNA from unamplified RNA and amplified RNA for 3 sputum samples which showed the greatest fold amplification in RNA concentration. Relatively more transcripts in cDNA from unamplified RNA (per ng of RNA) than in cDNA from amplified RNA (per ng of aRNA) (Table 1.5) were observed.

Table 10.5: Quantitative PCR for human β2-Microglobin A on cDNA

Quantitative PCR for human β2-Microglobin A on cDNA from amplified RNA and cDNA from unamplified RNA. To assume the reliability, the human β2-Microglobin A quantitative PCR result showed relatively more transcript in cDNA from unamplified RNA than cDNA from amplified RNA.

RNA (ng/µl)	Un aRNA	Vol cDNA	RNA/ cDNA	RelCos B2MA	RNA/ Amp	aRNA	Yiel/ aRNA	Fold/ aRNA	RNA/ cDNA	B2MA/ aRNA	Fold diff aRNA
17.6	0.53	10	176	24261	50	88.3	13.25	265x	500	26962	9.0
31.3	0.94	10	313	2158295	50	110.4	16.56	331x	500	233457	9.2
30.5	0.92	10	305	987	50	558.4	83.76	1678x	500	297	3.3

Un aRNA=Un amplified RNA in 30µl (ng); Vol cDNA= Volume used for cDNA preparation; RNA/cDNA=RNA used for cDNA preparation (ng); RelCos=Relative copies of B2Microglobulin A; RNA/Amp=RNA used for amplification (ng); aRNA=amplified RNA; Yiel/aRNA=Yield of amplified RNA; Fold/aRNA= Fold increase in RNA by amplification; RNA/cDNA= RNA used for cDNA preparation (ng); BEMA/aRNA= Relative copies of B2MA/ng of input aRNA; Fold diff aRNA= Fold difference of un amplified RNA versus amplified RNA.

10.8. Quality control on the MessageAmp^{TM}II-Bacterial Kit

To establish if the kit was working properly, a tube of control RNA consisting of 1mg/ml *E.coli* total RNA was provided with the kit. The control RNA was diluted 1:10 by adding 2µl of control RNA to 18µl Nuclease-free water. One µl of the diluted RNA (100 ng) was used in an amplification reaction following similar protocol as documented previously. After completing the aRNA purification, the absorbance was measured at 260nm. It was recommended that the positive control reaction should produce 100µg of aRNA. The control *E.coli* total RNA was163 ng/µl which indicates that the Kit we used for RNA amplification was correctly working based on the reference give in 100ng/µl. This forced us to go back to the reverse transcription procedures and set another protocol to maximize the amplification of RNA. Previously we used 0.5µg RNA for amplification and this was changed to 1µg of RNA because we were unable to detect hspX gene at 0.5µg amplification. The volume of amplified RNA and unamplified H37Rv RNA was determined after measuring the RNA concentration (1000 divided by X; where X is the concentration of RNA in ng/µl) (Table 1.6.).

Table 10.6: Reverse transcription using 1µg of aRNA

Volumes of amplified RNA, unamplified H37Rv RNA and water used for reverse transcription

Sample number	Amount RNA used for cDNA (µg)	Volume of aRNA (µl) for 'RT' and 'no RT'	Volume of water to add (µl)
2	1.0	18.8	2.2
3	1.0	11.7	9.3
4	1.0	6.4	14.6
6	1.0	11.4	9.6
7	1.0	18.2	2.8
12	1.0	15.8	5.2
19	1.0	16.4	4.6
20	1.0	1.8	19.2
21	1.0	4.9	16.1
22	1.0	16.0	5.0
Amplified H37Rv	1.0	3.2	17.8
Unmplified H37Rv	0.5	8.7	12.3

10.9. Reverse transcription of aRNA to form cDNA in a 40µl reaction volume

The reverse transcription of aRNA is much similar to the previously documented procedure with difference in the volume of reaction mixture. Samples were diluted with RNase-free water and the PCR master mix was prepared following Table 1.7.

Table 10.7: Reverse transcription of aRNA

Components of reverse transcription Master Mix used for reverse transcription of aRNA to generate cDNA with the corresponding volume of each reactant and the following reagents were added.

	RT	no RT contro
RNA (1µg)	X	X
Genome directec primers (25pmol	2.0	2.0
dNTPs (10mM)	2.0	2.0
RNase free wate:	21-x	21-x

Samples were incubated in the PCR machine at 65°C for 5 minutes and immediately transferred to ice without allowing samples to cool and the following reagents were added..

5xbuffer	8.0	8.0
0.1% DTT	4.0	4.0
RNase Out	1.5	1.5

In the PCR machine, samples were incubated at 25°C for 2 minutes.

Superscript II reverse transcriptase	2	---
RNAse free water	-----	2

In the PCR machine samples were incubated at 25°C for 10 minutes, 42°C for 50 minutes and 70°C for 15 minutes and samples were stored at-20°C.