MYCOBACTERIUM TUBERCULOSIS:

MOLECULAR EPIDEMIOLOGY,

SUSCEPTIBILITY TO REACTIVE NITROGEN INTERMEDIATES

AND

ANTI TUBERCULOSIS DRUGS RASISTANCE IN NORTHWEST ETHIOPIA

By

MEKIDIM MEKONNEN, B.Sc

Department of Microbiology, Immunology and Parasitology,

Faculty of Medicine, Addis Ababa University

JUNE, 2012

MYCOBACTERIUM TUBERCULOSIS: MOLECULAR EPIDEMIOLOGY, SUSCEPTIBILITY TO REACTIVE NITROGEN INTERMEDIATES AND ANTI TUBERCULOSIS DRUGS RASISTANCE IN NORTHWEST ETHIOPIA

By

MEKIDIM MEKONNEN, B.Sc

A thesis submitted to the school of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the Degree of Master of Science in Medical Microbiology

JUNE 2012

ACKNOWLEDGMENTS

I am extremely grateful and would like to acknowledge Jimma University, Addis Ababa University Medical Faculty (AAU-MF), Aurmeur Hannsen Research Institute (AHRI/ALERT) and Swedish heart-lung foundation for financially supporting this project.

I also thank the study participants, all the advisors, co-investigators and staffs of AHRI/ALERT for their marvelous and crucial inputs, without their help this project would have remain being just a dream. Thank you, Dr. Thomas Shcon, you were the expert, mentor and everything for our project.

My acknowledgment also particularly goes to Dr Abrham Assefa, Dr Daniel Elias, Dr Yimtubezinash W/amanuel, Dr Daniel Asrat and Ebba Abate were corner stone of these project and I thank you all for the inspiration, professional advice, and for being considerate and wakeup calls during my hibernation.

Last but not least, I am very much grateful to my families (mom, dad and all my brothers) for the love, confidence, and patience and trust that they were holding on me and supporting me in all walks of life. My great friends: Embeitey, Sami, Z, Mafi, Misganaw, Sinte, Wosen, Sisay and many others; you were such wonderful and dependable backups and I thank you all for your huge inputs and encouragement you offered me.

TABLE OF CONTENTS **PAGE**

LIST OF TABLES **PAGE**

LIST OF FIGURES **PAGE**

ABBREVIATIONS

BMI : Body mass index

CR : Chest x-ray

DETA/NO : Diethylenetriamine/nitric oxide adduct

DOTs : Directly observed therapy of short course

DR : Direct repeat locus

EDTA : Ethylenediamine tetraacetic acid

iNOS : Inducible nitric oxide synthase

IUATLD : International union against tuberculosis and lung disease

MDR-TB : Multi drug resistant tuberculosis

MGIT : Mycobacterial growth indicator tube

MIRU-VNTR : Mycobacterial interspersed repetitive units-Variable number of tandem repeat

MTBC : Mycobacterium toberculosis complex

NO : Nitric oxide

OADC : Olic acid albumin deoxycholate citrate

PAS : Poly-amino salicylic acid

PPD : Purified protein derivatives

PRA : PCR restriction-enzymes assay

RDs : Region of difference

RFLP : Restricted fragment length polymorphism

ROIs : Reactive oxygen intermediates

RNIs : Reactive nitrogen intermediates

SSPE : Sodium chloride sodium phosphate with EDTA

SDS : Sodium dodycil sulphate

TBHBC : Tuberculosis high burden countries

TLCP : Tuberculosis and leprosy control program

TLRs : Toll like receptors

TNFs : Tumor necrosis factors

TST : Tuberculin skin test

XDR-TB : Extensively drug resistant tuberculosis

ABSTRACT

Background: *Tuberculosis, caused by M. tuberculosis, is the deadliest disease causing 3 million annual deaths globally. M. tuberculosis is an intracellular pathogen that survives and replicates within cells, primarily macrophages. An important part of the host defense against tuberculosis is nitric oxide and other reactive intermediates produced by activated macrophages. M. tuberculosis differ in their strain types, susceptibility to the conventional anti Tb drugs and to the effect of nitric oxide which in turn may influence clinical outcome of active tuberculosis patients.*

Objectives: *The aim of this project, therefore, was to investigate strains differences, the effect of the potent RNI, NO (nitric oxide), and the convectional anti-Tb drugs on the clinical isolate of M. tuberculosis, in vitro, from patients receiving arginine (Nitric oxide) in the form of peanuts in an ongoing clinical trial study and to correlate the findings with clinical outcome of those subjects.*

Methods: *Both, prospective and retrospective study had been conducted to investigate the molecular epidemiology, NO (nitric oxide) susceptibility and anti-mycobacterial sensitivity patterns of the clinical isolates MTB in order to asses correlation of results to clinical outcome of active tuberculosis patients. Smear-positive sputums from 180 study participants were cultured to obtain pure clinical isolates. Standard PCR method and spoligotyping were done to identify the strains of M. tuberculosis. The isolates were also tested for their drug susceptibility pattern using proportion method. The in vitro effects of RNI and susceptibility profile of those isolates were determined after series of optimizations experiments using DETA/NO (potent nitric oxide donor chemical).*

Results: *Of 176 smear-positive TB patients enrolled, 93 (52.8%) were female, 68 (38.6%) were HIV sero-positive and the mean age was 27.5 (SD = 9.55; R = 15 – 59). HIV sero-prevalence was high among age groups between 25 – 45 yrs of age (P=0.000). Of 176 smear-positive sputa 67.6% were culture positives. Conventional PCR of isolates revealed that all strains belong to M. tuberculosis. Spoligotyping was done for 108 strains, of which 83 (76.8%) were clustered in to 4 major M. tuberculosis families being CAS families the dominant ones (43%). The remaining 25 (23.2%) were unique and no non-tuberculos mycobcteria was detected. The overall proportion of drug resistant strains was 14.3% (14/98), poly-resistant 1% and no MDR-strains were identified.*

The clinical strains generally showed variability in percent survival to NO exposure with median survival rate of 14.1% (IQR, 3.0 – 30.3%), and exposure of strains to 1mM of DETA (NO donor) for 24hrs significantly reduced (P=0.000) the bacterial colony forming units (cfu). In logistic regression analysis, nitric oxide survival rate of clinical isolates was significantly associated with some of the clinical conditions of the patients like BMI (kg/m^2) (P= 0.04), smear conversion (P= 0.03) and cure rate (P= 0.04).

Conclusions: *Mycobacterium tuberculosis was the only mycobacterial strain found among consecutive smear positive isolates, the rate of anti-microbial resistance was high. Patients carry drug resistant strains significantly exhibited decreased cure rate and smear conversion rate at 2 months. Clinical isolates of M. tuberculosis exhibiting nitric oxide resistance showed significantly more anti mycobacterial drug resistance compared to nitric oxide susceptible strains. Further research is needed on the importance and use of nitric oxide to prevent and control of the spread of tuberculosis.*

Key words: *Tuberculosis, nitric oxide, drug resistance, spoligotypes, clinical outcome*

CHAPTER I: INTRODUCTION

1.1 General Introduction

The consequences of tuberculosis (TB) on society are immense and TB is still a significant threat to public health. Worldwide, one person out of three is infected with *Mycobacterium tuberculosis,* estimated to be two billion people in total, and TB accounts for 2.5 % of the global burden of diseases. Currently, it holds the seventh place in the global ranking of causes of death. Unless intensive efforts are made, it is likely to maintain that position until 2020 (Dye *et al.,* 1999; Smith, 2004).

The fact that only about 5–10% immune-competent individuals will develop active disease during the first few years following exposure while the majority elicit effective immunity and contain the infection in latent form. This is partly explained by efficiency of the host defense against microbial pathogens which involves a number of specialized cells of the immune system (Dye *et al.,* 1999; Toossi 2000; Raja, 2004). An important part of the host defense against tuberculosis is nitric oxide produced by activated macrophages from L-arginine, molecular oxygen, and NADPH, (Nathan and Shiloh, 2000; Chan *et al.,* 2001).

Several animal and macrophage experiments have shown that nitric oxide (NO) and related reactive nitrogen intermediates (RNI) constitute a major host defense mechanism against many intracellular pathogens including *M. tuberculosis* in both the acute and the persistent phases of infection (Flynn and Chan, 2001). It has been shown also that RNIs are actively produced in human tuberculosis although the relative importance of RNIs in human tuberculosis is controversial (Nicholson *et al* ., 1996, Rich *et al* ., 1997, Rockett *et al.,* 1998, Nathan and Shiloh, 2000; Chan *et al.,* 2001).

Previous studies have shown that, in tuberculosis infected mice models where there is extensive nutritional depletion and protein-malnutrition, the production of NO and expression of iNOS (Inducible Nitric Oxide Synthase) was impaired (Chan *et al* 1996; Macallan 1999).

However, restoring full protein diet and provision of nutritional supplements such as arginine have shown to reverse the serious course of the disease and mediate clinical improvement in HIV negative active tuberculosis patients (Macallan, 1999; Schon *et al.,* 2003) even though no direct causality was proven between arginine supplementation and increased NO production versus clinical improvement (Schon *et al.,* 2003).

There are no previous reports regarding the effect of NO (nitric oxide) on the clinical isolates of *Mycobacterium tuberculosis* from active smear positive TB patients in relation to their clinical outcome. Furthermore, the limited existing data also showed that the susceptibility of mycobacteria to RNI (or nitric oxide) was strain-dependent (Shiratsuchi *et al.,* 1990, Doi *et a.,* 1993; O'Brien *et al*., 1994, Rhoades, 1997). Therefore, there is a need to investigate the nitric oxide susceptibility profile of the clinical isolates of *Mycobacterium tuberculosis* and see whether this difference could attribute to variability in the clinical outcome of active tuberculosis patients.

1.2 Background

Tuberculosis (TB), one of the oldest diseases known to affect humans, is caused by bacteria belonging to the *Mycobacterium tuberculosis* complex, and the disease usually affects the lungs, although almost any organ could be involved. This infectious disease was sporadic until the 1700s and became epidemic afterward because of the industrial revolution, the increase in population density, and unfavorable living conditions. Furthermore, human migrations and colonization of countries and continents helped to spread TB, which became an endemic disease in many settings (Raviglione, 1995).

1.2.1 Historical Overview of Tuberculosis

Though the modern members of *M. tuberculosis* complex seem to have originated from a common progenitor about 15,000 - 35,000 years ago, the early progenitor of *M. tuberculosis* was believed to probably co-evolved with early human creatures in East Africa three million years ago as TB was documented in Egypt, India, and China as early as 5,000, 3,300, and 2,300 years ago, respectively (Daniel, 2006).

In 1882, Robert Koch managed to isolate the tubercle bacillus (called also Koch bacillus), the bacterium responsible for TB, and he established TB as an infectious disease. Over the last 100 years, it was estimated that TB has probably killed 100 million people (Frieden, 2003).

1.2.2 Microbiology of the *Mycobacterium* species

There are more than 70 species so far described within the *Mycobacterium* genus based on their biochemical and other molecular based specific characteristics. The rapidly growing *Mycobacterium* species (producing visible colonies within 7 days under optimal culture conditions) are mainly common saprotrophs of natural habitats, and only a few of them can be pathogenic for humans or animals (e.g., *Mycobacterium abscessus*, *M. fortuitum*, *M. porcinum*) whereas the majority are non-pathogenic (e.g., *M. smegmatis*, *M. agri*). In contrast, the majority of the slowly growing *Mycobacterium* species are pathogenic for humans and/or animals (e.g., all the species of the MTB complex as well as *M. leprae*, *M. ulcerans* and *M. avium-intracellulare complex* (MAC), and only a few of them are non-pathogenic (e.g., *M. terrae*, *M. gordonae*) (Rogall *et al.,* 1990, Vincent and Portaels, 1992, Shinnick and Good, 1994).

The Mycobacterium tuberculosis complex (MTBC), the main etiology of tuberculosis, is composed of seven different species, *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. canettii*, and recently, *M. caprae* and *M. pinnipedii*; and each member is associated with a specific primary host, although infection is known to occur in various alternative hosts. The species responsible for TB in humans and for which no animal reservoirs was found are *M. tuberculosis*, *M. africanum*, and *M. canettii* (Shinnick and Good, 1994, Aranaz A *et al*., 2003, Cousins *et al.,* 2003). Tuberculosis due to *M. africanum* was most commonly observed in West Africa (Viana *et al.,* 2001). *Mycobacterium bovis* is principally the agent of bovine TB, but this species can also be pathogenic for humans, with the number of cases related to such infection probably underestimated (Ayele *et al.,* 2004).

The members of the MTBC, as well as all mycobacteria species, are rod-shaped bacteria (0.2–0.6 µm wide, 1–10 µm long), non-motile, non-encapsulated, non-spore forming, aerobes (growing most successfully in tissues with a high oxygen content such as in the lungs), or facultative anaerobes which stain poorly with the gram stain. They are facultative intracellular pathogens, usually infecting mononuclear phagocytes (e.g., macrophages) (Wayne 1994; Haas, 2000).

M. tuberculosis, specifically, is classically described as fastidious, slow-growing, aerobic, lipid-rich, hydrophobic, acid-fast bacterial rod measuring 1-4 µm long and 0.3-0.6 µm in diameter (Wayne, 1994; Haas, 2000). The aerobic requirement for *M. tuberculosis* was reported to be variable as it may survive anaerobic conditions too (Segal, 1984). Under optimal conditions, *M. tuberculosis* requires 16-18 hours to undergo one cycle of replication and a single bacillus may yield a visible colony within 3-6 weeks or less after inoculation on mycobacterial media. The bacilli are best isolated from clinical specimen on rich and fairly complex media because of the cell wall of *M. tuberculosis,* which is thick and consisting of a plasma membrane surrounded by a complex wall structure including arabinogalactans, mycolic acids, peptidoglycan and lipoarabinomannans (LAM) (Brennan, 1994; Wayne, 1994; Haas, 2000).

The cell wall structure, in particular the high lipid content, forms the basis for the acid-fast properties of the mycobacterium, i.e. retention of the aryl methane dyes such as fuchsine and auramine O after treatment with acid alcohol (Nolte, 1995). MTB has an unusual cell wall, with an additional layer beyond the peptidoglycan layer, which is rich in unusual lipids, glycolipids and polysaccharides. Lipids constitute more than half of the dry weight of the mycobacteria probably the highest amount among all bacteria (Cole and Barrell, 1998). However, the lipid composition of the tubercle bacillus may vary during the life cycle in culture, depending on the availability of nutrients. The waxy coat confers the distinctive characteristics of the genus: acid fastness, extreme hydrophobicity, resistance to injury, including that of many antibiotics, and distinctive immunological properties. It probably also contributes to the slow growth rate of some species by restricting the uptake of nutrients (Nolte, 1995; Brennan, 1994; Wayne, 1994; Haas, 2000).

1.2.3 Molecular Biology of *M. tuberculosis*

Based on 16S rRNA studies, genus *Mycobacterium* belongs to the actinomycete bacteria hence Mycobacteria have typical features of the group, which is characterized by a high G-C content in their DNA, aerobic metabolism and a tendency towards mycelial growth, but differ from many members of the group in lacking a spore stage in their life cycle. Most of the actinomycete, including many non-pathogenic mycobacteria are soil saprophytes indicating the ancestor of *Mycobacterium tuberculosis* (Saunders and McFadden, 2003).

M. tuberculosis has a 4,411,529-bp circular genome which is *haploid*, as are all bacteria genomes. This genome is characterized by the presence of numerous repeated sequences and has no plasmid (Cole *et al* 1998a). The H37Rv genome contains 3,924 identified open reading frames, of which 40% were confidently assigned functions on the basis of similarity to known genes and another 44% were assigned a probable function, leaving 16% orphan genes (Cole *et al*., 1998b).

In 1997, Sreevatsan *et al.* studied 26 structural genes or loci, and they observed very low levels of genetic variation hence concluded that the genetic diversity of the species is localized, especially in transposable elements and in genes involved in host–pathogen interactions, particularly those related to host immunological responses. However, the latter point was disproved later for that most of the genes coding for targets of the host immune systems were monomorphic while the transposable elements show high levels of genetic polymorphism, thus they are widely used for studying the genetic variability in the MTB species (Sola *et al*., 1998, Fleischmann *et al*., 2002, Gutacker *et al.,* 2002). The comparison of the complete sequences of the two strains (H37Rv, which is the classical reference strain, and one recent virulent MTB isolate: CDC1551) confirms a much higher degree of polymorphism than previously thought; and it was also possible to identify large-sequence polymorphisms (LSPs) and single nucleotide polymorphisms (SNPs) though the molecular basis of variability in virulence and transmissibility remains undefined (Fleischmann *et al*., 2002, Gutacker *et al.,* 2002).

A total of 224 genes (5.5%), including genes in all major functional categories, were found to be partially or completely deleted. Deletions are not distributed randomly throughout the genome but instead tend to be aggregated. It was observed that the identified deletions were evidently unessential to the development of the disease, as they were found in active clinical cases. In contrast, their frequency spectrum suggested that most polymorphisms are weakly deleterious to the pathogen. These results raise numerous opportunities to advance in the study of drug resistance, virulence, and host–pathogen interactions (Tsolaki *et al.,* 2004).

1.3 Epidemiology, Clinical Diagnosis and Treatment of Tuberculosis

1.3.1 Global Epidemiology of Tuberculosis

Tuberculosis, still results in a massive global case load and it has been estimated that between 2002 and 2020, approximately 1000 million people will be newly infected with *M. tuberculosis*; of these, over 150 million will develop disease, and 36 million will die (WHO, 2001).

According to WHO report of 2008, there were an estimated 9.2 million new cases of TB in 2006 (139 per 100 000 population), including 4.1 million new smear-positive cases (44% of the total) and 0.7 million HIV-positive cases (8% of the total). The African Region has the highest incidence rate per capita (363 per 100 000 population) (Figure 1.1). There were an estimated 14.4 million prevalent cases of TB in 2006, of which an estimated 0.5 million cases were of multidrug-resistant TB (MDR-TB). In 2006 1.5 million deaths were caused by TB in HIV-negative people and 200,000 among people infected with HIV (WHO, 2008a).

In 2007, a total of 5.1 million new cases (out of the estimated 9.2 million new cases) were notified for 2006 among 202 countries and territories, of which 2.5 million (50%) were new smear-positive cases. The African, South-East Asia and Western Pacific regions accounted for 83% of total case notifications (WHO, 2008a).

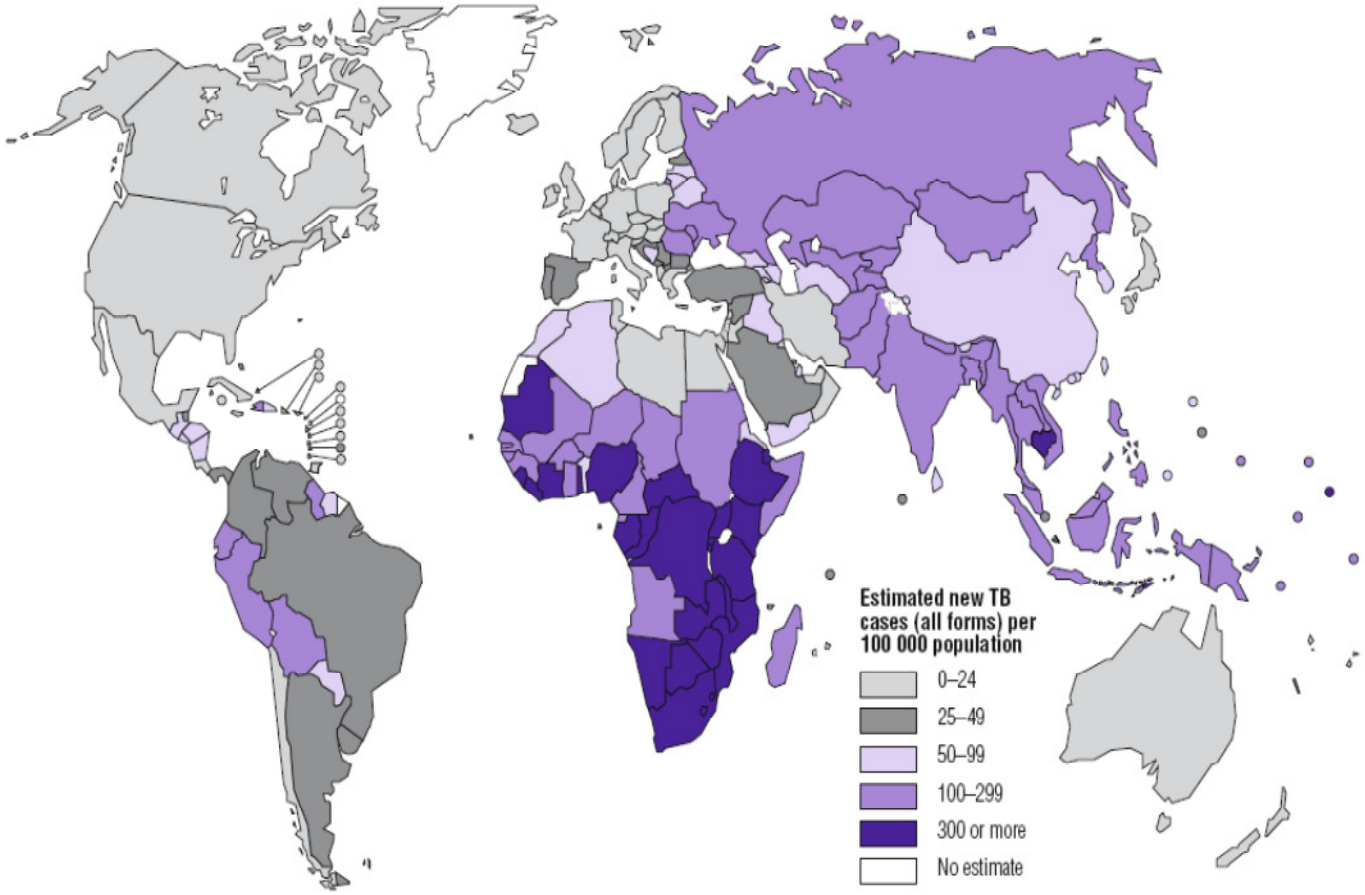


Figure 1.1 Estimated TB incidence rates, by country, 2006 (Source: WHO, 2008a)

Of the total estimated number of new TB cases arising annually, about 80 percent of new cases occur in the 22 top-ranking high burden countries (TBHBC); however, 90% of all cases and 99 % of deaths occur in developing countries, with the greatest burden in sub-Saharan Africa and South East Asia. These countries (Table 1) are the focus of intensified efforts in *Directly Observed Treatment Short-course* (DOTS) expansion (Dye, 2006, WHO, 2006a, and WHO, 2006b).

Table 1.1. List of TB-High burden countries and estimated epidemiological burden of TB, 2006 (Source: WHO, 2008a)

		INCIDENCE[a]				PREVALENCE ALL FORMS		MORTALITY ALL FORMS		HIV PREV. IN INCIDENT TB CASES[b]
		ALL FORMS		SMEAR-POSITIVE						
	POPULATION 1000s	NUMBER 1000s	PER 100 000 POP PER YEAR	NUMBER 1000s	PER 100 000 POP PER YEAR	NUMBER 1000s	PER 100 000 POP	NUMBER 1000s	PER 100 000 POP PER YEAR	%
1 India	1 151 751	1 933	168	867	75	3 445	299	325	28	1.2
2 China	1 320 864	1 311	99	590	45	2 658	201	201	15	0.3
3 Indonesia	228 864	534	234	240	105	578	253	88	38	0.6
4 South Africa	48 282	454	940	184	382	482	998	105	218	44
5 Nigeria	144 720	450	311	198	137	890	615	117	81	9.6
6 Bangladesh	155 991	351	225	158	101	610	391	70	45	0.0
7 Ethiopia	81 021	306	378	136	168	520	641	68	83	6.3
8 Pakistan	160 943	292	181	131	82	423	263	55	34	0.3
9 Philippines	86 264	248	287	111	129	373	432	39	45	0.1
10 DR Congo	60 644	237	392	105	173	391	645	51	84	9.2
11 Russian Federation	143 221	153	107	68	48	179	125	24	17	3.8
12 Viet Nam	86 206	149	173	66	77	194	225	20	23	5.0
13 Kenya	36 553	141	384	56	153	122	334	26	72	52
14 UR Tanzania	39 459	123	312	53	135	181	459	26	66	18
15 Uganda	29 899	106	355	46	154	168	561	25	84	16
16 Brazil	189 323	94	50	59	31	104	55	7.6	4.0	12
17 Mozambique	20 971	93	443	39	186	131	624	24	117	30
18 Thailand	63 444	90	142	40	62	125	197	13	20	11
19 Myanmar	48 379	83	171	37	76	82	169	6.1	13	2.6
20 Zimbabwe	13 228	74	557	30	227	79	597	17	131	43
21 Cambodia	14 197	71	500	31	220	94	665	13	92	9.6
22 Afghanistan	26 088	42	161	19	73	60	231	8.3	32	0.0
High-burden countries	**4 150 313**	**7 334**	**177**	**3 265**	**79**	**11 889**	**286**	**1 330**	**32**	**11**

According to the WHO, most of the HIV-positive cases (85%) in 2006 were from African regions. South Africa, for example, had 0.7% of the world's population but 28% of the global number of HIV-positive TB cases and 33% of HIV positive cases in the African Region. Furthermore, among the 15 countries with the highest estimated TB incidence rates, 12 were in Africa (Table 1.1) (WHO, 2008a).

1.3.2 Tuberculosis in Ethiopia

According to WHO, Ethiopia is still top on the list of tuberculosis high-burden countries of the world so far recognized. Ethiopia stands 7th in the list of High Burden Countries (HBCs) for TB in 2006 (WHO, 2008a) (Figure 1.2). According to the WHO report of 2008, the incidence of TB of all forms and smear positive TB in 2006 was 379 and 168 per 100,000 population, respectively; and the prevalence and mortality of tuberculosis of all forms was estimated to be 643 and 84 per 100,000 populations respectively (WHO, 2008a). According to the MOH's hospital statistics, tuberculosis is the leading cause of morbidity, the third cause of hospital admission (after deliveries and malaria), and the second cause of death in Ethiopia, after malaria (MOH, 2008).

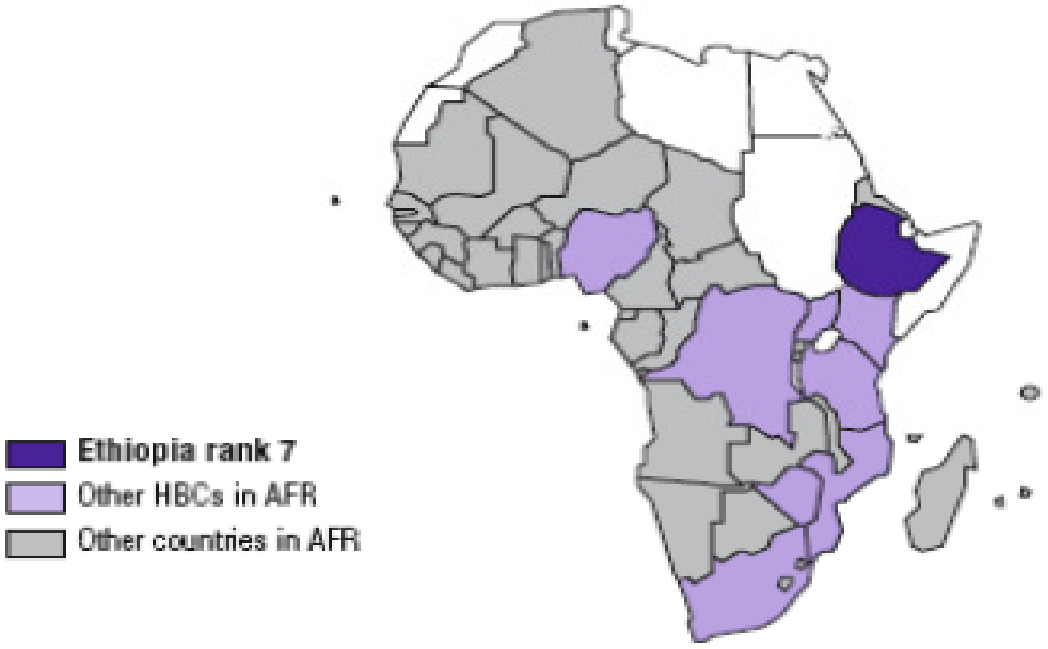


Figure 1.2 WHO African Region; Rank based on estimated number of incident cases (all forms) in 2006 (Source: WHO, 2008a)

There is also steady rise in case notifications (Table 2) because of increasing DOTS coverage, improved reporting and the impact of HIV/AIDS. However, the national smear-positive case detection rate by the DOTS program has remained to be less than 30%, which is far below the national and global target of 60% and 70% respectively. This is due largely to the small proportion of the population (only 50% - 60%) thought to have access to health services of any kind, including for TB (WHO, 2007; WHO 2008a; MOH, 2008).

Table 1.2 Eight years overview of TB case notification in Ethiopia (TBL Data, TLCT, FMOH) 2006/07

Year (G.C.)	Total New Cases	Smear Positive	%	EPTB	%	Case notification rate per 100,000 population		Treatment success rate
						Smear Positive	All forms	
1999/2000	83,334	26,459	32	26,542	31	42	131	
2000/01	90,729	32,423	36	29,312	32	50	139	
2001/02	105,250	35,915	34	37,138	35	53	157	
2002/03	108,488	37,014	34	38,818	36	54	157	
2003/04	121,026	41,430	34	42,477	35	59	173	
2004/05	123,090	38,800	31	44,021	36	53	169	81%
2005/06	120,163	36,674	31	43,255	36	49	160	78%
2006/07	125,552	37,645	30	45,045	36	49	163	85%

In Ethiopia, among all new TB cases the highest proportion of cases are reported as extra-pulmonary TB (>34% in 2003 and 36% in 2006/07, regional variation 29–54%) and the vast majority, about 75% of extra-pulmonary cases are reported as lymph node TB (MOH 2003, Yasin *et al.,* 2003; WHO, 2005b; MOH, 2008). In 2005 and 2006/07 in Ethiopia, of new pulmonary cases notified under DOTS, 49% were smear-positive and of these 46 % were women (WHO, 2008a). DOTS treatment success among new patients was only 76% and 79% in the 2002 and 2004 cohorts respectively (WHO, 2005b; WHO, 2007) and the national data indicated 85% success rate (Table 1.2) in 2006/07 which is set as the global target (MOH, 2008). The WHO report also indicated that of the new adult TB cases of age between 15 and 49, HIV-positivity was 11% (ranging 7 – 14%) in 2005 (WHO, 2007), however the national data and WHO's 2008 report indicated 31% and 40% respectively (MOH, 2008; WHO, 2008a).

The first study on primary drug resistance of *Mycobacterium tuberculosis* in Ethiopia was reported in 1996 (MOH, 2008). The study showed primary resistance to Isoniazid and Streptomycin to be 14.8% and 4.9%, respectively. Several studies were done later in Addis Ababa and in some other parts of the country with results showing primary drug resistance to one or more drugs ranging from 7.6% to 32.5% (MOH, 2008). The first countrywide Drug Resistance Survey in Ethiopia between 2003 and 2006 was carried out on 880 sputum samples (804 new patients and 76 retreated patients) randomly collected using proportion method at national public health TB referral laboratory. MDR-TB strains isolated in 1.6% of new cases and in the 11.8% of previously treated cases (MOH 2008a; WHO, 2008b).

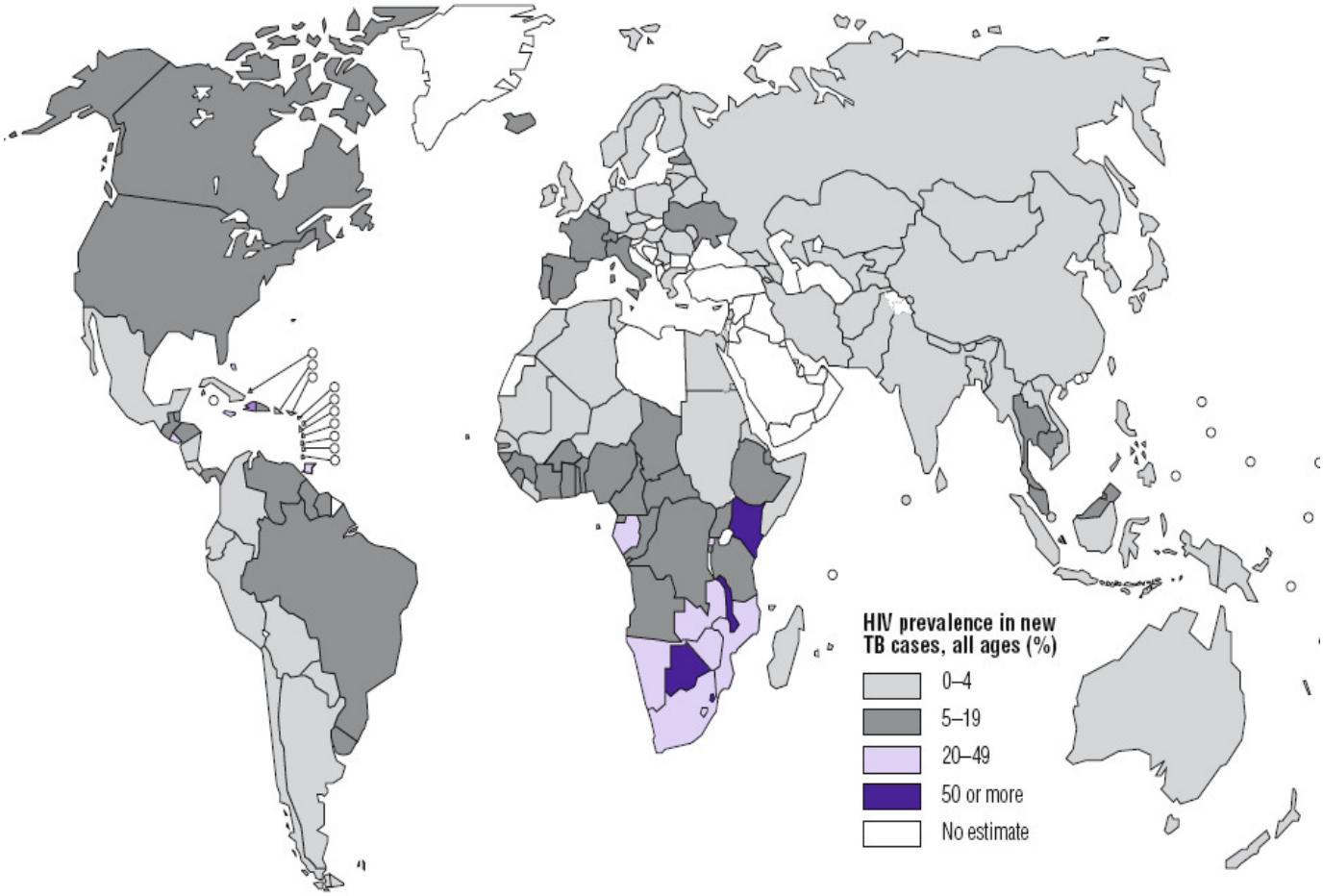
Hermans *et al.* (1995) and Bruchfeld *et al.* (2002) used DNA fingerprinting to describe Ethiopian *M. tuberculosis* strains and identified that 42-48% strains collected in different times appeared in clusters, indicating a high prevalence of recent transmission. Regarding the proportions of non-tuberculous mycobacteria among the smear positive cases limited data from studies in Ethiopia show a prevalence of 2% (Bruchfeld *et al.,* 2002)

1.3.3 Interaction of Tuberculosis and HIV Epidemic

HIV infection is the most fueling risk factor for TB as it increases the risk of reactivating latent *M. tuberculosis* infection and rapid TB progression soon after *M. tuberculosis* infection or re-infection. The link between TB and HIV and their involvement in aggravating each other is well noted. TB has shown to increase replication of HIV by activating CD_4^+ cells and enhancing production of pro-inflammatory cytokines, and HIV as well increases the susceptibility to TB by further depletion of protective CD_4^+ cells, besides the progression of HIV/AIDS disease in high endemic areas was considered as risk factor for developing TB (Lawn *et al.,* 2002; Wood *et al.,* 2002). The life time risk for a immune-competent individual to develop TB following exposure is estimated to 5-10 % which is increased to a yearly risk at the same level in HIV co-infected TB patients (Harries, 2001), and TB is one of the most common causes of morbidity and the most common cause of death in HIV-positive adults living in high endemic countries (Harries *et al.,* 2001; Corbett, 2003; Aaron, 2004, WHO, 2006b).

The TB burden in countries with a generalized HIV/AIDS epidemic has therefore increased rapidly over the past decade, especially in the severely affected countries of eastern and southern Africa. In addition to increasing individual susceptibility to TB following *M. tuberculosis* infection, a high burden of HIV-associated TB cases also expands *M. tuberculosis* transmission rates at the community level. Furthermore, HIV has been associated with epidemic outbreaks of TB in several countries in which the main reported outbreaks involved multidrug-resistant (MDR) strains which respond poorly to standard therapy and it is becoming the growing burden of TB (Corbett, 2003; Aaron, 2004; WHO, 2006a; WHO, 2006b).

The global increase in the incidence of TB since 1980 is attributable to the spread of HIV in Africa and the high incidence rates estimated for the African countries were partly explained by the relatively high rates of HIV co-infection as where HIV infection rates are higher in adult populations; they are also estimated to be higher among new TB patients as shown in Figure 1.3 which maps the distribution of HIV among TB patients, showing the relatively high rates in countries of eastern and southern Africa (WHO, 2008a).



Source: WHO, 2008

Figure 1.3. Estimated HIV prevalence in new TB cases by countries in 2006

1.3.4 Clinical Patterns and Diagnosis of Tuberculosis

Tuberculosis is classified as pulmonary or extra-pulmonary, which respectively involves the lungs or any organ other than the lungs. In order of frequency, the extra-pulmonary sites most commonly involved in tuberculosis are the lymph nodes, pleura, genitourinary tract, bones and joints, meninges, peritoneum, and pericardium. However, virtually all organ systems may be affected as a result of hematogenous dissemination. Less commonly, reactivation tuberculosis can also occur in most other organs and disease at these sites ranges from a localized tumor-like granuloma (tuberculoma) to a fatal chronic meningitis (Mario and Richard, 2005)

Primary tuberculosis is either asymptomatic or manifest only by fever and malaise. The universal clinical symptom is cough for more than two weeks, which is initially dry but bloody sputum could be produced as the disease progresses. The other most common clinical manifestations include fever, malaise, fatigue, sweating, and weight loss, and all progress with continuing disease. The methods of diagnosis of active disease include clinical suspicion, response to treatment, chest radiographs, staining for acid fast bacilli, culture for mycobacteria, and, more recently, nucleic acid amplification assays (Brodie and Schluger, 2005). The chest X-ray examination is traditionally considered as one of the most important tests, but its low specificity can lead to over-diagnosis, therefore, respiratory samples (expectorated sputum) are submitted to the bacteriological laboratory for microscopic examination by the Ziehl–Neelsen (ZN) method and for mycobacterial culture where available *t*o confirm the diagnosis of PTB. Biopsy can play a role in the confirmation of the diagnosis of EPTB, such as tubercular lymphadenitis. Multiplication of tubercle bacilli in any site of the human body causes a specific type of inflammation, with formation of a characteristic granuloma (Mario and Richard, 2005).

Until very recently, a skin reaction called Mantoux test or tuberculin skin test (TST) or PPD (purified protein derivative) skin test was the only available test to detect latent disease or to confirm the cases of active disease with negative sputum smear or culture. Nevertheless, this test presents various problems such as relatively poor sensitivity and specificity.

Recently, a new generation of tests such as QuantiFERON-TB and QuantiFERONTB Gold (QFN-Gold) tests (Cellestis Limited, St. Kilda, Australia) and the T SPOT-TB test (Oxford Immunotec, Oxford, UK) has been developed; and these tests are based on the detection in serum of either the release of IFN-γ (QuantiFERON) or detection (T SPOT-TB)-gamma with increased specificity as unspecific reaction caused by BCG vaccination or non-tuberculous mycobacteria could be ruled out (Brodie and Schluger, 2005).

In HIV co infected and smear negative active tuberculosis patients, atypical pulmonary features predominate and chest radiography changes may be atypical or attributable to other infections like PCP and bacterial pneumonia. Furthermore, the tuberculin skin testing could become false negative, especially in developing countries presenting a high rate of HIV/MTB co-infection, with the high coverage of BCG vaccination, with asymptomatic TB infection, with the presence of non-tuberculous mycobacteria, and with anergy due to HIV or malnutrition. Therefore, culture and PCR remain the most sensitive techniques, as they can produce a positive result for specimens containing as few as 10 bacilli. This is of a great interest, as HIV-positive patients generally produce sputum with low bacilli loads (Colebunders and Bastian, 2000).

1.3.5 National and International Treatment Guidelines for Tuberculosis

Several drug regimens are recommended depending on many factors, such as disease localization and severity, result of sputum smear microscopy, human immunodeficiency virus (HIV) co-infection, prevalence of drug resistance in the setting, availability of drugs, cost of treatment and medical supervision, whether the patient has previously received any anti-tuberculosis drug, and qualifications of health staff at the peripheral level. Therefore, in order to establish treatment priorities and maintain cure, WHO and other international organizations, recommended that tuberculosis patients should be classified into four categories and the respective treatment regimens (ATS/CDC/IDSA, 2003: WHO, 2003: WHO, 2004).

Category I comprises those patients with a high priority for treatment who are new smear-positive patients, new smear-negative pulmonary TB patients with extensive parenchymal involvement, and patients with concomitant HIV /acquired immunodeficiency syndrome (AIDS) disease or severe forms of extra-pulmonary TB. Patients with a lower priority for treatment are classified as follows: **Category II** (relapse, treatment failure or default), **Category III** (new smear-negative pulmonary TB other than in Category I and less severe forms of extra-pulmonary TB) and **Category IV** those exhibiting chronic sputum-positive TB after re-treatment and proven or suspected MDR-TB (ATS/CDC/IDSA, 200; WHO, 2003; WHO, 2004).

The current short-course treatment guideline aimed at complete elimination of active and dormant bacilli and involves two phases. The initial phase in which three or more drugs (usually isoniazid (H), rifampicin (R), pyrazinamide (Z) and ethambutol (E) or streptomycin (S)) are used for two months, and allow a rapid killing of actively dividing bacteria, resulting AFB negativity; and the continuation phase in which fewer drugs (usually isoniazid and rifampicin) are used for 4 to 7 months, aimed at killing any remaining or dormant bacilli and preventing recurrence. These above mentioned five drugs (H, R, E, Z, and S) are labeled as first-line drugs. When resistance to any of these first-line drugs is found or highly suspected, or when adverse effects to first-line drugs develop during therapy, the treatment should include other drugs known as second-line drugs and these are the aminoglycosides kanamycin and amikacin, the polypeptide capreomycin, PAS, cycloserine, the thioamides ethionamide and prothionamide and several fluoroquinolones such as moxifloxacin, levofloxacin and gatifloxacin (ATS/CDC/IDSA, 2003; WHO, 2003; WHO, 2004).

In Ethiopia the recommended treatment guideline for category I and III is the same. This regimen still consists of 2 months treatment with Ethambutol (E), Rifampicin (R), Isoniazid (H) and Pyrazinamide (Z) during the intensive phase, followed by six months with Ethambutol and Isoniazid, i.e 2ERHZ/6EH.
However, the latest international recommendations suggest replacing the regimen with 6EH for the continuation phase with 4 months of RH, which has been documented to be more

effective than EH. Many countries already adapted their treatment regimen to these recommendations and Ethiopia will also adapt them soon as indicated in TLCP manual (FMOH, 2008). And, the regimen for Category II patients, also called retreatment regimen incorporate streptomycine injection in the intensive phase, i.e 2S(ERHZ) / 1(ERHZ) / $5E_3(RH)_3$ (5 months of treatment with a combination of E, R and H, 3 times a week on alternate days) (FMOH, 2008).

As a routine, all sputum-positive patients on DOTS must have sputum specimens examined at the end of the 2^{nd}, 5^{th} and 7^{th} month to follow up treatment outcomes and manage the patients accordingly to declare cured, treatment failure or to determine the need for investigation of MDR-TB (MOH, 2008).

1.3.6 Drug Resistant Tuberculosis

Drug resistance in tuberculosis is a matter of great concern for TB control programs since there is no cure for some multidrug-resistant TB (MDR-TB) strains of *M. tuberculosis*. MDR-TB, defined as resistance to at least rifampicin (R) and isoniazid (H), hence patients harboring MDR strains of *M. tuberculosis* need to be entered into alternative treatment regimens involving second-line drugs that are more costly and more toxic. Moreover, the problem of extensively drug resistant (XDR) strains defined as strains showing resistance to at least rifampicin and isoniazid, in addition to any fluoroquinolone and to at least 1 of the 3 following second line injectables drugs in anti-TB treatment: capreomycin, kanamycin and amikacin (WHO, 2008b).

Based on the most recent (WHO, 2008b) data, the global population weighted proportion of any resistance among new, previously treated and all TB cases were 17.0% (95% CLs, 13.6-20.4), 35.0% (95% CLs, 24.1-45.8) and 20.0% (95% CLs, 16.1-23.9) respectively. And isoniazid resistance in the same report was 10.3% (95% CLs, 8.4-12.1), 27.7% (95% CLs, 18.7-36.7) and 13.3% (95% CLs, 10.9-15.8) among new, previously treated and all TB cases respectively. The proportion of MDR-TB for new and re-treatment cases were 2.9% (95% CLs, 2.2-3.6) and 15.3% (95% CLs, 9.6-21.1) (WHO, 2008b).

Drug resistance in tuberculosis is also a huge problem in Ethiopia. Based on the data from national survey in 2006/2007, resistance to the individual drug in new patients was found to be 7.7% (H), 2.7% (R), 2.3% (E) and 23.2% (S). And the national prevalence of MDR-TB was 1.6% and 11.8% for new and previously treated patients respectively (MOH, 2008; WHO, 2008b).

1.4. Laboratory Methods for Detection, Isolation and Identification of *Mycobacterium tuberculosis*

There are many diagnostic methods that have been developed to detect and identify M. tuberculosis complex in mycobacteriology laboratories. These include acid fast bacilli (AFB) smear microscopy, culture on Löwenstein-Jensen medium, biochemical characteristics of the strains and molecular techniques involving specific nucleic acid markers. However, microscopy and culture are still the "gold standards" for the diagnosis of active TB in low-resource countries to confirm TB in patients with a clinical presumption of active disease.

1.4.1 Microscopy

Smear microscopy is still among the most rapid and inexpensive ways to diagnose tuberculosis and a rapid means to identify the most contagious patients. For the diagnosis of pulmonary TB, three first-morning sputum or three consecutive spot-morning-spot sputum specimens, based on the DOTS guidelines by WHO (http://www.phppo.cdc.gov/dls/ila/documents/lstc2.pdf) and IUTLD (http://www.iuatld.org/pdf/en/guides_publications/microscopy_guide.pdf) technical guide obtained after a deep, productive cough are usually recommended, whereas specimens to be collected for the diagnosis of extra-pulmonary disease depend on the site of the disease.

To determine whether a clinical specimen contains AFB or not, the specimen is spread onto a microscope slide, heat-fixed, stained with a primary staining, decolorized with acid-alcohol solution and counterstained with a contrasting dye in order to obtain better differentiation between the microorganism and the background when the slide is observed under the microscope. Though several methods can be used for determining the acid-fast organisms,

Ziehl-Neelsen technique utilizing basic fuchsin in ethanol as primary staining is the widely method used in developing countries including Ethiopia. In this method, AFB appears red after decolorization with acid-alcohol (Somoskovi, 2001; Van Deun, 2005). The minimum number of bacilli needed to be detected in stained smears has been estimated to be 5,000-10,000 per mL of sputum, therefore, the sensitivity of AFB smear staining relative to culture has been estimated to vary from 10 to 50% at least in high endemic areas where HIV is prevalent.

Several studies have been published on improving smear microscopy performance using methods that concentrate the bacilli present in the sputum specimen. The methods consist of specimen liquefaction and concentration or sedimentation steps prior to preparation of the smear and staining. The routine known concentration procedure used for microscopy in many countries including Ethiopia, is the 'bleach microscopy method', in which the sputum is liquefied with sodium hypochlorite (NaOCl or household bleach), and concentrated by centrifugation before AFB staining. In this method a significant improvement in the proportion of positive AFB smear results has been reported, ranging from 7% to 23% (Angeby, 2004).

1.4.2 Culture and Identification of Mycobacteria

Culture is used to detect cases with low mycobacterial loads and is also requested in cases at risk of drug-resistant TB for drug susceptibility testing, or in cases where disease due to another member of the *Mycobacterium* genus is suspected. Also, it is the only reliable means to monitor the effectiveness of therapy in TB patients (Vidal *et al.,* 1996). Mycobacterial culture detects as few as 10 –100 viable organisms/ml of specimen; hence it is more sensitive than smear microscopy. For detection of mycobacteria in clinical specimens, use of a combination of solid and liquid media is recommended as liquid media significantly shorter times for detection. The solid media widely used are generally of two types, egg-based and agar-based media.

The egg-based media such as Löwenstein-Jensen (LJ), Ogawa (more economic as asparagine is replaced by sodium glutamate which is more readily available and much cheaper), Modified Ogawa medium (pH 6.4) and Stonebrink medium (similar to LJ medium in which sodium pyruvate is replaced glycerol to promote growth of *M. bovis*), usually contain malachite green dye which inhibits growth of contaminating organisms and can bind and neutralize toxic compounds encountered in clinical specimens. Egg-based media support good growth of *M. tuberculosis* but need relatively longer period, 18–24 days on average, for growth. Besides, they are non-synthetic so that the quality of the ingredients may vary considerably which may affect the reproducibility of results (Jacobus and Robledo, 2007)

Agar-based media, e.g., Middlebrook 7H10 or 7H11, on the other hands, are chemically better defined and show growth of *M. tuberculosis* complex colonies within 10–14 days and visual examination of the colonies is much easier. The drawbacks of agar-based media are their limited shelf-life, higher costs of preparation, and requirement for a CO_2-enriched atmosphere.

Conventional culture media should be examined for growth twice a week for the first four weeks starting on day 3 to 5 post-inoculation, and thereafter, once a week until the eighth week to declare culture negative. On egg-based media *M. tuberculosis* bacilli produce characteristic non-pigmented colonies, with a general rough and dry appearance; and the colonies appear flat, dry and rough with irregular edges on agar based media (Jacobus and Robledo, 2007).

M. tuberculosis is identified by features as slow-growing, non-pigmented colonies that are niacin positive, are inhibited by p-nitrobenzoic acid and display nitratase activity (Table 1.3). Additional tests that confirm an isolate as *M. tuberculosis* are susceptibility to pyrazinamide, growth on thiophene carboxylic acid hydrazide, absence of catalase production at 68°C and absence of iron uptake (Jacobus and Robledo, 2007).

Table 1.3 Summary of the differential characteristics colonies of species *in the M. tuberculosis complex*

Test	*M. tuberculosis*	*M. bovis*	*M. bovis BCG*	*M. africanum*	*M. microti*	*"M. canettii"*
Morphology	rough	rough	rough	rough	rough	smooth
Pyruvate rather than glycerol as carbon source	-	+	+	-	-	-
Pyrazimidase	+	-	-	+	+	+
Niacin	+	-	-	+/-	+	-
Nitratase	+	-	-	+/-	-	+
Urease	+/-	-	+	+/-	+/-	+
Susceptibility to TCH	R	S	S	S	S	R
O_2 requirement	aerobic	Micro-aerophilic	aerobic	Micro-aerophilic	Micro-aero-philic	Unknown

R= resistant, S= susceptible, TCH= Thiophene-2-carboxylic acid hydrazide

On the other hand, Liquid culture media like Middlebrook 7H9 and Middlebrook 7H12 are used both in manual and automated systems for the detection of Mycobacteria. The radiometric, semi-automated BACTEC 460TB System (Becton Dickinson Microbiology Systems, USA) represented the most efficient and rapid technique to culture mycobacteria; in this method, ^{14}C-labeled palmitic acid as a carbon source in the medium is metabolized by microorganisms to $^{14}CO_2$, the amount of $^{14}CO_2$ and the rate at which the gas is produced are directly proportional to the growth rate of the organisms in the medium. For *M. tuberculosis*, an average detection time of 8 days was found for smear-positive specimens, compared to 19 days in non-radioactive, conventional solid media (Roberts *et al.,* 1983). For smear-negative specimens, an average recovery time of *M. tuberculosis* between 14 days in the BACTEC 460TB and 26 days in conventional media has been reported (Morgan *et al.,* 1983).

However, problems associated with the use of radioisotopes and the potential of needle punctures have implied the search for non radio-labeled and safer alternatives (Jacobus and Robledo, 2007).

The recently developed culture techniques involve non-radiometric liquid media, and range from manual systems utilizing simple tubes (MB Redox, Heipha Diagnostica Biotest, Heidelberg, Germany; Mycobacteria Growth Indicator Tube (MGIT), Becton Dickinson Microbiology Systems) to fully automated systems (BACTEC MGIT 960, Becton Dickinson Microbiology Systems; MB/BacT Alert 3D, Bio-Mérieux, Marcy-l'Etoile, France; ESP Culture System II, Trek Diagnostic Systems, Westlake, Ohio, USA). The non-automated MB Redox technique is based on a modified Kirchner medium which contains a colorless tetrazolium salt as a redox indicator which is reduced to colored formazan by actively growing mycobacteria. AFB can then be detected visually as pink-to-purple pinhead-size particles. Another manual culture method is represented by the MGIT (Becton Dickinson Microbiology Systems) which contains a modified 7H9 broth in conjunction with a fluorescence quenching-based oxygen sensor. Growth of mycobacteria or other microorganisms in the broth depletes the oxygen, and the indicator fluoresces brightly when tubes are illuminated with UV at 365 nm (Enrico and Palomino 2007).

Currently, there are several fully automated systems which allow continuous monitoring of mycobacterial cultures such as: the BACTEC MGIT 960 based on the MGIT technology; the MB/BacT Alert 3D utilizing a colorimetric carbon dioxide sensor in each bottle to detect growth of mycobacteria, and the ESP Culture System II which is based on the detection of pressure changes in the headspace above the broth medium resulting from gas production or consumption due to growth of microorganisms (Enrico and Palomino, 2007).

1.4.3 Molecular Methods for Identification Mycobacteria including *M. tuberculosis*

I. Molecular identification of MTBC

Most strains of *M. tuberculosis* complex encountered in a clinical mycobacteriology laboratory can easily be identified by physiological and biochemical characteristics. However, for strains with phenotypes which do not match fully with the type strain, additional procedures, mainly molecular ones, have to be considered.

The PCR restriction-enzyme analysis (PRA) method is based on the amplification of target fragment such as *hsp65, rpoB* and *gyrB* genes by PCR, followed by the digestion of the amplified product with two restriction enzymes (Lee, 2000; Goh, 2006). The products of the digestion reaction are then separated and visualized by agarose gel electrophoresis; and the restriction pattern obtained is then compared to an algorithm database available on the internet at http://app.chuv.ch/prasite/index.html which comprises 74 PRA patterns corresponding to 38 defined species of mycobacteria (Enrico and Palomino, 2007)

The DNA-probe technology for identification mycobacteria is still one of the most successful molecular diagnostic procedures worldwide. There are many commercial DNA probes methods used for mycobacterial identification in clinical laboratories. The pioneer system, AccuProbe (Gen-Probe, San Diego, CA) is still very popular due to its extremely simple procedure and it is the only DNA-probe system not requiring previous amplification of the target. The line-probe assay uses the reverse hybridization technology with differently-specific DNA-probes immobilized in parallel lines on a paper strip to detect target DNA. The commercial line probe methods available include INNO-LiPA MYCOBACTERIA (Innogenetics, Ghent, Belgium), GenoType Mycobacterium (Hain, Germany), and GenoType MTBC (Hain, Germany) (Enrico and Palomino, 2007).

The recently developed PCR-based MtbC typing method that makes use of MtbC chromosomal region-of-difference deletion (RD) loci (representing the loss of genetic material in *M. bovis* BCG compared to *M. tuberculosis* H37Rv is now becoming the popular procedure to identify species of mycobacteria as it is rapid, simple, and reliable methodology (Parson *et al.,* 2002; Richard *et al.,* 2003). In this method, different primer sets (RD primers), for example, which amplify within the RD loci such Rv1510 (RD4), Rv1970 (RD7), Rv3877/8 (RD1), and Rv3120 (RD12) are run in separate but simultaneous reactions. Each primer pair either specifically amplified a DNA fragment of a unique size or failed, depending upon the source mycobacterial DNA. The pattern of amplification products from all of the reactions, visualized by agarose gel electrophoresis, allowed immediate identification either as MtbC composed of *M. tuberculosis* (or *M. africanum* subtype II), *M. africanum* subtype I, *M. bovis*, *M. bovis* BCG, *M. caprae, M. microti*, or *M. canettii* or as a *Mycobacterium* other than MtbC (MOTT) (Parson *et al* 2002, Richard *et al* 2003).

II. Genotyping of *M. tuberculosis*

Several molecular techniques (genotyping) are available to explore the genetic diversity of MTB populations and are useful for epidemiological surveillance and understanding of TB transmissions. The three main techniques classically used in molecular epidemiology studies. All three of these molecular tools are based on the study of transposable and repetitive elements of the MTB genome: IS*6110* (Insertion Sequence *6110*) based restriction fragment length polymorphism (RFLP) genotyping, spoligotyping, and MIRU-VNTR (mycobacterial interspersed repetitive units-variable number tandem repeats) (van Embden *et al.,* 1993; Daley *et al.,* 2005).

Until recently, *IS6110*-based RFLP technique was the gold standard approach for genotyping MTB isolates which is based on the analysis of the locus called *insertion sequence-6110 (IS6110)*. Through a RFLP analysis, these insertion sequences have been used as epidemiological tools since they vary in copy number and may have different integration sites in different strains.

Briefly, extracted DNA from a bacterial culture is digested with the restriction endonuclease *Pvu-II*. DNA fragments are then separated according to their molecular weight by gel electrophoresis. The gel is then transferred and hybridized by a specific probe of IS*6110* elements, resulting in easily readable band patterns (van Embden *et al.,* 1993; Otal *et al.,* 1991).

However, this technique presents several disadvantages such as; requiring culture of MTB and a large amount of DNA, time-consuming, labor-intensive, technically demanding and it has relatively poor discriminatory power for isolates with fewer than five copies of IS*6110*.

Spoligotyping (spacer oligonucleotide typing) method is based on polymorphism of the chromosomal DR (direct repeat) locus. The DR elements contain multiple, well-conserved 36-bp DRs interspersed with non-repetitive spacer sequences (34–41 bp long). Strains vary in the number of DRs and in the presence or absence of particular spacers. Spoligotyping method detects the presence or absence of spacers of known sequence in an isolate in two steps; PCR based amplification of spacers between DRs and hybridization of biotin-labeled products on membrane containing covalently linked 43 oligonuclotides derived from spacers of *M. bovis* BCG and MTB H37Rv (Kamerbeek *et al.,* 1997; Dale *et al.,* 2001).

Contrary to the IS*6110* genotyping method, spoligotyping technique is polymerase chain reaction (PCR)-based, simple, rapid, and only small amounts of DNA are needed. It can be done on clinical samples or on strains shortly after inoculation into liquid culture. The results can be represented as a binary code (0 corresponding to absence, 1 to presence) and can be expressed in a digital format (Kamerbeek *et al.,* 1997, Dale *et al.,* 2001), which makes it easy to compare the data between laboratories and with data deposited in the international spoligotyping database SpolDB4 (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo) at the Pasteur Institute in Guadeloupe (Brudey *et al.,* 2006). Moreover, spoligotyping is useful not only to identify the species of the MTBC responsible for the infection, but also to characterize the MTB family at an intra-species level.

The disadvantage of this method is its paucity of discrimination for strains with larger IS6110 copy number, resulting in the need for another method for resolvent genotyping (Wilson *et al.,* 1998; Soini *et al.,* 2000). Recently, a second-generation spoligotyping that is more resolvent was developed to detect the presence of the 43 traditional spacers, as well as 51 novel spacers (van der Zanden, 2002).

Lately, a new genotyping technique was elaborated based on specific repetitive elements of *Mycobacterium tuberculosis*. A novel intergenic repetitive units dispersed throughout the mycobacterial chromosome have been identified in late 90's and called mycobacterial interspersed repetitive units (MIRUs) by Supply *et al.* (1997) and variable number of tandem repeats (VNTRs) by Frothingham *et al*. (1998).These structures are composed of 40–100-bp repetitive sequences organized in direct tandem repeats that are scattered in several locations throughout the chromosome of MTB H37Rv, and the total number of MIRUs or VNTRs is estimated to be about 40–50 per genome and some of these MIRU-VNTR loci have been tested so as to be used for molecular epidemiology studies (Supply *et al.,* 1997; Frothingham 1998; Supply *et al.,* 2004).

1.4.4 Detection of Drug Resistance in *Mycobacterium tuberculosis*

The detection of drug resistance (Drug susceptibility testing (DST)) in *Mycobacterium tuberculosis* is increasingly important procedure as drug resistance, including multidrug resistance, is a rapidly growing public health concern globally. Hence early detection of drug resistance constitutes one of the priorities of TB control programs as it allows initiation of the appropriate treatment in patients and also for surveillance of drug resistance (Dorman *et al.,* 2007).

In many settings, detection of drug resistance has been performed by the 'conventional methods' which is based on detection of growth of *M. tuberculosis* in the presence of the antibiotics. However, due to the laboriousness of some of these methods, and the long period of time necessary to obtain results, in recent years, new phenotypic and genotypic

methodologies and approaches have been proposed. DST on solid media such as Löwenstein Jensen or Middlebrook 7H10 is more widely used conventional phenotypic method in high endemic and resource limited countries. Most laboratories use the proportional method where the main principle is that resistance is defined as bacterial growth in the presence of an antibiotic at a higher level than in the 1:100 diluted controls. Other methods approved by the WHO include the absolute concentration method and the resistance ratio method (Schwoebel *et al.,* 2000; Kim, 2005; Drobinewski *et al.,* 2007).

Among the phenotype methods broth based methods such as BACTEC460 or Mycobacterial Growth Indicator Tube*,* BACTEC 960 MGIT (Becton Dickinson USA), are widely used in high resource countries both for conventional culture and AST (Drobinewski *et al.,* 2007; WHO, 2008c).

For AST, these methods, based on the proportion method, show a good agreement for the most important bactericidal drugs such as isoniazid and rifampicin but less so for ethambutol and streptomycin in inter-laboratory comparisons (Laszlo *et al.,* 2002).

Genotypic methods for drug resistance in TB look for the genetic determinants of resistance rather than the resistance phenotype, and involve two basic steps: nucleic acid amplification such as polymerase chain reaction (PCR), to amplify the sections of the *M. tuberculosis* genome known to be altered in resistant strains; and assessing the amplified products for specific mutations correlating with drug resistance (García de Viedma, 2003; Palomino, 2005).

In many cases, the genotypic methods in particular have been directed towards detection of RIF resistance, since it is considered a good surrogate marker for MDR-TB, especially in settings with a high prevalence of MDR-TB. New rapid PCR-based methods to detect MDR and XDR-TB on smear positive samples are now available such as GenoType MTBDR assay (Hain LifeScience GmbH, Nehren, Germany) which is an important contribution to rapid diagnosis of multidrug resistant strains (Ling *et al*., 2008).

Genotypic methods have the advantage of a shorter turnaround time, no need for growth of the organism, the possibility of direct application in clinical samples, lower biohazard risks, and the feasibility of automation; however, not all molecular mechanisms of drug resistance are known.

1.5 Pathogenesis of MTB

Tuberculous bacilli are spread out by infected patients coughing, sneezing, or speaking, and they can be inhaled by another individual in close contact. The inhalation of this aerosol presents a risk of tuberculous infection. The tubercle bacillus enters the human body mainly via the respiratory tract through the inhalation of the droplets in the air. The outcome following exposure to MTB can basically be divided to spontaneous healing, primary progressive tuberculosis which is more common in small children and immune-compromised children and subclinical primary infection leading to latent infection which can later give rise to reactivation and post-primary TB which is most common form of tuberculosis (Smith, 2003; Kaufmann and McMichael, 2005). The outcome is further complicated by the issue of re-infection which has been in to focus lately and could occur at any stage of the disease. In Africa and other areas where there is high rate of transmission, the risk of re-infection seems to be increased, especially in the immunocompromized patients; and it has also been shown that one patient could harbor different clinical strains simultaneously as identified by genotyping techniques (Robin *et al.,* 2004; van Rie *et al.,* 2005).

In the early event of the disease process, *M. tuberculosis* usually enters the alveolar passages of exposed humans in an aerosol droplet, where its first contact was thought to be with resident macrophages, but it is also possible that bacteria can be initially ingested by alveolar epithelial type II pneumocytes (Mario *et al.,* 2005). Dendritic cells also play a very important role in the early stages of infection since they are much better antigen presenters than are macrophages and play a key role in activating T cells with specific *M. tuberculosis* antigens and may also play an important role in dissemination of *M. tuberculosis* as they are migratory (Gonzalez and Orme, 2001).

The mycobacteria are phagocytosized in a process that is initiated by bacterial contact with macrophage mannose and/or complement receptors, hence complement receptors (CR1, CR2, CR3 and CR4), mannose receptors (MR) and other cell surface receptor molecules play an important role in attaching of the organisms to the phagocytes (Schlesinger, 1993).

Cholesterol, in cell plasma membranes, and the human toll-like receptors 2 (TLR2) also plays an important role for the binding and uptake of mycobacterium by phagocytes since their removal decreases the uptake and phagocytosis of mycobacteria (Gatfield and Pieters, 2000; Noss *et al.,* 2001).

On entry into a host macrophage, *M. tuberculosis* and other intracellular pathogens initially reside in an endocytic vacuoule called the phagosome. If the normal phagosomal maturation cycle, i.e. phagosome-lysosome fusion, occurs these bacteria can encounter a hostile environment that includes acid pH, reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNIs), lysosomal enzymes, and toxic peptides (O'Brien *et al*., 1994; Rich *et al.,* 1997; Nathan and Shiloh, 2000).

In the later stage, the infected macrophages in the lung, through their production of chemokines, attract inactivated monocytes, lymphocytes, and neutrophils forming granulomatous focal lesions composed of macrophage-derived giant cells and lymphocytes. As cellular immunity develops, macrophages loaded with bacilli are killed, and these results in the formation of the caseous center of the granuloma, surrounded by a cellular zone of fibroblasts, lymphocytes, and blood-derived monocytes, hence the impact of the host cellular immune response determines whether an infection is arrested or progresses to the next stages (Dannenberg and Rook, 1994).

In efficient cell-mediated immunity, the infection may be permanently eradicated at this stage. In yet some individuals, latent infection will be established through equilibrium between the bacilli and the host response, the granulomas subsequently heal, leaving small fibrous and calcified lesions as a sigh of previous primary infection. However, if an infected person cannot control the initial infection in the lung or if a latently infected person's immune system becomes weakened by immunosuppressive drugs, HIV infection, malnutrition, aging, or other factors, the granuloma center can become liquefied by which then serves as a rich medium in which the bacteria can replicate in an uncontrolled manner (Dannenberg and Rook, 1994; Kaufmann and McMichael, 2005). At this point, viable *M. tuberculosis* can

escape from the granuloma and spread within the lungs (active pulmonary TB) and even to other tissues via the lymphatic system and the blood (miliary or extrapulmonary TB) leaving the person infectious (Dannenberg and Rook, 1994; Fenton and Vermeulen, 1996; Kaufmann and McMichael, 2005).

1.6 Host Immune Response against Tuberculosis

Uptake of *M tuberculosis* by macrophages represents the first major host–pathogen interaction in tuberculosis, and presumably, macrophages of some hosts, upon initial contact with *M tuberculosis,* are able to kill the pathogen directly and completely eliminate it, never allowing a latent stage of infection to develop. Although there is of course no direct evidence for this, there are persons with repeated exposure to cases of active tuberculosis who neither develop positive tuberculin skin tests, nor active tuberculosis, providing indirect evidence that such an outcome is possible (Schluger, 2001).

1.6.1 Innate Immunity

Phagocytosis

The phagocytosis and the subsequent secretion of IL-12 are processes initiated in the absence of prior exposure to the antigen and hence should form a component of innate immunity. Among other components of innate immunity are natural resistance associated macrophage protein (Nramp), neutrophils, natural killer cells (NK), mast cells, dendritic cells, epithelial cells and defensin (Diamond *et al.,* 1998; Schluger, 2001; Fulton *et al* ., 2002). In addition plasma lysozyme and other enzymes may play an important role in the first line defense, of innate immunity to *M. tuberculosis* (Selvaraj *et al.,* 2001).

Reactive Oxygen Intermediates (ROI)

It was reviewed that Hydrogen peroxide (H_2O_2), one of the reactive oxygen intermediates (ROI), generated by macrophages via the oxidative burst, was the first identified effecter molecule that mediated mycobactericidal effects of mononuclear phagocytes. However, the ability of ROI to kill *M. tuberculosis* has been demonstrated only in mice and remains to be confirmed in humans (Nathan and Shiloh, 2000; Chan *et al.,* 2001; Thomas *et al.,* 2001; Rhoades *et al*., 1997).

Nitric Oxide (NO) and Reactive Nitrogen Intermediates (RNIs)

Phagocytes, upon activation by mycobacterial antigens together with IFN-γ and/or tumor necrosis factor-a (TNF-α), generate nitric oxide (NO) and related RNI via inducible nitric oxide synthase (iNOS2) using L-arginine as the substrate. The significance of these toxic nitrogen oxides in host defense against *M. tuberculosis* has been well documented, both *in vitro* and *in vivo*, particularly in the murine system (Nathan and Shiloh, 2000; Chan *et al.,* 2001; Zahrt and Deretic 2001; Chan *et al.,* 1992).

In contrast to the murine models of TB, there is some controversy on the role of NO in killing or limiting the growth of *M. tuberculosis* in humans. Nevertheless, there is a growing body of evidence that NO produced by TB-infected human macrophages and by epithelial cells is also antimycobacterial against *M .tuberculosis* (Chan *et al*., 1992; Chan *et al.,* 2001; Nicholson, 1996; Wang, 1998; Nicholson, 1996; Wang, 1998). Furthermore, the significance of NO production was shown by iNOS inhibition with L-NMMA (N^G-monomethyl-L-arginine) which resulted in enhanced *M. tuberculosis* growth in human macrophages (Chan *et al.,* 2001).

In some studies it was shown that in alveolar macrophages, from normal volunteers, iNOS and NO were detected after *M. tuberculosis* infection and that there was an inverse correlation between the magnitude of intracellular growth and the amount of NO produced. Furthermore it was also shown that iNOS was inducible in IFN- primed m onocyte-derived macrophages that are infected with *M. tuberculosis*, providing proof that there was high-output NO production in TB-infected macrophages (Nicholson *et al.,* 1996; Bonecini-Almeida *et al.,* 1998; Rich *et al.,* 1997).

In another study in the human promyelocytic cell line HL-60, vitamin D3, postulated to have a therapeutic although hitherto non proven effect against TB, suppressed the growth of *M. tuberculosis* via the production of NO (Rockett *et al.,* 1998). Kuo and colleagues (2000) showed that alveolar macrophages from TB patients produced increased amounts of NO compared to healthy control subjects.

Furthermore, NO played an auto-regulatory role in amplifying the synthesis of TNF-α and IL-1 as it was demonstrated that the increased direct NO levels in exhaled air of patients with TB was due to up-regulation of iNOS in alveolar macrophages (Wang *et al.,* 1998). In addition, the amount of exhaled NO correlated with the capacity of the alveolar macrophages *in vitro* to produce NO. Nitric Oxide is not only mycobactericidal but may also participate in the formation of protective tissue granulomas (Facchetti *et al.,* 1999).

In a randomized double-blind study in Gondar, Ethiopia, on the effects of arginine supplement on the clinical outcome of smear-positive tuberculosis, treatment with arginine in conjunction with anti-TB drugs increased exhaled NO production and enhanced clinical improvement during the intensive phase of anti-Tb therapy as indicated by increased sputum conversion and decreased cough although a direct causality was not proven (Schon *et al.,* 2003).

1.6.2 Adaptive immunity

It is frequently assumed that tubercle bacilli are not exposed to antibody, as they are intracellular pathogens, and therefore humoral immune response is considered to be non-protective. However, recent reports indicated that during the initial steps of infection, antibodies alone or in conjunction with the proper cytokines may provide important functions, such as prevention of entry of bacteria at mucosal surfaces. It was shown that specific antibodies increased the internalization and killing of BCG by neutrophils and monocytes/macrophages, and antibody-coated BCG bacilli were more effectively processed and presented by dendritic cells for stimulation of CD4+ and CD8+ T-cell responses (Salinas-Carmona, 2004; Williams, 2004; De Valliére *et al.,* 2005; Reljic, 2006).

The CD4- CD8- T cells, known as gamma/delta (γσ T cells) were proved to be relevant for the regulation of the immune response in that γσ T cells from human TB patients display a lytic activity, produce IL-17 which promotes the flow of cells towards infection sites during early infection in response to IL-23 secretion by dendritic cells infected with *M. tuberculosis* and, like dendritic cells, they can process and efficiently present antigens and give the co-

stimulating signals needed to induce proliferation of αβ T cells, hence T cells are noted to act as a link between the innate immune response and the adaptive immune response (De la Barrera, 2003; Brandes, 2005; Lockhart, 2006).

1.6.3. Mycobacterial Evasion Mechanism of the Host Immune Response

Pathogenic bacteria, including Mycobacteria, have various strategies for avoiding being killed by phagocytes. *M. tuberculosis* may be taken up via mannose receptors that fail to trigger killing events, and it also inhibits complement-receptor-mediated Ca^{2+} signaling, which may contribute to the failure of killing mechanisms (Chan *et al.,* 1991; Malik *et al.,* 2000).

Mycobacteria can inhibit acidification of the phagosome and modify intracellular trafficking of vacuoles, so that they behave like part of the endosomal recycling compartment rather than as toxic phagolysosomes (Sturgill-Koszycki *et al.,* 1994; Xu *et al.,* 1994). These vacuoles release quantities of LAM which insert into glycosylphosphatidylinositol (GPI)-rich domains in the cell membrane and LAM gain unusual glycan structure with the ability to modify numerous macrophage functions including the ability to respond to IFN-γ, and the ability to present antigen (Ilangumaran *et al*., 1995).

The pattern of cytokine release from infected macrophages changes so that macrophage activation is diminished, and T cell recruitment impaired as recruitment of Th1 lymphocytes requires cytokine like IL-12 production, which is inhibited by increased production of TGF-β and IL-10 (Ellner, 1997; Fulton *et al.,* 1998; Toossi *et al.,* 1997).

Again, TGFb and IL-10 also impair macrophage microbicidal function and the IL-10 contributes to increased release of TNF receptor-2, which blocks the activating role of TNF-α (Balcewicz-Sablinska *et al.,* 1998).

Induction of certain types of apoptosis appear to reduce the viability of the contained Mycobacteria, and it has been noted that the release of soluble Type 2 TNF receptors (sTNFRII) induced by virulent strains of *M. tuberculosis* may limit the apoptotic death of infected alveolar macrophages (Oddo *et al*., 1998; Balcewicz-Sablinska *et al.,* 1998).

Mycobacterial pathogens have also evolved a variety of mechanisms to counteract host cell production of ROI and RNI. A subset of these processes involve the expression of genes whose products interfere with synthesis of reactive intermediates, allow the direct catabolization of intermediary products, or participate in the repair of ROI- and RNI-generated DNA damage (Shiloh and Nathan, 2000).

1.7. Significance of the Study

Since there are no previous reports regarding the molecular epidemiology, effect of NO (nitric oxide) and anti-TB drugs on the clinical isolates of *Mycobacterium tuberculosis* from active smear positive TB patients in relation to their clinical outcome; and only limited existing data indicated that the susceptibility of mycobacteria to RNI (or nitric oxide) was strain-dependent. There was a need to investigate strain variability, the nitric oxide and drug susceptibility profile of the clinical isolates of *Mycobacterium tuberculosis* to see whether these differences could attribute to variability in the clinical outcome of active tuberculosis patients.

Therefore, beyond molecular epidemiology of MTB and prevalence of drug resistant TB, this study will give preliminary data concerning the importance of nitric oxide in host defense against tuberculosis and may also stimulate further study regarding the effect of arginine or arginine rich foods to improve the clinical outcome of conventional anti-Tb treatment to initiate its incorporation in TB control programs to combat pulmonary tuberculosis.

1.8. Objectives of the Study

General Objective

- To investigate the molecular epidemiology, drug and nitric oxide susceptibility of *M. tuberculosis* isolates obtained from active pulmonary tuberculosis patients

Specific Objectives

- To investigate anti-microbial susceptibility patterns of the isolates to conventional first line anti-tuberculosis drugs
- To investigate susceptibility patterns of the isolates to Nitric Oxide (NO)
- To identify factors associated with clinical outcome of active pulmonary TB
- To investigate the presence of non-tuberculous mycobacteria by molecular methods

CHAPTER II: MATERIALS AND METHODS

2.1 Study Area, Study Subjects and Sample size

This study was conducted on 180 smear positive stored sputum samples collected from consecutive pulmonary TB patients enrolled in the previous clinical trial study on arginine rich food supplementation (ClinicalTrials.gov identifier: NCT00857402) who treated at the Directly Observed Treatment Short course (DOTS) clinic in the referral and teaching hospital of the University of Gondar, North-west Ethiopia. All of 180 previously stored smear-positive sputum samples and the respective active pulmonary tuberculosis patients were considered in this study, hence no sampling technique or sample size calculation was employed. Active tuberculosis patients who were smear-positive, age between 15 and 60, and willing to take part in the study were taken as study participants. The exclusion criteria were history of previous treatment for tuberculosis, chronic diseases, pregnancy, lactation and malignancy.

2.2 Study Design

The study design combined prospective cross-sectional, *In vitro* experiment, and retrospective clinical data of 180 smear-positive sputum samples. The prospective and experimental design employed to investigate the molecular characterization, drug susceptibility testing and assessment of effect of nitric oxide on clinical isolates. And, retrospective data from the patients' chart such as sputum conversion at week 8, amount of NO in exhaled air, urinary nitrate/nitrite and other clinical and laboratory findings both at baseline and at 8 weeks were considered to relate with clinical outcome.

2.3 Laboratory Methods

The laboratory procedures consisted of Acid Fast Bacilli detection (using the conventional Ziehl-Neelson technique), Mycobacterial culture for isolation of *Mycobacterium* species, drug sensitivity testing (DST) to investigate drug resistance, conventional PCR technique for species determination, spoligotyping to identify the epidemiological patterns of the

circulating strains, and *in vitro* Nitric oxide killing assay (optimized experiments) as shown Figure 2.1.

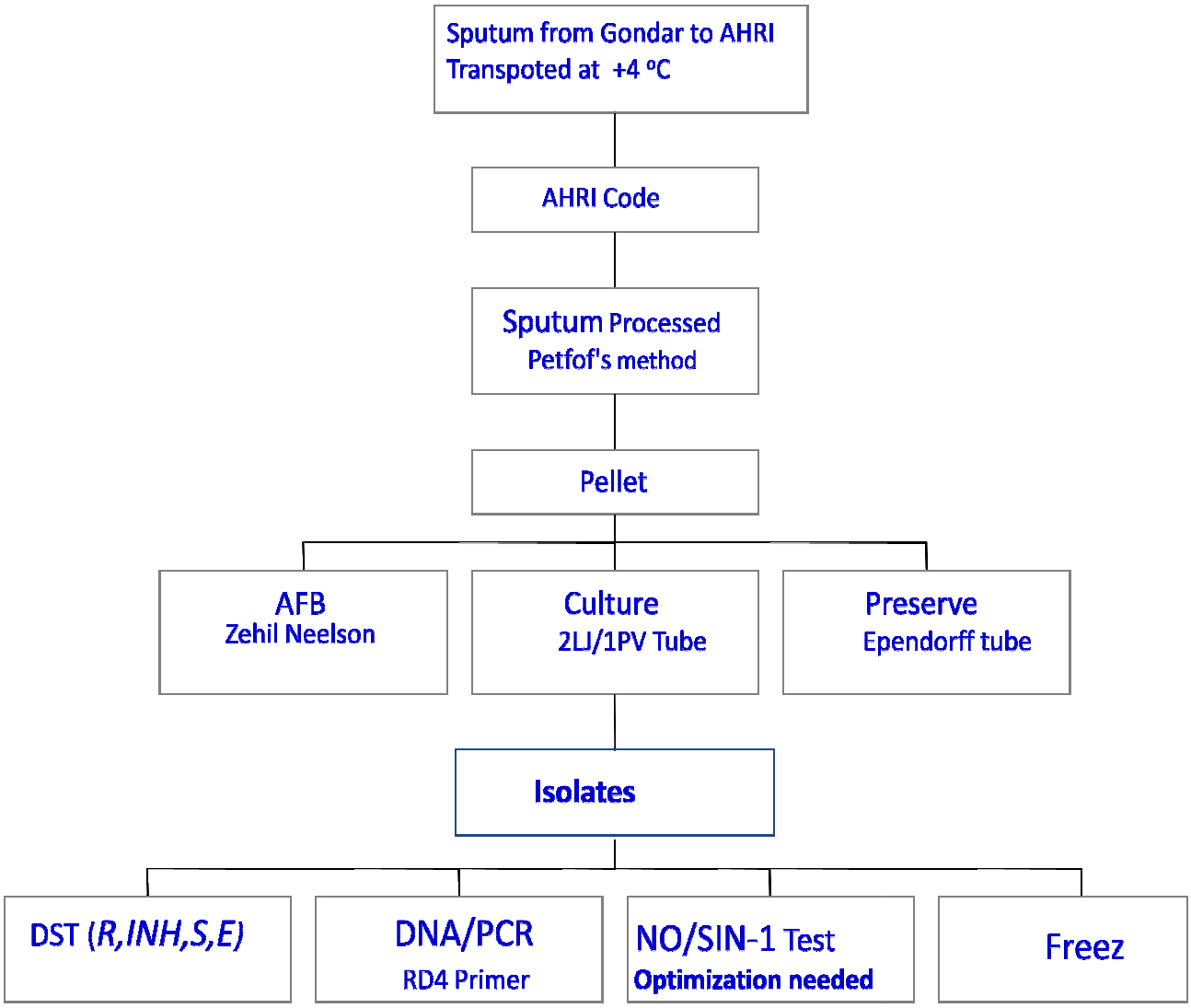


Figure 2.1 Profiles for Laboratory Protocols

2.3.1 AFB Detection and Isolation

I. Specimen collection, transport and processing

About 1.5ml crude sputum from the spot sputum sample from smear-positive TB patients was already collected with sterile tubes (Nunc, Denmark) and stored at -20^{o}C in the microbiology laboratory at Gondar University Hospital. The sputum samples were transported in cold chain at +4 ^{o}C to Addis Ababa, and processed with full precautions. For infection control and quality of the study, experiments were carried out in P3 (Biosafety level 3) tuberculosis laboratory at AHRI/ALERT, Addis Ababa, Ethiopia.

The specimens were processed using the adopted standard protocols (Kent *et al.,* 1985; Master, 1992; HPA, 2003) modified in AHRI TB laboratory. Sputum was digested and decontaminated by adding equal amount of 4% NaOH to the specimen, i.e. Modified Petroff's method (Annex-I). The mixture then kept in room temperature for 30 minutes with shaking every 10 minutes, and accompanied by centrifugation for 15 minutes at 3000g. After discarding the supernatant the sediment was neutralized with 2N HCL using phenol red as indicator. The pellet was resuspended with 3 ml Phosphate-buffer-saline (PBS) for subsequent culturing and Ziehl-Neelson staining.

II. Smear Test

A total of 100 µl of each decontaminated sample was placed on a microscope slide and stained with conventional Ziehl-Neelson technique (Kent *et al.,* 1985; Master, 1992; HPA, 2003). Slides were examined microscopically at 1000X magnification to check for the presence of the bacilli before culturing the sputum. A stain was considered positive if it contained at least five bacilli per 300 fields (Somoskovi *et al.,* 2001). The grading of the bacilli was as per IUATLD recommendation (Enarson *et al*., 1996) (Annex II).

III. MTB culture

From the above re-suspended pellet 250µl was inoculated on two conventional Löwenstien Jensen (LJ) slant media containing glycerol and one LJ slant containing sodium pyruvate. The tubes then were incubated at $37^{o}C$ and growth was monitored twice a week for at least eight weeks till labeled as culture-negative. A culture was considered positive if it contained at least one colony (Kent *et al*., 1985; Master, 1992; HPA, 2003) (Annex I).

2.3.2. PCR for Identification of Mycobacteria

PCR amplification primers conditions

M. tuberculosis was identified by a rapid and simple PCR-based approach built on genomic deletion analysis that make use of the presence or absence of *M. tuberculosis Complex (MtbC)* chromosomal region-of-difference deletion loci, RD4 region (Brosch, 1998) which represents a deletion of 12,7 kb which is primarily deleted in members of the *MtbC* other than *M. tuberculosis*. If RD4 is present (in case of *M. tuberculosis or M. africanum*) a product size of 335 bp is obtained, if deleted, however, (in case of *M. bovis*) amplicon product size of 446 bp will be obtained (Brosch *et al.,* 1998).

PCR was carried out as described by Parsons *et al.* (2002) and according to optimized protocol in AHRI PCR lab, as briefly described below, using multi-primer PCR assay with three RD4 primers with the following sequences: RD4 internal (RD4 Inter-ACACGCTGGGGAAGTATAGC), RD4 flanking reverse primer (RD4 FR-AAGGCGAACAGATTCAGCAT) and RD4 flanking forward primer (RD4 FF-CTCGTCGAAGGCCACCTAAAG).

PCR reaction mixture was prepared in 25 µl as follows : 12.5 µl Hotstart PCR master mix (Qiagen GmbH), 2 µl of heat killed cell DNA template (bacterial cells were kept at 80^{0}C for 1 hour), 10 mM of RD4 flank R , 10 mM of RD4 flank F, 50 mM of RD4 int .8.0 µl of each primer at a concentration 10 mM for RD4 flank R and RD4 flank F, and 50 mM of RD4 internal ones (5 µl internal, 1.5µl forward and 1.5µl reverse primers). PCR amplifications was performed in a PCR machine (GeneAmp 9700, Perkin-Elmer Biosystems, Foster City,

Calif.), using program (with an initial denaturation step of 15 min at 95°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, ending with a final elongation step for 10 min at 72°C and holding at 4°C. For the analysis of PCR product, 8 µl of the PCR product was mixed with 3 µl of DNA loading dye and analyzed on 1.5% agarose gel containing ethidium bromide, and electrophoresis was run for 1 hour at 100 V. Amplification of 335 bp confirmed *M. tuberculosis*, whereas, the presence of 446 bp product was interpreted as *M. bovis* (Brosch *et al.,* 1998; Parsons *et al.,* 2002) (Annex III).

2.3.3. Strain characterization

Spoligotyping

For Spoligotyping method the oligonucleotides DRa and DRb (having the sequence GGTTTTGGGTCTGACGAC-5'biotinylated and CCGAGAGGGGACGGAAAC, respectively) were used as primers to amplify the whole DR region by PCR.

Approximately 10 ng of genomic mycobacterial DNA heat extracted from cultured cells was used as a target for spoligotyping. Fifty micro-liters of the following reaction mixture were used for the PCR: *Tth* buffer (5 mM Tris z HCl, 5 mM KCl, 0.7 mM MgCl2, pH 9.0), each deoxynucleoside triphosphate at 200 mM, 20 pmol each of primers DRa and DRb, 10 ng of DNA, and 0.5 U of *Tth* polymerase (HT Biotechnology Ltd., Cambridge, United Kingdom). The mixture was heated for 3 min at 96^{o}C and subjected to 20 cycles of 1 min at 96^{o}C, 1 min at 55^{o}C, and 30 sec at 72^{o}C. The amplified product was hybridized to a set of 43 immobilized oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus. These oligonucleotides were covalently bound to a membrane as follows; a membrane (Biodyne C; Pall Biosupport, Portsmouth, United Kingdom) was activated by using 16% (wt/vol) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma). The oligonucleotides were applied to the membrane in parallel by using a miniblotter system (MN45; Immunetics, Cambridge, Mass.). After a short incubation, the membrane was inactivated by using 100 mM NaOH and washed in 2X SSPE (1X SSPE is 0.18 M NaCl, 10 mM NaH_2PO4, and 1 mM EDTA [pH 7.7]; Gibco BRL Life Technologies, Inc.) supplemented with 0.1% sodium dodecyl sulfate (SDS; Sigma).

For hybridization, 20 µl of the amplified PCR product was diluted in 150 µl of 2X SSPE–0.1% SDS and heat denatured. The diluted samples (130 ul) were pipette into the parallel channels in such a way that the channels of the mini-blotter apparatus were perpendicular to the rows of oligonucleotides deposited previously. Hybridization was done for 60 min at 60^{o}C. After hybridization, the membrane was washed twice in 250 ml of 2X SSPE–0.5% SDS for 10 min each time at 60^{o}C and then incubated in 1:4,000-diluted streptavidin-peroxidase conjugate (Boehringer) for 45 to 60 min at 42^{o}C. The membrane was washed twice, for 10 min each time, in 250 ml of 2X SSPE–0.5% SDS at 42^{o}C and rinsed with 250 ml of 23 SSPE for 5 min at room temperature.

Detection of hybridizing DNA was done by using chemiluminescent ECL (Amersham) detection liquid, followed by exposure to X-ray film (Hyperfilm ECL; Amersham) in accordance with the instructions of the manufacturer. For repeated use of membranes, the membranes were stripped by being washed two times for 30 min each time in 1% SDS at 80^{o}C and then incubated for 15 min in 20 nM EDTA (pH 8) at room temperature (Kamerbeek et al 1997).

2.3.4. Drug Susceptibility Testing

Drug susceptibility test was performed on initial isolates from all patients in order to identify resistant strains. The isolates were tested for their susceptibility to the primary drugs with the indicated critical concentration as recommended by WHO for streptomycin (SM), isoniazid (INH), ethambutol (EB), and rifampin (RIF) at concentration (µg/mL) of 2.0, 0.1, 5.0 and 1.0 respectively, using indirect agar proportion method (Canetti *et al*., 1969, NCCLS 2000).

In this method, Middle brook 7H10 supplemented with glycerol and 10% OADC was used. Loop-full of colony was taken and vortexed in a test tube that contains 7-10 beads and drops of PBS to homogenize the content. The bacterial suspension was adjusted to a McFarland turbidity standard-1 (OD value between 0.25 and 0.36 at 600 nm using Novaspec II photometer (Pharmacia Biotech Ltd, UK) with PBS. The bacterial suspension was diluted to 1:10 and 1:1000, and about 100µl from the former dilution transferred into all drug containing media and one drug free control media. Another 100µl from 1:1000 dilutions was transferred in to one drug free control media.

All the tubes were then incubated at 37^{o}C along with one reference strain (ATCC 35835, Isoniazid resistant) and known clinical isolates, as a control, and tubes were monitored for colony formation every week until the third week. The proportion of growth was calculated by dividing the number of colonies in a drug containing medium with the number in the corresponding drug free medium when the number of colonies in drug free medium is between 50 and 150. A bacterial growth of more than 1% was taken as resistant (Annex IV)

2.3.5. Optimization Experiments for RNI Killing Assay

RNI killing assay was done to determine the percentage survival of the isolates to nitric oxide, and the assay was developed after undertaking a series of independent standardizing experiments that help to set the early log period and bacterial concentration, the optimum exposure time of the isolates to RNI, and the optimum concentration of the RNI donor's for bacterial killing (Figure 2.2).

I. Determination of Early Log phase and in relation to Concentration of *M. tuberculosis* (Pilot study 1)

To determine the early log phase of the bacteria and the corresponding concentration, two loop full of a fresh clinical isolate from Löwenstein-Jenson agar was transferred to 20ml of 7H9 broth supplemented with OADC in a small Erlenmeyer flask and incubated for 14 days at 37°C in an atmosphere of 5% CO_2. One ml of the above 14 day old culture was transferred to 14 ml fresh 7H9 with OADC to 18 flat-bottomed small bottles (bijoux/universal bottles) labelled day1, day3, day5, up to day17 in duplicate and incubated at 37°C in an atmosphere of 5% CO_2. At each day from day 1, at the same time of the day (+/- 1 hour) until the 17th day, an aliquot of 200ul from the above culture was serially diluted 1:10 (for 8 dilution steps) in 1800µl sterile PBS/0.05% Tween 80 after thorough mixing. Then, 100ul from each serially diluted culture was inoculated to Middlebrook 7H10 agar to plate out and CFU was counted for each tube after incubating at 37°C an atmosphere of 5% CO_2 for 3 weeks. Finally the early log phase period and the corresponding bacterial concentrations were determined by plotting CFU/ml versus incubation period (IP) graph (Annex V).

II. Optimal incubation Time for RNI mediated killing of *M. tuberculosis* (Pilot study 2)

A loop full of fresh clinical isolate from the same isolates used for pilot experiment 1 was transferred to 10ml of 7H9 broth supplemented with OADC in a small Erlenmeyer flask and incubated for 14 days at 37°C in an atmosphere of 5% CO_2. After the 14^{th} day, 1ml of the above culture was transferred to 14 ml fresh 7H9 with OADC and incubated at 37°C in an atmosphere of 5% CO_2 for 11 days to reach the early log phase (app 1.5 x 10^9 bacteria/ml) as determined by pilot experiment 1. Then 100ul of the 11 day old culture was further diluted 1:15 in sterile PBS/Tween80 (1400ml PBS) to prepare starting concentration of approximately 10^8 CFU/ml. Then 400ul of the diluted bacteria (final concentration of 10^7 CFU/ml) was exposed to 200ul of 20mM DETA/NO [final concentration of 1mM DETA/NO (Diethylenetriamine/nitric oxide adduct, nitric oxide donor chemical from Sigma)] and 200ul sterile PBS (as RNI free control) in 3400µl Middlebrook 7H9 medium without OADC for 4, 24, and 72 hours in separate tubes. At the specified time period 100ul of 10-fold serially diluted aliquot from each tube was inoculated to Middlebrook 7H10 plate agar until 3 weeks for CFU count. The percent survival of the isolates for the DETA/NO after exposing for the specified time was calculated as outlined below (Annex VI).

$$\% \text{ survival in DETANO} = \frac{\text{CFU/ml in DETA/NO}}{\text{CFU/ml in PBS}} \times 100$$

III. Dose-Response investigation of DETA/NO exposure to *M. tuberculosis* during 24h incubation (Pilot study 3)

A loop full of fresh clinical isolate from the same isolates used for pilot experiment 1 was transferred to 10ml of 7H9 broth supplemented with OADC in a small Erlenmeyer flask and incubated for 14 days at 37°C in an atmosphere of 5% CO_2. After the 14^{th} day, 1ml of the above culture was transferred to 14 ml fresh 7H9 with OADC and incubated at 37°C in an atmosphere of 5% CO_2 for 11 days to reach the early log phase (app 1.5 x 10^9 bacteria/ml) as determined by pilot experiment 1. Then 100µl of the 11 day old culture was further diluted in 1400ul sterile PBS/Tween80 to prepare starting concentration of approximately 10^8 CFU/ml. Then 400µl of diluted bacteria (final concentration of 10^7 CFU/ml) was exposed separately to five duplicate tubes each containing 200µl DETA/NO of final concentration 10mM, 1mM, 100µM, 10µM, 1µM and PBS (0M) in 3400µl Middlebrook 7H9 medium without OADC for 24h (set by pilot experiment 2). After 24 hrs 100µl of 10-fold serially diluted aliquot from each tube was inoculated to Middlebrook 7H10 plate agar until 3 weeks for CFU count. The percent survival of the isolates for the DETA/NO after 24 hour exposure was calculated as outlined below (Annex VII).

$$\% \text{ survival in DETANO} = \frac{\text{CFU/ml in DETA/NO}}{\text{CFU/ml in PBS}} \times 100$$

IV. Optimized Assay for determining NO susceptibility in *M. tuberculosis*

Loopful of fresh clinical isolate from LJ medium was transferred to 10ml of 7H9 broth supplemented with OADC in a small Erlenmeyer flask and incubated for 14 days at 37°C in an atmosphere of 5% CO_2. After the 14^{th} day, 1ml of the above culture was transferred to 14 ml fresh 7H9 with OADC and incubate at 37°C in an atmosphere of 5% CO_2 for 11 days to reach the early log phase (app 1.5 x 10^9 bacteria) as determined by pilot experiment 1. Then 100µl of the 11 day old culture was further diluted 1:15 in 1400µl sterile PBS/Tween80 to prepare starting concentration of approximately 10^8 CFU/ml. Then 400µl of diluted bacteria (final concentration of 10^7 CFU/ml) were exposed in duplicate tubes to 200µl of 20mM DETA/NO (final concentration 1mM) and DETA free PBS (control) separately in 3400µl Middlebrook 7H9 medium without OADC for 24h (set by pilot experiment 2) at 37 ^{0}C in a 5% CO_2 atmosphere. After 24 hrs incubation, 100µl of 10-fold serially diluted aliquot from each tube was inoculated to Middlebrook 7H10 plate agar for 3 weeks for CFU count. The percent survival of the isolates for the DETA/NO after 24 hour exposure was calculated by the following formula. (Annex VIII)

$$\% \text{ survival in DETA/NO} = \frac{\text{CFU/ml in DETA/NO}}{\text{CFU/ml in PBS}} \times 100$$

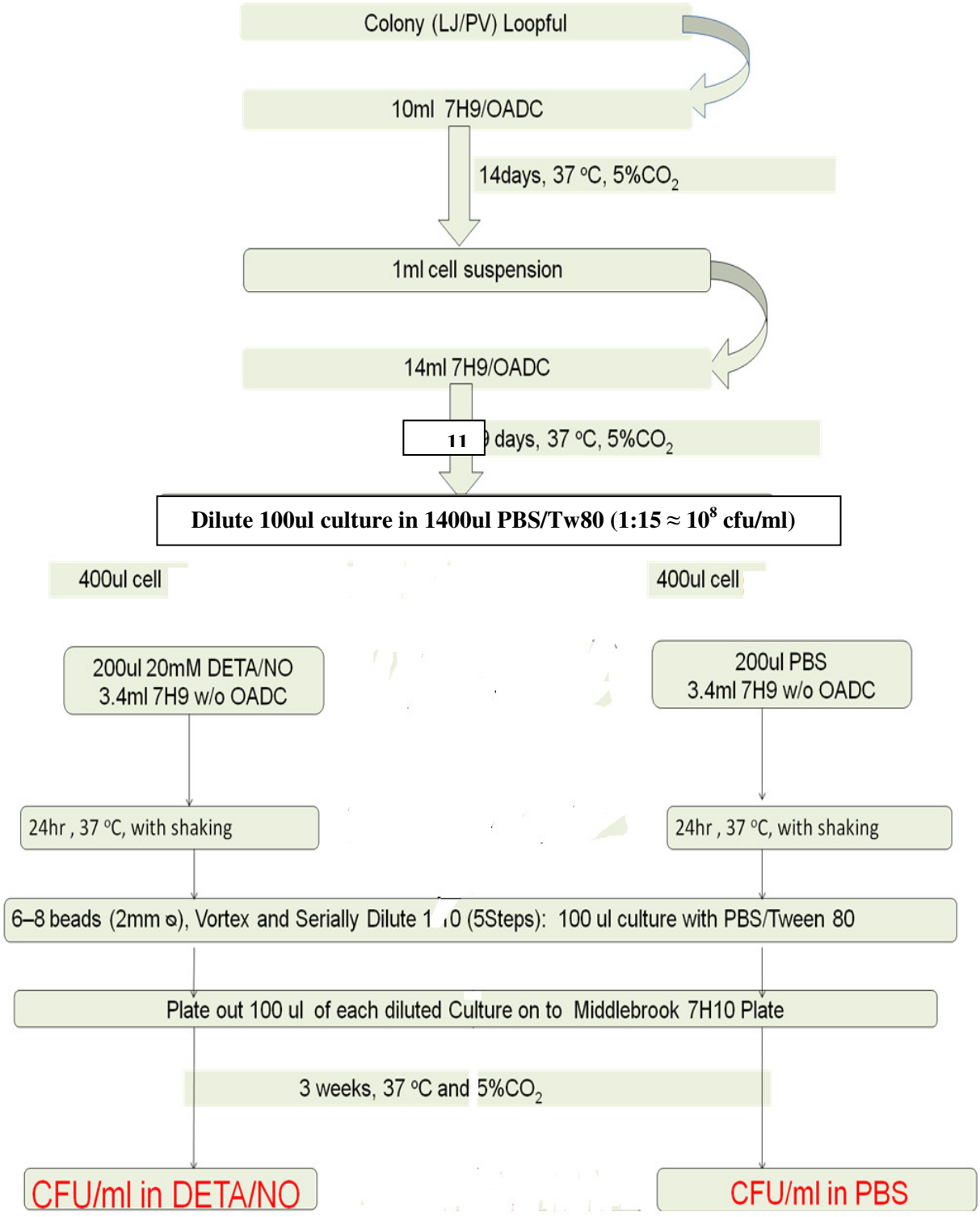


Figure 2.2 Flowchart of Optimized RNI Susceptibility Assay

2.4. Retrospective Clinical and Laboratory Data

For this study a number of clinical and laboratory data were retrospectively collected from patients' records obtained at baseline and while on treatment to follow up the clinical outcome of each patient. These variables include; age, sex, HIV sero-status, BCG vaccination, body mass index (BMI), reported weight loss, weight gain from baseline to 8^{th} week, duration of cough and fever in weeks, smoking habit, presence of hemoptysis, erythrocyte sedimentation rate (ESR), baseline AFB grade, AFB smear conversion at 8^{th} week, chest X-ray improvement, urinary nitrite and exhaled nitric oxide concentration in ppb (parts per billion) at week 0 and 5 months. The results of these clinical parameters were analyzed with the nitric oxide and drug susceptibility profile of the clinical isolates harbored by the respective patients to look for possible association with clinical outcome of active smear positive pulmonary tuberculosis patients.

2.5 Quality Control and Quality Assurance

The quality of the study was assured, as much as possible, by strictly following all the Standard Operating Procedures (SOPs) and incorporating quality control or reference strains, known laboratory strain (H37Rv) supplied by reference laboratories from national (specify the name of the lab) and abroad (specify the name of the lab) throughout the work. All the reagents, chemicals and media were collected from reputable sources and all the indicated protocols were optimized and assisted by expertise. Moreover, the work has been done in P3 level facility (Biosafety level 3).

2.6 Statistical Analysis

All the laboratory data was registered in a lab log book and entered to a computer data software program for relevant analysis. Other retrospective data were obtained from the previous laboratory and clinical records and results were double entered into SPSS version 16.0 statistical software (SPSS Inc., Chicago, Patent No. 7,023,453) and STATISTICA version 8.0 (StatSoft; Maisons-Alfort, France). Mean values, proportions, and other relevant statistics were obtained, analyzed and presented in the form of tables and graphs.

The continuous data are tested with student t-test and discrete data (all percentages) were tested with Fishers exact test, and p-value of less than 0.05 was considered as statistically significant.

In order to correlate nitric oxide susceptibility to clinical data, multiple logistic regression analysis was applied entering variables with a p-value less than 0.1 from the univariate analysis.

2.7. Ethical Issues

The ethical clearance for using the stored sputum samples, retrospective demographic, clinical and laboratory data was obtained from the Faculty Research Publication Committee-II (FRPC-II), Faculty of Medicine, Addis Ababa University, AHRI/ALERT Ethical Review Committee, Gondar University and Linköping University, Sweden. The stored sputum samples were collected from patients with active pulmonary tuberculosis during the previous clinical trial study on arginine rich food supplementation (ClinicalTrials.gov identifier: NCT00857402). There was not any direct contact between the study subjects and investigator. Results of this analysis have not been linked to the study subjects' identification in any form.

CHAPTER III RESULTS

3.1 Patients' Characteristics from Retrospective Records

One hundred eighty new smear positive active pulmonary tuberculosis (PTB) patients were enrolled for the previous clinical trial study on arginine rich food supplementation (ClinicalTrials.gov identifier: NCT00857402). Of which, four of the participants were already registered as smear negative, hence excluded from the analysis; therefore there were 176 eligible participants for final analysis.

Out of the 176 participants, 93 (52.8%) were males and 68 (38.6%) were HIV sero-positives (Table 3.1). The mean age and average value of BMI was 27.4 (n=175, SD=9.5, R; 15-59) and 16.6 (n=173, SD=2.2, R; 12.5-23.5) respectively. The proportions of BCG vaccinated, smokers and patients exhibited with haemoptysis were 10.5%, 13.1% and 29.3% respectively (Table 3.1). Their average smear AFB grade was 2.1 (SD=0.64).

There was strong association between age and HIV sero-status as HIV sero-positivity was more prevalent (70.6%) in age group between 25 and 45 years old (P=0.000). The other factors as sex, BMI, smear AFB grade and BCG vaccination were not linked with HIV sero-status (Table 3.2).

Table 3.1 Characteristics and frequency of new pulmonary patients in Gondar, 2005-2007

Variable	Frequency	Percent
Sex (n=176)		
Male	93	52.8
Female	83	47.2
HIV (n=176)		
Positive	68	38.6
Negative	108	61.4
Age, yrs (n=175)		
<25	90	51.1
25-45	78	44.6
>45	7	4.0
BMI (kg/m^2) (n=173)		
<16	77	44.5
16-18	58	33.5
>18	38	22.0
Temperature (^{o}C) (n=175)		
<37.8	96	54.9
>37.8	79	45.1
BCG vaccinated (n=172)		
Yes	18	10.5
No	154	89.5
Smokers (n=175)		
Yes	23	13.1
No	152	86.9
Hemoptysis at wk 0 (n=174)		
Present	51	29.3
Absent	123	70.7
AFB grade at wk 0 (n=176)		
+	26	14.8
++	84	47.7
+++	66	37.5

Table 3.2 Characteristics and distribution of PTB patients by HIV sero-status in Gondar, 2005-2007

	HIV Sero-status			
Variables	**Positive n (%)**	**Negative**	**Total**	**P-value**
Sex				
Male	36 (52.9)	51	93	
Female	32 (47.1)	57	83	0.980
Total	68	108	176	
Age (yrs)				
<25	16 (23.5)	74	90	
25-45	48 (70.6)	30	78	**0.000**
>45	4 (5.9)	3	7	
Total	68	107	175	
Temperature (^{o}C)				
<37.8	33 (48.5)	63	96	
>37.8	35 (51.5)	44	79	0.180
Total	68	107	175	
BCG vaccinated				
Yes	6 (9.1)	12	18	
No	60 (90.9)	94	154	0.642
Total	66	106	172	
Haemoptysis at wk 0				
Present	18 (26.9)	33	51	
Absent	49 (73.1)	74	123	0.575
Total	67	107	174	
AFB grade at wk 0 (n=176)				
+	12 (18.2)	14		
++	35 (53.0)	49		0.326
+++	21 (31.8)	45		
Total	66	108		

There was remarkable statistical difference (P = 0.000) in HIV sero-prevalence among different age groups of new smear positive PTB patients as only 23.5% of young adults (< 25 yrs) and 5.9% of the older (> 45 yrs) were HIV sero-positives when compared to the adults between 25 and 45 yrs which constitute more than 70% of the HIV-TB co-infected patients (Figure 3.1).

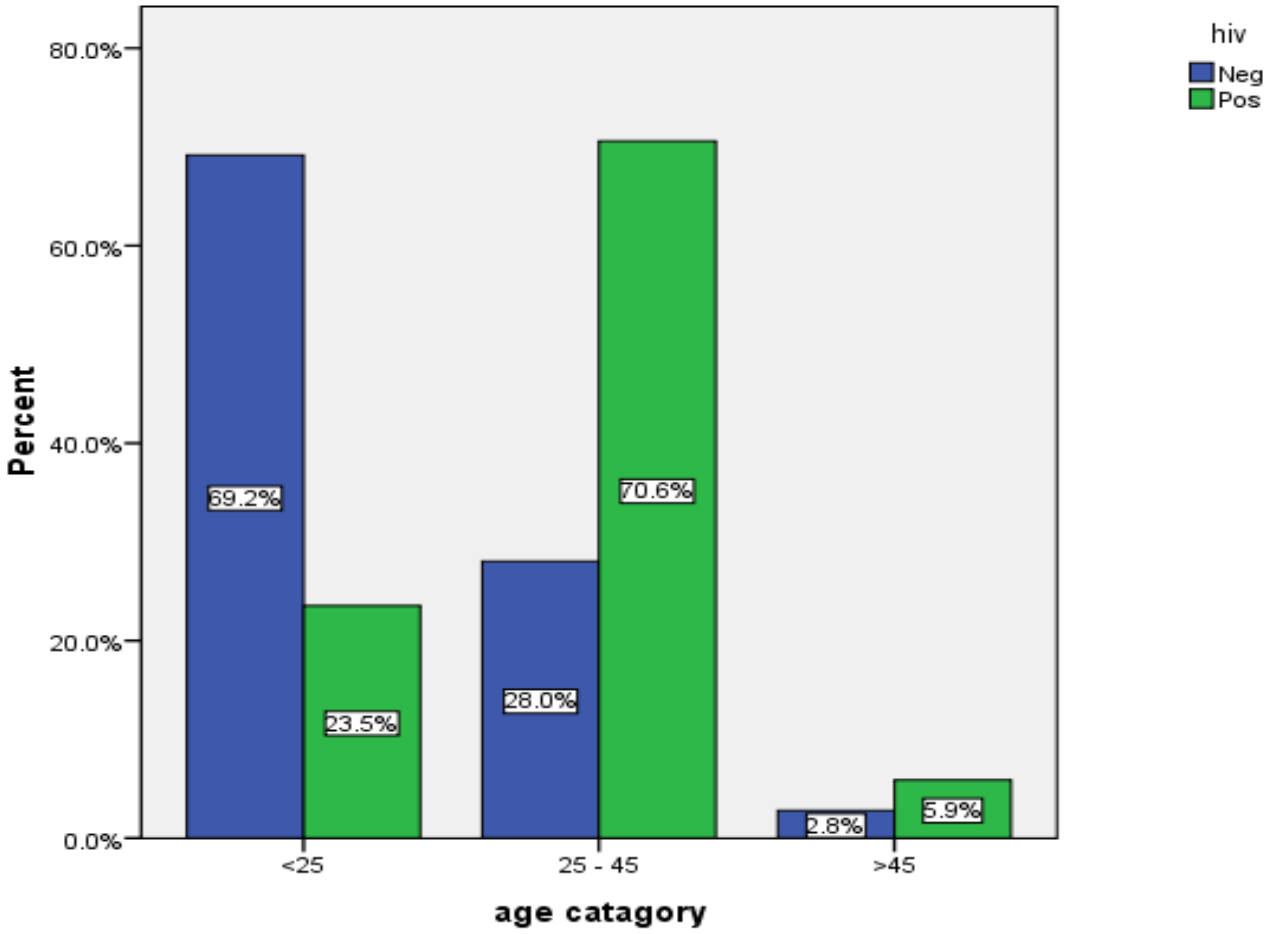


Figure 3.1 HIV Sero-positivity among Smear positive TB patients by age in Gondar, 2005-2007

3.2 Mycobacterial Culture

A 2ml portion of sputum stored at -20^{o}C of the first among the three sputum smear samples (n=180) were cultured on the conventional LJ medium to isolate *Mycobacterium species.* Of these, 117 sputum samples were culture positive making the recovery rate **65.0%,** however the true recovery rate was 66.5% as 4 of the 180 sputa were smear negatives, and one isolate failed to recover for subsequent experiments. The rest of samples were lost due to either contamination, drying out during incubation process, or were culture negative. The contamination rate was 4.4 % (8 of the 180).

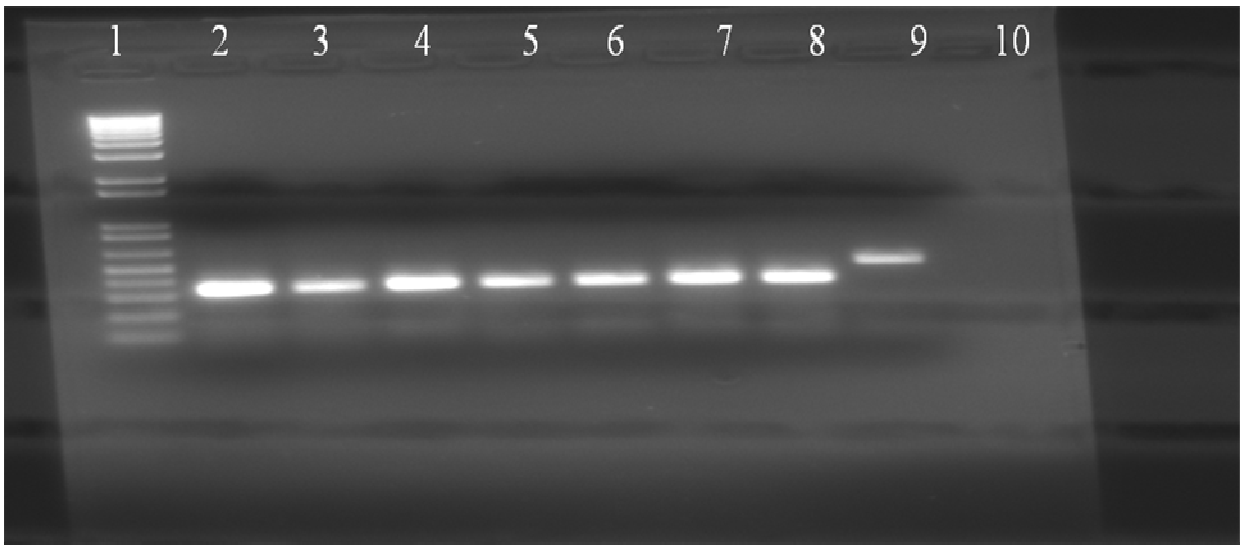
In this study culture positivity was not related to the patients' clinical records such as HIV sero-status and sputum smear AFB grade as there was no statistical difference in culture positivity among both HIV positive and negatives (P=0.490) and smear AFB grade 1+, 2+ and 3+ (P= 0. 517). However, of the 47 culture negative sputum samples processed for AFB 20 (42.5%), 21 (44.7%), 5 (10.6%) and 1(2.1%) were found to be AFB negative, 1+, 2+ and 3+ respectively up on re-checking showing statistical difference (P=0.000) between groups in that having smear negative and AFB 1+ constitute more than 85% of the culture negatives. Again, having AFB smear negative and grade 1+ result has a larger chance of also being culture negative (OR= 12.2; 95% CI, 1.8 – 80.9).

Table 3.3. Culture positivity by HIV status and AFB grade of PTB patients in Gondar, 2005-2007

Variable	Culture on LJ			
	Negative n (%)	**Positive n (%)**	**Total**	**P-value**
HIV Sero-status (n=164)				
Negative	27 (57.4)	74 (63.2)	101(61.6)	
Positive	20 (42.6)	43 (38.6)	63 (38.4)	0.490
Total	4 7	117	164	
AFB grade Record (n=164)				
+	9 (18.2)	16 (13.7)	25 (15.2)	
++	23 (53.0)	54 (46.2)	77 (40.0)	0.517
+++	15 (31.8)	47 (40.2)	62 (37.8)	
Total	47	117	164	
AFB grade Recheck (n=61)				
Negative	20 (42.6)	0	20	
+	21 (44.7)	1 (7.1)	22	
++	5 (10.6)	10 (71.4)	15	0.000
+++	1 (2.1)	3 (21.5)	4	
Total	47	14	61	

3.3 PCR Based Identification of *Mycobacterium tuberculosis*

All the 116 mycobacterial isolates were tested with conventional PCR techniques to identify and differentiate at the species using the RD4 primers that amplify the region of difference deleted locus, and results revealed that all the isolate tested have similar pattern of bands with *Mycobacterium tuberculosis* H37Rv strain with amplification signal at band size of 335bp indicating that all strain identified were *Mycobactrium tuberculosis* (or *M. africanum* subtype II which is not prevalent in Ethiopia). The absence of the indicated amplification signal indicates that none of the strains were from members of non-tuberculoses mycobacteria (NTM) or atypical mycobacteria.



Lane1= ladder, Lane 2-7= Clinical samples, Lane8= M. tuberculosis H37Rv (positive control), Lane9= M. bovis (positive control) and Lane10= NTC (non template control)

Figure 3.2 Image of some of PCR amplified bands of selected strains using RD4 primers, AHRI, 2007/08

3.4. Spoligotyping Pattern of the Clinical Isolates

Of the 116 isolated strains 108 (93%) were successfully typed by the conventional spoligotyping technique as described by Kamerbeek *et al.* (1997). Of these, 108 strains 83 (76.8 %) were found to be clustered as they had matching shared type identification (STI) number in the international spoligotype database while the remaining 25 strains (23.2%) were unique (non clustered) as they do not have a shared spoligotype in the database. These 84 shared types were distributed in to 12 clusters as indicated in the table below (Table 3.4), STI number 25, 149, 54 and 357 were the predominate ones constituting 58.3% (49/84) of the clustered isolates. The identified strains were grouped in to 4 major families and 9 sub-families, CAS and T families being the most common strains having the proportion of 42.8% and 40.5 respectively (Figure 3.4).

Samlpe ID	Pattern	SIT number	Family
13	nnnoooonnonnnnnnnnnnoooooooooooooooonnnnnnnn	21	CAS1_KILI
14	nnnoooonnnnnnnnnnnnnnnoooooooooooonnnnnnnnn	26	CAS1_DELHI
15	nnnnnnnnnoooooooonoonnnnnnnnnnnnnnoooonnnnnnn	unique	
16	nnnnnnnnnnnnnonnnnnnnnnnnnnnnnnnnnnnoooonnnnnnn	37	T3
17	nnnnnnnnnnnnnonnnnnnnnnnnnnnnnnnnnnnoooonnnnnnn	37	T3
19	nnnoooonnonnnnnnnnnnoooooooooooooooonnnnnnnn	21	CAS1_KILI
21	nnnoooonnonnnnnnnnnnoooooooooooooooonnnnnnnn	21	CAS1_KILI
22	nnnoooonnonnnnnnnnnnoooooooooooooooonnnnnnnn	21	CAS1_KILI
24	nnnnnnnnnnnnnnnnnnnnnnnnnnnnnoooonoooonnnnnnn	unique	
25	nnnoooonnonnnnnnnnnnoooooooooooooooonnnnnnnn	21	CAS1_KILI
26	nnnoooonnonnnnnnnnnnoooooooooooooooonnnnnnnn	21	CAS1_KILI
27	nnnoooonnnnnnnnnnnnnnnoooooooooooooonnnnnnn	357	CAS
28	nnnnnnnnnnoooooooooonnnnnnnnnnnnnnnoooonnnnnnn	149	T3_ETH
29	nnnnnnnnnnoooooooooonnnnnnnnnnnnnnnoooonnnnnnn	149	T3_ETH
31	nnnnnnnnnnoooooooooonnnnnnnnnnnnnnnoooonnnnnnn	149	T3_ETH
33	nnnoooonnnnnnnnnnnnnnnnoooooooooooonnnnnnnnn	26	CAS1_DELHI
34	nnnoooonnnnnnnnnnnnnnnnoooooooooooonnnnnnnnn	26	CAS1_DELHI
35	nnnnnnnnnnoooooooooonnnnnnnnnnnnnnnoooonnnnnnn	149	T3_ETH
36	nnnoooonnnnnnnnnnnnnnnnoooooooooooonnoonnnnn	25	CAS1_DELHI
37	nnnnnnnnnnoooooooonoonnnnnnnnnnnnnnoooonnnnnnn	unique	
39	nnnoooonnnnnnnnnnnnnnnnoooooooooooooonnnnnnn	357	CAS

* n and o respectively represents the presence and absence of the respective spacers

Figure 3.3*Binary patterns of spoligotypes of some of the isolates from PTB patients in Gondar, 2008

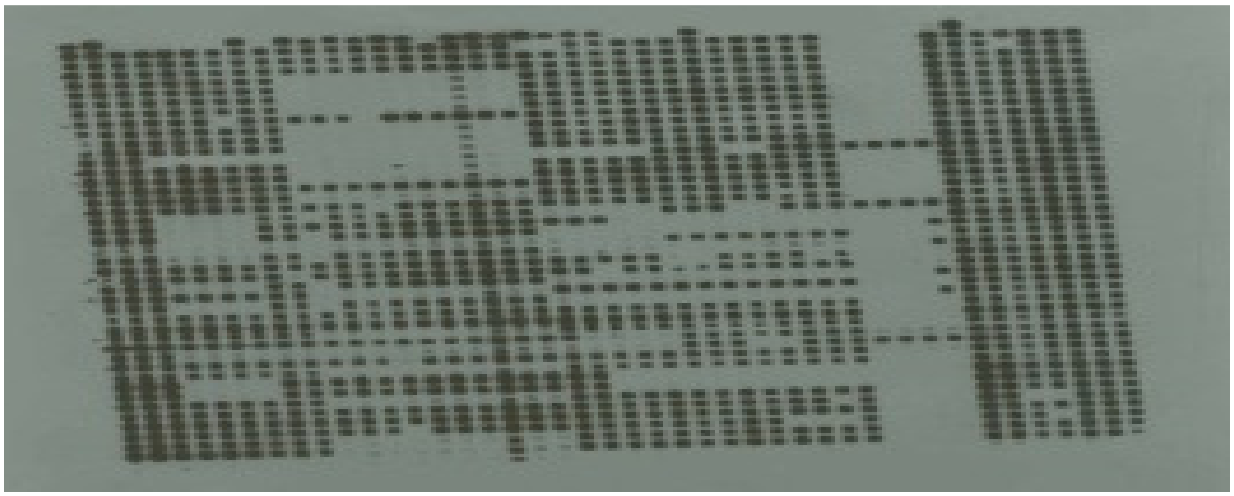


Figure 3.4 Photographic image of amplified spacer nucleotides signaled by hybridized dots.

Table 3.. The frequency and proportions of MTB families in PTB patients from Gondar, 2008

STI[†] (Family)	N	%
5 (CAS1_DELHI)	16	14.8
149 (T3_ETH)	13	12
53 (T1)	11	10.2
357 (CAS)	9	8.3
37 (T3)	8	7.4
21 (CAS1_KILI)	7	6.5
46 (U (likely H))	6	5.55
777 (H4)	5	4.6
26 (CAS1_DELHI)	4	3.7
35 (H4)	2	1.85
52 (T2)	1	0.93
910 (U)	1	0.93
Unique	25	23.1
Total	**108**	**100**

[†] Shared type identification number

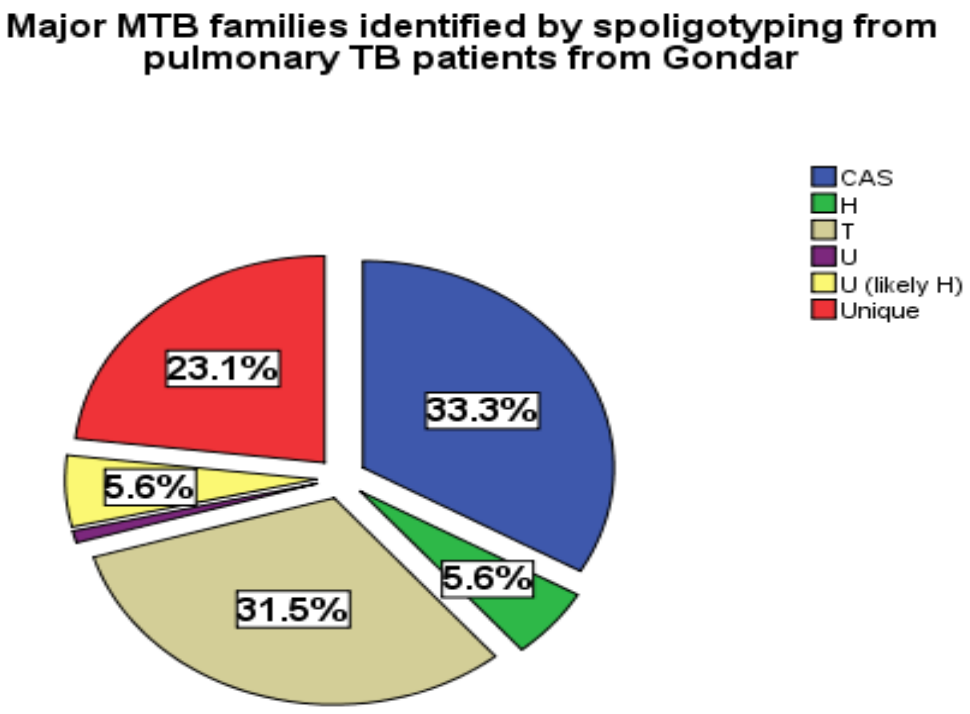


Figure 3.5 Distribution of major MTB strains by spoligotyping

Our spoligotyping result also confirms that all of the isolated strains were *M. tuberculosis* as none of them exhibited spoligotyping patterns specific to *M. bovis* (lack of spacers 39 to 43) or *M. africanum* (simultaneous lack of spacers 8, 9, and 39).

To identify the possible association between clustering and clinical and demographic characteristics such as age, sex, HIV status, drug resistance, and nitric oxide resistance data were entered in univariate analysis and none of them were associated with clustering (Table 3.5).

Table 3.5 Factors associated with clustering of strains by spoligotyping

Variable	Total N	Clustered	SD	N	Non clustered	SD	N	P value
Mean age, yrs	107	26.7	8.7	82	28.7	12.7	25	0.06
%NO survival	48	19.9	21.4	41	11.8	12.6	7	0.05
Mean BMI, Kg/m^2	108	16.6	2.1	83	16.8	2.8	25	0.24
Female, %	108	53	-	83	44	-	25	0.43
HIV +, %	108	34.9	-	83	44	-	25	0.41
Drug resistant, %	90	14.5	-	69	14.3	-	21	0.98

3.5 Drug Susceptibility Results and Associated Factors

Drug sensitivity testing was done for 98 of the 116 (84.5%) of the strains obtained and the overall prevalence of drug resistant was found to be 14.3% (14 out of 98). The proportion of resistance to INH, STM, and RIF was found to be 8.2 (8/98), 6.1 (6/98) and 1.0% (1/98) respectively. One out of 98 strains tested was resistant to more than one drug (INH and STM) making the proportion of poly-resistance isolate to be 1.0%. No MDR-TB strains were isolated in this study.

Table 3.6 Susceptibility pattern of *Mycobacterium tuberculosis* against antituberculosis drugs

Drug resistance (n=98)	No. (%) of isolates
Resistance to any drug	**14 (14.3)**
Resistance to any of the following drugs	
INH	**8 (8.2)**
STM	**6 (6.1)**
RIF	**1 (1.0)**
ETM	**0**
Resistance to one drug only	
INH	**7 (7.1)**
STM	**5 (5.1)**
RIF	**1 (1.0)**
Resistant to more than one drug	
INH/STM	**1 (1.0)**

INH: Isozianide *STM: Streptomycin* *RIF: Rifampcin* *ETM: Etambutol*

In logistic regression analysis used to identify possible clinical and demographic data possibly associated with drug resistance, only BMI (kg/m^2), sputum smear conversion, treatment outcome, bacterial resistance to nitric oxide were significantly associated (Table 3.7).

Table 3.7: Clinical data in relation to drug susceptibility pattern of 1st line drugs (INH, STM, EMB and RIF) for PTB patients in Gondar, 2008

Variables	Total No.	Fully Susceptible	SD	n	Any resistance	SD	n	p
Age	95	28.3	10.5	83	26.8	5.3	12	0.61
Sex (% males)	95	48.2	0.5	83	66.7	0.5	12	0.24
HIV (% positive)	95	42.2	1.0	83	33.3	1.0	12	0.57
BMI (kg/m2)	95	16.9	2.3	83	15.5	1.3	12	**0.046**
Temperature (^{o}C)	94	37.7	1.1	82	38.2	1.2	12	0.16
ESR (mm/h)	95	74.8	25.2	83	68.7	19.6	12	0.42
Weight gain at wk 8 (%)	90	91	0.3	78	91.7	0.3	12	0.94
Cough week 8 (%)	90	52.6	0.5	78	58.3	0.5	12	0.72
Hemoptysis wk 8 (%)	90	6.4	0.2	78	8.3	0.3	12	0.81
Smear conversion wk 8 (%)	92	91.2	0.3	80	66.7	0.5	12	**0.034**
Cured (%)	95	78.3	0.4	83	50	0.5	12	**0.045**
NO-survival (%)	48	15.7	19.1	40	33.9	21.3	8	**0.02**
uNO wk 0 (mM)	48	1520	1378.5	83	2661	4009.0	12	**0.054**
eNO wk 0 (ppb)	53	16.2	8.0	47	14.5	9.5	6	0.63
eNO month 5 (ppb)	40	17.9	10.5	36	17.4	8.1	4	NS
uNO = Urinary nitric oxide metabolites; eNO = Exhaled nitric oxide; wk = week; SD = standard deviation; n = number of isolates; NS= not significant								

3.6 Results of RNI Optimization Experiments

Three pilot experiments were designed to optimize and set protocol for nitric oxide survival assay. These optimization experiments were designed to determine the bacterial early logarithmic phase and the corresponding bacterial concentration (Pilot 1), to determine the optimum bacterial exposure time to nitric oxide (Pilot 2), and to determine bacterial dose-response trend to various concentration of nitric oxide (Pilot 3). Therefore, three randomly selected fresh clinical isolates (coded RNI027, RNI065 and RNI066) and two laboratory strains (*Mycobacterium tuberculosis* H37Rv and *M. bovis* BCG) were used for the entire experiments to minimize inter-species bias.

Based on the data from pilot experiment 1, therefore, the period for early logarithmic phase was determined to be day 11th and the average bacterial concentration for the three clinical isolates at the specified period was calculated to be approximately 1.50 X 10^9 CFU/ml (Figure 3.5). The cfu/ml of all the strains included in the experiment from day 1 to 17 is summarized and presented in the table in the annex part (Annex V).

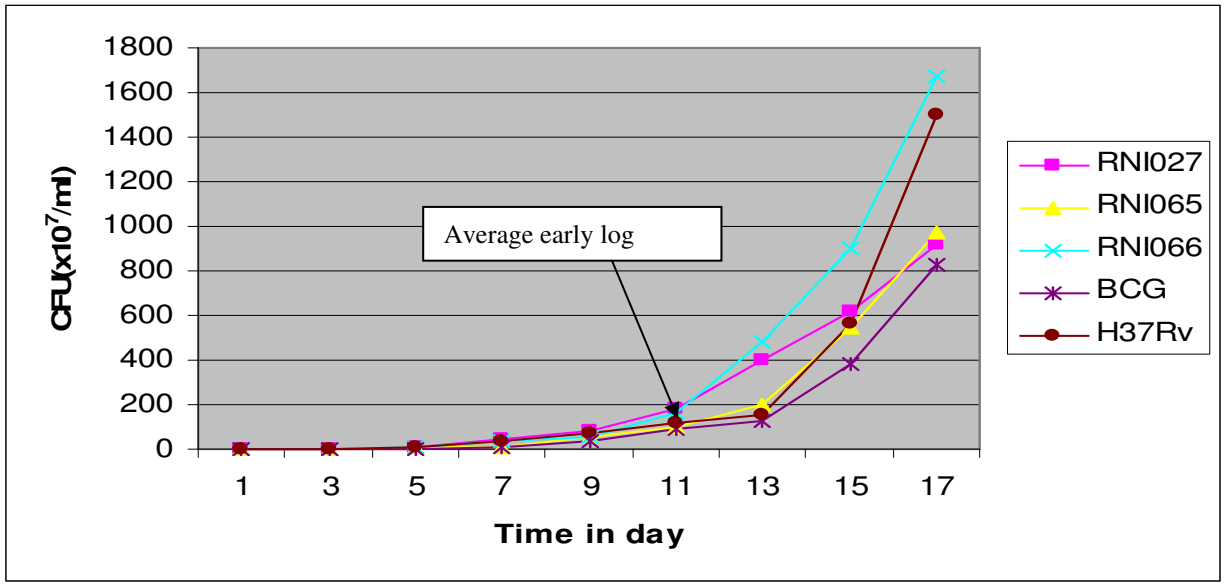


Figure 3.5 Graphical presentations of early log period and corresponding bacterial concentration

To set an exposure time for optimum mycobacterial killing by a final concentration of 1mM DETA/NO, three different incubation periods (i.e. 4hr, 24hr and 72hrs) were tested (Pilot 2). The CFU/ml of in duplicate for each isolates tested was recorded and compared to the CFU/ml of the control tubes, and the average survival (% survived) was calculated for each isolates after exposure to the three specified time. Therefore, based on the data, **24 hour** was set as the optimal exposure time to run the main experiments as the 4hr and 72hr incubation results about more than 60% and less than 1% survival respectively (Figure 3.6).

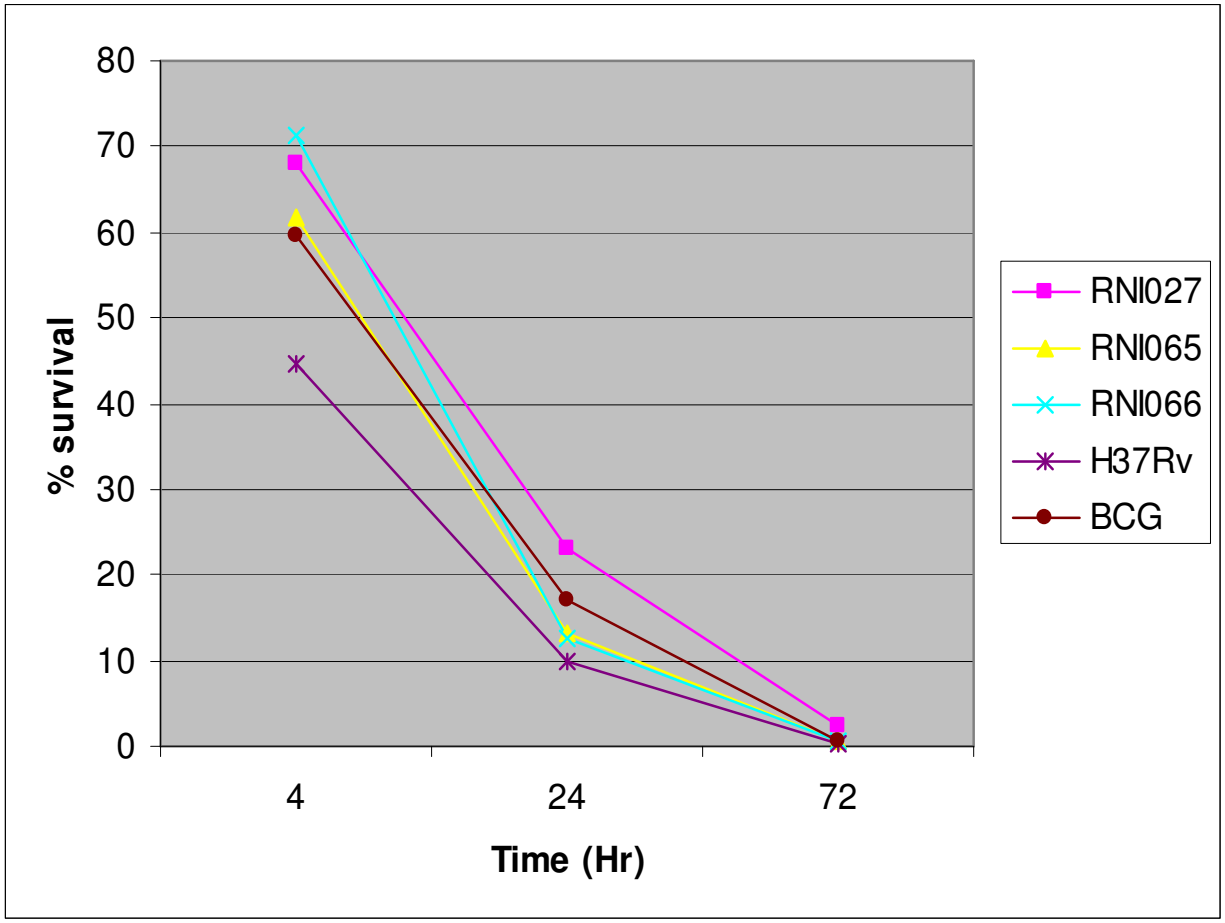


Figure 3.6 Rate of Mycobacterial survival after exposure to 1mM DETA/NO for variable times

To observe the trend bacterial response for variable nitric oxide concentration and examine whether the efficiency of RNI killing is a function of dose, the following final concentration of DETA/NO was tested on the specified three clinical isolates. The number and proportion of the strains survived greatly varied in different concentration of DETA/NO compared to the control (0mM DETA/NO). In 10mM DATE/NO, the proportion of strains survived on average was approximately 1.0% while up to 40% survived in 0.01mM DETA/NO.

Table 3.8 The cfu/ml and percentage survived of three clinical isolates (RNI027, RNI065 and RNI066) to exposure of variable concentration of nitric oxide donor (DETA/NO) for 24hrs.

	RNI 027		**RNI065**		**RNI066**	
DETA/NO	**cfu/ml ($x10^4$)**	**%Survive**	**cfu/ml ($x10^4$)**	**%Survive**	**cfu/ml ($x10^4$)**	**%Survive**
10mM	30	1.2	21.5	0.8	32	0.95
1mM	450	18	360	14.4	410	12
0.1mM	700	28	620	23.1	920	27
0.01mM	980	39.2	940	35	1350	40.5
0.001mM	1730	69	1700	64	2500	73.5
0mM	2500	100	2680	100	3400	100

As clearly indicated by the graph the percentage survival of all isolates was increased as the concentration of DETA/NO (nitric oxide) decreased. (Figure 3.7) Base on the data, a 10-fold decrease in nitric oxide concentration results an average increase of bacterial survival by 10 to 35%.

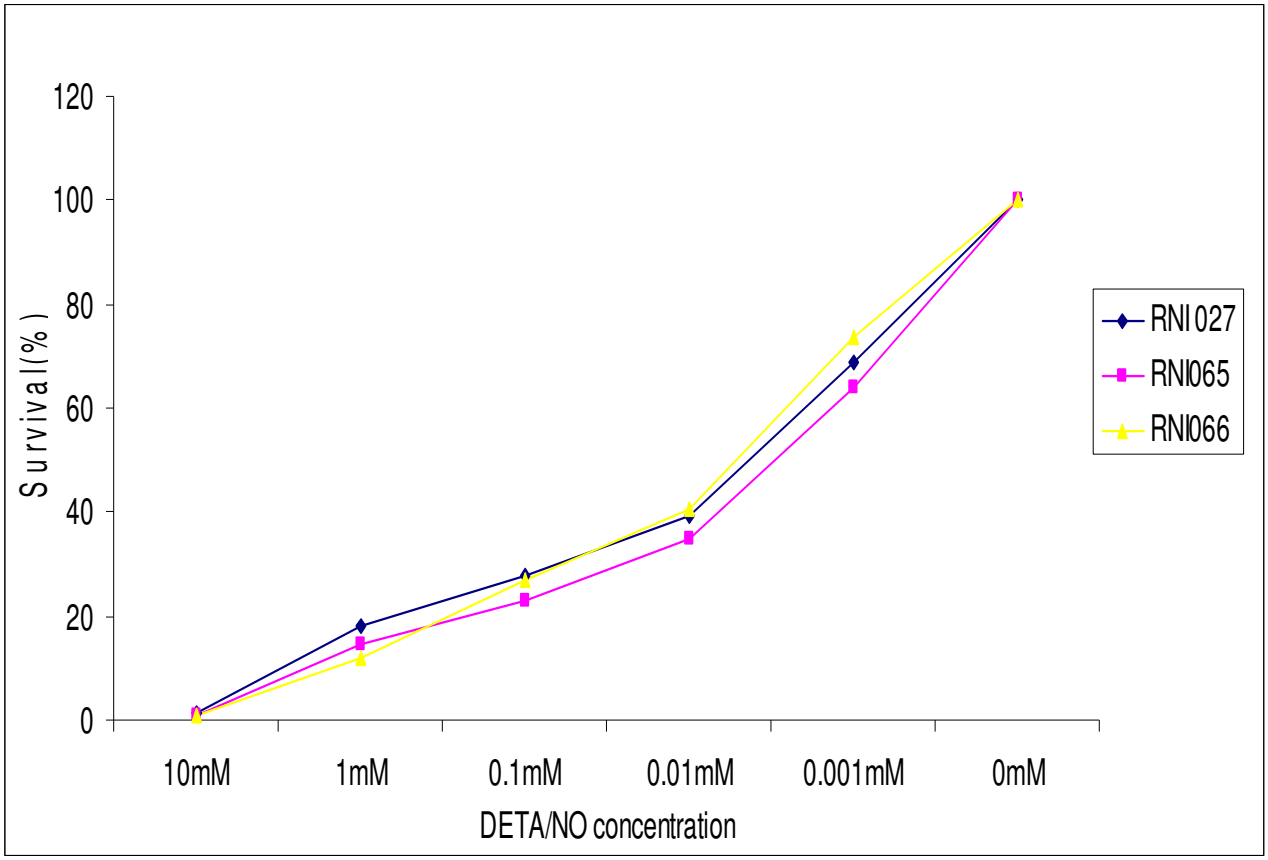


Figure 3.7: Dose response trend of mycobacteria for variable concentration of nitric oxide

CHAPTER IV: DISCUSSION

Since tuberculosis (TB) is still a significant threat to public health, there is a need to undertake massive research projects to investigate and reveal various aspects of the disease and its etiologies. Therefore, we believe that some of the findings in the present study, along with the existing and subsequent studies, may have significant contributions to fill the gap in prevention and control of tuberculosis.

The main findings and scientific contributions of the present study are that clinical isolates of *M. tuberculosis* had a variable nitric oxide susceptibility pattern where nitric oxide (NO) resistance is significantly correlated to the antibiotic drug resistance pattern. Moreover, *M. tuberculosis* was the only mycobacterial strain isolated as confirmed by PCR against the RD4 locus and spoligotyping.

The co-infection rate with HIV among TB patients was high (38.6%) (Table 3.3). This is slightly lower than in previous reports of smear positive TB patients in Addis Ababa (49%) by Demisse *et al*. (2000) and from Gondar (52.1%) by Kassu *et al.* (2007). However, the co infection rate in the present study was significantly lower ($p<0.02$, chi square test) than in a previous prospective study conducted in Gondar in 2000-2001 where 52.5 % of smear positive TB patients were co infected with HIV (Schön *et al.,* 2003). The reason for this difference may be due to a true overall decrease of HIV infection because of increase awareness in combination with the natural course of the HIV epidemic where co infected patients are becoming more common among patients with other clinical manifestations than smear positive pulmonary TB such as extra pulmonary or smear negative TB.

Almost two thirds of the HIV co infected TB patients were found in the age group between 25-45 years and this indicates that HIV infection in this age group is associated with a high risk for developing TB (Table 3.2 and Figure 3.1). Thus, a minority of the HIV co infected patients are found in the younger age group (15-25 years old) which could be due to a shorter time at risk or maybe because of increasing caution in using protection during sexual activity among the young population. As many as 44.5% of the TB patients had body mass

index (BMI) less than 16 kg/m^2 which is the lower range for severe malnutrition (WHO expert group, 2004).

This data indicates that TB patients have low nutritional margins before being diagnosed with TB which has also been shown to be a risk factor for mortality in a study from Guinea Bissau (Gustafsson *et al*., 2004). Therefore, based on this result one can suggest that nutritional supplementation is likely to be of benefit and such studies are underway (Abba, *et al.,* 2008). In this study, almost half (45.1%) of the patients were febrile (defined as ear temperature above 37.8^{o}C), which has been described to be more common in HIV co-infected patients (Schön, 2002) but was not confirmed in the present study.

Out of the three sputum samples provided by each patient, only one sample (1.5ml) was stored at -20^{o}C by the laboratory. Although we only used this small amount of one of the sputum samples for mycobacterial culture, the bacteria was recovered from 66.5% of those samples collected. The recovery rate is relatively high considering the prolonged storage at -20°C and the small amount of sputum stored. There was no correlation in the retrieval rate with regards to HIV status but an initial low smear grade was associated to being culture negative (Table 3.2). Thus, samples with a low smear grade of AFB were less likely to become culture positive.

One of the limitations of the present study is small portions (1.5ml) of the three sputum samples were used for culture which further decreases the sensitivity of culture. Moreover, freeze-thawing of the bacteria might lead to reduced viability. It has been reported that prolonged storage at room temperature or +4°C decreases the culture retrieval to 37-67% within four weeks (Paramasiva *et al*., 1983, Banda *et al*., 2000). Our approach could most likely be improved by storing pooled sputum samples in -20^o C which are concentrated before culture. In order to markedly improve the management of TB in referral hospital in sub-Saharan Africa, it would be of great benefit to develop local capacity for culture facilities using novel methods adopted for low income areas such as microscopic-observation of drug-susceptibility (MODS) (Moore *et al.,* 2004).

The PCR analysis using RD4 as the target for detection can be used to confirm that a cultured mycobacterial isolate is belonging to either of the two members of the *M. tuberculosis* complex; *M. tuberculosis* and *M. africanum.* In the present study all 116 cultured isolates were identified as *M. tuberculosis* by PCR against the RD4 locus (Figure 3.2). We interpreted this as a confirmation that *M. tuberculosis* is the most common or even only etiology for active pulmonary TB in Gondar as epidemiological data have shown that *M. africanum* is very uncommon in Ethiopia and is mainly present in West Africa. Our result is consistent with the findings most of the studies in Ethiopia where very low numbers of non-tuberculous mycobacteria (1-2 %) such as *M. avium-intracellulare* were observed (Hermans *et al.,* 1995; Bruchfeld *et al.,* 2002).

The spoligotype pattern may be used to discriminate species other than *M. tuberculosis* and our spoligotype results done on 93% of the isolates confirm that all of the isolated strains were *M. tuberculosis* as none of them exhibited spoligotyping patterns specific to *M. bovis* (lacks spacers 39 to 43) or *M. africanum* (lacks spacers 8, 9, and 39) (Figure 3.4). Thus, our data have shown that mycobacteria other than *M. tuberculosis* such as non-tuberculous mycobacteria are not present among sputum samples collected from Gondar, Ethiopia. This has clinical implications as even in HIV co infected smear positive TB patients, mycobacterial species other than *M. tuberculosis* are very rare. Thus, the DOTS regimen which is directed against tuberculosis is sufficient for treatment of smear positive patients, which is of clinical importance as other treatment regimens including claritromycin which might be necessary for the treatment of non-tuberculous mycobacteria are not indicated. However, it should still be a major long term goal for most areas that are managing TB patients to implement culture facilities for resistance surveillance and improving diagnostic support of TB infection at large.

Spoligotyping results showed that 76.8% of the isolates got a match for a reported pattern in the spoligotype database spoldb3 (Kamerbeek *et al.,* 1997). The most prevalent spoligotypes were CAS and T families which constitute more than 60% of the matched isolates (about 30% each) (Table 3.4 and Figure 3.5). Similar to our finding, the CAS family lineage was also shown to be endemic in Sudan and other sub-Saharan countries (Brudey *et al.*, 2006). The movement of people across the borders of the neighboring nations may attribute for the dominance of this same strain around Gondar and Sudan.

The second most common spoligotype (12%) was T3_Eth which is a sub strain of the T3 family originally found in Ethiopia ((Table 3.4 and Figure 3.5) (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo, Brudey *et al.,* 2006). The clustering of strains indicate that local spread is present in the Gondar area which calls for increasing efforts of early case finding and treatment as resistant strains have the potential to spread rapidly in the society. As many as 25 strains (23.2%) were unique and did not have a match in the spoligotyping database version spoldb4 (http://www.pasteur-guadeloupe.fr). These unique strains are interesting and need to be confirmed by restricted fragment length polymorphism (RFLP) as there seem to be a lack of systematic investigations of prevailing spoligotypes in Ethiopia including the Gondar area. The advantages of spoligotyping are the relatively low cost and a high reproducibility (Driscoll, 2009). The main disadvantage is its paucity of discrimination which in some circumstances calls for the use of a second method with higher resolution such as RFLP or MIRU VNTR.

We found an overall drug resistance rate of 13.3% and the majority of strains were single drug resistant against either isoniazid or streptomycin ((Table 3.6). The isoniazid resistance rate is in particular important as isoniazid and ethambutol are still used in the follow up phase of DOTS treatment in many areas of sub Saharan Africa although a switch to rifampicin and ethambutol has been recommended in many national, WHO based recommendations (FMoH 2008, WHO 2008b). The overall resistance was slightly lower than in a previous study conducted in Addis Ababa (Eyob *et al*, 2004). This group conducted a study on samples collected from individuals with extra pulmonary and smear negative TB while in the present study we only collected sputum smear positive samples and this might

be the reason for having a lower rate of over all drug resistance TB in our study. On the other hand our finding is comparable to previous studies conducted in Addis Ababa where a 14% and 15.6% resistance to one or more first line drugs from samples collected were detected (Demissie *et al.,* 1997; Bruchfeldt *et al.,* 2002).

Interestingly, patients infected with drug resistant strains showed significantly lower body mass index (BMI) than patients with drug susceptible strains. The individual performing the susceptible testing was blinded to the clinical follow up of the patients, thus, as an external validation of our data there was a clear difference in the smear conversion rate (91.2% vs. 66.7%, p=0.034) and cure rate (78.3% vs. 50%, p=0.02) between patients with drug susceptible or drug resistant strains (Table 3.5 and Table 3.7). Moreover, we found that in patients infected with drug resistant strains, nitric oxide susceptibility was impaired compared to susceptible strains (Table 3.5 and Table 3.7).

In this study, multi drug resistance strains (defined as resistance to both isoniazid and rifampicin) were not observed. The possible explanation could be the relatively small sample size smear positive sputum samples that had been collected for one and a half years from the DOTS centre of Gondar University Hospital. In the first nationwide study in Ethiopia among isolates collected between 2003 and 2006, the prevalence of MDR TB was 1.6 % among new TB cases and 12 % among retreatment cases (www.who.int/tb). Compared to all these previous studies conducted in Ethiopia (FMoH 2008, WHO 2008b), drug resistance seems not to be increasing at a high rate in Gondar where the samples collected for the present study.

In Ethiopia, although being in the top ten list of high endemic countries for TB, drug susceptibility results for M.tb are not widely available, which is particularly the case for areas outside Addis Ababa. However, the initiation of culture facilities in big teaching hospitals like Gondar and in regional laboratories available in the different regional cities in the country is of paramount importance. A continuous, yearly surveillance of resistant patterns from several areas of the country is essential as multi drug resistant strains are an increasing trend globally and could rapidly spread in countries like Ethiopia where low

socio economic status, overcrowding, inadequate health facility and poverty are the major problems of the country.

It has clearly been established that nitric oxide and reactive nitrogen intermediates are produced in immune-activated macrophages during infection of pulmonary tuberculosis but its relative importance has been debated (Schön *et al*., 2002, Voskuil *et al.,* 2003, Nathan *et al.,* 2000). There are very few reports on the variation of nitric oxide susceptibility in clinical isolates of *M. tuberculosis*. Thus, there was a need to develop an in house method for this purpose built on previous experiences (Schön *et al.,* 2002). Several optimization experiments were performed in order to establish the optimal dose and exposure time of the NO donor DETA/NO. This NO donor was used because it has a relatively uncomplicated chemical structure and releases low levels of NO for a prolonged time period similar to production *in vivo*. In this study, the bacteria was cultured in the presence of albumin and catalase but the NO exposure was performed without these additives as these substances could interfere with the testing by reacting and buffering the true effect of NO. The early logarithmic phase in our experimental system was defined as day 11. All clinical isolates grew more rapidly than the laboratory strains BCG and H37Rv which probably reflects the virulence of the clinical strains. It has previously been described that rapidly growing strains such as CDC1551 are virulent (Manca *et al.,* 1999). In subsequent experiments, we analysed the optimal incubation period with DETA/NO and although some killing was present already at 4h of exposure, 24 h was regarded as the optimal time of exposure as nearly total killing was observed at 72 h (Figure 3.6).

The half life of DETA is about 24 hours and the inhibiting effect at 4h should have been more pronounced if the bacteria would have been further cultured in the absence of a NO donor up to 24h. In another report, the addition of 0.5 mM DETA/NO led to decomposition below the 50-μM threshold concentration required for dormancy regulon induction (dosR) and for inhibition of respiration and growth 16–17h after addition (Voskuil et *al.,* 2003). Interestingly, we found that H37Rv was more susceptible against DETA/NO compared to clinical isolates similar to previous reports (Firmani *et al.,* 2002). In dose response

experiments, we found that there was a dose dependent killing by DETA/NO by one log10 at 1 mM DETA/NO and by 2 log10 for 10mM DETA/NO for three clinical strains ((Table 3.8 and Figure 3.7).

The dose response cure was linear confirming a concentration dependent killing at these concentrations. The dose of 1mM DETA/NO was selected as this is in the range previously used by other groups both for *M. tuberculosis* and other bacterial strains (Voskuil *et al.,* 2003). Lower doses of DETA/NO have been used to induce expression of latency associated genes such as *dosR* but this was not the purpose of this study (Voskuil *et al.,* 2003). High doses (>0.5 mM) induces over 400 genes in Mtb. (Voskuil *et al.,* 2003), this gene expression reaction being totally different than that observed at lower levels. The inhibiting effect of DETA/NO is most likely mediated by respiratory inhibition in the bacteria after NO exposure (Voskuil *et al.,* 2003) and the effect is most likely primarily bacteriostatic.

Over all, a nitric oxide induced bacterial killing in comparison to PBS at 18.1% was detected. There are very limited if any similar studies so far in the scientific literature to study the susceptibility against NO in a large selection of clinical strains by using a modern NO donator. One brief previous report using acidified nitrite (Firmani *et al.,* 2002) established a variation between a few selected laboratories *M. tuberculosis* strains and found a small variation among them. However, the use of acidified nitrite as an experimental system to generate nitric oxide has been questioned. It may not be the most optimal way for NO exposure as the acidity itself may add to the effect and also that other RNIs are produced other than NO. In our study, the growth of most *M. tuberculosis* isolates were inhibited by nitric oxide in comparison to the vehicle (PBS) which was used as a control in all experiments. The level of inhibition was up to 10-fold which is relatively low compared to the bactericidal drugs used against TB but considering the long replication time of *M. tuberculosis,* the effect is likely to be greater if investigated at later time points.

As for anti-TB drugs, killing of *M. tuberculosis* takes more time relative to other bacteria with higher replication rates such as *E. coli*. There was a difference in NO susceptibility between the isolates and similar to the threshold used for the drug susceptibility testing we

defined a threshold for "wild type" isolates. NO resistance was defined as a reduction in survival compared to control of 0-10 %. The analysis was blinded to antibiotic resistance data and interestingly, a high level of antibiotic resistance was observed in the isolates with NO resistance (Table 3.7). Multiple logistic regression analysis showed that there was a highly significant association between relative NO resistance and antibiotic resistance independent of age, sex or HIV co-coinfection. Strikingly, no nitric oxide resistant strains were detected among isoniazid susceptible strains. Interestingly, there are recent reports which suggest that nitric oxide is produced upon activation of isoniazid from the mycobacterial gene *katG* (Timmins et al., 2004).

From this data and our results, it could be speculated that nitric oxide resistant strains have deficiencies in the bacterial deactivation systems and oxidative stress defences for neutralisation of reactive nitrogen intermediates and such as (catalase) *katG* and (alkyl hydroperoxide reductase) *ahpC*. These genes are together with *inhA* constitutes most of the isoniazide resistance and *katG* and *ahpC* are induced by high concentrations of NO such as above 0.5mM DETA/NO (Voskuil IE et al 2003). As a continuation of this study it would be of interest to investigate the expression of such defence factors against nitric oxide as they are also involved in the activation and resistance against isoniazid (Timmins *et al.,* 2004). Such defence mechanisms could be targets for new treatment strategies. Other reasons for the association between relative NO resistance and isoniazid resistance could be that in isoniazid susceptible strains, the nitric oxide generated from activated macrophages together with the nitric oxide generated from the activation of isoniazid has to be produced in order to initiate effective killing.

CONCLUSIONS

In conclusion, the results of the present study show:

- *Mycobacterium tuberculosis* is the only mycobacterial strain found among consecutive smear positive isolates as detected by PCR against the RD4 locus and confirmed by spoligotyping.
- The rate of anti-microbial resistance against any first line anti-tuberculosis drug was 13.3%.
- In patients with drug resistant strains there was a clearly significant decrease in the final cure rate and smear conversion rate at 2 months.
- No multi drug resistant strains were identified in the cultured isolates.
- A method to detect susceptibility patterns of clinical Mtb isolates against nitric oxide (NO) was developed and optimized which showed a clear variability in the susceptibility against NO.
- Clinical isolates of *M. tuberculosis* exhibiting nitric oxide resistance showed significantly more anti mycobacterial drug resistance compared to nitric oxide susceptible strains.

RECOMMENDATIONS

Based on findings of the present study and previous related studies in Gondar the following recommendations have been drawn;

- Since most of the TB patients were malnourished, nutrition supplement should be considered in the patients' management.
- Most of the strains were sensitive to nitric oxide, hence boosting its concentration of and/or relevance of nitric oxide for treatment and control of tuberculosis should be substantiate with further studies.
- DOTS regimen is sufficient for treatment of smear positive active tuberculosis patients as the clinical significance of non-tuberculous mycobacteria was not indicated.
- Clustering of strains and presence of rifampcin resistant strain call for local spread and potentials of emergence of drug resistant TB, hence there is a need to strengthen and implement drug resistance surveillance for early case finding and treatments.
- The identified unique spoligotypes should be confirmed and well characterized with better discriminating methods, like RFLP.
- Further studies should be done with large sample size and pooled sputum in order to increase the quality of the data and draw powerful conclusion.

REFERENCES

Aaron L, Saadoun D, Calatroni I, *et al*. (2004). Tuberculosis in HIV-infected patients: a comprehensive review. *Clin Microbiol Infect;* 10: 388-98.

Abba K, Sudarsanam TD, Grobler L, Volmink J.Nutritional supplements for people being treated for active tuberculosis. Cochrane Database Syst Rev. 2008 Oct 8;(4):CD006086. Review.

Kassu A, Mengistu G, Ayele B, Diro E, Mekonnen F, Ketema D, Moges F, Mesfin T, Getachew A, Ergicho B, Elias D, Aseffa A, Wondmikun Y, Fusao O (2007). Co-ninfection and clinical manifestations of tuberculosis in human immunodeficiency virus –infected and uninfected adults at a teaching hospital, northwest Ethiopia. *J Microbiol Immunol Infect.* 40:116-122

Altare F, Durandy A, Lammas D, *et al*. Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. *Science* 1998; 280: 1432 – 1435.

American Thoracic Society/Centers for Disease Control and Prevention/Infectious Diseases Society of America: Treatment of Tuberculosis. *Am J Respir Crit Care Med* 2003; 167: 603-62.

Angeby KA, Hoffner SE, Diwan VK. (2004). Should the ´bleach microscopy method´ be recommended for improved case detection of tuberculosis? Literature review and key person analysis. *Int J Tuberc Lung Dis*; 8: 806-15.

Aranaz A, Cousins D, Mateos A, Dominguez L (2003). Elevation of *Mycobacterium tuberculosis* subsp. caprae to species rank as *Mycobacterium caprae* comb. nov., sp. nov. *Int J Syst Evol Microbiol*; 53:1785–9.

Altare F, Durandy A, Lammas D, *et al*. (1998) Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. Science 280: 1432 – 1435.

Asamoah- Odei E, García Calleja JM, Boerma JT. (2004). HIV prevalence and trends in sub-Saharan Africa: no decline and large sub-regional differences. Lancet 364: 35-40.

Asiimwe BB, Koivula T, Källenius G, Huard RC, Ghebremichael S, Asiimwe J, Joloba ML (2008). Mycobacterium tuberculosis Uganda genotype is the predominant cause of TB in Kampala, Uganda. *Int J Tuberc Lung Dis*. 12:386-91.

Ayele WY, Neill SD, Zinsstag J, Weiss MG, Pavlik I (2004). Bovine tuberculosis: an old disease but a new threat to Africa. *Int J Tuberc Lung Dis* ; 8:924–37.

Balcewicz-Sablinska MK, Keane J, Kornfeld H, Remold HG. (1998). Pathogenic *Mycobacterium tuberculosis* evades apoptosis of host macrophages by release of TNF-R2, resulting in inactivation of TNF-alpha. J. Immunol 161: 2636 – 2641.

Banda HT, Harries AD, Boeree MJ, Nyirenda TE, Banerjee A, Salaniponi FM. Viability of stored sputum specimens for smear microscopy and culture. Int J Tuberc Lung Dis 2000:4(3):272-4.

Bellamy R. (2000). Identifying genetic susceptibility factors for tuberculosis in africans: a combined approach using a candidate gene study and a genome-wide screen. Clin Sci (Colch); 98: 245 – 250.

Bermudez, L. E., and J. Goodman. (1996). Mycobacterium tuberculosis invades and replicates within type II alveolar cells. Infect. Immun., 64:1400-1406

Bonecini-Almeida, M. G., S. Chitale, I. Boutsikakis, J. Geng, H. Doo, S. He, and J. L. Ho. (1998). Induction of in vitro human macrophage anti-Mycobacterium tuberculosis activity: requirement for IFN-γ and primed lymphocytes. J. Immunol. 160:4490–4499.

Brandes M, Willimann K, Moser B. (2005) Professional antigen-presentation function by human gammadelta T Cells. Science; 309: 264-8.

Brennan, P.J. and Draper, P. Ultra structure of Mycobacterium tuberculosis. In: B.R. Bloom (Eds) Tuberculosis: Pathogenesis, protection and Control. American Society for Microbiology, Washington, DC, 1994, p. 271-284.

Brodie D, Schluger NW(2005). The diagnosis of tuberculosis. *ClinChest Med* 2005; **26**(2):247–71

Brosh, R., Gordon, S.V., Billaut, A., Garnier, T., Eiglmeier, K., Soravito, C., Barrell, B.G., Cole, S.T. (1998) Use of a Mycobacterium tuberculosis H37Rv Bacterial artificial chromosome (BAC) library for genome mapping, sequencing and comparative genomocs. Infect. Immun. 66, 2221-2229

Bruchfeld, J., G. Aderaye, I. Berggren Palme, B. Bjorvatn, S. Gebremichael, S. Hoffner, and L.Lindquist. Molecular Epidemiology and Drug Resistance of Mycobacterium

tuberculosis Isolates from Ethiopian Pulmonary Tuberculosis Patients with and without Human Immunodeficiency Virus Infection. J. Clin. Microbiol. 2002: 5: 1636–1643

Brudey K, Driscoll JR, Rigouts L, et al. (2006) *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol. 6: 23.

Canetti, G., W. Fox, A. Khomesnko, *et al*. (1969). Advances in techniques of testing mycobacterial drug sensitivity, and the use of sensitivity tests in tuberculosis programmes. Bull. World health Org. 41: 21-43.

Chan J, Fan X, Hunter SW, Brennan PJ, Bloom BR. (1991). Lipoarabinomannan, a possible virulence factor involved in persistence of Mycobacterium tuberculosis within macrophages. Infect Immun 59: 1755 – 1761.

Chan, E. D., Chan, J., and Schluger, N. W. (2001). **W**hat is the Role of Nitric Oxide in Murine and Human Host Defense against Tuberculosis? Current knowledge. Am. J. Respir. Cell Mol. Biol., 25: 606-612.

Chan, J., Tian, Y., Tanaka, K.E,. Tsang, M.S., Yu, K., Salgame, P., Carroll, D., Kress, Y.,Teitelbaum, R., and Bloom, B. R. (1996). Effects of protein calorie malnutrition on tuberculosis in mice. Proc Natl Acad Sci USA 93: 14857 – 14861

Chan, J., Y. Xing, R. S. Magliozzo, and B. R. Bloom. Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages. J. Exp. Med. 1992. 175: 1111-*1122*

Colebunders R, Bastian I. A review of the diagnosis and treatment of smear-negative pulmonary tuberculosis. *Int J Tuberc Lung Dis* 2000;**4**(2):97–107

Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al (1998a): Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature;393:537.

Cole, S.T., and Barrell, B.G (1998b). Analysis of genome of Mycobacterium tuberculosis H37Rv. Novartis Found Symp. 217, 160-172.

Cooper A, Poinar HN. (2000). Ancient DNA: do it right or not at all. Science 289: 1139.

Corbett EL, Watt CJ, Walker N, *et al*. (2003). The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Arch Intern Med 163: 1009-21.

Cousins DV, Bastida R, Cataldi A, *et al*. Tuberculosis in seals caused by a novel member of the *Mycobacterium tuberculosis* complex: *Mycobacterium pinnipedii* sp. nov. *Int J Syst Evol Microbiol* 2003; 53:1305–14.

Dale JW, Brittain D, Cataldi AA, et al. Spacer oligonucleotide typing of bacteria of the *Mycobacterium tuberculosis* complex: recommendations for standardised nomenclature. *Int J Tuberc Lung Dis* 2001;**5**(3):216–9.

Daley CL. Molecular epidemiology: a tool for understanding control of tuberculosis transmission. *Clin Chest Med* 2005;**26**(2):217–31, vi.

Daniel TM. (2006). The history of tuberculosis. Respir Med 100: 1862-70.

Dannenberg, A. M. (1989). Immune mechanisms in the pathogenesis of pulmonary tuberculosis. Rev. Infect. Dis. 11: 369-378

Dannenberg, A. M., Jr., and J. A. Rook. (1994). Pathogenesis of pulmonary tuberculosis: interplay of tissue-damaging and macrophage-activating immune responses. Dual mechanisms that control bacillary multiplication, 1994: p. 459-483. In B. R. Bloom (ed.), Tuberculosis: pathogenesis, protection, and control. American Society for Microbiology, Washington, D.C

De La Barrera SS, Finiasz M, Frias A, et al. (2003) Specific lytic activity against mycobacterial antigens is inversely correlated with the severity of tuberculosis. Clin Exp Immunol 132: 450-61.

de Valliere S, Abate G, Blazevic A, Heuertz RM, Hoft DF.(2005) Enhancement of innate and cell-mediated immunity by antimycobacterial antibodies. Infect Immun; 73: 6711-20

Demissie M, Lindtjorn B, Tegbaru B. Human immunodeficiency virus (HIV) infection in tuberculosis patients in Addis Ababa. Eth J Health Dev 2000; 14: 277–282.

Demissie, M., M. Gebeyehu, and Y. Berhane.. Primary resistance to anti-tuberculosis drugs in Addis Ababa, Ethiopia. Int. J. Tuberc. Lung Dis.1997:1:64–67.

http://www.who.int/tb_beijingmeeting/country_level/mdrtb_afrcountryprofile2007_eth.pdf

Diamond G, Bevins CL. (1998). beta-Defensins: endogenous antibiotics of the innate host defense response. Clin Immunol Immunopathol; 88: 221-5.

Doi, T., M. Ando, T. Akaike, M. Suga, K. Sato, and H. Maeda. (1993). Resistance to nitric oxide in *Mycobacterium avium* complex and its implication in pathogenesis. Infect. Immun. 61:1980–1989.

Donoghue HD, Spigelman M, Zias J, Gernaey-Child AM, Minnikin DE. (1998). *Mycobacterium tuberculosis* complex DNA in calcified pleura from remains 1400 years old. Lett Appl Microbiol; 27: 265-9.

Dorman SE, Chaisson RE. (2007) From magic bullets back to the magic mountain: the rise of extensively drug resistant tuberculosis. *Nat Med*; **13(3)**: 295-298.

Driscoll JR. Spoligotyping for molecular epidemiology of the Mycobacterium tuberculosis complex. Methods Mol Biol. 2009;551:117-28.

Drobinewski F. Rüsch-Gerdes S, Hoffner S. (2007) Antimicrobial susceptibility testing of Mycobacterium tuberculosis (EUCAST document E.DEF 8.1) - Report of the Subcommittee on Antimicrobial Susceptibility Testing of Mycobacterium tuberculosis of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID). *Eur J Clin Microb*; **13(12)**:1144-56

Dye C, Watt CJ, Bleed DM, Mehran Hosseini S, Raviglione MC. (2005). Evolution of tuberculosis control and prospects for reducing tuberculosis incidence, prevalence, and deaths globally. JAMA 293: 2767-75.

Dye C. (2006). Global epidemiology of tuberculosis. Lancet; 367: 938-40.

Dye, C., Scheele, S., Dolin, P., Pathania, V., and Raviglione, M.C. (1999). Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. *JAMA* 282: 677-86.

Ellner JJ. (1997). Regulation of the human immune response during tuberculosis. J Lab Clin Med 130: 469 – 475.

Enarson, DA, Rieder HL, Arnadottir T, Trebucq A. (1996). Tuberculosis Guide for Low Income Countries, 4th ed.,Paris: International Union against Tuberculosis and Lung Disease, 1996.

Enrico T, Juan C Palomino (2007). New Diagnostic Methods; In JC Palomino, SC Leao and V Ritacco. (Eds) Tuberculosis 2007: From basic science to patient care (1st ed, pp 441-459), Institute of tropical medicine, Antewerp, Bourcillier-Kamps

Eyob G, Guebrexabher H, Lemma E, Wolday D, Gebeyehu M, Abate G, Rigouts L, van Soolingen D, Fontanet A, Sanders E, Dorigo-Zetsma JW. Drug susceptibility of Mycobacterium tuberculosis in HIV-infected and -uninfected Ethiopians and its impact on outcome after 24 months of follow-up. Int J Tuberc Lung Dis. 2004:8(11):1388-91.

Facchetti, F., W. Vermi, S. Fiorentini, M. Chilosi, A. Caruso, M. Duse, L. D. Notarangelo, and R. Badolato. (1999) Expression of inducible nitric oxide synthase in human granulomas and histiocytic reactions. Am. J. Pathol. 154:145–152.

Fenton, M. J., and M. W. Vermeulen. (1996) Immunopathology of tuberculosis: roles of macrophages and monocytes. Infect. Immun., 64:683-690

Ferguson, J. S., D. R. Voelker, F. X. McCormack, and L. S. Schlesinger. (1999). Surfactant protein D binds to Mycobacterium tuberculosis bacilli and lipoarabinomannan via carbohydrate-lectin interactions resulting in reduced phagocytosis of the bacteria by macrophages. J. Immunol. 163:312-321.

Filliol I, Driscoll JR,Van Soolingen D, et al (2002). Global distribution of *Mycobacterium tuberculosis* spoligotypes. *Emerg Infect Dis*;**8**(11):1347–9.

Firmani MA, Riley LW. Reactive nitrogen intermediates have a bacteriostatic effect on Mycobacterium tuberculosis in vitro. J Clin Microbiol. 2002 Sep;40(9):3162-6.

Fleischmann RD, Alland D, Eisen JA, et al. Whole-genome comparison of *Mycobacterium tuberculosis* clinical and laboratory strains. *J Bacteriol* 2002;**184**(19):5479–90.

Flynn JL, Chan J. (2001). Immunology of tuberculosis. Annu Rev Immunol; 19: 93-129.

Frieden TR, Sterling TR, Munsiff SS, Watt CJ, Dye C. (2003) Tuberculosis. *Lancet* 9387:887–99.

Frothingham R, Meeker-O'Connell WA. (1998) Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. *Microbiology* **144** :1189–96.

Fulton SA, Cross JV, Toossi ZT, Boom WH. (1998). Regulation of interleukin-12 by interleukin-10, transforming growth factor-beta, tumor necrosis factor-alpha, and interferon-gamma in human monocytes infected with Mycobacterium tuberculosis H37Ra. J Infect Dis 178: 1105 – 1114.

Fulton SA, Reba SM, Martin TD, Boom WH. (2002). Neutrophil-mediated mycobacteriocidal immunity in the lung during *Mycobacterium bovis* BCG infection in C57BL/6 mice. Infect Immun 70: 5322-7.

Garcia de Viedma D. (2003) Rapid detection of resistance in *Mycobacterium tuberculosis*: a review discussing molecular approaches. Clin Microbiol Infect; 9: 349-59.

Gatfield, J., and J. Pieters. (2000). Essential role for cholesterol in entry of mycobacteria into macrophages. Science 288:1647-1650

Gaynor, C. D., F. X. McCormack, D. R. Voelker, S. E. McGowan, and L. S. Schlesinger. (1995). Pulmonary surfactant protein A mediates enhanced phagocytosis of Mycobacterium tuberculosis by a direct interaction with human macrophages. J. Immunol.. 155:5343-5351

Ghatole M, Sable C, Kamale P, Kandle S, Jahgirdar V. (2005). Evaluation of Biphasic Culture System for Mycobacterial Isolation fom the Sputum of Patient with Pulmonary Tuberculosis. Indian Jurmal of Medical Microbiology 23 (2): 111-113.

Glatman-Freedman A, Casadevall A. (1998). Serum therapy for tuberculosis revisited: reappraisal of the role of antibody-mediated immunity against Mycobacterium tuberculosis. Clin Microbiol Rev 11: 514 – 532.

Glatman-Freedman, F. A., Martin JM, Riska PF, Bloom BR, Casadevall A. (1996). Monoclonal antibodies to surface antigens of Mycobacterium tuberculosis and their use in a modified enzyme-linked immunosorbent spot assay for detection of mycobacteria. J Clin Microbiol. 34:2795-2802.

Goh KS, Fabre M, Huard RC, Schmid S, Sola C, Rastogi N. Study of the *gyrB* gene polymorphism as a tool to differentiate among *Mycobacterium tuberculosis* complex subspecies further underlines the older evolutionary age of 'Mycobacterium canettii'. Mol Cell Probes 2006; 20: 182-90.

Gonzalez-Juarrero, M., and I. M. Orme. (2001). Characterization of murine lung dendritic cells infected with Mycobacterium tuberculosis. Infect. Immun., 69:1127-1133

Gustafson P, Victor F Gomes, Cesaltina S Vieira, Paulo Rabna, Rémonie Seng, Peter Johansson, Anita Sandström, Renée Norberg, Ida Lisse,1 Badara Samb, Peter Aaby (2004). Tuberculosis in Bissau: incidence and risk factors in an urban community in sub-Saharan Africa. International Journal of Epidemiology; 33:163–172

Gutacker MM, Smoot JC,Migliaccio CA, et al. Genome-wide analysis of synonymous single nucleotide polymorphisms in *Mycobacterium tuberculosis* complex organisms: resolution of genetic relationships among closely related microbial strains. *Genetics* 2002;**162**:1533–43.

Haas, D.W. Mycobacterium tuberculosis. In: Principles and practices of infectious diseases., B.J. Mandel GL, Dolin R, (Eds), Philadelphia, Churchil Livingstone. 2000. pp 2576-2607.

Harries, A. D., Hargreaves, N. J., Kemp, J., Jindani, A., Enarson, D. A., Maher, D. and Salaniponi, F. M. (2001). Deaths from tuberculosis in Sub-Saharan African countries with high prevalence of HIV-1. Lancet 357: 1519-1523.

Health Protection Agency (HPA) (2003). Standard Operating Procedure: investigation of specimens for mycobacterium species. Issued by Standards Unit, Evaluations and Standards Laboratory. Specialist and Reference Microbiology Division, UK.

Hermans, P. W. M., F. Messadi, H. Guebrexabher, D. van Soolingen, P. E. W. de Haas, H. Heersma, H. de Neeling, A. Ayoub, F. Portaels, D. Frommel, M. Zribi, and J. D. A. van Embden. (1995). Analysis of the population structure of *Mycobacterium tuberculosis* in Ethiopia, Tunisia and the Netherlands: usefulness of DNA typing for global tuberculosis epidemiology.. J. Infect. Dis. 171:1504–1513.

Ilangumaran S, Arni S, Poincelet M, *et al*. (1995). Integration of mycobacterial lipoarabinomannans into glycosylphosphatidylinositol-rich domains of lymphomonocytic cell plasma membranes. J Immunol 155: 1334 – 1342.

Jacobus H. de Waard, J. Robledo (2007). Conventional Diagnostic Methods; In JC Palomino, SC Leao and V Ritacco. (Eds) Tuberculosis 2007: From basic science to

patient care (1st ed, pp 441-459) Bourcillier-Kamps, Institute of tropical medicine, Antewerp, Belgium.

Johnson, C.M., Cooper, A.M., Frank, A.A., Bonorino, C.B., Wysoki, L.J., Orme, I.M. (1997). Mycobacterium tuberculosis aerogenic rechallenge infections in B cell-deficient mice. Tuber Lung Dis; 78: 257 – 261.

Kamerbeek J., L. Schouls, A. Kolk, M. van Agterveld, D. van Soolingen et al (1997). Simultaneous Detection and Strain Differentiation of *Mycobacterium tuberculosis* for Diagnosis and Epidemiology. Journal of Clinical Microbiology, Apr. 1997, p. 907–914

Kaufmann, S.H., and McMichael, A.J. (2005). Annulling a dangerous liaison: vaccination strategies against AIDS and tuberculosis. *Nat Med* 4: 33–44.

Kent PT, Kubica GP. (1985) Public Health Mycobacteriology: A Guide for the Level III Laboratory. US Department of Health and Human Services, 1985. CDC, Atlanta, Georgia.

Kim J. Drug-susceptibility testing in tuberculosis: methods and reliability of results. *Eur Respir J* 2005; **25**:564-569.

Kramnik I, Dietrich WF, Demant P, Bloom BR.(2000). Genetic control of resistance to experimental infection with virulent *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A 97: 8560-5.

Kuo H.P., Wang C.H, Huang K.S, Lin C.H, Yu C.T, Liu C.Y, and L.C. Lu. (2000). Nitric oxide modulates interleukin-1 and tumor necrosis factor synthesis by alveolar macrophages in pulmonary tuberculosis. Am. J. Respir. Crit. Care Med., 161:192–199

Laszlo A. Rahman M, Espinal M et al. (2002) Quality assurance programme for drug susceptibility testing of Mycobacterium tuberculosis in the WHO/IUATLD supranational reference laboratory network: five rounds of proficiency testing, 1994-1998. *Int J Tuberc Lung Dis* **6**:748-756.

Lawn, S. D., Butera, S. T., and Shinnick, T. M. (2002). Tuberculosis unleashed: the impact of human immunodeficiency virus infection on the host granulomatous response to mycobacterium tuberculosis. Microbes Infect 4: 635-646.

Lee H, Park HJ, Cho SN, Bai GH, Kim SJ. Species identification of mycobacteria by PCR-restriction fragment length polymorphism of the *rpo*B gene. J Clin Microbiol 2000; 38: 2966-71.

Ling DI, Zwerling AA, Pai M. (2008) GenoType MTBDR assays for the diagnosis of multidrug-resistant tuberculosis: a meta-analysis. *Eur Respir J* **32(5)**:1165-74.

Lockhart E, Green AM, Flynn JL. (2006) IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during *Mycobacterium tuberculosis* infection. J Immunol, 177, 4662-9.

Macallan, D.C. (1999). Nutrition and immune function in Human Immunodeficiency Virus infection. Proc Natl Soc 58: 743-748.

MacMicking, J., R. North, R. LaCourse, J. Mudgett, S. Shah, and C. Nathan. Identification of nitric oxide synthase as a protective locus against tuberculosis. Proc. Natl. Acad. Sci. USA. 1997. 94:5243-5248

Malik ZA, Denning GM, Kusner DJ. (2000). Inhibition of $Ca^{(2+)}$ signaling by Mycobacterium tuberculosis is associated with reduced phagosome-lysosome fusion and increased survival within human macrophages. J Exp Med 191: 287 – 302.

Manca C, Tsenova L, Barry CE 3rd, Bergtold A, Freeman S, Haslett PA, Musser JM, Freedman VH, Kaplan G. Mycobacterium tuberculosis CDC1551 induces a more vigorous host response in vivo and in vitro, but is not more virulent than other clinical isolates. J Immunol. 1999 Jun 1;162(11):6740-6

Mario C. Raviglione, Richard J. O'Brien (2005). Tuberculosis: Clinical Manifestations. In Dennis LK, Eugene B, Anthony SF, Stephen LH, Dan LL and J. Larry J. (Eds) Harrison's Principlr of Internal Medicine (16^{th} ed, pp 956-960) McGraw-Hill Mrdical Publisher, New York.

Master, R.N. (1992) "Mycobacteriology". Clinical Microbiology Procedures Handbook, Vol. 1, ASM, 1992, Washington, D.C.

Ministry of Health (1998). Epidemiology and AIDS Department, Ministry of Health. 1998. *In* AIDS in Ethiopia: background, projections, impacts, interventions, 2nd ed., p. 3–4. Epidemiology and AIDS Department, Ministry of Health, Addis Ababa, Ethiopia.

Ministry of Health TB and Leprosy Control Team. (2003). Annual Joint Review Mission Report. October 2003, Addis Ababa.

Ministry of Health Ethiopia (2008). Tuberculosis, Leprosy and TB/HIV Prevention and Control Programme Manual (Fourth Edition) 2008.

Mitike, G., D. Kebede and H. Yeneneh. (1997). Prevalence of antituberculosis drug resistance in Harar tuberculosis centre, Ethiopia. East Afr. Med. J. 74:158–161.

Moore DA, Mendoza D, Gilman RH, et al. Microscopic observation drug susceptibility assay, a rapid, reliable diagnostic test for multidrug-resistant tuberculosis suitable for use in resource-poor settings. J Clin Microbiol 2004; 42:4432-7.

Morgan MA, Horstmeier CD, DeYoung DR, Roberts GD: Comparison of a radiometric method (BACTEC) and conventional culture media for recovery of mycobacteria from smear-negative specimens. J Clin Microbiol 1983;18:384–388.

Murray, C.J., Styblo, K., and Rouillon, A. (1990). Tuberculosis in developing countries: burden, intervention and cost. *Bull Int Union Tuberc Lung Dis* 65: 6-24.

Nandan D, Knutson KL, Lo R, Reiner NE. (2000). Exploitation of host cell signaling machinery: activation of macrophage phosphotyrosine phosphatases as a novel mechanism of molecular microbial pathogenesis. J Leukoc Biol 67: 464 – 470.

Nardell EA. (1993). Environmental control of tuberculosis. Med Clin North Am; 77: 1315-34.

Nathan, C. and Shiloh, M.U. (2000). Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. PNAS . 97 : 8841–8848

Nathan, C. F., and J. J. B. Hibbs. (1991) Role of nitric oxide synthesis in macrophage antimicrobial activity. Curr. Opin. Immunol. 1991, 3:65

NCCLS. (2000) Susceptibility testing of Mycobacteria, Nocardia, and other aerobic actinomycetes; tentative standard – second edition. NCCLS document M24-T2 [ISBN 1-56238-423-6]

Newport M, Huxley CM, Huston S, *et al*. (1996) A mutation in the interferon-c receptor gene and susceptibility to mycobacterial infection. New Engl J Med 335: 1941 – 1949.

Nicholson, S., M. da G. Bonecini-Almeida, J. R. Lapa e Silva, C. Nathan, Q. W. Xie, R. Mumford, J. R. Weidner, J. Calaycay, J. Geng, N. Boechat, C. Linhares, W. Rom, and J. L. Ho. (1996). Inducible nitricoxide synthase in pulmonary alveolar macrophages in patients with active pulmonary tuberculosis. J. Exp. Med. 183:2293-2302

Nolte, F.S and Metchock, B. (1995) Mycobacterium. In: P.R. Murray, E.J. Baron, M.A Pfaller, F.T. Tenover and R.H Yolken (Eds), Manual of clinical microbiology. American Society for Microbiology, Washington.

Noss, E. H., R. K. Pai, T. J. Sellati, J. D. Radolf, J. Belisle, D. T. Golenbock, W. H. Boom, and C. V. Harding. (2001). Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of Mycobacterium tuberculosis. J. Immunol..167:910-918

O'Brien, L., J. Carmichael, D. B. Lowrie, and P. W. Andrew. (1994). Strains of *Mycobacterium tuberculosis* differ in susceptibility to reactive nitrogen intermediates in vitro. Infect. Immun. 62:5187–5190.

Oddo M, Renno T, Attinger A, Bakker T, MacDonald HR, Meylan PR. (1998). Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular Mycobacterium tuberculosis. J Immunol 160: 5448 – 5454.

Otal I, Martin C,Vincent-Levy-Frebault V,Thierry D, Gicquel B. (1991) Restriction fragment length polymorphism analysis using IS6110 as an epidemiological marker in tuberculosis. *J Clin Microbiol*; **29**(6):1252–4.

Palomino JC. Nonconventional and new methods in the diagnosis of tuberculosis: feasibility and applicability in the field. Eur Respir J 2005; 26: 1-12.

Pancholi P, Mirza A, Bhardwaj N, Steinman RM. (1993). Sequstration from immune $CD4^+$ T cells of mycobacteria growing in human macrophages. Science 260: 984 – 986.

Paramasivan CN, Narayana AS, Prabhakar R, Rajagopal MS, Somasundaram PR, Tripathy SP. Effect of storage of sputum specimens at room temperature on smear and culture results. Tubercule 1983: 64(2):119-24

Parsons L. M, Brosch R., Cole S. T., *et al*. (2002). Rapid and Simple Approach for Identification of Mycobacterium tuberculosis Complex Isolates by PCR-Based Genomic Deletion Analysis. J. Clin. Microbiol. 40: 2339-2345.

Raja, A. (2004). Immunology of Tuberculosis. *Indian J of Med Res* 120 : 213 – 232

Raviglione MC, Snider DE, Jr., Kochi A.(1995) Global epidemiology of tuberculosis. Morbidity and mortality of a worldwide epidemic. *JAMA*; 273(3):220–6.

Rhoades, E. R., and I. M. Orme. (1997). Susceptibility of a panel of virulent strains of *Mycobacterium tuberculosis* to reactive nitrogen intermediates. Infect. Immun. 65:1189–1195.

Rich, E. A., M. Torres, E. Sada, C. K. Finegan, B. D. Hamilton, and Z. Toossi. 1997. *Mycobacterium tuberculosis* (MTB) stimulated production of nitric oxide by human alveolar macrophages and relationship of nitric oxide production to growth inhibition of MTB. *Tuberc. Lung. Dis.* 78:247–255.

Reljic R, Clark SO, Williams A, et al. (2006). Intranasal IFNgamma extends passive IgA antibody protection of mice against *Mycobacterium tuberculosis* lung infection. Clin Exp Immunol; 143: 467-73.

Richard C. Huard, Luiz Claudio de Oliveira Lazzarini, W. Ray Butler, Dick van Soolingen, and John L. Ho (2003). PCR-Based Method To Differentiate the Subspecies of the *Mycobacterium tuberculosis* Complex on the Basis of Genomic Deletions. J. Clin. Microbiol. 41: 1637–1650

Robin M. Warren, Thomas C. Victor, Elizabeth M. Streicher et al. (2004) Patients with Active Tuberculosis often Have Different Strains in the Same Sputum Specimen. Am J Respir Crit Care Med; 169: 610-14

Roberts GD, Goodman NL, Heifets L, Larsh HW, Lindner TH, McClatchy JK, McGinnis MR, Siddiqi SH, Wright P: Evaluation of the BACTEC radiometric method for recovery of mycobacteria and drug susceptibility testing of *Mycobacterium tuberculosis* from acid-fast smear-positive specimens. J Clin Microbiol 1983;18:689–696.

Rockett, K. A., R. Brookes, I. Udalova, V. Vidal, A. V. Hill, and D. Kwiatkowski. 1998. 1,25-Dihydroxyvitamin D3 induces nitric oxide synthase and suppresses growth of *Mycobacterium tuberculosis* in a human macrophage-like cell line. *Infect. Immun.* 66:5314–5321.

Rogall T, Flohr T, Bottger EC.Differentiation of *Mycobacterium* species by direct sequencing of amplified DNA. *J Gen Microbiol* 1990;**136** (9):1915–20.

Salinas-Carmona MC, Perez-Rivera I.(2004). Humoral immunity through immunoglobulin M protects mice from an experimental actinomycetoma infection by *Nocardia brasiliensis*. Infect Immun; 72: 5597-604.

Saunders G and McFadden J: Molecular biology of mycobacterium tuberculosis; in Kaufmann SHE, Hahn H (eds): Mycobacteria and TB. Issues Infect Dis. Basel, Karger, 2003, vol 2, pp 97–111.

Schlesinger, L. S. (1993). Macrophage phagocytosis of virulent but not attenuated strains of Mycobacterium tuberculosis is mediated by mannose receptors in addition to complement receptors. J. Immunol., 150:2920-2930

Schluger, N.W. (2001). Recent advances in our understanding of human host responses to tuberculosis. Respiratory Research, 2:157-163

Schluger, N.W., Rom, W.N. (1998). The host immune response to tuberculosis. Am J Respir Crit Care Med, 157:679-691

Schon T, Elias D, Moges F., Melese, E., Tessema, T., Stendahl, O., Britton, S., Sundqvist, T. (2003). Arginine as an adjuvant to chemotherapy improves the clinical outcome in active tuberculosis. Eur Resp J 21: 483-488

Schön T (2002). Nitric oxide in tuberculosis and leprosy. PhD Thesis. Linköping University 2002.

Schwoebel V, Lambregts-van Weezenbeek CSB, Moro M-L, et al. (2000) Standardization of antituberculosis drug resistance surveillance in Europe. Recommendations of a World Health Organization (WHO) and International Union Against Tuberculosis and Lung Disease (IUATLD) Working Group. *Eur Respir J*; **16**:364-371.

Segal W. Growth dynamics of in vivo and in vitro grown mycobacterial pathogens. In: G.P. Kubica and L.J Wayne (Eds), The Mycobacteria-a course book. Marcel Dekker Inc., New York, N.Y. 1984. p. 547-574.

Selvaraj P, Kannapiran M, Kurian SM, Narayanan, PR. (2001). Effect of plasma lysozyme on live Mycobacterium tuberculosis. Curr Sci 81: 201-3

Shiloh MU and Nathan CF. (2000). Reactive nitrogen intermediates and the pathogenesis of Salmonella and mycobacteria. Curr Opin Microbiol. 3: 35–42

Shinnick TM, Good RC. (1994). Mycobacterial taxonomy. *Eur J Clin Microbiol Infect Dis* **13**: 884–901.

Shiratsuchi, H., J. J. Johnson, H. Toba, and J. J. Ellner.(1990). Strain- and donor-related differences in the interaction of *Mycobacterium avium* with human monocytes and its modulation by interferon-g. J. Infect. Dis. 162: 932–938.

Smith, I. (2003). *Mycobacterium tuberculosis* Pathogenesis and Molecular Determinants of Virulence. Clinical Microbiology Reviews 3: 463–496

Smith, I. (2004). What is the health, social, and economic burden of tuberculosis. p. 233-7, In: Frieden T. (ed). Toman´s tuberculosis case detection, treatment, and monitoring: questions and answers. 2nd ed. Geneva, WHO, 2004. WHO/HTM/TB/2004.334.

Somoskovi A, Hotaling JE, Fitzgerald M, O´Donnell D, Parsons LM, Salfinger M. (2001) Lessons from a proficiency testing event for acid-fast microscopy. Chest 2001; 120: 250-7.

Soini H, Pan X, Amin A, Graviss EA, Siddiqui A, Musser JM. (2000) Characterization of *Mycobacterium tuberculosis* isolates from patients in Houston,Texas, by spoligotyping. *J Clin Microbiol*; **38**(2):669–76.

Sola C, Horgen L, Devallois A, Rastogi N. Combined numerical analysis based on the molecular description of *Mycobacterium tuberculosis* by four repetitive sequence-based DNA typing systems. *Res Microbiol* 1998;**149**(5):349–60.

Somoskovi A, Hotaling JE, Fitzgerald M, O´Donnell D, Parsons LM, Salfinger M. (2001) Lessons from a proficiency testing event for acid-fast microscopy. Chest ; 120: 250-7

Sreevatsan S, Pan X, Stockbauer KE, et al (1997). Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. *Proc Natl Acad Sci USA* 1997;**94**(18):9869–74.

Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, *et al*. (1994). Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science 263: 678 – 681.

Supply P, Magdalena J, Himpens S, Locht C. (1997) Identification of novel intergenic repetitive units in a mycobacterial two-component system operon. *Mol Microbiol*;**26**(5):991–1003

Supply P, Mazars E, Lesjean S,Vincent V, Gicquel B, Locht C. (2000) Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. *Mol Microbiol* **36**(3):762–71.

Taylor GM, Young DB, and Mays SA. (2005). Genotypic analysis of the earliest known prehistoric case of tuberculosis in Britain. J Clin Microbiol; 43: 2236-40.

Teitelbaum R, Glatman-Freedman A, Chen B, Robbins JB, Unanue E, Casadevall A, Bloom BR. (1998). A mAb recognizing a surface antigen of Mycobacterium tuberculosis enhances host survival. Proc Natl Acad Sci USA, 95:15688-15693

Timmins GS, Master S, Rusnak F, Deretic V. Nitric oxide generated from isoniazid activation by KatG: Source of nitric oxide and activity against Mycobacterium tuberculosis. Antimicrob, Agents Chemother. 2004. 48(8):3006-3009.

Toossi Z, Mincek M, Seeholtzer E, Fulton SA, Hamilton BD, Hirsch CS. (1997) Modulation of IL-12 by transforming growth factor-beta (TGF-beta) in Mycobacterium tuberculosis-infected mononuclear phagocytes and in patients with active tuberculosis. J Clin Lab Immunol 49: 59 – 75.

Toossi, Z., and Ellner, J. J. (2000). Pathogenesis of tuberculosis. *In* Tuberculosis: Current Concepts and Treatment. L. N. Friedman, editor. CRC Press LLC, Boca Raton, FL. p. 19–47.

Tsolaki AG, Hirsh AE, DeRiemer K, et al. Functional and evolutionary genomics of *Mycobacterium tuberculosis*: insights from genomic deletions in 100 strains. *Proc Natl Acad Sci USA* 2004;**101**(14):4865–70.

Valway SE, Sanchez MPC, Shinnick TF, *et al*. An outbreak involving extensive transmission of a virulent strain of *Mycobacterium tuberculosis*. N Engl J Med 1998; 338**:** 633-9.

Van Deun A, Hamid Salim A, Aung KJ, et al.(2005) Performance of variations of carbol fuchsin staining of sputum smears for AFB under field conditions. Int J Tuberc Lung Dis 9:1127-33.

van der Zanden AG, Kremer K, Schouls LM, et al. (2002) Improvement of differentiation and interpretability of spoligotyping for *Mycobacterium tuberculosis* complex isolates by introduction of new spacer oligonucleotides. *J Clin Microbiol*; **40**(12):4628–39.

van Embden JD, Cave MD, Crawford J, *et al*. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting recommendation for a standardized methodology. J Clin Microbiol 1993; 31: 406-9.

van Rie A, Victor TC, Richardson M, et al. Reinfection and mixed infection cause changing *Mycobacterium tuberculosis* drug-resistance patterns. Am J Respir Crit Care Med 2005; 172: 636-42.

Verver S, Warren RM, Beyers N, et al. Rate of reinfection tuberculosis after successful treatment is higher than rate of new tuberculosis. Am J Respir Crit Care Med 2005; 171: 1430-5.

Viana NC, Gutierrez C, Sola C, Filliol I, Boulahbal F, Vincent V, Rastogi N: Genetic diversity of *Mycobacterium africanum* clinical isolates based on IS*6110*-restriction fragment length polymorphism analysis, spoligotyping, and variable number of tandem DNA repeats. J Clin Microbiol 2001;39:57–65.

Vidal R, Martin-Casabona N, Juan A, Falgueras T, Miravitlles M (1996) Incidence and significance of acid-fast bacilli in sputum smears at the end of antituberculous treatment. Chest 109: 1562 - 1565.

Vincent V, and Portaels F. (1992). Proposed minimal standards for the genus *Mycobacterium* and for description of new slowly growing *Mycobacterium* species. *Int J Syst Bacteriol*;**42** (2):315–23.

Voskuil MI, Schnappinger D, Visconti KC, Harrell MI, Dolganov GM, Sherman DR, Schoolnik GK. Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program. J Exp Med. 2003 Sep 1;198(5):705-13

Wang C.H., C.Y. Liu, H.C. Lin, C.T. Yu, K.F. Chung, and H.P. Kuo. (1998). Increased exhaled nitric oxide in active pulmonary tuberculosis due to inducible NO synthase up regulation in alveolar macrophages. Eur. Respir. J. 11:809–815.

Wayne, L.G. Cultivation of Mycobacterium tuberculosis for research purposes. In: B.R. Bloom (Eds) Tuberculosis: Pathogenesis, protection and Control. American Society for Microbiology, Washington, DC, 1994. p. 73-83.

WHO expert consultation* Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies Lancet 2004; 363: 157–63

http://apps.who.int/bmi/index.jsp?introPage=intro_3.html last accessed 2010-01-11.

Williams A, Reljic R, Naylor I, et al. (2004). Passive protection with immunoglobulin A antibodies against tuberculous early infection of the lungs. Immunology; 111: 328-33.

Wilkinson RJ, Llewelyn M, Toossi Z, *et al*. Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case-control study. Lancet 2000; 355: 618 – 621.

Wilson SM, Goss S, Drobniewski F. (1998) Evaluation of strategies for molecular fingerprinting for use in the routine work of a *Mycobacterium* reference unit. *J Clin Microbiol*;**36**(11): 3385–8.

Wood, R., Maartens, G., and Lombard, C. J. (2002). Risk factors for developing tuberculosis in HIV-1 infected adults from communities with very low or very high incidence of tuberculosis. J Acquir Immune Defic Syndr 23: 75-80.

World Health Organization (1994). WHO Tuberculosis Control Programme: framework for effective tuberculosis control. Geneva, WHO, (WHO/TB/94.179).

World Health Organization.(1993) Resolution WHA44.8. Tuberculosis control programme. In: Handbook of resolutions and decisions of the World Health Assembly and the Executive Board. Vol III, 3rd ed. (1985-1992). Geneva, WHO, 1993 (WHA44/1991/REC/1): 116.

World Health Organization (2001). Global tuberculosis control. WHO report 2001 Geneva, Switzerland

World Health Organization.(2003) Treatment of tuberculosis. Guidelines for National Programmes, Geneva 2003. WHO/CDS/TB/2003.313

World Health Organization (2004). TB/HIV. A clinical manual. WHO/HTM/TB/2004.329.

World Health Organization (2005a). Addressing poverty in tuberculosis control: options for national TB control programmes. Geneva, WHO, 2005 (WHO/HTM/TB/2005.352).

World Health Organization (2005b). Profile of high burden countries: WHO report 2005, Annex I, pp 83-86.WHO, Geneva , Switherland.

World Health Organization (2006a). Global tuberculosis control: surveillance, planning and financing. Geneva, Switzerland: WHO; 2006. Publication WHO/HTM/TB/2006.362.

World Health Organization (2006b), Fact sheet, No 104, March 2006.

World Health Organization (2007). Profile of high burden countries: WHO report 2007, Annex I, pp 97-100.WHO, Geneva , Switherland.

World Health Organization (2008). Global tuberculosis control: surveillance, planning, financing; WHO report 2008. "WHO/HTM/TB/2008.393".

World Health Organization (2008b). The WHO/IUATLD Global Project on Anti-tuberculosis Drug Resistance Surveillance 2002-2007; The fourth global report. WHO/HTM/TB/2008.394

World Health Organization. (2008c) Policy guidance on drug susceptibility testing (DST) of second line antituberculosis drugs. Geneva. WHO 2008. http://www.who.int/tb/publications/2008/who_htm_tb_2008_392.pdf (27th of May 2009, date last accsessed).

Xu S, Cooper A, Sturgill-Koszycki S, *et al*. (1994). Intracellular trafficking in Mycobacterium tuberculosis and Mycobacterium avium-infected macrophages. J Immunol; 153: 2568 – 2578.

Yassin, M. A., Olobo, J. O., Kidane, D., Negesse, Y., Shimelis, E., Tadesse, A. Demissie, A., Britton, S., Harboe, M., Aseffa, A., Abate, G. (2003). Diagnosis of tuberculosis lymphadenitis in Butajira, rural Ethiopia. Scand J Infect Dis 35: 240-243

Zahrt, T.C., and V. Deretic. (2001). Reactive Nitrogen and Oxygen Intermediates and Bacterial Defenses: Unusual Adaptations in Mycobacterium tuberculosis. Antioxid. Redox Signal 1: 141–159.

ANNEXES

ANNEX I: Mycobacterium Isolation (Petroff's Method) (SOP1)

1. Reagents, media and materials needed

- 2-4% sterile NaOH
- Sterile Phenol red indicator
- 1N HCl
- 2N HCl
- 2LJ/1PV media for each sample
- Sterile plastic pasture pipette (3mm)
- Conical tube of 10ml
- Eppendorff tubes to preserve the left-over sample

2. Procedure

1. Transfer the sputum from its container to the corresponding labled conical tube.
2. Add an equal volume of 4% w/v NaOH to the specimen and agitate by vortexing.
3. Incubate at ambient temperature for 15-30mins agitating at least every 10 mins.
4. Centrifuge appropriately with 3000g for 15 minutes.
5. Decant the supernatant into a discard jar/ case-role in the hood taking care that none of the supernatant contaminates the outside of the container and that the deposit is not lost.
6. Homogenize the pellet with remaining supernatant, add a drop of phenol red indicator and neutralize with a drop of 2N HCl. (NB: Drop-with additon of 1N HCl prevent sudden over nutralization)
7. Add 3ml of PBS and inoculate 200 µl to the culture media (two-LJ with glycerol and one-LJ with pyruvate) with the deposit. **(NB: a drop of this deposite should be preserved for AFB check)**
8. Incubate the tubes at 37^{o}C at horizontal position for one week then to be upright in the second week.
9. Monitor the growth every week for up to the 8^{th} week before report it negative.

NB: We should do AFB test on the culture negative specimens

Culture result entry form

			2nd wk		3rd wk		4th wk		5th wk		6th wk		7th wk		8th wk		rmrk	AFB
S.n	**RNI**	**Date**	**LJ**	**PV**	**LJ**	**PV**	**LJ**	**PV**	**LJ**	**PV**	**LJ**	**PV**	**LJ**	**PV**	**LJ**	**PV**	**+ / -**	**+ / -**
1	**001**	**3/11/2006**																
2	**002**																	
3	**003**																	

ANNEX II SOP 2: Smear Test with Ziehl-Neelsen staining

I. Reagents and Preparation

Fuchsin

Basic fuchsin 3.0g

95% ethanol (technical grade) 100ml

Dissolve basic fuchsin in ethanol.....Solution 1

Phenol

Phenol crystals 5g

Distilled water 100ml

Dissolve phenol crystals in distilled water

(gentle heat may be required).....Solution 2

Working solution

Combine 10ml of solution 1 with 90ml of solution 2 and store in an amber bottle. Label bottle with name of reagent as well as preparation and expiry dates. Can be stored at room temperature for six to twelve months and filter before use.

Decolourising agent:

3% acid-alcohol

Concentrated hydrochloric acid (technical grade) ……..3ml

95% ethanol (technical grade) ……97ml

Carefully add concentrated hydrochloric acid to 95% ethanol. *Always add acid slowly to alcohol, not vice versa*. The mixture will heat up. Store it in an amber bottle. Label bottle with name of reagent and dates of preparation and expiry. It can be stored at room temperature for six to twelve months.

Decolourising agent:

25% sulphuric acid

Concentrated sulphuric acid (technical grade)…… 25ml

Sterile distilled water……. 100ml

Carefully add concentrated sulphuric acid to water. *Always add acid slowly to water, not vice versa*. The mixture will heat up. Store it in an amber bottle. Label bottle with name

of reagent and dates of preparation and expiry. Can be stored at room temperature for six to twelve months.

Counterstain:

Methylene blue

Methylene blue chloride…….. 0.3g

Distilled water……... 100ml

Dissolve methylene blue chloride in distilled water and store in an amber bottle. Label bottle with name of reagent and dates of preparation and expiry. It can be stored at room temperature for six to twelve months.

II. Ziehl-Neelsen staining Procedure

- Place the numbered slides on a staining rack in batches (maximum 12).
- Ensure that slides do not touch each other
- Flood entire slide with Ziehl-Neelsen carbolfuchsin, which has been filtered prior to use, or cover each slide with a piece of filter paper if unfiltered carbolfuchsin is used
- Heat the slide slowly until it is steaming. *Do not boil*. Maintain steaming for three to five minutes by using low or intermittent heat.
- Rinse each slide individually in a gentle stream of running water until all free stain is washed away
- Flood the slide with the decolourising solution for a maximum of three minutes
- Rinse the slide thoroughly with water. Drain excess water from the slide
- Flood the slide with counterstain
- Allow the smear to counterstain for 60 seconds
- Rinse the slide thoroughly with water. Drain excess water from the slide.
- Allow smear to air dry.

III. RECORDING AND REPORTING OF RESULTS

It is recommended that a uniform pattern of reading is followed, observing 100 useful fields. A useful microscopic field is regarded as one in which cellular elements of bronchial origin (leucocytes, mucuous fibres and ciliated cells) are observed. The fields in which there are no such elements should not be included in the reading.

Negative

Indicate the staining method. Report "negative for acid-fast bacilli" for all smears in which no acid-fast bacilli have been seen in 100 fields.

Positive

Indicate the staining method. The number of acid-fast bacilli found is an indication of the degree of infectivity of the patient as well as the severity of tuberculosis disease. Results should therefore be quantified.

For *Ziehl-Neelsen stained* smears the following semi-quantitative method of reporting is recommended:

No of acid-fast bacilli (AFB)	Field	Report
No AFB	100 immersion fields	No acid-fast bacilli observed
1-9 AFB	100 immersion fields	record exact figure
10 to 99 AFB	100 immersion fields	1+
1 to 10 AFB	per field	2+
more than 10 AFB	per field	3+

ANNEX III: PCR-Based Identification of *M. tuberculosis*

2.1 Preparation of DNA for PCR

DNA from mycobacterial cultures should be extracted and purified as follows.

Transfer loopful of colony in to about 1.5 ml of 1XTE buffer in eppendorff tube and heat it at 80^{o}C for 20 minutes to kill the cell.

Spin down the culture suspension in microcentifuge for 5 min at 12,000g at RT and discard the supernatant.

Re-suspend in 1 ml of Tris-EDTA buffer (10 mM Tris-Cl [pH 8.0], 1 mM EDTA) by vortexing, and transfer to a 2-ml Eppendorf tube.

Add 100 µl Lysozyme solution (10 mg/ml in Tris-EDTA buffer) and five 3mm-diameter glass beads will be then added, vortexed, sonicated for 10 min, vortexed again, and then incubated at 37°C for 2 h with brief vortexing every 30 min.

To the resultant suspension, after water bath sonication for 10 min and division into two 1.5-ml Eppendorf tubes, added 70 µl of 10% sodium dodecyl sulfate and 10 µl of proteinase K (10 mg/ml).

The mixture will then be vortexed and incubated for 2h at 65°C, with brief vortexing every 30 min, and cool for few minutes.

Afterwards, 100 µl of 5 M NaCl will be added and vortexed, and following the addition of 80 µl of 10% hexadecyltrimethyl ammonium bromide, CTAB, (Sigma, St. Louis, Mo.) in pure water and wait for few minutes and vortex until the liquid become white-milky, the mixture will be incubated at 65°C for 10-30 min.

For DNA extraction 750 µl of chloroform/isoamyl alcohol(24:1) will be added, mixed well, and centrifuged at 12,000 g for 5 min at RT in a Microfuge. The resultant upper phase will

be transferred to a clean tube with 420 µl of isopropanol and mix it gently to precipitate the nucleic acid.

The tubes will be then cooled on ice and spun in a Microfuge for 30 min at 15,000 g and 4°C.

Remove the supernatant, the DNA pellet will be washed with 1ml 75% ethanol to remove residual CTAB and NaCl, respin for 5min at RT at 12000g to repellet and air-dry it for about 5min.

The DNA will be then resuspended in RNase- and DNase-free water, quantified, diluted to 50 to 500 µg/ml, and store at -20 ^{o}C until used for PCR.

2.2 PCR amplification primers and conditions

1. According to optimized protocol of AHRI lab each PCR reaction mixture is prepared in 25 µl of PCR Master mix as follows :

3.5 µl of water

12.5 µl Hotstart

7 µl of each primer at 20 µM (5 µl internal, 1µl forward and 1µl reverse primers)

2 µl of DNA (equaling 25 to 250 ng) .

PCR amplifications is performed in a PCR system machine, using program (with an initial denaturation step of 15 min at 95°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, ending with a final elongation step for 10 min at 72°C and holding at 4°C).

Run the 100-bp ladder, PCR products, *M. Tuberculosis* and *M. bovis* strains, as positive control for amplification of the target locus just to check that the PCR is giving the correct

sizes, and a on agarose (1.5%) gel electrophoresis incorporating ethidium bromide staining. and capture the image.. The primer sequences that is used here is

RD 10 (region present, 308 bp; region absent, 202 bp)

RD10 FF .....5_-CTG-CAA-CCA-TCC-GGT-ACA-C-3_

RD10 Int .....5_-GAA-GTC-GTA-ACT-CAC-CGG-GA-5_

RD10 Rv.....5_-AAG-CGC-TAC-ATC-GCC-AAG-3_

PCR Result Entry Form

SN	Date	Sample ID	Result (Mtb/NMtb)	remark
1				
2				
3				
4				
Etc				

ANNEX IV: Preparation of Drug Solution and susceptibility test

I. Drug Solutions Preparation

Reagents: *Isoniazid (INH), Rifampicin (R), Streptomycin (S), Ethambutol (E), Ethanol, Middlebrook 7H10, Oleic acid Albumin Dextrose Catalase (OADC), PBS/Middlbrook 7H9, ATCC35836 or ATCC35838.*

Isoniazid: - Prepare two stocks of drug solutions

- Prepare Stock-1 at a concentration of 1mg/ml in 20 ml PBS and into the medium to get final concentration of 1mg/L.
- Dilute 2ml of stock-1 (2ml Stock-1 + 8ml PBS) to get Stock-2, and add this drug into the medium to get the final concentration of 0.2mg/L.

Rifampicin: Prepare only one stock

- Dissolve 20mg of the drug in 2ml of concentrated ethanol by vigorous vortexing
- Add 18ml of PBS and dilute the drug solution, and add this solution into the medium to get a final concentration of 1mg/L

Streptomycin: Prepare two stocks

- Prepare stock-1 by dissolving 200mg of the drug in 20ml PBS. This drug solution is added in to the medium to get a final concentration of 10mg/L
- Dilute stock-1 one in five (2ml of stock-1 + 8ml of PBS) to get stock-2 with concentration of 2.5mg/ml, and add this solution to the medium to get final concentration of 5mg/L.

Ethambutol: Prepare two stocks

- Prepare stock-1 of ethambuol by dissolving 200mg of the drug in 20ml of PBS and add this drug into the medium to get a final concentration of 10mg/L.
- Dilute stock-1 one in two (5ml of the stock-1 + 5ml PBS) to get stock-2 with a concentration of 6.25mg/ml, and add this solution in to the medium to get a final concentration of 5mg/L.

II. Protocol for Drug Susceptibility Test

- Add a drop of PBS to 10ml empty glass tube containing 7-9 glass beads.
- Transfer a loop full of colony from LJ into the tube containing the beads and vortex it thoroughly. Add 3 drops of PBS and re-vortex and leave to settle.
- Transfer the upper homogeneous suspension and measure the OD.
- Prepare a bacterial suspension with turbidity corresponds to McFarland turbidity standard-1 (OD value between 0.25 and 0.3 at 600nm) using Novaspec II photometer (Pharmacia Biotech Ltd, UK)
- Make 1:10 and 1:1000 dilutions from the above bacterial suspension.
- Transfer 100µl (4 drops with 1ml disposable plastic pipette) from 1:10 bacterial dilution into all drug containing tubes and one drug free control.
- Incubate all the tubes a 37^{o}C and examine the colony formation every week until the third week.
- Calculate the proportion of the growth by dividing the number of colonies in a drug medium with the drug free medium when the numbers of colonies in drug free medium are between 50 and 150.
- A bacterial growth more than 1% is taken as resistant, and the proportion of bacteria less than 1% is considered as susceptible. It is also possible to compare the number of colonies in a drug containing tube inoculated with 1:10 dilution with a control containing 1:1000 diluted bacterial suspension. A higher number of colonies in a drug containing medium indicate resistance.

Remarks:

- The test is done based on the modified proportion method using Middlebrook 7H10 agar supplemented with 10% OADC and glycerol.
- Stock solutions will be prepared for each drugs in PBS at different concentration, and after filter sterilization using 0.2µM filter, aliquots of the drug solutions are kept in cryotubes and stored at -70^{o}C.
- One reference strain (ATCC35836, S sensitive or ATCC35838, R resistant) should be included in each test batch as a control.

ANNEX V SOPs for the optimization Experiments (Pilot Experiment 1)

5.1 Determination of early log phase and bacterial concentration)

1. Using a fresh clinical isolate, take a loop of colonies from Löwenstein-agar and transfer to the 20ml of 7H9 supplemented with OADC in a small Erlenmeyer flask for 14 days at 37°C in an atmosphere of 5% CO_2.

2. Prepare 18 flat-bottomed small bottles (bujox/universal bottles) and label day1, day3, day5....up to day17 in duplicate.

3. Transfer 1ml of the above 14 day old culture to 14 ml fresh 7H9 with OADC to 18 flat-bottomed small bottles (bijoux/universal bottles) and label day1, day3, day5....up to day17 in duplicate and incubate at 37°C in an atmosphere of 5% CO_2.

4. At each day from day 1 at the same time of the day (+/- 1 hour) until 17 days, determine the bacterial concentration by CFU counting as follows:

    4.1 Following thorough mixing of the culture, take out the duplicate day 1 labelled bottle and transfer 1ml of the culture to a tube containing 6-7 2.5mm sterile glass beads and vortex it. Pellet the remaining bacterial suspension at 3000g and preserve it at -20^{o}C in pre-labelled cryotubes.

    4.2 Take an aliquot of 200ul from the above culture and serially diluted it by 1:10 (for 8 dilution steps) in 1800ul sterile PBS/Tween 80 (0.05 %) and thoroughly mix by repeated pipetting.

    4.3 Inoculate 100ul from each serially diluted culture to Middlebrook 7H10 agar labelled day1, sample code and date of inoculation. (NB: **Use sterile steel-spreader to evenly seed the culture for ease of plate counting.**)

    4.4 Seal the plates with aluminium foil and incubate them at 37°C in an atmosphere of 5% CO_2 for 3 weeks.

    4.5 Count and determine the colony forming units (CFUs) per ml after 3 weeks from plates containing 30-300 discrete colonies.

5. **Repeat step 4.1 to 4.5 for the cultures in bottles labelled day3 through day17 on the indicated period.**

6. **Enter the data, plot IP (incubation period) versus cfu/ml graph and determine the early log phase period and the corresponding bacterial concentration.**

Result Entry Form for Early Log Phase and Concentration

Day (IP)	Sample	SD1	SD2	Cfu/ml 1	Cfu/ml 2	Cfu/ml avrg	remark
01	RNI027						
	RNI065						
	RNI066						
	BCG						
	H37Rv						
03	RNI027						
	RNI065						
	RNI066						
	BCG						
	H37Rv						
05	RNI027						
	RNI065						
	RNI066						
	BCG						
	H37Rv						
07	RNI027						
	RNI065						
	RNI066						
	BCG						
	H37Rv						
09	RNI027						
	RNI065						

	RNI066						
	BCG						
	H37Rv						
11	RNI027						
	RNI065						
	RNI066						
	BCG						
	H37Rv						
13	RNI027						
	RNI065						
	RNI066						
	BCG						
	H37Rv						
15	RNI027						
	RNI065						
	RNI066						
	BCG						
	H37Rv						
17	RNI027						
	RNI065						
	RNI066						
	BCG						
	H37Rv						

ANNEX VI: Optimization experiment (Pilot 2)

6.1 Pilot experiment 2: Setting Incubation Time for Optimum RNI killing and Dilution Range for Viable CFU Count

1. From the same isolates used for pilot experiment1, take a loopful of colonies and transfer to the corresponding labelled 10ml Middlebrook 7H9 broth medium supplemented with OADC in a small bijoux bottle and incubate it for 14 days at 37°C in an atmosphere of 5% CO_2.

2. After the 14^{th} day, transfer 1ml of the above culture to 14 ml fresh 7H9 with OADC and incubate at 37°C in an atmosphere of 5% CO_2 for 9 days to reach the early log phase (app 6.4 x 10^8 bacteria) as determined in pilot experiment 1.

3. On day 9^{th}, take out the culture tubes and dilute 100ul of the culture with 6.4ml sterile PBS/Tween80 to dilute the log phase culture to approximately 10^7 CFU/ml.

4. Prepare 18 small bijoux bottle (6 for DETA/NO, 6 for SIN-1 and 6 for PBS) and label with 4hr, 24hr and 72hr for each group in duplicate.

5. Add 400 ul of the bacterial suspension and 3.4 ml 7H9 **without** OADC to each tube above.

6. Then add the following supplements *in duplicates* to the bacterial suspensions above:
    - 200 ul of 20 mM DETA/NO (at RT) diluted in PBS to the DETA/NO labelled tubes
    - 200 ul of 20 mM SIN-1 (at RT) diluted in PBS to the SIN-1 labelled tubes
    - 200ul sterile PBS (at RT) as control to the PBS labelled tubes

7. Incubate for 4, 24 and 72 h according to the label at 37^oC and 5% CO_2.

8. At the specified time period, take out the tubes and thoroughly mix the culture. And serially dilute (**1:10)** aliquot of 200ul from each culture at the specified time point with 1800ul PBS/Tween 80 (0.05 %) in 8 dilutions steps. And pellet the remaining bacterial suspensions at 3000 g for 10 min and store the pellets at -20°C in pre labelled eppendorf tubes.

9. Inoculate 100ul of each serially diluted culture on the corresponding pre labelled Middlebrook 7H10 plate and incubate for 3 weeks in 5% CO_2 atmosphere at 37^{o}C.

10. After 3 weeks culturing, count the CFUs on the feasible plates containing 30-300cfus and record the dilutions.

11. Calculate and record the percentage survival of the isolates by computing the proportion of viable bacteria on DETA/NO and SIN-1 containing medium compared to the PBS containing (control).

Formula for calculating percent (%) survival of the isolates after DETA/NO exposure

$$\textbf{\% survival in DETA/NO} = \frac{\textbf{CFU/ml in DETA X 100}}{\textbf{CFU/ml in PBS(control)}}$$

ANNEX VII: Dose-Response trend experiment to DETA/NO, 24h incubation (**Pilot experiment 3**)

3. From the same isolates used for pilot experiment1, take a loopful of colonies and transfer to the corresponding labelled 10ml Middlebrook 7H9 broth medium supplemented with OADC in a small bijoux bottle and incubate it for 14 days at 37°C in an atmosphere of 5% CO_2.

4. After the 14^{th} day, transfer 1ml of the above culture to 14 ml fresh 7H9 with OADC and incubate at 37°C in an atmosphere of 5% CO_2 for 9 days to reach the early log phase (app 6.4 x 10^8 bacteria) as determined in pilot experiment 1.

6. On day 9^{th}, take out the culture tubes and dilute 100ul of the culture with 6.4ml sterile PBS/Tween80 to dilute the log phase culture to approximately 10^7 CFU/ml.

7. Prepare 12(six duplicates) small bijoux bottle and label them 200mM, 20mM, 2mM, 0.2mM, 0.02mM and PBS in duplicates.

8. Add 3.4 ml Middlebrook 7H9 **without** OADC and 400 ul of the bacterial suspension to each tube above.

9. Then add the following supplements *in duplicates* to the bacterial suspensions in the corresponding labelled tube:
   - 200 ul of 200 mM Deta/NO (undiluted stock solution) (Final concentration 10mM).
   - 200 ul of 20 mM Deta/NO diluted in PBS (Final concentration 1mM).
   - 200 ul of 2 mM Deta/NO diluted in PBS (Final concentration 100uM).
   - 200 ul of 0.2 mM Deta/NO diluted in PBS (Final concentration 10uM).
   - 200 ul of 0.02 mM Deta/NO diluted in PBS (Final concentration 1uM).
   - 200ul PBS as control.

10. Incubate all the tubes for 24h at 37°C and 5% CO_2.

11. After 24h incubation, take out the tubes and thoroughly mix the culture. And serially dilute (**1:10**) aliquot of 200ul from each culture with 1800ul PBS/Tween 80 (0.05 %) in 4 dilutions steps. And pellet the remaining bacterial suspensions at 3000 g for 10 min and store the pellets at -20°C in pre labelled eppendorf tubes.

9. Inoculate 100ul of each serially diluted culture on the corresponding pre labelled Middlebrook 7H10 plate and incubate for 3 weeks in 5% CO_2 atmosphere at 37°C.

10. After 3 weeks culturing, count the CFUs on the feasible plates containing 30-300cfus and calculate and record the percentage survival to each concentration by computing the proportion of viable bacteria on DETA/NO containing tube against the control (PBS containing tubes.

$$\textbf{\% survival in DETA/NO} = \frac{\textbf{CFU/ml in DETA X 100}}{\textbf{CFU/ml in PBS(control)}}$$

Result Entry Form for Dose-Response Experiment

Date	ID		200mM	20mM	2mM	0.2mM	0.02mM	PBS
		CFU1						
		CFU2						
		CFUavg						
		%Survive						
		CFU1						
		CFU2						
		CFUavg						
		%Survive						
		CFU1						
		CFU2						
		CFUavg						
		%Survive						
		CFU1						
		CFU2						
		CFUavg						
		%Survive						
		CFU1						
		CFU2						
		CFUavg						
		%Survive						
		CFU1						
		CFU2						
		CFUavg						
		%Survive						

ANNEX VIII: Protocol for Nitric Oxide Susceptibility Test

1. Take a loopful of fresh colonies from labled LJ medium and immerse it in to 10ml of 7H9 supplemented with OADC in a small screw capped flat bottomed tube at 37°C in 5% CO_2 incubator for 14 days.

2. Transfer 1ml of the above bacterial culture to 14 ml fresh 7H9 with OADC and incubate at 37°C in an atmosphere of 5% CO_2 for extra 9 days ie early log phase culture of app 6 X 10^8 bacterial/ml.

3. Add 200 ul of the above culture to a total volume of 12ml PBS/Tween 80 (0.05 %) to dilute the log phase culture to approximately 10^7 CFU/ml. (dilution 1:60)

4. Prepare 6 small flat bottomed screw-capped bottles containing 500 ul of the bacterial suspension added to fresh 4.25 ml 7H9 **without** OADC and add the following supplements *in duplicates* to this bacterial suspensions:
    a) aliqoute of 250 ul of 20mM Deta/NO (at RT) diluted in PBS in the first 2 bottles
    b) aliqoute of 250 ul of 20mM SIN-1 (at RT) diluted in PBS in the second 2 bottles
    c) sterile 250 ul PBS (at RT) as control

5. Incubate the bacterial suspensions for 24 h at 37°C in 5% CO_2 incubator and gently shake the bottels at intervals.

6. Following thorough mixing of the culture take 100ul from each culture and serially diluted by 1:10 in PBS/Tween 80 (0.05 %) four steps. Pellet the remaining bacterial suspensions at 3000g for 10 min and store the pellets at -70°C in pre labelled eppendorf tubes.

7. Inoculate 100ul of the serially diluted culture on 7H10 Middlebrook agar and count the colony forming units (CFUs) after 3 weeks.

8. Determine the percentage survival of the isolates by calculating the proportion of viable bacteria on DETA/NO and SIN-1 containing medium compared to the control (PBS containing medium)

$$\% \text{ survival in DETA/NO} = \frac{\text{CFU/ml in DETA/NO} \quad X\ 100}{\text{CFU/ml in PBS/control}}$$

.

Notes:

No OADC should be used here as albumin is a known and potent chelator of NO and ONOO.

Aliquoted stock solutions of 52 ul made from DETA/NO 50mg prepared at 200 mM in PBS and stock solutions of 52 ul of 100 mM SIN-1 (pre diluted in vial at 100mM in PBS) both from Cayman and then stored at-70. These stock dilutions are diluted 1:10 (DETA) and 1:5 (SIN-1) before use. It is important that the DETA/NO powder is totally dissolved before aliquoting but that exposure beyond 15 min at RT is avoided.

The range for dilution should be adjusted according to pilot experiment 2.

DECLARATION

I, under signed, declare that this M.Sc. thesis is my original work, has not been presented for a degree in any other University and that all sources of materials used for this thesis have been duly acknowledged.

M.Sc. Candidate Mekdim Mekonnen Teklu

Signature _____

Date and place of submission _____

Addis Ababa, Ethiopia

Supervisor Yimtubezinash W/Amanuel (MD, M.Sc., PhD)

Signature _____

Date and place of submission _____

Addis Ababa, Ethiopia

Supervisor Daniel Asrat (MD, M.Sc., PhD)

Signature _____

Date and place of submission _____

Addis Ababa, Ethiopia