In vitro Immune Response to Vaccine Candidate *Mycobacterium tuberculosis* Antigens in Healthy Adult Ethiopians

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ABBREVIATIONS

AAU	Addis Ababa University
AAU-MF	Addis Ababa University, Medical Faculty
Ag	Antigens
Ag85B	Antigen 85B
AHRI	Armauer Hansen Research Institute
AIDS	Acquired Immunodeficiency Syndrome
ALERT	All African Leprosy, TB Rehabilitation and Training center
ALP (AP)	Alkaline Phosphatase
APC	Antigen-presenting cells
ATS	American Thoracic Society
BCG	Bacille Calmette Guerin
BMI	Body Mass Index
CD	Cluster Designation
CDC	Centers for Disease Control and Prevention
CFP	Culture Filtrate Protein
DMSO	Dimethyl sulfoxide
DNA	Deoxyribo Nucleic Acid
DTH	Delayed type hypersensitivity
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
ESAT-6	Early secretory antigenic target
FACS	Fluorescence-activated cell sorter
FRPC	Faculty Research and Publication Committee
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
INH	Isoniazid
LT	Laboratory Technology
LTBI	Latent Tuberculosis Infection
MDR-TB	Multidrug resistant tuberculosis
MTB	Mycobacterium tuberculosis
NERC	National Ethical Research Committee
OD	Optical density
OPD	O-Phenylenediamine Dihydrochloride
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PBST	Tween in PBS
PHA	Phytohaemagglutinin
PPD	Purified Protein Derivative
PVDF	Polyvinyl-difluoride
QFT-G	QuantiFERON®-TB Gold InTube
RD	Region of Differences
RPMI-1640	Roswell Pack Memorial Institute

RT	Room Temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SD	Standard Deviation
SFC	Spot Forming Cells
SSI	Sateen Serum Institute
TB	Tuberculosis
TGF-β	Transforming Growth Factor Beta
Th	T-helper cell
TNF	Tumor Necrosis Factor
TST	Tuberculin Skin Test
WHO	World Health Organization

ABSTRACT

Introduction: The global burden of disease due to tuberculosis (TB) is huge and is one of the challenges in the fight against infectious diseases. The widespread use BCG vaccine was for many years believed to be one of the key control measures for TB, but its efficacy has been questioned following the results of trials conducted in developing countries. Therefore, there is an urgent need particularly in developing countries, for a vaccine with acceptable efficacy to combat the TB epidemic. However, protective immunity in TB is incompletely understood. In murine models, cell-subset depletion experiments in vivo showed that CD4 and CD8 άβ as well as γδ T cells all have a role in protective immunity. Studies demonstrated that immunization of mice with live *M. tuberculosis* induces protection and delayed type hypersensitivity (DTH), whereas heat-killed organisms induce DTH only. This experimental observation has sparked interest in antigens released or secreted by mycobacteria in culture filtrates as vaccine candidates and different studies on these antigens showed promising results. Ethiopia being one of the high TB burden countries, this project is designed with the objective to generate background immunological data on immune response to selected candidate TB vaccine antigens of healthy adult Ethiopians in preparation for a TB vaccine clinical trial.

Methods: Tuberculin skin test (TST) and QuantiFERON®-TB-Gold (QFT-G) assay were done. Peripheral blood monocytic cells **(**PBMCs) were isolated from healthy adults and the immune response following PBMC stimulation with the different TB antigens, ESAT-6, Ag85B and fused Ag85B-ESAT-6, was measured by the *in vitro* assays, IFN–γ ELISPOT assay and IFN–γ ELISA.

Results: Our findings showed that 46.7 % and 43.9% of the study participants had positive TST (TST≥10 mm) and QFT-G assay results respectively. The overall strength of agreement between TST (≥10mm) and QFT-G assay was very good (Kappa = 0.83), with concordant results in 98/107 (91.6%). Concordance between the two tests was good in participants with BCG scar (85.4%, Kappa = 0.71), but it was found to be excellent (96.6%, Kappa =0.93) in those who had not BCG scar. By using IFN–γ ELISPOT assay, the median (IQR) responses to Ag85B, ESAT-6 and fused Ag85B-ESAT-6 were found to be 256 (147-408), 305 (133-474) and 429 (267-601) SFC/Million of PBMC respectively. The median (IQR) responses to Ag85B, ESAT-6 and fused Ag85B-ESAT-6 were found to be 0(0.0-189.0), 328.0 (0.0- 2351.0) and 96.0(0.0- 1220.0) pg/ml of IFN-*γ* respectively as it was measured by IFN–γ ELISA.

Conclusion: An overall strong agreement between TST and QFT-G was observed at both TST≥10mm and TST>5 mm cutoff values in spite of the routine BCG vaccination at birth. We also have demonstrated the possibility to have false TST positivity in adults as the result of BCG vaccine administered in infancy, at cutoff of TST>5mm, but the possibility of true Latent TB infection (LTBI) is highly likely at larger indurations (TST≥10mm). QFT-G assay was not able to differentiate recent infection from remote one. By using ELISPOT assay and ELISA, we were able to demonstrate the existence of effector and central memory T-cells specific to the fused antigens, Ag85B-ESAT-6, and its components, ESAT-6 and Ag85B, in healthy adult populations. The presence of significant ESAT-6 specific memory T-cells in the latent group makes the fused vaccine valuable to be used as post-exposure vaccine. The persistence of Ag85B specific T-cells as the result of childhood BCG vaccination enables the fused vaccine to be used as booster vaccine besides its potential use as post-exposure vaccine.

CHAPTER I: INTRODUCTION

1.1 General Introduction

The global burden of tuberculosis (TB), which is caused by *Mycobacterium tuberculosis* (MTB), is huge and is one of the challenges in the fight against infectious diseases. The burden has increased because of its association with human immunodeficiency virus (HIV) and AIDS (Selwyn *et al*., 1989), the increase of multidrug-resistant strains of MTB (Snider *et al*., 1993), and poor socioeconomic condition especially in developing countries.

The widespread use of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) vaccine was for many years believed to be one of the key control measures for TB in the world, but its efficacy has been questioned following the results of trials conducted in developing countries many years after its use worldwide (Fine, 1989). Therefore, there is an urgent need particularly in developing countries, for a vaccine with acceptable efficacy to combat the TB epidemic.

In recent years, TB vaccine research has focused on antigens released by live MTB in culture medium, as these antigens are believed to be at least partially responsible for the efficacy of live vaccines (Andersen, 1997; Horwitz *et al*., 1995). Some of the extracellular antigens have been tested in animal models for their potential use as vaccines and many studies have demonstrated substantial levels of protection (Andersen, 1994; Lindblad *et al*., 1997; Pal *et al*., 1992; Roberts *et al*., 1995).

Ethiopia being one of the high TB burden countries (WHO, 2009), studying the immune response to the different antigens in our country has great significance in TB vaccine clinical trials. Hence, this project aims at generating background immunological data on immune response to selected candidate TB vaccine antigens of healthy adult Ethiopians in preparation for a TB vaccine clinical trial.

1.2 Epidemiology of Tuberculosis

Annually about 9 million people develop active TB and 1.8 million die from all forms of the disease in the world. The global prevalence of all forms of TB is 206 per 100,000 populations (WHO, 2009).

According to recent estimate by WHO, Ethiopia, having estimated incidence of 378 and 163 per 100,000 populations, for all forms of TB and new smear positive cases,

respectively, is ranked 7th out of the high burden countries globally. And the prevalence is 579 per 100,000 populations (WHO, 2009).

Clinical studies have demonstrated that human immunodeficiency virus type 1 (HIV-1) infection is one of the most important risk factors for susceptibility to tuberculosis and reactivation tuberculosis. People with latent tuberculosis infection (LTBI), Tuberculin Skin Test (TST) positive, not infected with HIV-1 have a 10% lifetime risk of developing active tuberculosis, whereas co infection with MTB and HIV-1 is associated with a 5 to15% yearly risk of active tuberculosis (Selwyn *et al*., 1989).

1.3 Infection and Immunity to M. tuberculosis

The nature of the cells responding to *M. tuberculosis* (MTB) infection and how their relative contribution changes over time is an area of obvious importance for rational vaccine development.

1.3.1 Pathogenesis and Pathology

MTB normally enters the host via the mucosal surface of the lung after inhalation of infectious droplets from an infected individual. The droplets are deposited in the alveolar spaces, where the bacteria are taken up by phagocytic cells. Ingestion of the bacteria induces a rapid inflammatory response which results in the accumulation of a variety of immune cells and, with time, the formation of a granuloma, characterized by a relatively small number of infected phagocytes surrounded by activated monocytes/macrophages and, farther out, activated lymphocytes. If the infection is successfully contained, the granuloma shrinks and may eventually calcify. If however, the immune response does not successfully control the bacterial replication, the granulomas increase in size and cellularity. Eventually, cell death in the hypoxic center of the granuloma leads to necrosis. If the granuloma is close to the surface of the lung, the tissue destruction caused by necrosis can breach the mucosal surface, giving rise to the prototype symptom of TB, a persistent cough with blood in the sputum, a process referred to as cavitation. At this point the patient is highly infectious, spreading the bacteria by aerosol (Gonzalez-Juarrero *et al.*, 2001).

The outcome of infection with MTB is determined by a dynamic balance of host and pathogen factors. In resistant individuals a high level of activity may be reached rapidly and the disease is consequently controlled in the asymptomatic stage. In susceptible host, by contrast, the process of bacterial multiplication and cellular recruitment continues, the

primary lesion enlarges and some bacteria are transported to the regional lymph nodes, giving rise to a granulomatous reaction. The combination of the primary lesion and changes in the regional lymph nodes is termed a primary complex. As the disease continues, the amplified immune reaction leads to intense inflammation, tissue destruction, caseous necrosis and the formation of cavitary lesions (Dannenberg, 1991). At this stage, lysis of the macrophages may result in the release of viable bacteria into the blood and the generation of metastatic foci in various organs (Wiegeshaus *et al*., 1989). From studies of the location of single cavitary lesions in the lung, the apical lung zone appears to be a particularly vulnerable site that allows bacterial multiplication either after haematogenous dissemination or directly after primary implant. If the host controls the infection, the lesions are encapsulated, sterilized and left as calcified scars. The bacteria may be held in stasis, but survive for many years until an eventual later down-regulation of host activity, e.g. as a consequence of immunosuppresion, allows a continued multiplication. This event is called the endogenous reactivation of TB (Smith *et al*., 1989).

1.3.2 Immunity

Protective immunity in TB is incompletely understood. The dogma is that cellular immunity is essential for protection and that humoral immune responses are of little or no importance. There is no doubt that cellular immunity represents the cornerstone of protective immunity, but the detailed correlates of protection remains to be elucidated (Kaufmann, 2006).

Monocytes-macrophages, particularly the alveolar macrophages, are the natural hosts for MTB. They have a limited intrinsic capacity to reduce the growth of mycobacteria but additional acquired immune activation by CD4 T-cells is necessary to control the infection (Rich, 1996). In order to get the help from CD4 T-cells, MTB antigens (Ag) have to be presented to specific CD4 T-cells by professional antigen-presenting cells (APC), including dendritic cells and other cells of the monocytes-macrophage lineage. The CD4 T-cells are induced to secrete interleukin-2 (IL-2), the main T-cell growth factor, and interferon-gamma (IFN-γ), an important activating signal for monocytes-macrophage. The production of IFN-γ is regulated by APC-derived factors, including

stimulatory IL-12 and suppressive IL-10 and transforming growth factor beta (TGF-β) (Bermudez *et al*., 1995).

Acquired deficiency of type-l responses (Th1 responses), e.g. HIV infection, dramatically increases the chances of clinical reactivation of MTB infection (Eriki *et al*., 1991). Genetic deficiencies in IFN-γ production result in increased susceptibility to mycobacterial diseases (Flynn *et al*., 1993).

Mechanisms, different from, but related to classical monocyte-CD4 T-cell interactions, contribute to MTB control as well. In murine models, cell subset depletion experiments *in vivo* showed that CD4 and CD8 άβ receptor (+) as well as γδ receptor (+) T cells all have a role in protective immunity in the order CD4 > CD8> γδ T cells. Interferon-γ is secreted by MTB activated γδ T-cells before άβ T-cells come in to play. Both activated CD4 and CD8 T-cells and γδ T cells can eliminate infected Monocytes-macrophages by their potent cytotoxic activity (Boom, 1996). In addition, there is evidence that double (άβ) negative T-cells, other than γδ T-cells, may recognize non-protein mycobacterial antigens through mechanisms involving presentation via CD1 receptor (Beckman *et al*., 1994).

Mycobacterial products induce the production of tumor necrosis factor-ά (TNF-ά) by monocytes-macrophage. TNF-ά has a complex role in the pathogenesis of TB as it can either increase phagocytic and killing capacities of monocytes-macrophage or promote the growth of MTB inside the cells, depending on the presence of other factors, including IFN-γ and 1,25 di-hydroxy vitamin D (Rook *et al*., 1996). TNF-ά appears to be required, together with IFN-γ, for the formation of granuloma, which limit the spread of the infection. This has been clearly demonstrated in the murine model of Bacille Calmette Guerin (BCG) infection, in which animals pretreated with neutralizing antibody to TNF-ά fail to contain the infection and develop progressive BCG disease (Flynn *et al*., 1995).

The role of Th2 cells in TB has been controversial, in part because the prototype Th2 cytokine, IL-4, is highly bioactive and generally produced at very low levels, making its detection problematic. Some work, using indirect markers such as immunoglobulin E or soluble CD30 has suggested that progression to TB is associated with a relative increase in the production of IL-4 or a shift in the Th1/Th2 balance (Lienhardt *et al*., 2002). More compellingly, recent work has shown that elevated expression of IL-4 in recall responses

by peripheral blood mononuclear cells from health care workers exposed to MTB in the course of their duties correlated with later development of TB (Ordway *et al*., 2004).

The cellular mechanism underlying the protective immune response against MTB appears to involve a T helper 1 (Th1)-type response, including the production of IFN-γ by sensitized CD4 (Orme *et al*., 1993) and CD8 (Tascon *et al*., 1998) T-cells to induce macrophage activation.

These days, there are *in vitro* assays, using IFN-γ production as an indicator of a protective response, that are used as an indicator of natural and vaccine-induced protection (Lein et al., 1997) as well as a diagnostic tool for infection with MTB and other species of mycobacteria (Van Pinxteren *et al*., 2000).

Methods to measure IFN-γ production by cultured peripheral blood cells in response to mycobacterial antigens include reverse transcription polymerase chain reaction (RT-PCR) of mRNA, fluorescence-activated cell sorter (FACS) analysis of stained intracellular cytokines (Smith *et al*., 2002) and enzyme-linked immunospot assay (ELISPOT) (Ewer *et al*., 2003) or enzyme-linked immunosorbent assay (ELISA) of supernatants from undiluted whole-blood culture to detect production after overnight stimulation (Streeton *et al*., 1998).

1.4 Control of Tuberculosis

Reduction of incidence of active TB should target on reduction of the rate of transmission of the disease. There are two main strategies for reducing the transmission rate: (i) early diagnosis and treatment of tuberculosis; (ii) preventing development of disease by vaccination. These two strategies complement each other, but they are not as effective in practical life as needed to combat the disease (Kaufmann, 2005).

1.4.1 Diagnosis

Tuberculin skin test (TST) using purified protein derivative (PPD), in the diagnosis of TB have proved to be unreliable because PPD is a poorly defined mycobacterial antigen mixture that contains antigens which are common to strains from the *Mycobacterium tuberculosis complex* , environmental nontuberculous strains and the vaccine sub-strain *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) (Harboe, 1981).

A meta-analysis of the effect of BCG vaccination on TST measurements showed the effect of BCG vaccination on TST results to be less after 15 years; and positive skin tests

with indurations of >15 mm are more likely to be the result of tuberculous infection than of BCG vaccination (Wang *et al*., 2002).

According to a guide line developed by American Thoracic Society (ATS) in collaboration with Centers for Disease Control and Prevention (CDC), there are three cut offs based on different risk factors in order to consider a ttest positive. A reaction of 5 mm is considered positive for: household contacts of persons with active tuberculosis; AIDS patients; fibrotic changes on chest radiograph consistent with prior TB; and Patients with organ transplants and other immunosuppressed patients (receiving the equivalent of >15 mg/d of prednisone for 1 mo or more). A reaction of 10 mm or more is considered positive in individuals with one or more of the risk factors: recent immigrants from high prevalence country; intravenous drug users; residents of long-term care facilities; and individuals with certain medical conditions that increase the risk of developing tuberculosis. These medical conditions include being 10% or more below ideal body weight, chronic renal failure, diabetes mellitus, high dose corticosteroid or other immunosuppressive therapy, some blood disorders like leukemia and lymphomas, and other cancers. A reaction of 15 mm is considered positive for a person with no risk factor for TB (ATS/CDC, 2000).

Routine contact investigation and treatment for LTBI is not part of the nation TB control programs in many developing countries including Ethiopia. The National TB Control Programs in these countries focus on early detection and treatment of active cases, and the diagnosis of TB relies largely on microscopic detection of acid-fast bacilli in smears from clinical specimens. However, this low cost and rapid method is characterized by a high variable sensitivity (22–80%), particularly low for paucibacillary and extrapulmonary TB patients (Metchock *et al*., 1999). Culture methods are more sensitive and specific but are time consuming, take several weeks to become positive and are not available in many peripheral diagnostic services. The new rapid diagnostic tests based on molecular techniques, such as polymerase chain reaction or nucleic probes, are sensitive and specific. However, their use in developing countries is not practicable due to the high cost and the need of well-trained staff and sophisticated laboratory facilities (Claridge *et al*., 1993).

1.4.2 Treatment

Although regimens exist for treating tuberculosis, they are far from ideal. Treatment usually involves a combination of drugs, isoniazid (INH) and rifampin, which are given for at least 6 months, and pyrazinamide and ethambutol (or streptomycin), which are used only in the first 2 months of treatment. Because this regimen is extremely difficult to adhere to, partial treatment leads to the development and spread of drug-resistant strains. These strains have a much lower cure rate and are more expensive to treat. There is thus an urgent need for shorter, simpler therapeutic and prophylactic regimens to increase adherence (ATS/CDC, 2003). In addition, new drugs are needed to combat the increasing number of multi-drug-resistant strains (MDR-TB). Treatment for MDR-TB often requires the use of second line TB drugs, all of which can produce serious side effects. Therapy for 18 months to 2 years may be necessary, and patients should receive at least three drugs to which the bacteria are susceptible.

1.4.3 BCG Vaccine

The first people to succeed in TB vaccine development were Albert Calmette and Camille Gue´rin of the Pasteur Institute, who attenuated a mycobacterium related to *M. tuberculosis* (*Mycobacterium bovis* bacillus Calmette-Gue´rin [BCG]) by growing it in culture medium for 13 years, monitoring its decrease in virulence in animals through this period (Calmette *et al*., 1929).

The number of new cases of TB declined steadily in western countries from nineteen fifties until the early eighties. The consequence of this apparent control was that the remaining problem became largely ignored and research into the disease was almost abandoned. In most developing countries, however, the incidence rate remained high and the disease today still accounts for nearly 20% of all deaths in adults (Bloom *et al*., 1992).

For several years BCG vaccination program was believed to be the key to solve the TB problem in developing countries. Presently, the only available vaccine is *M bovis* Bacille Calmette-Güerin, the BCG vaccine. This vaccine generally induces high levels of acquired resistance in animal models of TB (Smith, 1985). The efficacy of this vaccine in human adults, however, varies from 0 to 80% in different populations, with a consistently low efficacy in many tropical regions of the world where the vaccine is most needed (Fine, 1995).

In the late seventies a WHO concerted large scale efficacy trial with the BCG vaccine in South India demonstrated no protective efficacy of the vaccine in adults (WHO, 1980). This alarming finding was the background for the WHO initiative to stimulate research that would exploit the new biomedical advances in the development of an improved vaccine against TB (WHO, 1983). According to WHO, TB is the world's most neglected health crisis and in 1993 the resurgence of the disease in industrialized countries led to the statement 'TB does not stop at national borders. It will be impossible to control TB in industrialized nations unless it is reduced as a health threat in Africa, Asia and Latin America'. WHO declared TB a global health emergency and in November the same year launched the new TB programme aimed at cutting the 3 million deaths a year to 1.6 million a year within the next 10 years (WHO, 1993). Currently, a new and improved TB vaccine therefore represents an urgent international research priority.

The reason for the failure of BCG in some populations has been a subject of debate since the 1950s, and many different hypotheses have been suggested to explain the observed variation. Some investigators have suggested that differences in the strain of BCG (Lagranderie *et al*., 1996), the age at vaccination (Tripathy, 1983), or methodological differences are important factors for the variation reported (Clemens et al., 1983). The most widely accepted hypothesis relates the efficacy of BCG to geographic location, with low to non detectable levels of protection against pulmonary TB seen in tropical regions such as Africa and India, where exposure to nontuberculous mycobacteria is common (Fine, 1989). One exception from this general rule is the consistent high efficacy when BCG is used to vaccinate newborns. Neonatal vaccination with BCG imparts protection against the childhood manifestations of TB (in particular, meningitis) (Colditz *et al*., 1995), but the efficacy wanes over a period of 10 to 15 years, and therefore it does not prevent against the later breakdown with pulmonary TB in the adult population in the third world (Sterne *et al*., 1998).

A review by Doherty et al suggested options for new TB vaccine design. The options are either to replace BCG with a vaccine that gives a longer duration of protection or to design a vaccine that can be given at a later time point to boost existing immunity and provide protection in adults. Both of these approaches have advantages and disadvantages, and the vaccines now entering clinical trials include proponents of both

approaches. However, one important problem of the late booster strategy is the fact that in highly TB endemic regions such a vaccine will in many cases be given to already latently infected individuals. It is therefore necessary not only to develop such a vaccine as a BCG booster vaccine but also as a post exposure vaccine. The only practical outcome might be a hybrid approach, what can be termed a multiphase vaccine that can be administered regardless of the infectious status of the individual and with activity both in naïve and already-infected individuals (Doherty *et al*., 2005).

1.5 Tuberculosis Vaccine Candidate Antigens

Immunization of mice with live MTB induces protection and DTH, whereas heat-killed organisms induce DTH only. This experimental observation has sparked interest in antigens released or secreted by mycobacteria in culture filtrates as vaccine candidates (Orme, 1998). Among the antigens the focus of this study was on ESAT-6, Ag85B and fused Ag85B-ESAT-6.

The new TB vaccines that are under development include recombinant BCG vaccines, attenuated MTB vaccines, DNA-based vaccines and subunit vaccines (Doherty, 2004). The subunit vaccine approach builds on the concept of stimulating the immune response to a number of selected antigens delivered in the form of recombinant antigens. Statens Serum Institute (SSI), Denmark has cloned and screened 250 antigens from MTB and isolated a small number. Among these are the early secretory antigenic target (ESAT-6) and antigen 85 (Ag85B). The two antigens are strongly recognized T-cell antigens in the first phase of infection; they have demonstrated protective efficacy in animal models; and they contain numerous well recognized epitopes in TB patients (Brandt *et al*., 2000; Ravn *et al*., 1999).

ESAT-6 is a secreted protein of major importance. A mouse model was infected with MTB, treated and reinfected. Upon reinfection, this mouse elicited strong T cell responses to ESAT-6, therefore making this protein relevant as a means of providing protective immunity. ESAT-6 has also been demonstrated to be present only in *M. tuberculosis* and *M. bovis*. This protein could be of great value as a diagnostic reagent for TB infection because the TST has limitation diagnostically because of its inability to differentiate between *M. tuberculosis* infection and sensitization after BCG vaccination (Harboe *et al*., 1996).

The number of IFN-γ producing cells reactive to ESAT-6 antigen is increased in recent converters, TST positives, and in TB patients but not in unvaccinated or BCG vaccinated healthy donors. ESAT-6-reactive IFN-γ producing cells in recent converters and TB patients recognized similar synthetic peptides. Thus, ESAT- 6 is a potential candidate for use in the detection of early as well as active TB (Ulrichs *et al*., 2000). Finding from follow up studies linked the magnitude of the ESAT-6 response to the degree of exposure and subsequent development of active TB in subjects with higher ESAT-6 response (Doherty *et al*, 2002) which also supports the use of ESAT-6 in early diagnosis of LTBI especially helpful for developed countries where treating LTBI is a policy in their TB control policy.

The 6-kDa early secretory antigenic target (ESAT-6) and the 10-kDa culture filtrate protein (CFP-10) from MTB are two dominant targets for T-cells in the early phases of infection (Skjøt *et al*., 2000). Both antigens are contained within the RD1 region (region of differences) of the mycobacterial genome, which is absent from BCG, *M. avium*, and most other nontuberculous mycobacteria (Harboe *et al*., 1996). Identification and characterization of the two MTB specific antigens, ESAT-6 and CFP-10, has led to the development of the new diagnostic tests for infection with MTB, Quantiferon-TB Gold (QFT-G).

The latest version of QFT-G, QuantiFERON®-TB Gold InTube (QFT-G), employs whole blood collected and incubated with overlapping peptides representing the TB antigens ESAT-6 and CFP-10 along with a peptide from another TB-specific antigen, TB7.7 (Rv 2654). Evacuated tubes are pre-coated with control and test antigens and the blood-collection tubes also serve as the incubation vessels. The QFT-G test measures the amount of IFN-*γ* produced by T-cells previously exposed to MTB when they are stimulated with the TB specific antigen during overnight incubation (Cellestis, 2006).

The antigen 85 (Ag85) complex of Mycobacterium bovis BCG was the first group of secreted antigens initially identified in culture supernatants (Closs *et al*., 1980). Ag85 complex comprises three closely related proteins (A, B, and C) encoded by separate genes (Content et al., 1991). This complex is present in a number of mycobacterial species, including M. bovis (both bovine and BCG strains), M. tuberculosis (Wiker *et al*., 1990), and Mycobacterium leprae (Rinke de Wit *et al*., 1993; Thole *et al*., 1992). Such

secreted antigens are likely to play a vital role in the induction of protective immunity (Andersen *et al*., 1993). Indeed, Ag85B induces strong T-cell proliferation and IFN- γ secretion in most healthy individuals exposed to *M. tuberculosis* and in BCG-vaccinated donors (Lalvani *et al*., 2001). Moreover, subunit vaccines expressing Ag85B and ESAT-6 (Hybrid1) (Brandt *et al*., 2000) were able to induce protection in a murine model of TB.

1.6 Significance of the Study

The lack of rapid, robust and specific diagnostic methods; the emergence of MDR-TB and lack of an effective vaccine has hampered the global control of TB. In addition, the Human Immunodeficiency Virus (HIV) pandemic makes TB control very much difficult. Therefore, there is a strong need for the development of better diagnostic methods and effective vaccines.

Quantifying the TST and QuantiFERON®-TB-Gold assay positivity rate; and the response following stimulation of PBMCs with the antigens ESAT-6, Ag85B, fused Ag85B-ESAT-6 and PPD in the context of TST response and QuantiFERON®-TB-Gold assay would have great significance in future TB vaccine clinical trial in Ethiopia. Therefore, this project is designed with the objective to generate background immunological data on immune response to selected candidate TB vaccine antigens of healthy adult Ethiopians in preparation for a TB vaccine clinical trial.

1.7 Objective

1.7.1 General objective

To generate background immunological data on immune response to selected candidate TB vaccine antigens of healthy adult Ethiopians in preparation for a TB vaccine clinical trial.

1.7.2 Specific objectives

1. To determine the antigen specific T cell response profile of healthy adult Ethiopians to selected mycobacterial antigens constituting a candidate TB vaccine (QuantiFERON®-TB-Gold test; and following stimulation of PBMCs with the antigens: ESAT-6,Ag85B, fused Ag85B-ESAT-6 and PPD).
2. To compare *in vivo* TST response to *in vitro* QuantiFERON®-TB-Gold assay in determining latent TB infection in healthy adult Ethiopians.

CHAPTER II: MATERIALS AND METHODS

2.1 Study Design

The study design is cross-sectional and study participants were prospectively enrolled into the study from December 2008 to February 2009.

2.2 Study Population

The study participants included were consecutive Medical and paramedical students (AAU-MF) participating in a TB vaccine trial entitled "A safety and immunogenicity phase 1 trial of an adjuvanted TB subunit vaccine (Ag85B-ESAT-6+IC31) administered in different antigen/adjuvant formulations in TST negative and TST positive volunteers at 0 and 2 months" after giving additional consent for this specific study. The study participants were recruited based on the following inclusion and exclusion criteria during the screening phase of the vaccine trial:

Inclusion criteria

1. Willingness to participate in the study
2. Healthy based on history and physical examination at the inclusion
3. Age 18 to 45 yrs of both sexes
4. No past or current history of chronic illness like diabetes mellitus, hypertension, renal failure, hematological or other malignancies

Exclusion criteria

1. Clinical or radiological evidence of active tuberculosis
2. Those on immune modulating drugs (steroids, immunosuppressive drugs or immunoglobulins) within the last 3 months
3. Positive for HIV serology test
4. History of previous treatment for TB

The sample size was calculated by using Epi Info version 6.04D, with 5% precision it was found to be 90. Together with 20% contingency the total sample size was 108. It was calculated based on a study done in Brazil, where positivity after PBMCs stimulation with ESAT-6, Ag85B and PPD was measured by IFN–ɣ ELISA. Since ESAT-6 antigen is specific for MTB, the positive response to ESAT-6 (100% and 80% among TST positive and TST negative healthy Brazilians respectively) was used in the calculation. Positive response was defined as an IFN–ɣ concentration above the mean plus 3 standard deviations of the values measured in nonstimulated control wells (Cardoso *et al.*, 2002).

TST was considered to be positive when the induration following the skin test was greater than 10mm.

According to Elias D (unpublished data), he reported 30% PPD skin test positivity at a specific rural area in western Ethiopia, Ginchi (90 km west of Addis), versus 70% in Addis Ababa (Kotebe college of teacher's education where students are supposed to come from both rural and urban areas like our study participans). From Elias D data we expect low frequency of TST negative study participants in Addis Ababa; hence the ratio of TST positives to TST negatives was taken to be 2:1 in sample size determination.

Because of the high cost of the different reagents, *in vitro* antigen stimulation for IFN–γ ELISA and IFN–γ ELISPOT assay was done only for randomly chosen half of the sample size calculated using the PBMCs samples collected from the vaccine trial.

The above two studies were used in sample size calculation because of lack of published local data.

2.3 Data collection and blood sample handling

Data on medical history and physical examination was collected by using a pretested study questionnaire. For those participants who satisfied both the inclusion and exclusion criteria of the clinical trial based on medical history and physical examination, blood sample was collected followed by TST; and finally Chest x-ray was taken. The inclusion and exclusion criteria of the vaccine trial were inclusive of the inclusion and exclusion criteria of this study except that the inclusion criteria pertaining to which allowed only participation of males.

Blood was collected by using BD Heparin tubes for isolation of PBMC, QuantiFERON®-TB Gold test tubes (Cellistis) for QFT-G test and BD EDTA tubes for HIV test and other specific parameters of the above mentioned clinical trial. Based on the pre freezing count PBMCs for this study were reserved in two Cryo tubes (BioGreiner).

2.4 Laboratory Investigations

2.4.1 Isolation of PBMC

Peripheral blood mononuclear cells (PBMCs) were isolated within 6 hours of heparinized venous blood sample collection and the blood sample was maintained at room temperature (RT) (20^0C-25^0C) until processed. The blood sample was layered in Ficoll-paque (Amersham) and PBMCs were isolated by density gradient centrifugation using Leucosep® tubes (BioGreiner). The cells were then counted, re-suspended in freezing

medium [RPMI-1640 (Invitrogen), 10% FCS (Invitrogen) and 10% DMSO (Sigma)], aliquoted into cryotubes tubes, and stored in liquid nitrogen until assayed.

2.4.2 IFN-γ ELISPOT Assay

The *in vitro* ELISPOT assays for IFN-γ were performed using standard protocol as described elsewhere (Lalvani *et al.,* 1997). A plate lay out which indicates the position of individual antigens, mitogen and negative control in a 96 - well ELISPOT plate was used throughout the assay.

On day one, 96-well microtiter plate with a polyvinyl-difluoride (PVDF) membrane (Millipore, MAIP S 4510) was pre-wetted with 35% ethanol for one minute. After washing with sterile water (200ul/well) for 5X, the plate was coated with capture anti-IFN-γ monoclonal antibody (Mab1-D1K, MABTECH) 50 µl per well in carbonate buffer solution (Sigma-Aldrich) at the final concentration of 10µg/ml. Then the plate was incubated overnight at 4^0C.

On day two (after 19 hours of incubation), the coating solution was flicked off and the plate was washed 3X with sterile PBS to remove unbound antibodies and blocked with 100µl/well of complete medium [RPMI-1640 supplemented with 10% FCS, 1% L-glutamine (Sigma) and 1% penicillin/streptomycin (Sigma)] and incubated for 1 hr at 37^oC to avoid non specific binding. Meanwhile frozen PBMC samples were thawed at 37^0C in water bath and washed two times with complete medium. Then cells were counted and adjusted to the desired concentration with complete medium. After 1 hour incubation, the blocking solution was flicked off and 50µl/well of the antigens Ag85B, ESAT-6 and PPD at a final concentration of 5µg/ml and fused Ag85B-ESAT-6 at a final concentration of 10 µg/ml were added in duplicates. The antigens were donated by Statens Serum institute (Copenhagen, Denmark). PHA (Sigma) at a final concentration of 2µg/ml as a positive control and complete medium as a negative control were also added in duplicate wells. Each of the antigens and the mitogen were diluted in complete medium. Finally 2 x 10^5 PBMC suspended in 50 µl of complete medium were seeded into each well and the plate was incubated for 19 hours in humidified 5% CO_2 incubator at 37^oC.

On day three, the plate was washed 6X with PBST (PBS with 0.05%Tween 20) and biotinylated detector antibody (mAb-7-B6-1-Biotin, Mabtech) was added at the

concentration of 1:1000 diluted in PBS and incubated for 3 hours at RT. To remove unbound detector antibody the plate was washed 6X with PBST and 50µl of 1:1000 PBS diluted streptavidin-ALP (Mabtech) was added and incubated for 1h at RT in the dark. Then the plate was washed 6X with PBST and 50µl/well of chromogenic alkaline phosphatase conjugate substrate [prepared as per manufacturer's specifications, for one plate using 200µl of 25x development buffer (BIORAD), 4.8ml of 0.22µl filtered deionised water, 50µl of each AP Colour Reagent A and B (BIORAD)] added and incubated at RT in dark until the negative control wells start to give faint background. The colour development was stopped by washing the plate in running tap water and then soaking it in water for 10 minutes. Finally the plate was air dried and spots were counted using an ELIspot reader (AID, GmBH, Strasburg, Germany).

The mean spot count from the duplicate wells was calculated using Microsoft Excel spread sheet. Quantitative counts were reported as spot-forming cells per million of PBMC (SFCs/10^6 PBMC) above the negative control wells which was found by multiplying with 5 (5x SFC / 5x 200,000 PBMC = SFC/10^6 PBMC). The qualitative response (positive or negative results) was pre defined as positive if test wells contained a mean of at least 5 spot-forming cells more than the mean of the negative control wells and were at least twice the mean of the negative control wells in numbers (Lalvani *et al.*, 2001).

2.4.3 IFN–γ ELISA of the supernatant after antigen stimulation

2.4.3.1 Stimulation assay/Cell culture

A plate lay out which indicates the position of individual antigens, mitogen and negative control in a round (U)-bottomed, 96-well plate was used throughout the assay.

The U-bottomed 96-well plate was labeled with plate number; and 100µl/well of the antigens Ag85B, ESAT-6 and PPD at a final concentration of 5µg/ml and fused Ag85B-ESAT-6 at a final concentration of 10 µg/ml were added in duplicates. PHA at a final concentration of 2µg/ml as a positive control and complete medium as a negative control were also added in duplicate wells. Each of the antigens and the mitogen were diluted in complete medium.

PBMC samples were thawed in water bath at 37^0C and washed two times with complete medium. Then cells were counted and adjusted to the desired concentration with complete medium. Finally 2 x 10^5 PBMC suspended in100 µl of complete medium were

seeded in to each well and the plate was incubated in humidified 5% CO_2 incubator at 37^{o}C for 6 days.

After the 6 days incubation time, the supernatant was collected (150 µl) from each well; transferred to another labeled microplate; and stored at -20^{o}C until assayed by ELISA.

2.4.3.2 Sandwich ELISA

A plate lay out which indicates the position of individual supernatants after 6days stimulation with the different antigens, mitogen and negative control in flat-bottomed, 96-well plates was used throughout the assay.

On day one, the 96-well microtiter plate (ThermoLab 4 HBX) was coated with purified anti-human IFN-γ monoclonal antibody (Pharmingen 55122, lot 72093) 50 µl per well in carbonate buffer solution (Sigma-Aldrich) at the final concentration of 2µg/ml except the blank. Then the plate was covered with plate sealer and incubated overnight at 4^{0}C.

On day two (after 20 hour incubation), the coating solution was flicked off; the plate was washed 4X with 300 µl of PBST (PBS with 0.05%Tween 20); and then the plate was blocked with 200 µl/well of blocking solution (PBS with 10% heat-inactivated Fetal calf Serum) except the blank and incubated for 2 hours at room temperature. After washing the plate with PBST for 2X; 50 µl of sample, standard (Pharmingen 554616, lot 33306) and diluent (blank) was added into each well in duplicate as per the plate layout. The standard was serially diluted with lowest detection limit of 31pg/mL. Finally, plate was covered with plate sealer and incubated overnight at 4^{0}C.

On day three, the plate was washed 4X with PBST and 100µl per well of the detector antibody biotin mouse anti-human IFN-γ mAb (Pharmingen 554550, lot 48629) was added at the final concentration of 2µg/ml diluted in blocking solution except the blank and incubated for 45 minutes at room temperature. Then the plate was washed 4X with PBST and 100µl per well of Avidin peroxidase at the final concentration of 2.5µg/ml diluted in blocking solution was added and incubated for 30 min at RT followed by another 4X wash with PBST. Finally, 100 µl/well of the substrate o-Phenylenediamine Dihydrochloride (OPD) fast (Sigma) was added and incubated for 40 minutes.The reaction was stopped by adding 50 µl stop solution (2M H_2SO_4) to each well. The optical density (OD) was read at 492 nm wave length using an ELISA reader (Thermo Lab systems Multiskan Ex).

All samples, standard and blank were run in duplicate. The average optical density was then calculated and the concentration of IFN-γ for each sample was calculated from standard curve using a soft ware called Wlogit version Feb 25 1996 S53, FO, M53, C925. IFN- γ values (pg/mL) for the TB-specific antigen stimulated wells were corrected for background by subtracting the value of the subject's respective negative (non-stimulated) control from all results. A "positive" IFN-γ response was defined as >62 pg/ml (Black *et al*., 2002).

2.4.4 QuantiFERON®-TB Gold In-Tube (QFT-G)

The QuantiFERON®-TB Gold In-Tube (IT) test (Cellestis Ltd, Australia) was performed in two stages. First, whole blood was collected into each of the QuantiFERON®-TB Gold blood collection tubes, which include a Nil Control tube, TB Antigen tube, and a Mitogen Control tube. The Nil Control tube contained only heparin, as a negative control; the heparinised TB antigen tube contained a peptide cocktail containing ESAT-6, CFP-10 and TB7.7 (p4) proteins; and the tube that contains Mitogen was used as a control for correct blood handling and incubation.

Venous blood was collected from each subject before administration of Mantoux TST into each of the QuantiFERON®-TB Gold blood collection tubes calibrated to draw 1 ml of blood. The tubes were shaken and immediately incubated at 37°C for16–24 hours, after which they were centrifuged and plasma harvested. Plasma was kept refrigerated at 4–6°C until the ELISA was performed.

The IFN-γ ELISA was performed using the method recommended by the manufacturer. IFN-γ values (IU/ml) for the TB-specific antigen stimulated plasmas were corrected for background by subtracting the value of the subject's respective negative control. QuantiFERON®-TB Gold IT Analysis Software was used to analyze raw data and calculate results which were reported as Negative, Positive and Indeterminate.

2.5 HIV Test

Upon consent, HIV test was done for all of the study participants at ALERT Hospital and International Clinical laboratories, AA, Ethiopia. The test was done according to the national guideline for HIV testing (Rapid HIV test Algorithm) that includes a combination of three tests namely, Determine® HIV -1/2, CapillusTM HIV-1/HIV-2 and Uni-GoldTM.

2.6 Tuberculin Skin Test (TST)

Tuberculin (0.1 ml Tuberculin PPD RT 23; Statens Serum Institute, Copenhagen, Denmark) was injected intradermally on the ventral aspect of the left forearm. After 48-72 hours, the transverse diameter of the skin induration was measured. For accurate measurement of induration, the margin of induration was marked by moving a ball point pen on the skin from the outer edge of the erythema or swelling inwards until resistance was felt where this point was marked as the margin on one side. The same procedure was applied on the opposite side then the transverse diameter of the indurations was measured using plastic ruler graduated in millimeters.

A positive TST result was defined as an induration of ≥10mm (ATS/CDC, 2000).

2.7 Statistical analysis

The collected data were double entered by two different data entry clerks into Microsoft access spread sheet, cleaned, verified and prepared for analysis.

Categorical data were reported as absolute or relative frequencies and compared between groups using Fisher's exact test. Continuous data were described using means, medians and percentiles where appropriate and compared within groups using either the student-t test or Mann-Whitney test. Comparisons between groups formed based on the antigen-specific T cell response to each of the antigens were assessed by the Kruskal-Wallis and Dunnett multiple-comparison tests.

Logistic regression was used to estimate crude and adjusted odds ratios (OR) that measures the effect of selected factors on the responses to each of TST and QFT-G tests. Variables included as potential influential factors were age, department (Laboratory Technology, Nursing and Others), year of study (First, Second and Third year), place of birth (Addis ababa, Amhara, Oromia, Southern Nations and Others), religion (orthodox, Muslim and Others), Khat consumption, BCG scar and BMI. Marital status,Cigarette smoking and history of contact with TB patient were excluded from the logistic regression because of their low frequency.

Concordance between the results of each of the TST and QFT-G tests was assessed by using Kappa, for the overall and both for study participants with and without BCG scar. Following previous recommenations regarding the interpration of Kappa values (Byrt, 1996) values of 0.93-1.00 indicate excellent agreement, 0.81-0.92 indicate very good agreement, 0.61-0.80 indicate good agreement, 0.41-0.60 indicate fair agreement, 0.21-

0.40 indicate slight agreement, 0.01-0.20 indicate poor agreement and 0.00 or less indicate no agreement.

STATA statistical software version 8.0 (STATA Corporation, Texas, USA.) and GraphPad Prism 4 statistical software were used for analyses. All p values reported are based on two-tailed comparisons, with statistical significance set at $p < 0.05$.

2.8 Ethical considerations

Before the conduct of this research project, the proposal was approved by Department of Microbiology, Parasitology and Immunology, and ethically cleared by Faculty Research Publications Committee-II (FRPC-II), Faculty of Medicine, Addis Ababa University. Ethical clearance was also obtained from AHRI/ALERT Ethics Review Committee, and the National Ethics Review Committee (NERC)..

All of the subjects were provided with all relevant information about the study before they gave consent (See Annex). Pre-test and post-test counseling for HIV was given and all information including their HIV status is kept confidential. None of the investigators were instructors of the participating students.

CHAPTER III: RESULTS

3.1 Study Participants' Characteristics

The characteristics of the study participants are presented in Table 1. A total of 107 students from Addis Ababa University Medical Faculty, who fulfilled the inclusion and exclusion criteria were included in the final analysis. All were male and the mean age was 20.9 (±2.0) years.

Table 1: **Characteristics of study participants (n =107)**

Characteristics	***No (%)***
Age in years (Mean ± *SD)*	*20.9 (±2.0)*
Department	
Laboratory Technology (LT)	*50 (46.7)*
Nursing	*34 (31.8)*
Midwifery	*9 (8.4)*
Radiography	*3 (2.8)*
Medicine	*3 (2.8)*
Dentistry	*3 (2.8)*
Pharmacy	*5 (4.7)*
Year of study	
First year	*43 (40.2)*
Second year	*47 (43.9)*
Third year	*17 (15.9)*
Place of birth (Region)	
Addis Ababa (AA)	*17 (15.9)*
Amhara	*30 (28.0)*
Oromia	*29 (27.1)*
Southern Nations	*22(20.6)*
Tigray	*8 (7.5)*
Somali	*1 (0.9)*
Religion	
Orthodox	*73 (68.2)*
Muslim	*22 (20.6)*
Protestant	*11 (10.3)*
Hawariat	*1 (0.9)*
Married	*2 (1.9)*
Khat Consumption	*9 (8.4)*
Cigarette Smoking	*2 (1.9)*
History of contact with TB patient	*4 (3.7)*
BCG scar	*48 (44.9)*
BMI (Kg/m^2) (Mean ± SD)	*18.9 (±1.8)*

3.2. Tuberculin Skin Test (TST) Result

Out of 110 participants who fulfilled the inclusion and exclusion criteria, one participant did not return for TST reading, 2 did not undergo the TST because of withdrawal of their consent after physical examination was done for one and after blood for QFT-G was drawn for the other. A total of 107 participants were ready for TST result analysis. Fifty of the 107 participants (46.7 %) had positive test result (TST≥10 mm).

The mean age (± SD) for study participants with positive and negative TST results was 21.4 (±2.6) and 20.4 (± 1.3) years respectively (P<0.05).

Forty eight of 107 study participants (44.9%) had BCG scar and of these 26 (54.1%) gave a positive TST result whereas of the 59 study participants who had no BCG scar, 24 (40.7%) gave a positive TST result (difference not significant). Employing another TST cut off, 54/107 (50.5%) had an induration of more than 5 mm, and at this cut off too, the impact of BCG scar was not significant..

The median size of induration for TST in participants with BCG scar was10 mm ranging from 0 to 35 mm and for participants with no BCG scar was 0 mm ranging from 0 to 25 mm (difference not significant).

There were no significant difference in TST positivity (TST≥10 mm) by school department, year of study, place of birth, religion, marital status, Khat consumption, cigarette smoking, history of contact with TB patient, BMI or BCG scar. However, in the logistic regression analysis (Table 2), the estimated odds of having a positive TST result (at a cut off of ≥10 mm) were nearly 2 times higher (OR 1.6, 95% CI 1.11-2.20; p=0.01) as age increased, while khat consumption led to 9 fold increase in risk (OR 9.2, 95% CI 1.41-59.69, p =0.02) and a unit increment in body mass index (BMI) led to 1.4 fold decrease in risk (OR 0.7, 95% CI 0.54-0.98, p =0.036).

Table 2: **Logistic regression for a positive TST (TST≥10 mm) result**

	Univariate analysis		Multivariate analysis	
	Crude OR	[95% CI]	Adjusted OR	[95% CI]
Age	1.3	1.03-1.68	1.6	1.11-2.20
Nursing/LT	1.5	0.62-3.61	1.1	0.33-3.43
Others[1]/LT	2.0	0.72-5.30	1.0	0.22-4.81
Second year/ First year	0.6	0.28-1.49	0.3	0.08-1.30
Third year/ First year	1.1	0.35-3.31	0.5	0.07-3.18
Amhara/AA	0.9	0.26-2.84	1.2	0.24-5.65
Oromia/AA	1.4	0.42-4.60	1.4	0.31-5.92
Southern Nations /AA	0.1	0.01-1.38	0.1	0.01-1.63
others[2]/AA	1.4	0.38-4.80	1.0	0.22-4.82
Muslim/Christian[3]	1.2	0.46-3.01	0.7	0.19-2.83
Khat consumption(Yes/No)	2.5	0.58-10.38	9.2	1.41-59.69
BCG Scar (Yes/No)	1.7	0.80-3.72	1.5	0.57-3.82
BMI	1.0	0.79-1.20	0.7	0.54-0 .98

[1] Midwifery, Radiography, Medicine, Dentistry and Pharmacy

[2] Tigray and Somali

[3] Orthodox, Protestant and Hawariat

3.3. QuantiFERON®-TB Gold In-Tube (QFT-G) assay Result

Forty seven of the 107 participants (43.9%) had positive result using QFT-G assay.

The mean age (± SD) for study participants with positive and negative results was 21.4 (±2.7) and 20.4 (± 1.2) years respectively (P< 0.05).

Forty eight of 107 study participants (44.9%) had BCG scar and of these 23 (47.9%) gave a positive QFT-G assay result whereas of the 59 study participants who had not BCG scar, 24 (40.7%) gave a positive result (difference not significant).

There were no significant difference in QFT-G assay positivity by school department, year of study, place of birth, religion, marital status, Khat consumption, cigarette smoking, history of contact with TB patient, BMI or BCG scar. However, in the logistic regression analysis, the estimated odds of having a positive QFT-G assay results were nearly 2 times higher (OR 1.7, 95% CI 1.18-2.35; p =0.004) as age increased, while khat consumption led to 10 fold increase in risk (OR 9.6, 95% CI 1.36-68.13, p = 0.02) and a unit increment in BMI led to 1.4 fold decrease in risk (OR 0.7, 95% CI 0.49-0.92, p=0.01).

Table 3: **Logistic regression for a positive QFT-G assay result**

	Univariate analysis		Multivariate analysis	
	Crude OR	[95% CI]	Adjusted OR	[95% CI]
Age	1.3	1.04-1.69	1.7	1.17-2.35
Nursing/LT	1.9	0.80-4.73	1.4	0.43-4.76
Others[1]/LT	2.5	0.92-6.94	2.1	0.43-10.10
Second year/ First year	0.7	0.28-1.50	0.5	0.11-1.95
Third year/ First year	0.9	0.30-2.87	0.8	0.11-5.43
Amhara/AA	1.1	0.33-3.65	1.5	0.30-7.62
Oromia/AA	1.3	0.40-4.47	1.2	0.28-5.60
Southern Nations /AA	0.2	0.02-1.77	0.2	0.02-2.34
others[2]/AA	1.7	0.48-6.16	1.1	0.22-5.42
Muslim/Christian[3]	1.7	0.67-4.41	1.4	0.36-5.57
Khat consumption(Yes/No)	2.8	0.66-11.77	9.6	1.36-68.13
BCG Scar (Yes/No)	1.3	0.62-2.89	1.0	0.39-2.80
BMI	0.9	0.75-1.16	0.7	0.49-0.92

[1] Midwifery, Radiography, Medicine, Dentistry and Pharmacy

[2] Tigray and Somali

[3] Orthodox, Protestant and Hawariat

3.4 Agreement between TST and QFT-G assay in the diagnosis of latent Tuberculosis Infection

The overall strength of agreement between TST (≥10mm) and QFT-G assay was very good (Kappa = 0.83, *p* value=0.000), with concordant results in 98/107 (91.6%). Concordance between the two tests was good in participants with BCG scar (85.4%, Kappa = 0.71, *p* value =0.000), but it was found to be excellent (96.6%, Kappa =0.93, *p* value =0.000) in those who had not BCG scar (Table 4).

By applying a 5mm induration TST cut off, the overall strength of agreement between TST and QFT-G was also very good (Kappa = 0.83, P value =0.000), with concordant results in 98/107 (91.6%%). Concordance between the two tests was also good in participants with BCG scar (83.3%, Kappa = 0.67, P value =0.000), and was excellent (98.3%, Kappa =0.97, P value =0.000) in those who had no BCG scar (Table 4).

Table 4: **The agreement between TST at two cut offs and QFT-G assay**

		QFT		Kappa	*p* value
BCG scar	TST	Positive	Negative		
Yes					
	≥10 mm	21	5	0.71	0.000
	<10	2	20		
No					
	≥10 mm	23	1	0.93	0.000
	<10	1	34		
Total					
	≥10 mm	44	6	0.83	0.000
	<10	3	54		
Yes					
	>5	22	7	0.67	0.000
	<5	1	18		
No					
	>5	24	1	0.97	0.000
	<5	0	34		
Total					
	>5	46	8	0.83	0.000
	<5	1	52		

By taking QFT-G assay as gold standard for the diagnosis of latent TB infection, TST positivity at > or =10 mm cutoff in participants with BCG scar and with no BCG scar among QFT negatives is not significant. However, 7 out of 25 (28.0%) participants with BCG scar were TST positive as compared to 1 out of 35 (2.9%) participants with no BCG scar at TST cutoff of 5mm or more among participants with QFT-G negative results (p = 0.007).

The correlation between TST (mm) and QFT-G assay (IU/ml) was assessed and found to be significant (spearman correlation coefficient=0.81, P<0.0001) as shown in Fig1.

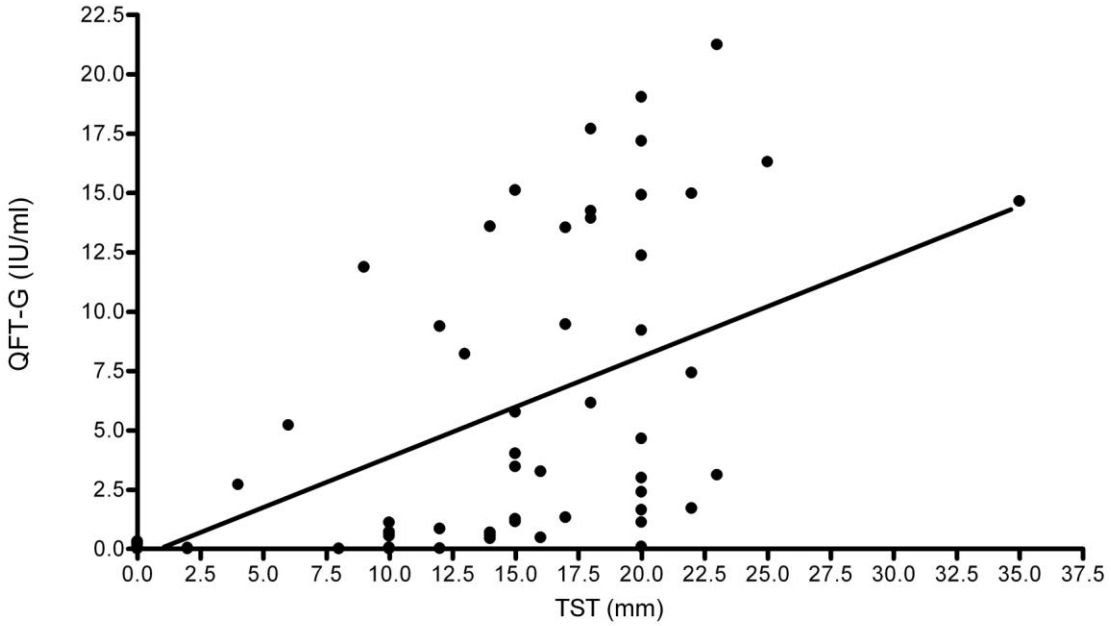

Figure 1: Correlation between TST (mm) and QFT-G (IU/ml)

3.5 Determination of antigen specific T-cell memory response by using ELISPOT assay and ELISA

A total of 57 PBMC samples were randomly selected out of the110 stored samples for *in vitro* stimulation with TB antigens Ag85B, ESAT-6, fused Ag85B-ESAT-6, PPD and PHA to measure IFN-γ response using ELISPOT assay and ELISA. Out of the 57, antigen stimulation for ELISA was not done for 10 of the thawed PBMC samples because of insufficient number of cells for both assays. One of the participants who did not return for TST reading is among the 57 participants. Hence, 56 results for ELISPOT assay and 46 for ELISA were ready for final statistical analysis.

3.5.1 ELISPOT assay Result

The antigen-specific T cell responses were assessed taking the mean number of spots in duplicate wells, each seeded with 200,000 cells and calculated as spot forming cells (SFC) per million of PBMC above the negative control wells which was found by multiplying with 5.

The overall median (IQR) responses to Ag85B, ESAT-6, fused Ag85B-ESAT-6 and PPD were found to be 256 (147-408), 305 (133-474), 429 (267-601) and 451 (305-706) SFC/Million of PBMC respectively. The response to PHA was beyond the upper limit of detection of the ELISPOT reader.

The median antigen-specific T cell response among the four antigens was compared (Fig 2). The median response to PPD was significantly higher as compared to either Ag85B or ESAT-6 response; and response to the fused Ag85B-ESAT-6 was also found significantly higher as compared to Ag85B response.

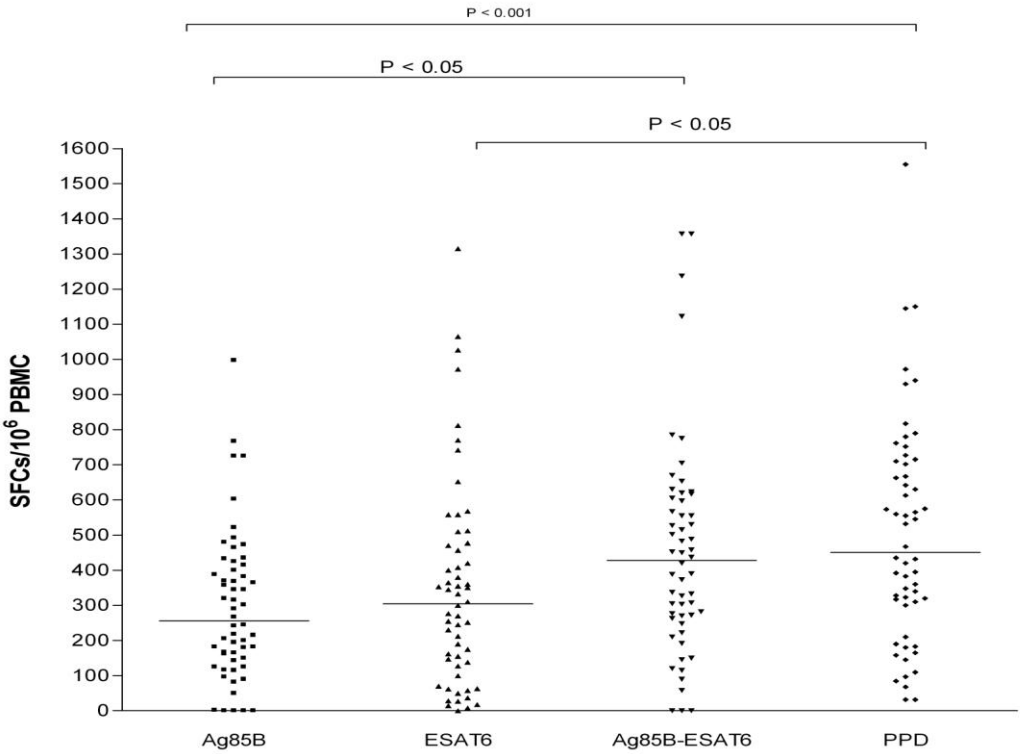

Figure 2: *In vitro* IFN-γ responses to stimulation with Ag85B, ESAT-6, fused Ag85B-ESAT-6 or PPD of PBMC from healthy adult Ethiopians as assed by ELISPOT. The medians of the groups are shown (bars), and levels of cytokine which were significantly different between groups are indicated.

The median antigen-specific T cell response to Ag85B was significantly higher in participants with BCG scar as compared to participants with no BCG scar whereas the response to the rest of the antigens was not significantly different between the two groups as shown in fig 3.

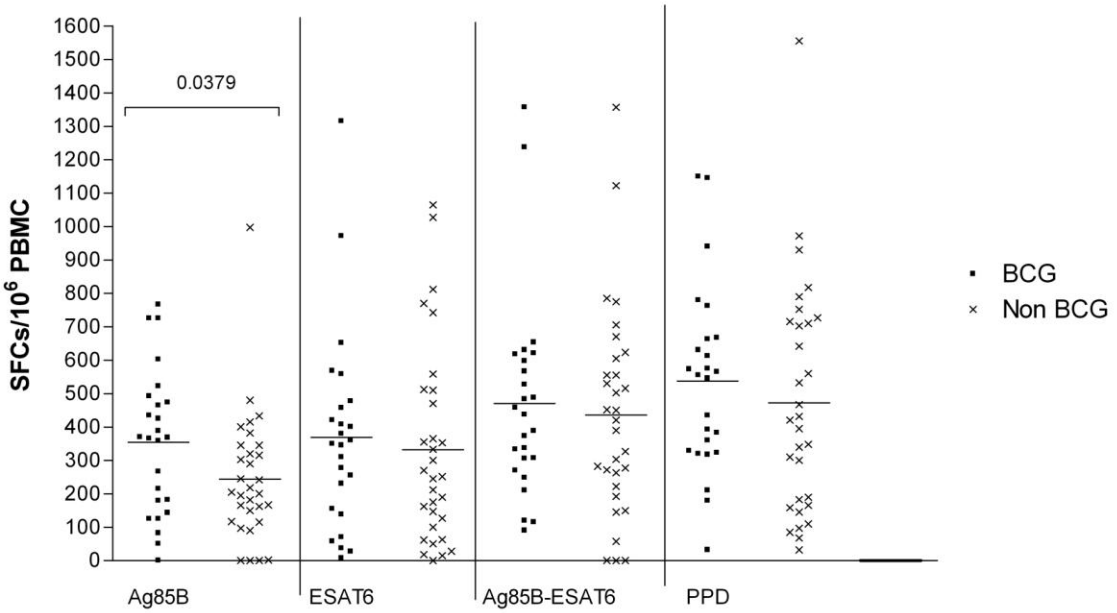

Figure 3: Comparison of *in vitro* IFN-γ responses to stimulation with Ag85B, ESAT-6, fused Ag85B-ESAT-6 or PPD of PBMC from healthy adult Ethiopians with BCG scar and with no BCG scar as assed by ELISPOT. The medians of the sub-groups are shown (bars), and levels of cytokine which were significantly different within groups are indicated.

The median antigen-specific T cell response to ESAT-6, Ag85B, fused Ag85B-ESAT-6 and PPD was significantly higher in TST positive participants as compared to the TST negative counterparts (Fig 4).

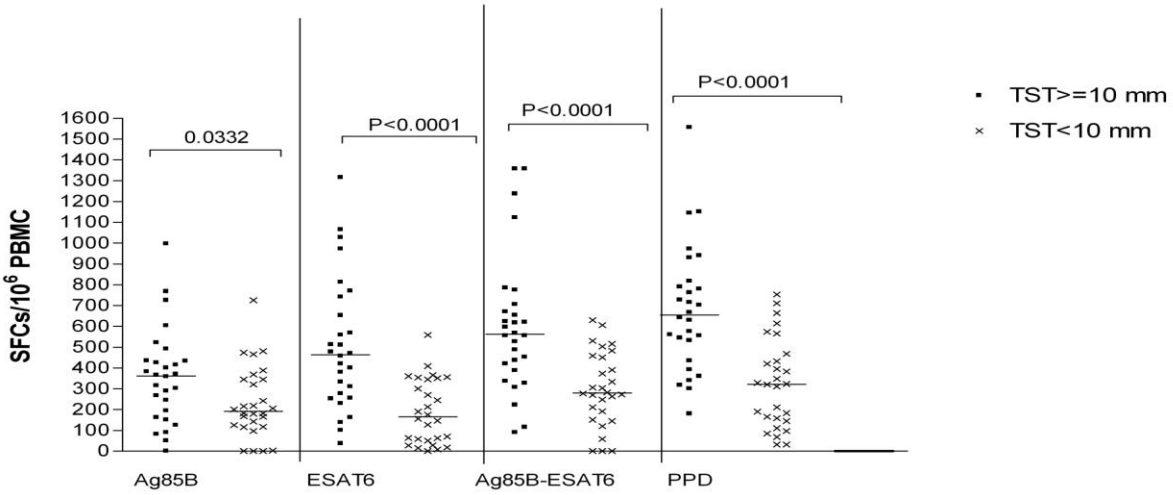

Figure 4: Comparison of *in vitro* IFN-γ responses to stimulation with Ag85B, ESAT-6, fused Ag85B-ESAT-6 or PPD of PBMC from TST positive and TST negative healthy adult Ethiopians as assed by ELISPOT. The medians of the sub-groups are shown (bars), and levels of cytokine which were significantly different within groups are indicated.

Finally, comparison in the median antigen-specific T cell response to Ag85B, ESAT-6, fused Ag85B-ESAT-6 and PPD was also found to be significantly higher in QFT-G positive participants as compared to the QFT-G negative counterparts to all of the antigens except Ag85B as shown in Fig 5.

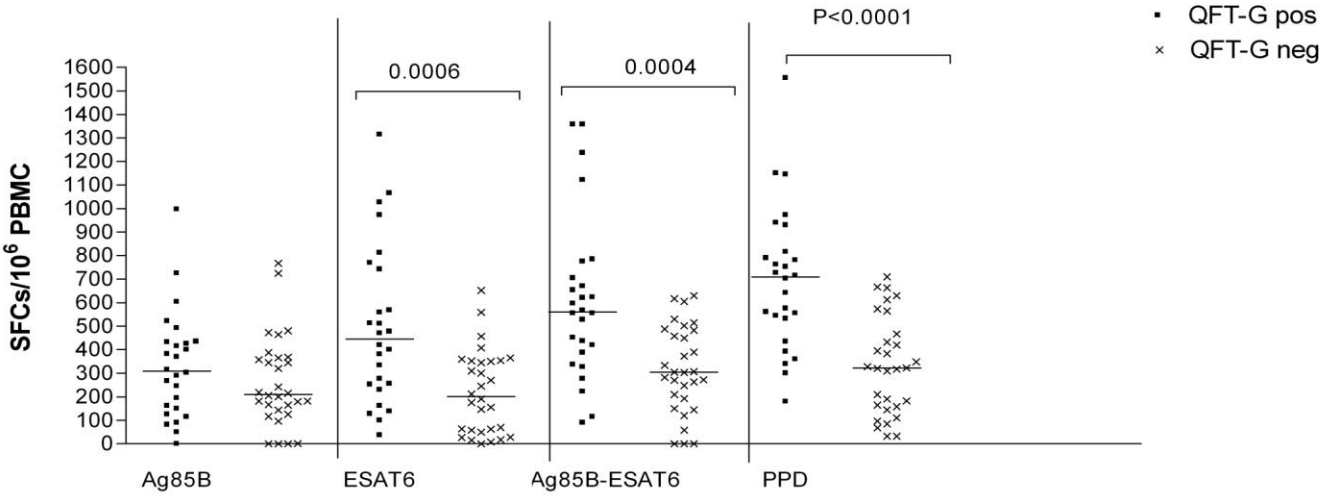

Figure 5: Comparison of *in vitro* IFN-γ responses to stimulation with Ag85B, ESAT-6, fused Ag85B-ESAT-6 or PPD of PBMC from QFT-G positive and QFT-G negative healthy adult Ethiopians as assed by ELISPOT. The medians of the sub-groups are shown (bars), and levels of cytokine which were significantly different within groups are indicated.

Qualitative antigen-specific T cell response based on ELISPOT was assessed. Out of the 56 participants, positive response to Ag85B, ESAT-6, fused Ag85B-ESAT-6 and PPD was found in 35(62.5%), 39 (69.6%), 47(83.9%) and 49(87.5%) respectively based on the pre defined criteria. This showed that response to these antigens was in the order of PPD>Ag85B-ESAT-6>ESAT-6>Ag85B.

3.5.2 ELISA Result

The antigen-specific T cell responses were assessed by taking the average Optical density from duplicate wells of each sample and calculating the concentration of IFN-γ(pg/ml) from the standard curve. IFN- γ values (pg/ml) for the TB-specific antigen stimulated wells were corrected for background by subtracting the value of the subject's respective negative (non-stimulated) control from all results.

The overall median (IQR) responses to Ag85B, ESAT-6, fused Ag85B-ESAT-6, PPD and PHA were found to be 0(0.0-189.0), 328.0 (0.0- 2351.0), 96.0(0.0- 1220.0), 3231.0 (157.5- 5266.0) and 5599.0 (4527.0- 7265.0) pg/ml of IFN-γ respectively.

The median response to PPD was significantly superior to either Ag85B or fused Ag85B-ESAT-6 response; and the response to ESAT-6 was also found to be significantly higher than Ag85B (Fig 6).

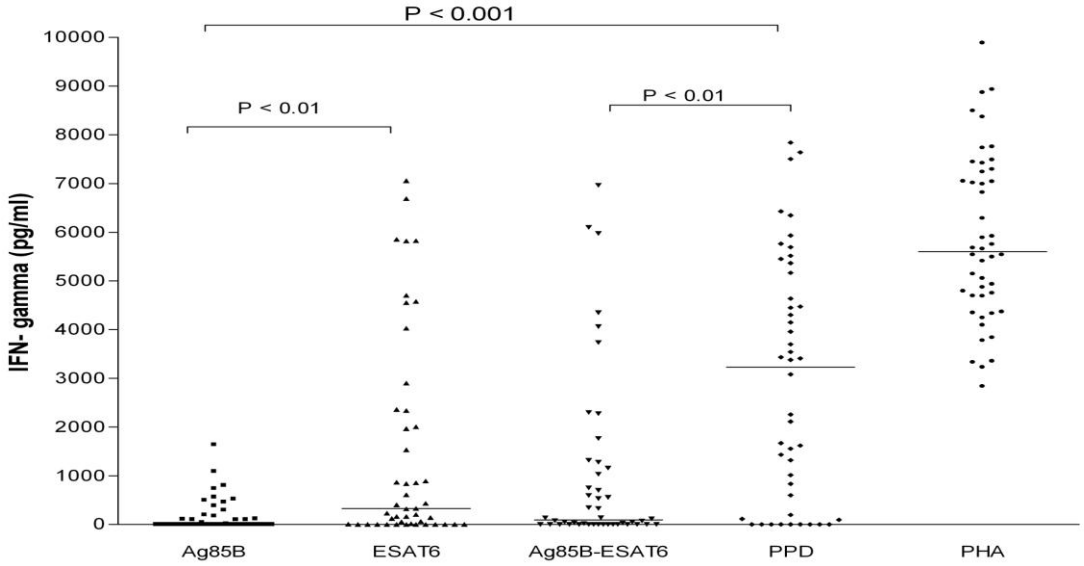

Figure 6: *In vitro* IFN-γ responses to stimulation with Ag85B, ESAT-6, fused Ag85B-ESAT-6, PPD or PHA of PBMC from healthy adult Ethiopians as assed by ELISA. The medians of the groups are shown (bars), and levels of cytokine which were significantly different between groups are indicated.

The median T cell response to the four antigens and PHA in participants with BCG scar as compared to participants with no BCG scar was not significantly different (Fig 7).

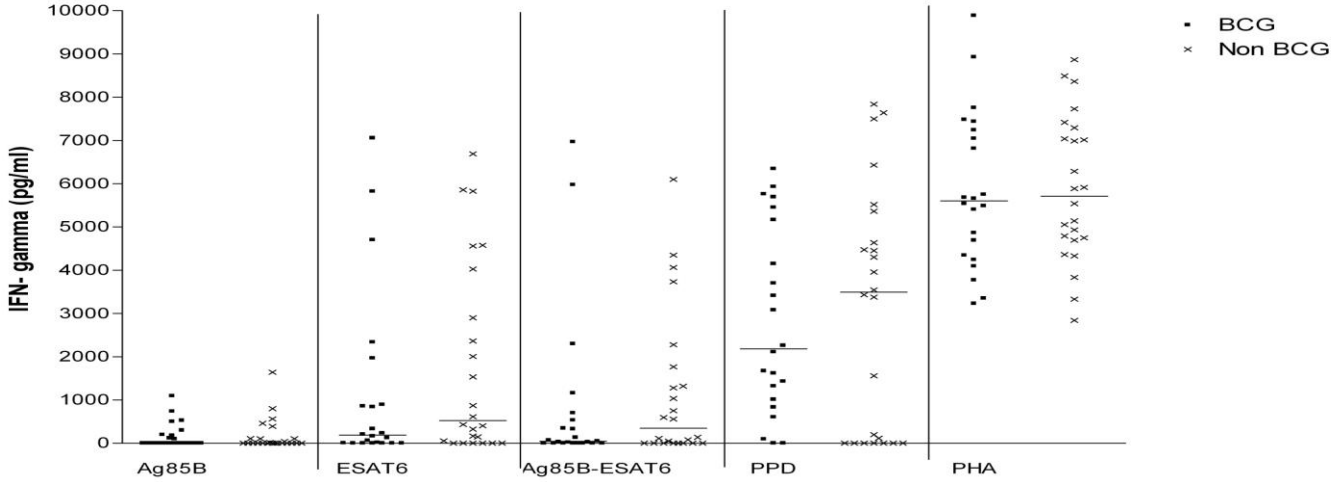

Figure 7: Comparison of *in vitro* IFN-γ responses to stimulation with Ag85B, ESAT-6, fused Ag85B-ESAT-6, PPD or PHA of PBMC from healthy adult Ethiopians with BCG scar and with no BCG scar as assed by ELISA. The medians of the sub-groups are shown (bars), and levels of cytokine which were significantly different within groups are indicated.

The median T cell response to ESAT-6, fused Ag85B-ESAT-6 and PPD was significantly higher in TST positive participants as compared to the TST negative counterparts as shown in Fig 8.

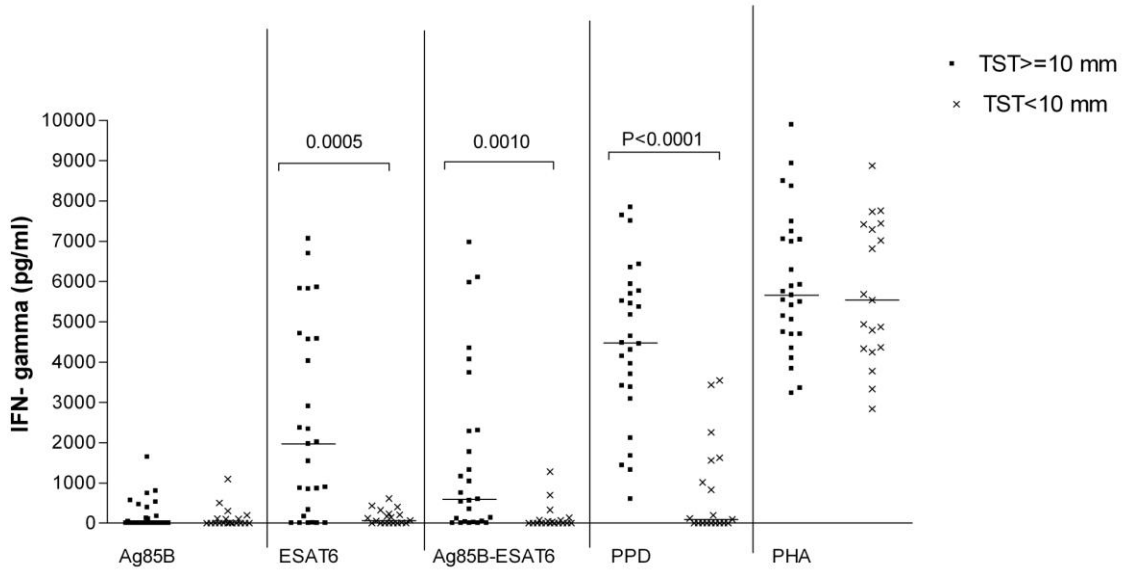

Figure 8: Comparison of *in vitro* IFN-γ responses to stimulation with Ag85B, ESAT-6, fused Ag85B-ESAT-6, PPD or PHA of PBMC from TST positive and TST negative healthy adult Ethiopians as assed by ELISA. The medians of the sub-groups are shown (bars), and levels of cytokine which were significantly different within groups are indicated.

The median T cell response to ESAT-6, fused Ag85B-ESAT-6 and PPD was also found to be significantly higher in QFT-G positive participants as compared to the QFT-G negative counterparts (Fig 9).

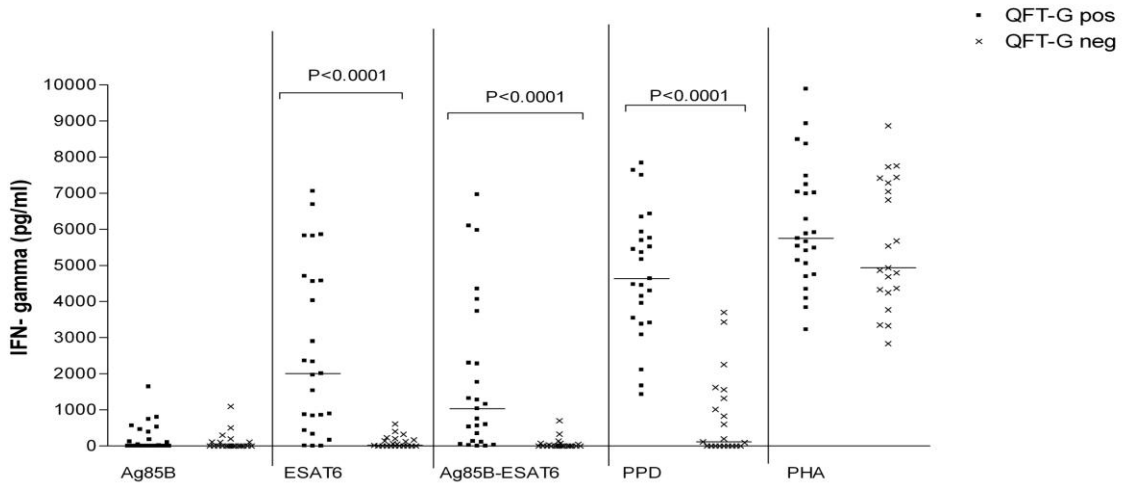

Figure 9: Comparison of *in vitro* IFN-γ responses to stimulation with Ag85B, ESAT-6, fused Ag85B-ESAT-6, PPD or PHA of PBMC from QFT-G positive and QFT-G negative healthy adult Ethiopians as assed by ELISA. The medians of the sub-groups are shown (bars), and levels of cytokine which were significantly different within groups are indicated.

Qualitative antigen-specific T cell response based on ELISA also was assessed. Out of the 46 participants, positive response to Ag85B, ESAT-6, fused Ag85B-ESAT-6 and PPD was found in 17(37.0%), 31 (67.4%), 25(54.4%) and 37(80.4%) respectively based on the pre defined criteria.

CHAPTER IV: DISCUSSION

4.1 Latent Tuberculosis Infection (LTBI) Diagnosis: TST and QFT-G

In this study, we tried to look at if there is a difference in prevalence of LTBI based on *in vivo* Tuberculin Skin Test (TST) and *in vitro* QuantiFERON®-TB-Gold (QFT-G) assay. Our findings showed that 46.7 % and 43.9% of the study participants had positive TST (TST≥10 mm) and QFT-G assay results respectively. The overall strength of agreement between TST (≥10mm) and QFT-G assay was very good (Kappa = 0.83), with concordant results in 98/107 (91.6%). Concordance between the two tests was good in participants with BCG scar (85.4%, Kappa = 0.71), but it was found to be excellent (96.6%, Kappa =0.93) in those who had not BCG scar.

The prevalence of LTBI is usually similar in males and females until adolescence, after which prevalence is higher among males (Roelsgaard *et al*., 1964; NTI, 1974; Lienhardt *et al*., 2003). Our prevalence estimation based on both TST and QFT-G assay might therefore be inflated since all of the study participants in this study are adult males, but still this will not be a problem on our aim of evaluating the two tests for use in a country with routine BCG vaccine use at birth and high burden of TB.

Despite the difference in the immunologic mechanism of the two tests: TST and QFT-G, we have found strong agreement between them. The TST is a classic example of delayed hypersensitivity reaction (Anderson *et al*., 2007), which cannot distinguish recent from remote infections. On the other hand, the QFT-G assay is suggested to detect more recent or active, rather than remote TB infections because it relies on the presence of antigen dependent immediate effector T-cells that produce IFN-γ up on stimulation (Pathan *et al*., 2001; Dheda *et al*., 2007; Kaech *et al*., 2002), yet only 4 of our study participants out of 107 (3.7 %) claimed to have history of contact with TB patient in their life time. However, a Meta analysis of many studies has shown that none of the currently available diagnostic tests for LTBI are able to differentiate adequately between recent and remote infections (Menzies *et al*., 2007). In addition, a recent study done among immigrants from high TB incidence country showed QFT-G assay not to be able to differentiate recent infection from remote infection (Kiki *et al*., 2009) which looks against the immunologic mechanism.

Although the study participants in this study were students in different health departments (See Table 1), we did not see significant difference in LTBI based on their department

and year of study, using both TST and QFT-G. In the multivariate analysis, department and year of study were not identified as risk factors for LTBI either. Therefore, we do not expect that TB patients in the hospital are the primary source of LTBI for our case, but could be source of continuous challenge to the immune system in previously infected ones resulting in booster effect. In view of the immunologic mechanism, the best explanation for the high prevalence of LTBI using QFT-G assay in high TB incidence countries in the absence of known recent exposure to active pulmonary TB patients could be the highly likelihood of having repeated casual contacts with active pulmonary TB patients who are mostly diagnosed late. The repeated exposure can then result in boosting of the immune response to MTB antigens resulting in a persistent pool of circulating effector T-cells, which can be activated within the short incubation period of the QFT-G assay.

In our study, the strength of agreement between TST (TST≥10 mm) and QFT-G in BCG vaccinated group is weaker as compared to the group without BCG vaccination. We also have looked the strength of agreement between TST and QFT-G by setting the cutoff for TST at >5mm. Like we have found at TST≥10 mm, the strength of agreement between the two tests in BCG vaccinated group is weaker than the group without BCG vaccination (See Table 3.4). The weak strength of agreement could have resulted because of the false positive TST happening as the result of the nature of the antigen used in the test, the Purified Protein Derivative (PPD), which contains mixture of antigens that are common to the MTB *complex,* environmental non-tuberculous mycobacterium strain and BCG (Harboe, 1981). Some studies have also shown that BCG-vaccinated individuals are more likely to have positive TST (Menzies *et al*., 1992; Tissot *et al*., 2005). However, we did not find significance difference in TST positivity rate, at TST≥10 mm and TST>5 mm, in BCG vaccinated and BCG unvaccinated participants. This finding could suggest either BCG vaccine administered in infancy has had minimal effect on false TST positivity in young adults ,or the high rate of TST positivity due to true LTBI resulting from the high incidence and late treatment of active pulmonary TB in our setting have confounded the effect of BCG.

Obviously, BCG vaccine is not expected to have an effect on QFT-G assay since the antigens that are contained in it are not present in BCG strains (Mahairas *et al*., 1996).

We have also noticed insignificant difference in QFT-G assay positivity rate between BCG vaccinated group (47.9%) and non-vaccinated group (40.7%). This is consistent with studies elsewhere (Mori *et al*., 2004; Brock *et al*., 2004). In addition to its specificity, studies have shown that QFT-G assay is as sensitive as TST by correlating its results with the degree of exposure (duration and proximity) to a source patient and the likelihood of acquiring infection from that source (Diel *et al*., 2008; Mori *et al*., 2004). By taking these in to account, we considered QFT-G assay as gold standard in order to evaluate whether the TST positivity that would have occurred because of BCG is possibly confounded by the high rate of TST positivity due to true LTBI.

Considering QFT-G assay as gold standard for the diagnosis of LTBI, we found that among QFT-G negatives, the rate of TST positivity in participants with BCG vaccination, 28.0% (7/25), was significantly higher than those without, 2.9% (1/35), at cutoff of >5mm, but at ≥10mm there was no significant difference. These observations suggest the effect of BCG vaccine administered in infancy on TST in adults at cutoff of >5mm that would be confounded in high incidence countries by high rate of LTBI, but the possibility of true LTBI is highly likely at larger indurations (≥10mm). Our observation is in agreement with what was indicated by a comprehensive meta-analysis of several studies demonstrating the effect of BCG vaccination to be less likely after 15 years especially on positive results with larger indurations (Wang *et al*., 2002). A more recent Meta analysis has also shown that the effect of BCG received in infancy is minimal 10 years after vaccination (Farhat *et al*., 2006).

Moreover, we have found high correlation between the amount of IFN-γ (IU/ml) in the QFT-G assay and TST induration (mm), spearman correlation coefficient=0.81 and $P<0.0001$, which also implies true LTBI at larger TST induration. This has been indicated by others as well (Pottumarthy *et al*., 1999; Ozdemir *et al*., 2007).

In the multivariate analysis, age increment and Khat chewing are found to be risk factors for LTBI whereas high BMI showed protective effect, based on both TST (TST≥10 mm) and QFT-G assay. In highly endemic areas, age increment is usually associated with an increment in LTBI rate (Roelsgaard *et al*., 1964; NTI, 1974; Lienhardt *et al*., 2003) that could result from cumulative exposure to MTB with time.

Khat (*Catha edulis*) is a plant which grows at high altitudes extending from East to Southern Africa, as well as Afghanistan, Yemen, and Madagascar (Pantelis *et al*., 1989). It is chewed for its stimulant effect. Historically, khat chewing was common in certain countries of East Africa and the Arabian Peninsula, but these days its use is becoming very common in different parts of the world including Western countries (Gebissa, 2010). The prevalence of khat use in Ethiopia is found to be 32% resulting in physical and mental ill-health (Belew et al., 2000). It is usually chewed in group sitting together in small crowded rooms for long hours especially in the cities and if there is one TB patient within the group the chance of infecting others is highly likely. One recent study conducted in Afar region, Ethiopia where Khat use is a common practice, has described it as risk factor for the development of pulmonary TB and its expansion (Legesse *et al*., 2010).

Although smoking has been associated with LTBI (Anderson et al., 1997; Leung *et al*., 2007; Leung *et al*., 2008), the number of smokers in our study were very low, 2 out of 107 study participants, and hence we do not expect it to confound the effect of khat chewing even if both habits are usually found in the same individuals. Thus, Khat chewing in our study can be considered as an independent risk factor for LTBI.

BMI is the most established anthropometric indicator used for the assessment of adult nutrition status (Lee *et al*., 2003). One of the best explanations for our finding on the association between high BMI and low rate of LTBI, based on both TST and QFT assay, could be the fact that poor nutritional status is associated with higher risk of TB infection and development of disease after infection (Lienhardt *et al*., 2001). The other explanation could be its relatedness with socioeconomic status especially in developing countries where low BMI is associated with low socioeconomic status (Nube *et al*., 1998, Bose *et al*., 2007; Kimhi, 2003), which in turn is an established risk factor for TB infection (Lienhardt *et al*., 2001).

4.2 Determination of antigen specific T-cell memory response by using ELISPOT assay and ELISA

Many new TB vaccines are under development including post exposure vaccine to latently infected individuals and BCG booster vaccine (Doherty *et al*., 2005). In both cases the vaccine will be given at later age and there has to be existing memory T-cells for the vaccine to work. In a population of healthy adults we evaluated the existence of memory T-cells for a fused subunit vaccine, Ag85B-ESAT-6, and its components, Ag85B and ESAT-6, by measuring IFN-γ production using ELISPOT assay and ELISA. At the development phase and for use after licensure of a post exposure vaccine, the exposure status has to be determined by using the available diagnostic methods for LTBI. TST and QFT-G are used here in this study to define LTBI, and both are not able to differentiate recent from remote infection (discussed in the first section of this thesis).

Lower overall median immune response to ESAT-6, subunit antigen, by ELISPOT assay was observed as compared to the *in vitro* immune response to PPD, crude antigen that can be considered as the total mycobacteria-specific immune response which includes the effects of *M. tuberculosis* infection, BCG vaccination and non-tuberculous mycobacterial infection (Harboe, 1981). By ELISA its response was comparable to PPD response. However, based on predefined criteria the proportion of participants with positive response to ESAT-6 by ELISA, 67.4%, was not higher than the proportion by ELISPOT, 69.6%. Since the correlate of protection for TB is unknown, depending on the definition of the immune response used in a study the interpretation will change as it is witnessed in our study.

By using both ELISPOT assay and ELISA, difference in immune response to ESAT-6 was not noticed based on BCG vaccination status, but its response was superior in QFT-G or TST positive, LTBI group, as compared to the negative counterparts. Our observation in relation to BCG vaccination status happened as the result of the differential presence of ESAT-6 among the different mycobacterial species as its presence was indicated in MTB, not in the BCG vaccine or most environmental mycobacteria (Harboe et al., 1996). Furthermore, the effect of BCG on TST in adults is minimal (discussed in the previous section of this thesis), therefore positive TST almost always indicates LTBI and there will be higher ESAT-6 response in TST positives than TST negatives.

Two types of memory T-cells, central and effector, with different functional and surface marker characteristics are already indentified, but their exact relationship remains to be elucidated. According to one of the different models proposed (Lanzavecchia *et al*., 2000), effector memory T cells migrate to inflamed peripheral tissues and induce immediate effector function, whereas central memory T cells that home in the secondary lymphoid organs, have little or no effector function, but readily proliferate and differentiate to effector cells in response to antigenic stimulation. Overnight ELISPOT assay is used to assess effector memory T cell responses (Goletti *et al*., 2006; Kaech *et al*., 2002) where as longer term antigen stimulation like our six days stimulation and quantification of the IFN-γ by ELISA measures central memory responses which is thought to be critical for long-term protection induced by vaccines (Lauvau *et al*., 2001; Migueles *et al*., 2002; Sallusto *et al*., 1999). Hence, by using ELISPOT assay and ELISA we were able to demonstrate the existence of ESAT-6 specific effector and central memory T-cells in healthy adult populations respectively. The reason why we observed high rate of ELISPOT response in the absence of recent exposure to TB patient in our study participants could have resulted because of possible repeated casual contact with TB patients resulting in immunity boost and increment of effector memory T-cells as in the case of our QFT-G assay finding (discussed in the first section).

The overall median immune responses to Ag85B by ELISPOT assay and ELISA were lower compared to the responses to PPD. The difference in response to Ag85B using both ELISPOT assay and ELISA was insignificant based on QFT-G assay which is specific to MTB (Liu *et al*., 2004), but was significantly higher for PPD in QFT-G assay positives than negatives. Moreover the BCG vaccinated group showed significantly higher AG85B-specific T-cell response by ELISPOT assay, as compared to non vaccinated group, but no difference for ESAT-6 and PPD was observed based on BCG vaccination status. Basically, it is expected to see higher response to Ag85B among QFT-G positives than negatives, and BCG vaccinated than non-BCG vaccinated since it is secreted by all *Mycobacterium* species including MTB and BCG (Wiker *et al*., 1990). However, many studies have shown that BCG-vaccinated individuals responded frequently to the N-terminal peptides of Ag85B (Roche *et al*., 1994, Mustefa *et al*., 2000) unlike T-cell lines from TB patients which recognizes epitopes scattered throughout the Ag85B sequence

(Silver *et al*., 1995, Mustefa *et al*., 2000). Based on all these we are tempted to conclude that the antigen we used in this study is partial sequence of the mature antigenAg85B containing only the immunodominant region of *M. bovis* BCG. Therefore BCG vaccinated but not latently infected groups showed significantly higher number of effector memory T cells specific to our Ag85B. The specificity of our Ag85B solely to BCG might be the reason why we have got lower overall ELISPOT assay and ELISA responses to Ag85B compared to PPD. In addition, ELISPOT response to Ag85B in TST positives was found to be higher than negatives which could be because of the effect of BCG vaccination even though its effect on TST at adulthood is minimal as previously stated in the first section of this thesis.

Positive response to Ag85B based on predefined criteria was seen in 62.5% and 37.0% of the participants using ELISPOT assay and ELISA respectively. The positive response using the two assays tells us the existence of Ag85B specific effector and central memory T-cells. However, significant difference for Ag85B-specific response by ELISA was not observed based on BCG vaccination status unlike the ELISPOT assay response. The discrepancy could be because of the functional difference between the two memory subtypes. Central memory T cells readily produce large amounts of IL-2, but very little IFN-γ, while effector memory T-cells often produce large amounts of IFN-γ and perforin, but very little IL-2 (Seder *et al*., 2003). Therefore we observed lower response in the case of ELISA since we were measuring central memory response using IFN-γ ELISA which is produced in a very little amount by these cells. The other important point worth discussing is the high ELISPOT response observed following BCG vaccination at birth in the absence of subsequent booster. We do not have clear answer to this. A recent study done in subjects treated for TB decades ago with modern anti-tuberculosis chemotherapy have shown the persistence of effector memory T-cells for longer duration (Tapaninen *et al*., 2010), but the driving factor is not clear whether it is persistence of antigens or bacteria.

Even though the efficacy of BCG wanes over a period of 10 to 15 years, and therefore it does not prevent against the later breakdown with pulmonary TB in the adult population in the third world (Sterne et al., 1998), the presence of Ag85B specific memory T-cells as

the result of childhood BCG vaccination is evident from this study that can possibly be boosted with a booster vaccine.

The responses to the fused Ag85B-ESAT-6 by ELISPOT were comparable to the *in vitro* PPD response. In both ELISPOT and ELISA, difference in response to Ag85B-ESAT-6 and PPD was not observed based on BCG vaccination status, but the response to both was superior in LTBI based on QFT or TST. This indicates us that the high rate of LTBI, QFT or TST, may have resulted higher response in the ESAT-6 component of the fused antigen and confounded the impact of Ag85B. For the same reason given for Ag85B, the overall median ELISA response for the fused Ag85B-ESAT-6 was also lower as compared to PPD. The ELISPOT assay and ELISA findings indicate the presence of memory T-cells against the fused vaccine, Ag85B-ESAT-6, and the comparable response using ELISPOT demonstrates that the vaccine contains the immunodominant epitopes which can then boost the TB specific immunity sufficiently when used in humans.

LIMITATIONS OF THE STUDY

- All of the study participants were males.
- The sample size was not large enough to see the impact of BCG vaccination on TST as the result of the confounding effect of real LTBI in our setting.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS:

- We found an overall strong agreement between TST and QFT-G in adults at both TST≥10mm and TST>5 mm cutoff values in high TB incidence countries in spite of the routine BCG vaccination at birth. We also have demonstrated the possibility to have false TST positivity in adults as the result of BCG vaccine administered in infancy, at cutoff of TST>5mm, that could be confounded in high TB incidence countries by the high rate of true LTBI, but the possibility of true LTBI is highly likely at larger indurations (TST≥10mm).
- QFT-G assay was not able to differentiate recent infection from remote one.
- Age and Khat use were independent risk factors for LTBI whereas higher BMI, which is related to better socioeconomic status, was found to be a protective factor for LTBI.
- By using ELISPOT assay and ELISA, we were able to demonstrate the existence of effector and central memory T-cells specific to the fused vaccine, Ag85B-ESAT-6, and its components, ESAT-6 and Ag85B, in healthy adult populations.
- The presence of significant ESAT-6 specific memory T-cells in the latent group makes the fused vaccine valuable to be used as post-exposure vaccine.
- The persistence of Ag85B specific T-cells as the result of childhood BCG vaccination is evident from our study which enables the fused vaccine to be used as booster vaccine besides its potential use as post-exposure vaccine.

RECOMMENDATIONS:

- Either TST or QFT-G assay can be used in the diagnosis of LTBI in adult populations residing in high TB incidence countries or immigrants from those countries living in low incidence country, but the choice between the two can be decided based on the purpose of LTBI diagnosis:
    - For screening participants in clinical trials, since there is usually the temptation to be more stringent, we recommend the use of QFT-G assay.
    - The judgment for routine use of LTBI diagnosis for new immigrants should depend on the cost-benefit analysis of the two tests in each country.

- The decision for routine use of LTBI diagnosis in order to vaccinate with post-exposure TB vaccine if those in the pipe line turnout to be safe and effective should also depend on the cost-benefit analysis of the two tests in each country.

- We recommend further investigation to be conducted on the association between Khat use and LTBI.

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APPENDIX I: QUESTIONNAIRE

I. General Information

Participant No|___|___|___|

University/Department/Year----------

Age (years) ☐☐

Sex

1. Male ☐ *2. Female* ☐

Place of birth----------

Religion

1. Orthodox ☐ 2. Muslim ☐ 3. Protestant ☐

4. Catholic ☐ 5. Others ☐

Marital status

1. Never married ☐ 2. Married ☐ 3. Separated ☐

4. Divorced ☐ 5. Widow/ widower ☐

II. Behavior

Have you ever Chewed Khat?

1. Yes ☐ *2.No* ☐

If yes, please give details on the year, months and frequency----------

---

Have you ever smoked Cigarette*?*

1. Yes ☐ *2.No* ☐

If yes, please give details on the year, months and number of Cigarette----------

---

Participant No|___|___|___|

III. Clinical data

Do you have Cough lasting for more than 3 weeks? *1.Yes* ☐ *2.No* ☐

Do you have past or present chronic illnesses? *1.Yes* ☐ *2.No* ☐

*If yes, please give details*_____

_____

*Do you have h*istory of previous treatment for TB? *1.Yes* ☐ *2.No* ☐

Do you have history of contact with TB Patient living within the same Household continuously?

1.Yes ☐ *2.No* ☐

*If yes, please give details*_____

_____

Are you on any drug treatment? *1.Yes* ☐ *2. No* ☐

*If yes, specify*_____

_____

Do you history of BCG Vaccination?

1.Yes ☐ *2.No* ☐ *3. Do not know* ☐

IV. Physical examination

Weight (Kg) |____|____|____|. |___| Height (cm) |____|____|____|

BMI|____|____|.|____|

Vital signs: Blood pressure (mmHg) Systolic |___|___|___|/Diastolic|___|___|___|

Any abnormal physical finding 1.Yes ☐ 2.No ☐

*If yes, please give details*_____

_____

Presences of BCG scar *1. Yes* ☐ *2. No* ☐

Participant No|___|___|___|

V. Chest x-ray

1. Normal ☐

2. Abnormal ☐ *(Please give details of radiology report)*----------

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VI. TST

Size of induration (mm) |____|____|

VII.. QuantiFERON TB® –Gold assay

Test result |____|____| . |____|____| IU/ml

Participant No|___|___|___|

VIII. Antigen Stimulation Tests

Measurement method	IMMUNOLOGY VARIABLE	RESULT/ Units
ELISPOT for IFNγ	Ag85B SPOT	|___|___|___|___| SFU/10^6 PBMCs
	ESAT6 SPOT	|___|___|___|___| SFU/10^6 PBMCs
	Fused Ag85B-ESAT-6 SPOT	|___|___|___|___| SFU/10^6 PBMCs
	PHA SPOT	|___|___|___|___| SFU/10^6 PBMCs
	PPD SPOT	|___|___|___|___| SFU/10^6 PBMCs
	Medium SPOT	|___|___|___|___| SFU/10^6 PBMCs
ELISA for IFNγ	Ag85B PROD	|___|___|___|___| pg/ml
	ESAT6 PROD	|___|___|___|___| pg/ml
	Fused Ag85B-ESAT-6 PROD	|___|___|___|___| pg/ml
	PHA PROD	|___|___|___|___| pg/ml
	PPD PROD	|___|___|___|___| pg/ml
	Medium PROD	|___|___|___|___| pg/ml

IX. General Remarks

_____ _____

Name of the physician Date Signature

APPENDIX II: INFORMATION SHEET AND CONSENT FORM

Armauer Hansen Research Institute (AHRI)
School of Graduate Studies (AAU-Medical Faculty)

Name of the Principal Investigator: Alemnew Fitayehu (MD)

Name of the Organization: Addis Ababa University-Medical Faculty.
Name of the sponsor: School of Graduate Studies (AAU) and Armauer Hansen Research Institute (AHRI)

Information sheet prepared for participants from Addis Ababa University-Medical Faculty in research project that is entitled as Immune Response to Vaccine Candidate Antigens in Healthy Adult Ethiopians.

We, the investigators, are planning to conduct a study that is aimed at determining the immune response to vaccine candidate MTB antigens in preclinical medical and paramedical students (AAU-MF). Investigators involved are final year M.Sc. graduate student from Medical Faculty/AAU, researchers from AHRI, an advisor from Medical Faculty/AAU, clinician and nurse.

Purpose

The purpose of this research is determining the immune response to vaccine candidate MTB antigens in healthy adult Ethiopians. The incidence and prevalence of TB is in Ethiopia and in the world at large is increasing because of many factors. The factors are the HIV/AIDS pandemic, the emergence of drug resistant TB; and lack of good diagnostic methods and effective vaccine. Vaccine being one of TB control measures, understanding the immune response to promising determining the immune response to vaccine candidate MTB antigens is of very much important in future vaccine development.

Procedure

To assess the immune response to vaccine candidate MTB antigens in healthy adult Ethiopians, we invite you to take part in our project. If you are willing to participate in

this project, you need to understand and sign the consent form. At enrollment a medical history will be taken & physical examination conducted; and chest X-ray, complete blood count, random or fating blood sugar, renal and liver function tests will be done. Blood samples (48 ml) will be obtained from all groups of study population. Upon consent, all study participants will be tested for HIV using different HIV testing kits as recommended by the Ethiopian Ministry of Health. (*See the section on ethical considerations*). Tuberculin skin testing will also be done after blood sample is taken for the above mentioned investigations including HIV test which will be read after 48-72 hours.

Risk and Discomfort

By participating in this research project, you may feel that it has some discomfort but this will not be too much. We will arrange places for physical, laboratory and chest x-ray examinations.

You will not face any risk by participating in this research, but there could be minor pain at the time of blood drawing and tuberculin skin testing. The risk of an adverse reaction following TST is very low. Occasionally, an individual who has been exposed to the TB bacteria will develop a large reaction in which the arm swells and is uncomfortable. This reaction should disappear in two weeks. A sore might develop where the injection was given, or a fever could occur, but these are extremely rare reactions. Risks will be avoided as the procedure is carried out by experienced health professional based on the standard good clinical practice

Benefits

As a participant, though you may not get direct benefit, you will have the chance to known your general health status from the physical examination, laboratory and chest X-ray results. In addition, the result of this study may help in future vaccine development where the world population especially people in developing countries like ours will benefit.

Incentives

You will not be provided any incentives to take part in this research. However, if the place of physical, laboratory or chest X-ray examinations is outside of the campus where you are living, the transportation cost and compensation for the lost time will be given to you.

Confidentiality

The information that we collect from this research project will be kept confidential. Information about you that will be collected from the study will be stored in a file, which will not have your name on it, but a code number assigned to it.

Right to refuse or withdraw

You have full right to refuse from participating in this research if you do not wish to participate. You also have full right to withdraw at any time you wish.

Whom to contact

This research project will be reviewed and approved by AHRI/ALERT ethical committee and Department of Microbiology, Addis Ababa University Faculty of Medicine.

This study plan will also be reviewed and approved by National Ethical Research Committee (NERC). This committee is the highest body in the country to approve such research plans. The main task of both institutional and national ethical committees is to make sure that research participants are protected from harm. If you want more information and check about this project, you can contact the chairman of the NERC.

If you have any questions contact any of the following individuals and you may ask at any time you want:

1. **Dr. Alemnew Fitayehu, Tel. 251911897357, Addis Ababa.**
   E-mail: fitayehu@yahoo.com
2. **Prof. Asrat Hailu, AAU, Medical Faculty. Tel. 251911480993, Addis Ababa.**
3. **Dr. Abraham Aseffa, AHRI, Ethiopia, Tel. 251113211334**

Consent form
(English Version)

Code_____

I have been requested to participate in a research project that aims at determining the immune response to vaccine candidate MTB antigens in healthy adult Ethiopians. And I am informed that the incidence and prevalence of TB in Ethiopia is increasing because of HIV/AIDS pandemic and the emergence of drug resistant TB; and lack of good diagnostic methods and effective vaccines. Vaccine being one of TB control measures, I agree that understanding the immune response to vaccine candidate antigens to be very much important in future vaccine development. For the study proper and prerequisite laboratory investigations 48ml of blood will be taken from me. The prerequisite laboratory investigations include CBC, liver function test, renal function test, RBS/FBS, chest X-ray and HIV testing- after pretest counseling.

I have been informed that all information I will be giving, the physical examination findings and investigation results will be kept confidential. I understand that the risks I am exposed to would be the discomfort from blood drawing and TST; and the rare and minor side effects of TST which subsides in short duration.

I also know that I have the right to withhold information or drop out of the study with out any need to explain to any one. I also know that I will not get antiretroviral drugs paid for by the project. But I will be linked to ART center where I can get support if I turned to be seropositive for HIV and interested to know my result; the result to be revealed after posttest counseling. And I have the right to know the results of other investigations and physical examination findings but treatment will not be arranged other than advising to get treatment and follow up in case of abnormal findings.

I have asked for clarification and received satisfactory response in a language I understood. And the investigator can use the sample taken, for TB Vaccine study. I finally confirm my agreement by putting my signature below.

Signature of participant_____Date_____

Signature of HIV counselor_____Date_____

Signature of investigator_____Date_____

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¾S/u?~ eU : ›`T¨` N"c" ¾U`U` ›="c+ƒżƒ (›l]) '

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ÃI Tw^]Á □ " Sð^]T>Á pî u}S^T]-‹ u<É" ¾}²ÒË c=J" ¯LT¨<U ¾c¨<'□ ‹" ui□ " ¾SŸLŸM ›ìó K+u= ¡ƒvƒ'ƒ K□ Û ¾v¡,]Á (MTB) ¨<lÊ uÖ?'— ›=ƒÄâ=Á¨<Á" ²"É U" □ "ÅT>SeM T¨p '¨<:: u<É'< ¾T>Á"ƒ}¨< Ÿ›Ç=e ›uv ż"u`c=+ Él[U[n ƒ/u?ƒ l¡U" óŸ<M+ ¾Te}`e }T] }S^T]-‹ Ÿ›l]" ›Ç=e ›uv ż'>y`c=+ l¡U" óŸ<K=+ □ "Ç=G<U '`e Ÿ›l] '¨< ::

¾Ø"~ ›LT

¾²=l Ø"ƒ ›LT ¾W¨<'□ ‹" ui□ " ¾SŸLŸM ›ìó K+u ¡ƒvƒ'ƒ K□ Û ¾v¡,]Á(MTB) ¨<lÊ‹ uÖ?'— ›=ƒÄâ=Á¨<Á" U" □ "ÅT>SeM S[Çƒ '¨<:: ¾+u= ui□ c`ßØ u}KÁż U¡"Á„‹ ¾}'d u›=ƒÄåÁ □ "Ç=G<U u›KU LÃ u›}nLÃ □ ¾ÚS[ SØ…M :& U¡"Á„‡U :- ¾›?‹ ›Ãy= c`ßØ Seóóƒ ' ¾ui□ ¨< ›Uß v¡,]¾ ì[+u= SÉ□ '>„‹" SKTSÉ ' Ÿ²=l kÅU ØpU LÃ Ã¨<M ¾'u`¨< ¾+u= ¡ƒvƒ wl ›KSJ" □ " ›S`m ¾J'< ui□ ¨<" ¾SS`S]Á ²È-‹ ›KS•` "†¨< ::eK²=l ¡ƒvƒ ›"Æ ui□ ¨<" ¾S„×Ö]¾ S"ÑÉ uSJ'< ¾W¨<'□ ‹" ui□ " ¾SŸLŸM ›ìó }eó K}×Kv†¨< ¾v¡,]Á(MTB) ¨<lÊ‹ U" □ "ÅT>SeM S[Çƒ ¨Å òƒ ›Ç=e ¾+u= ¡ƒvƒ KT²ÒËƒ u×U ÖnT> '¨<::

›"H@É

ŸLÃ u}Ökc¨< Ø"ƒ □ "Ç=d}ñ }Òw²ºM :: uØ"~ KSd}õ ónÅ— ŸJ'< upÉT>Á eKØ"~ ›LT" pÉS G<'@□ -‹" uT>Ñv □ "Ç=[ÆM"" Ÿ²=ÁU SeTTƒ-" uò`T- □ "Ç=Á[ÒÓÖ<M" □ "hK":: }d□ ò¨< u}S^T]¨< ¾T>k`u<Kƒ" ØÁo-‹ ÃSMdM ' uHŸ=U U`S^ □ "Ç=G<U ¾}KÁż ¾ÅU" ¾Ï ¾i"ƒ" ¾WÑ^ U`S^<->‹

ÃÅ[ÓK□ M:: Kª‘¨< Ø‘ƒ □ ‘ upÉS G<’@□ ’ƒ ›eðLÑ> KJ’< ¾}KÁ¿ ¾ÅU U`S^-‹ ¾T>¨<M 48 T>M ¾ÅU “S<“ Ã¨cÇM::¾ÅU U`S^-‹ ¾N?T„KAÍ= ' Ñ<uƒ ' Ÿ<LK=ƒ ' e"D` □ ‘ ¾›?‹ ›?Ãy= “†¨<:: ¾›?‹ ›Ãy= U`S^ ¾T>Å[Ñ¨< ŸpÉS U`S^ ¾U¡` ›ÑMÓKAƒ u□ L ’¨< ::¾ÅU “S<“ u’`e Ÿ}¨cÅ u□L ¾qÇ LÃ U`S^ (TST) u’`dD ÃÅ[ÒM :: ¾qÇ LÃ S`U^¨< Ÿ48 -72 c¯ƒ u□ L Ã’uvM:: ŸLÃ Ÿ}Ökc<ƒ U`S^-‹ ¾}[ð¨<” ¾ÅU “S<“ }S^T]¨< }SddÃ KJ’< Ø“„‹ K=Á¨<K¨< Ã‹LM ::

K=Ÿc~ ¾T>‹K< eÒ„‹“ ¾UŒƒ SÕÅKA‹

u²=I Ø“ƒ LÃ Sd}õ- ¾}¨c’ ›KSS†ƒ K=•[¨< Ã‹LM ’Ñ` Ó” Á” ÁIK ¾}Ò’’ ›ÃÅKU :: uHŸ=U KT>Å[Ó U`S^ □ ”Ç=G<U KLw^„]“ ^Ï U`S^ x□ -‹” □ “S‰‰K” :: uØ“~ ¨pƒ K=ÁÒØS<-ƒ ¾T>‹K<ƒ ‹Óa‹ S”ŸM ¾ÅU“S<“ uT>¨eÉuƒ □ ‘ ¾qÇ LÃ U`S^ (TST) uT>Å[Óuƒ ¨pƒ ¾T>•[¨< ¾ISU eT@ƒ ’¨< :: ¾qÇ LÃ U`S^¨< ›Mö ›Mö ´p}— ¾J’< Ñ<Ç„‹ K=ÁeŸƒM Ã‹LM ’Ñ` Ó” u›ß` Ñ>²? ¨eØ ¾T>Öó “†¨< :: ›Mö ›Mö K=Ÿc~ ¾T>‹K<ƒ Ñ<Ç„‹ ¾□ Ï TuØ ¨ÃU ›”Ç”É Ñ>²? ÅÓV SlcM □ ‘ ƒŸ<dƒ “†¨<:: “S<“¨< ¾T>¨cÅ¨< □ ‘ ¾qÇ LÃ U`S^¨< ¾T>Å[Ñ¨< ucKÖ’< ¾Ö?“ vKS<Á-‹ eKJ’ K=Ÿc~ ¾T>‹K<ƒ Ñ<Ç„‹ ›Ã•\U :: G<K<U e^-‹ ¾T>c\ƒ ¾Ö?“ e’UÓv`“ Å”w uT>Á²¨< Sc[ƒ ÃJ“M ::

ØpV‹

}d□ ò uSJ”- kØ}— ¾J’< ØpTØpV‹ LÁÑ–< Ã‹LK< ’Ñ` Ó” uHŸ=U uT>Å[Ó U`S^ □ ”Ç=G<U u^Ï“ Lw^„] U`S^-‹ ›ÖnLÃ ¾Ö?“ G<’@□ -” T¨p Ã‹LK< :: u}ÚT]U ¾²=I Ø“ƒ ¨Ö?ƒ ¨Åòƒ ›Ç=e ¾+u= ¡ƒvƒ KT²ÒËƒ K=ÖpU Ã‹LM u²=IU ¾›KU I´w u}KÃU uTÅÓ LÃ ÁK< □ ”Å ›=ƒÄâ=Á ÁK< GÑa‹ }ÖnT> ÃJ“K<::

T""h

u²=I Ø“ƒ uSd}õ- K=cØ-ƒ ¾T>‹M T""h ›Ã•`U ’Ñ` Ó” ¾HŸ=U ¾Lw^„]“ ¾^Ï U`S^-‡ ¾T>"H@Æƒ ŸUƒ•\uƒ ¢K?Ï ¨<ß ŸJ’ ¾□ ¡c= ¨ß □ ‘ KT>Öó¨< Ñ>²? T""h ÃcÖ-□M ::

UeÖ=` SÖup

Ãl Ø“ƒ }Ö“q“ ¨<Ö?~ }Ö“pa }Ñu=¨<]þ`ƒ □ ”ÅT>²ÒÏ □ S<” ’¨< :: Ãl ]þ`ƒ c=²ÒÏ □ `e¨” u}ScŸ} ¾}Ñ+< ¾ÓM S[Í-‹ □ ‘ M¿ M¿ ¨Ö?„‹ G<K< u}“ÖM ]þ`ƒ

□ "ÅTÃÅ[Ñ< □ "Ç=G<U ¾□ `e- T"'ƒ SÓKÝ ¾J'< S[Í-‹ KUdK? -:eU ' ¾S}¨mÁ lØ` ' ¾ÓM ›É^h' ¨ ² } u]þ`~ □ "ÅTÃ"}~ □ "[ÒÓ×K" ::

uØ"~ ÁKSd}õ ¨ÃU ^e" ¾TÓKM Swƒ

¾□ `e- }dƒö uðnÅ˜'ƒ LÃ ¾}Sc[} '¨<:: ¾›KSd}õ Swƒ- ¾}ÖUk '¨< □ "Ç=G<U □ ^e-" ŸØ"~ TÓKM ŸðKÑ< uT"—¨<U c¯ƒ" Ñ>²? ÁK T"U ðnÉ }dƒö-" Ts[Ø Ã‹LK<::

S[Í KTÓ–ƒ

ÃI Ø"ƒ u›l]" ›.›¿ □ "Ç=G<U uwN?^© e'UÓv`" U`U` ¢T>, ÉÒõ ›Ó˜…M :: ¾□ '²=l ¢T>,-‹ e^ ¾Ø"~ }d□ ò-‹ ŸÑ<Çƒ SÖun†¨<" T[ÒÑØ'¨< ::

ØÁo ¨ÃU Tw^]Á "eðKÑ-ƒ ¾T>Ÿ}K<ƒ" c-‹ Á'ÒÓ\

1. ›KU'¨< õ□¾G< :- ›/›¿ /l¡U" óŸ<M+
   e.l :- 251911877357 ›/›uv
2. ý/` ›e^ƒ HÃK< :- ›/›¿ /l¡U" óŸ<M+
   e.l :- 251911480993' ›/›uv
3. Ê/` ›w[HU ›có &- ›l]
   e.l :- 25111 3211334 ' ›/›uv

¾eUU'ƒ Sð^[T>Á pî

¾UeÖ=` lØ`----------□ '@ ¾Ø"~ }d□ ò ¾+u= e`ßƒ u}KÁ¿ U¡"Á„‹ ¾}'d u›=ƒÄâ=Á □ "Ç=G<U u›KU LÃ u›ÖnLÃ □ ¾ÚS[ □ "ÅS× }'Óɑ—M:: U¡"Á„‡U ¾›?‹ ›?Ãy= Seóóƒ ' ¾ui□ ¨< ›Uß v¡,]Á ì[ +u= SÉH'>„‹" SKTSÉ' ›S`m ¾J'< ¾U`S^ ²È-‹ ›KS•` □ " ØpU LÃ Ã¨<M ¾'u[¨< ¾+u= ¡ƒvƒ wl ›KSJ" □ "ÅJ'< ›¨<o›KG<:: ¡ƒvƒ ›"Æ ui□ ¨<" ¾Sɑ]Á S"ÑÉ □ "ÅJ' □ " }eó K}×Kv†¨< K¡ƒvƒ'ƒ ¾□ Û ¾v¡,]Á (MTB) ¨<lÊ‹ ¾c¨<'□ ‹" ›ìó U" □ "ÅT>SeM S[Çƒ ¨Åòƒ KT>Å[Ó ›Ç=e ¡ƒv„‹" ¾T²ÒËƒ e^ ÖnT> SJ'<" }[ÉŠ }eTU‰KG< ::

K$‹"¨< Ø"ƒ □ " upÉS G<'@□ 'ƒ ›eðLÑ> KJ'< ¾}KÁ¿ ¾ÅU U`S^-‹ ¾T>¨<M 48 T>M ¾ÅU "S<" Ÿ□ '@ Ã¨eÇM::¾ÅU U`S^-‹ ¾N?T„KAÍ= ' Ñ<uƒ ' Ÿ<LK=ƒ '

e"D` □ “ ¾›?‹ ›?Ãy= SJ“†¨” □ “ ¾^Ï U`S^U □ ”ÅT>Å[ÓM˜ }’Óa˜ }eTU‰KG<:: ¾›?‹ ›Ãy= U`S^ ¾T>Å[ÓM˜ pÉS U`S^ ¾U¡` ›ÑMÓKAƒ Ÿ}cÖ˜ u%EL SJ’<” }eTU‰u□KG<::

¾UeÖ¨< S[Í □ ”Ç=G<U ¾HŸ=U“ ¾}KÁę (¾ÅU“ ^Ï) U`S^ ¨<Ö?„‹ uT>eÖ=` □ ”ÅT>ÖupM˜ }’Óa—M:: uØ“~ ¨pƒ K=ÁÒØS< ¾T>‹K< ‹Óa‹ ¾ÅU “S<“ uT>kÇuƒ □ “ qÇ LÃ uT>Å[Ó U`S^ (TST) ¨pƒ ŸT>•[¨ ¾ISU eT@ƒ u}ÚT] ¾qÇ LÃ U`S^¨<” }ŸƒK¨< K=Ÿc~ ¾T>‹K< ‹Óa‹ ›’e}— ¾J’< ¾□Ï TuØ ¨ÃU SlcM □ “ ƒŸ<dƒ SJ“†¨<” }’Óa˜ □ ’²=IU u=J’< u›ß` Ñ>²? ¨<eØ □ ”ÅT>Öó }[É‰KG<:: ¾›?‹ ›Ãy= ¾U[S^ ¨<Ö?,” T¨p ŸðLÓŸ< ÉI[U`S^ ¾U¡` ›ÑMÓKAƒ □ ”ÅT>cÖ˜ □ “ zÃ[c< uÅT@ ¨<eØ Ÿ}Ñ– ì[ ›?‹ ›Ãy= SÉH’>ƒ ýaÏ¡~ □ ”ÅTÁk`wM˜ ’Ñ` Ó” ¾›?‹ ›Ãy= ¾I¡U“ T°ŸM Ò` □ ”ÉÑ“˜ □ ”ÅT>Å[Ó}’Óa—M :: □ ”Ç=G<U K?KA‹ ¾U`S^ ¨<Ö?„‹” T¨p ŸõKÓŸ<˜ Sw, ¾}Öuk u=J”U ui□ u=Ñ˜w˜ I¡U“ □ ”ÇÅ[Ó ŸSUŸ` ¨<ß ýaË¡~ I¡U“ □ ”ÅTÁk`wM˜ }’Óa˜ }eTU‰KG< : u}ÚT]U Sd}õ ”MðKŸ< ÁKU”U pÉS G<’@□ Ts[Ø □ ”ÅU‹M ›¨<o›KG<::

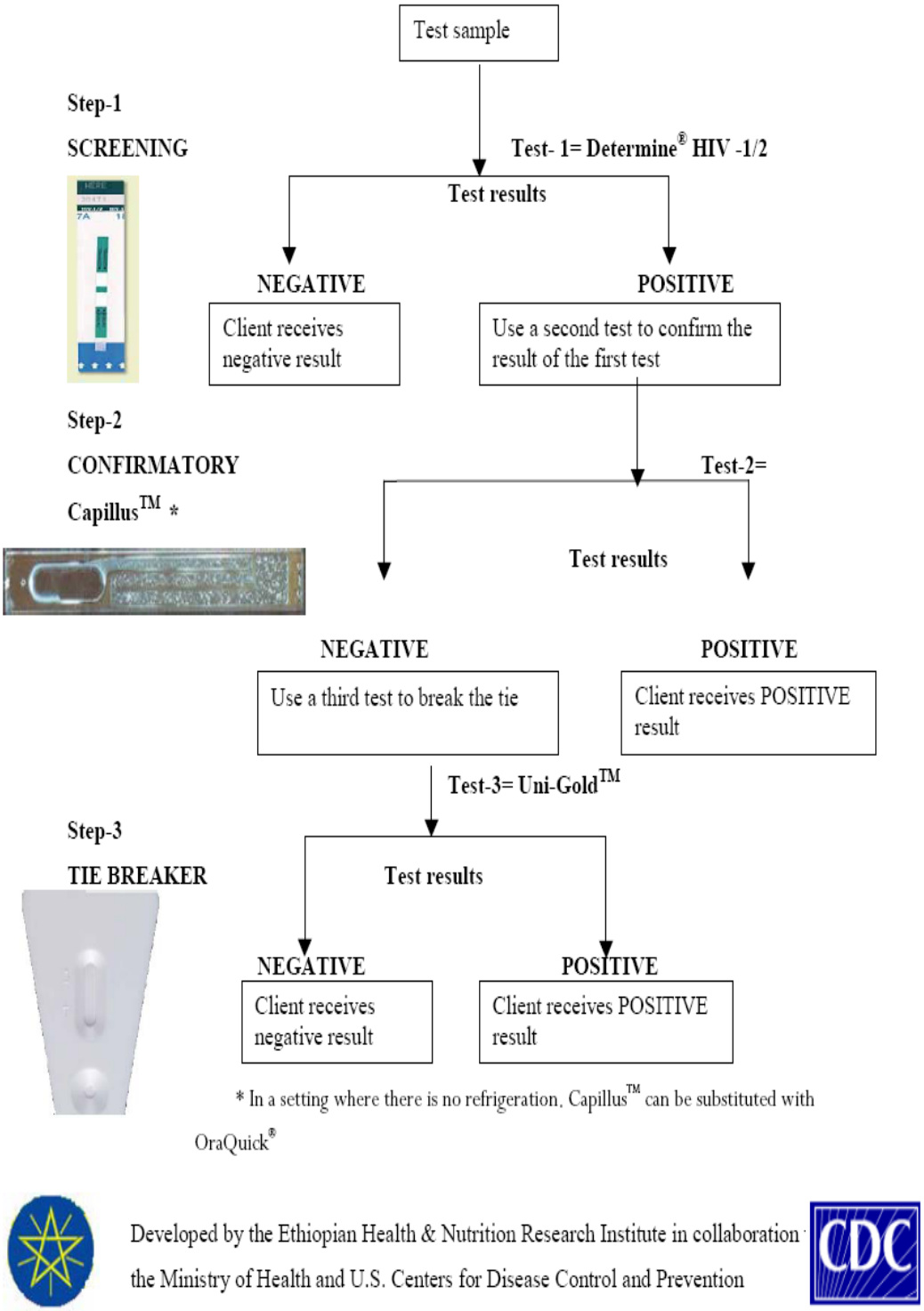
ØÁo ¾SÖ¾p °ÉK< }cØ„˜ uT>Ñv˜ s”s um SMe ›Ó”‰KG<:: ¾}¨cÅ¨<” “S<“ }S^T]¨< K+u= ¡ƒvƒ U`U`U □ ”Ç=Á¨<K¨< ðpÍKG<:: uSÚ[h uØ“~ KSd}õ S<K< uS<K< ðnÅ— SJ’@” uò`TÂ ›[ÒÓ×KG< ::

¾Ø“~ }d□ ò ò`T (eU ›ÃÑKîU)---------- k” ----------

¾›?‹ ›Ãy= U¡` ›ÑMÓKAƒ cÜ ò`T ----------k” ----------

¾}S^T] ò`T ---------- k” ----------

APPENDIX III: RAPID HIV TEST ALGORITHM



DECLARATION

I, the undersigned, declare that this thesis is my original work. It has not been presented for a degree in this or any other university and all the source materials used for this thesis have been duly acknowledged.

Name of the candidate Alemnew Fitayehu Dagnew

Signature _____

Place Addis Ababa

Date _____

The final version of the thesis is approved by the following supervisors:

1. Prof. Asrat Hailu

   Signature_____ date _____

2. Dr. Markos Abebe

   Signature_____ date _____